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Modulation of professional phagocyte activity

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TABLE OF CONTENTS

ABSTRACT

Neutrophils and monocytes/macrophages, so called professional phagocytes, as integral constituents of innate immunity serve as an essential first-line of defence against microbial pathogens and foreign substances at sites of inflammation. Upon activation, these cells among other functional manifestations produce reactive oxygen and nitrogen species. While respiratory burst is important for the elimination of invading pathogens, the overproduction of reactive oxygen species or the impairment of endogenous antioxidant defences may lead to a damage of membrane lipids, DNA, proteins, and lipoproteins which results in various autoimmune and inflammatory diseases. Thus, a modulation of professional phagocyte activity is very important task in physiology and medicine. We have been studying the possibilities of professional phagocyte modulation by both endogenous (serotonin) and exogenous (polyphenols and polysaccharides of plant origin as food constituents) mediators. Our studies bring the evidence that serotonin released from platelets is a very important modulator of professional phagocyte activity. Its inhibitory activity is manifested by the decrease in the generation of reactive oxygen species due to the inhibition of myeloperoxidase activity and by direct scavenging of reactive oxygen species. The effects of serotonin on professional phagocytes are also partially mediated by 5-HTR2 receptor expressed on monocytes/macrophages but not on neutrophils. We also introduced and optimized several methods to investigate antioxidant properties of plant extracts and individual plant polyphenols. We confirmed that many medicinal plants, vegetables and berries are rich sources of polyphenol compounds and free radical scavengers. Furthermore, we demonstrated that various plant polyphenols and polysaccharides exerted evident immunomodulatory activity. Thus, these compounds represent effective naturally occurring substances with potent pharmacological effects on respiratory burst of professional phagocytes useful for treatment of compromised immune system and control of inflammation.

1 INTRODUCTION

Innate immunity serves as an essential first-line of defence against microbial pathogens and foreign substances. Neutrophils are the typical effector cells of the innate immune response because they are the first leukocytes to be recruited to an inflammatory site. In response to a variety of stimuli, neutrophils adhere to and migrate through the endothelium to reach the inflamed tissue where they engulf invading microorganisms and destroy them through multiple oxidative and non-oxidative mechanisms (Futosi et al. 2013, Kolaczkowska and Kubes 2013, Sadik et al. 2011). Neutrophils together with monocytes and macrophages are called professional phagocytes because of their primary function – engulfing and destroying potentially injurious particles.

1.1 NEUTROPHILS

Neutrophils play a vital role in innate immune reactions. They are recruited to sites of infection and constitute the first line of defence (Selders et al. 2017, Timar et al. 2013). Based on the short life-span and predefined set of functions, neutrophils were most often considered as one population. Thus, functional and mechanistic studies on neutrophils generally deal with them as a homogenous population of terminally differentiated cells (Chakravarti et al. 2009). However, a growing number of studies are showing the phenotype and functional heterogeneity of neutrophils existing simultaneously both under healthy and acute immune response conditions. Rather than being an end-stage uniform cell population, neutrophils can show a great level of plasticity and develop distinct phenotypes or subpopulations under a wide range of physiological (e.g. age) and pathological (e.g. inflammation, infection, cancer) conditions. This makes neutrophils not only passive by-standers but showing their importance in orchestrating function of other immune cells and over all inflammatory response (Beyrau et al. 2012, Kamp et al. 2012, Sagiv et al. 2015, Welin et al. 2013).

Neutrophils are the most abundant white blood cell type in the blood stream of humans, comprising about 50–70% of all leukocytes (Kruger et al. 2015). In response to infection or injury they migrate to the site of inflammation to neutralize the invading microorganisms or to eliminate tissue injury (Serhan et al. 2007). Neutrophil recruitment from the blood stream is triggered by inflammatory stimuli such as chemokines, cytokines, or lipid mediators. The inflammatory microenvironment triggers a process leading to the transmigration of neutrophils through the vessel wall, known as extravasation (Mocsai et al. 2015). After the

phagocytosis of invading microorganisms, neutrophils die by programmed cell death and are ingested by tissue macrophages (Maderna and Godson 2003).

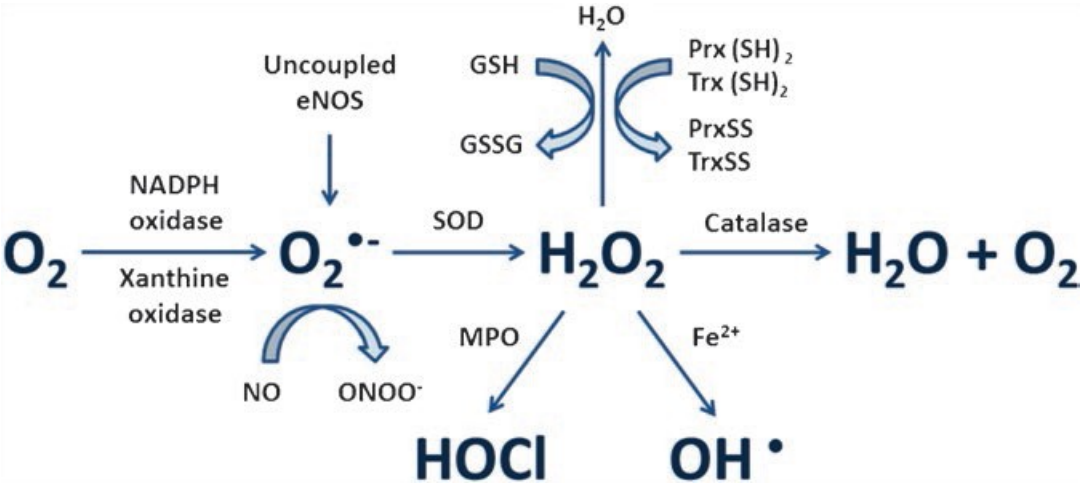

Fig. 1: *Cascade of reactive oxygen species generation. eNOS - endothelial nitric oxide synthase, GSH – reduced glutathione, GSSG - oxidized glutathione, MPO - myeloperoxidase, Prx - peroxiredoxin, SOD - superoxide dismutase, Trx - thioredoxin (Vasicek et al. 2017)*

Reactive oxygen species produced by phagocytes (Fig. 1) after their activation are one of the most important microbicidal mechanisms associated with protection against invading microorganisms and also seem to have important physiological roles in priming the immune system as second messengers (Filippin et al. 2008, Halliwell 2007, Oktyabrsky and Smirnova 2007). The process of reactive oxygen species production by neutrophils is known as the respiratory burst and follows an activation of the enzyme NADPH oxidase (Fig. 2). NADPH oxidase is a multicomponent enzyme system composed of heterodimeric membrane-associated flavocytochrome b588 protein, which is composed of gp91phox and p22phox subunits, three cytosolic proteins (p47phox, p67phox, and p40phox) and small GTPase (Rac1 or Rac2) (Babior 1999). NADPH oxidase produces superoxide anion radical which is rapidly reduced, either spontaneously or enzymatically, to hydrogen peroxide. Hydrogen peroxide is the principal substrate for another important enzyme myeloperoxidase which is stored in the azurophil granules and released by activated neutrophils to produce other reactive oxygen species such as hypochlorous acid with potent cytotoxic properties (Kolarova et al. 2013, Rudolph et al. 2012).

While respiratory burst is important for the elimination of invading microorganisms, the overproduction of reactive oxygen species or the impairment of endogenous antioxidant

defences may result in damaging effects on the host's own cells and tissues (Freitas et al. 2009). The inappropriate production of reactive oxygen species by neutrophils is associated with oxidative damage to membrane lipids, DNA, proteins, and lipoproteins which results in various autoimmune and inflammatory diseases (Perecko et al. 2013, Sadik et al. 2011).

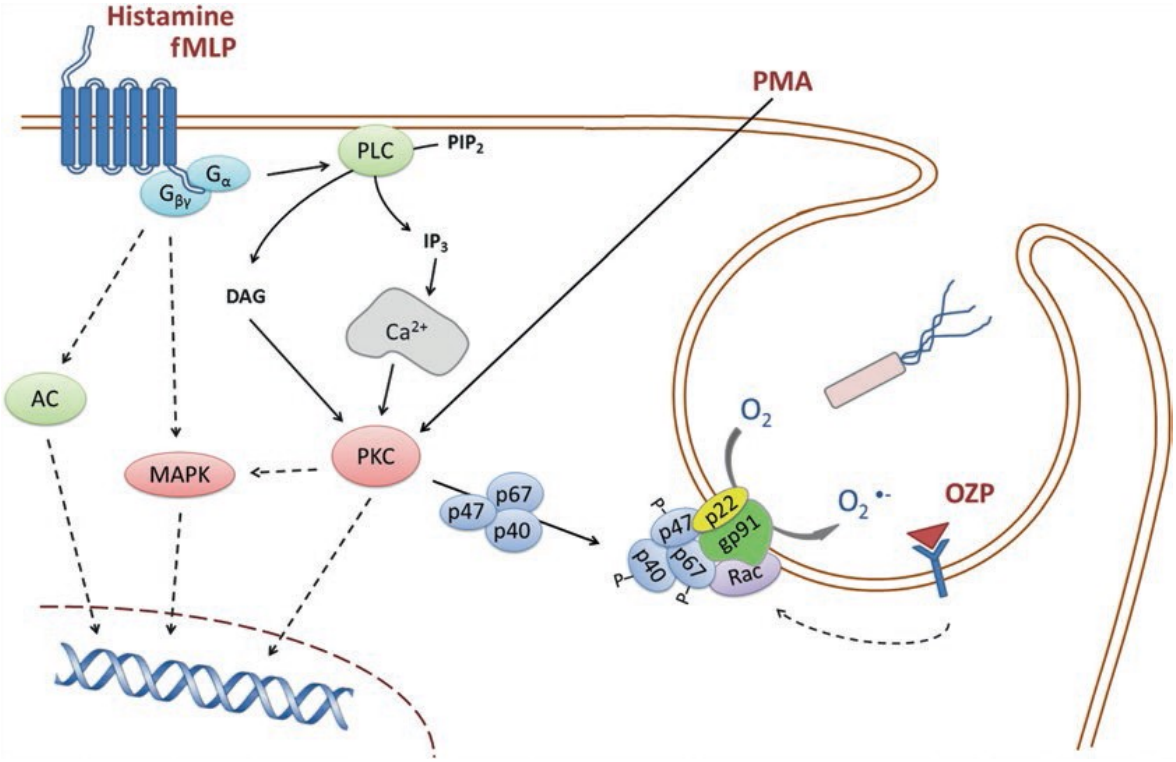

Fig. 2: *Signalling pathways leading to the assembly and activation of NADPH oxidase in neutrophils. AC - adenylate cyclase, DAG - diacyl-glycerol, fMLP - N-Formylmethionine-leucyl-phenylalanine, IP3 - inositol trisphosphate, MAPK - mitogen-activated protein kinases, OZP - opsonized zymosan particles, PIP2 – phosphatidylinositol 4,5-bisphosphate, PKC - protein kinase C, PLC - phospholipase C, PMA - 4-phorbol-12-myristate-13-acetate (Vasicek et al. 2017)*

1.2 MONOCYTES AND MACROPHAGES

Monocytes are generated from hematopoietic stem cells in bone marrow, released into circulation and further differentiated into tissue macrophages and dendritic cells (Murray and Wynn 2011, Shi and Pamer 2011). Cells of the monocyte/macrophage lineage are important for host antimicrobial defence (Serbina et al. 2008) and are implicated in many inflammatory diseases (Geissmann et al. 2008). Monocytes and macrophages together with neutrophils belong to professional phagocytes which express various types of receptors on

their surface to recognize signals that are not found in healthy cells and tissues (Gundra et al. 2014, Matzinger and Kamala 2011). Upon activation, mononuclear phagocytes produce reactive oxygen and nitrogen species by NADPH oxidase and inducible NO synthase, respectively (Ambrozova et al. 2011). Nevertheless, the complex mechanisms of respiratory burst and other functional manifestations of mononuclear phagocytes are different from those in neutrophils. Neutrophils exhibit more rapid rates of phagocytosis and a more intense respiratory burst response than macrophages. The endocytic and phagosome maturation pathways in macrophages are substituted in neutrophils by the rapid delivery of preformed granules to phagosomes (Nordenfelt and Tapper 2011).

2 EFFECTS OF SEROTONIN ON PROFESSIONAL PHAGOCYTES

Serotonin (5-hydroxytryptamine), known mainly as one of the neurotransmitters of the central nervous system, is also present in a variety of peripheral tissues, including several constituents of the immune system. As the major secretory product of activated platelets, it has been widely reported as a regulator of various constituents of the immune system and immune functions. This modulation is complex and the data available are rather controversial (Mohammad-Zadeh 2008, Olivier 2015, Wu et al. 2019, Xia et al. 2019).

We found that human platelets significantly decreased the amount of reactive oxygen species produced by stimulated neutrophils and that the reduction was partially mediated by serotonin released from platelets during their activation. We further investigated whether platelet-induced inhibition of neutrophil respiratory burst could occur independently of serotonin release and whether it could be pharmacologically modulated. We observed that the inhibition of reactive oxygen species production was not accompanied with release of serotonin by non-stimulated platelets and was more profound after addition of chloroquine (a serotonin liberating drug) to platelet-neutrophil samples. Chloroquine itself did not affect neutrophil reactive oxygen species production, but it caused a release of serotonin from platelets to concentrations sufficient for inhibition of neutrophil reactive oxygen species production. Our results indicated that non-stimulated platelets decreased neutrophil production of reactive oxygen species by a serotonin-independent mechanism, while their inhibitory effect could be enhanced pharmacologically through chloroquine-induced release of serotonin. Furthermore, the effectiveness of chloroquine was found to be altered in the presence of platelets. This indicated that under in vivo conditions, the effect of chloroquine on one type of cells could be modified in the presence of cells of another type (Jancinova et al. 2003).

Our next experiments clearly demonstrated that serotonin in a dose dependent manner was also a potent inhibitor of reactive oxygen species production by rat whole blood phagocytes. Since the effect of serotonin on phagocytes is complex, our experiments were focused on elucidating the possible individual mechanisms of serotonin activity. Our results suggested that the modulatory effect of serotonin on an respiratory burst of rat whole blood phagocytes might occur through the activation of the 5-HTR2 serotonin receptor subtype since DOI hydrochloride, a selective 5-HTR2 receptor agonist, had an inhibitory effect on the reactive oxygen species production similar to that of serotonin. However, the antagonist of 5-HTR2 receptor did not block the effect of serotonin. Therefore, we could speculate that other

mechanisms are involved in the serotonin dependent modulation of rat whole blood phagocyte activity. Another probable explanation could be the direct scavenging activity of serotonin against free radicals produced during respiratory burst of phagocytes (Okenkova et al. 2007). It was previously already shown that serotonin could act as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes (Schuff-Werner et al. 1995, Vial et al. 1995).

The aim of the following study was to clarify the mechanisms of serotonin effects on various parameters of respiratory burst of human phagocytes using various luminol-enhanced chemiluminescence methods. It was proved again that serotonin inhibited the chemiluminescence response of human phagocytes in a dose dependent manner. Serotonin was also found to exert a dose dependent inhibition of myeloperoxidase activity. The hypothesis that the inhibitory activity of serotonin might be also receptor mediated was evaluated using various serotonin receptor agonists and antagonists. Only DOI hydrochloride, a selective agonist of 5-HTR2 receptor, exerted similar effects on phagocytes as serotonin. It could be concluded that serotonin inhibitory activity on human phagocytes could be explained by the decrease in the generation of reactive oxygen species (due to the inhibition of myeloperoxidase activity) and direct scavenging of reactive oxygen species already produced. The effect of serotonin on phagocytes might be also partially mediated by 5-HTR2 receptor (Ciz et al. 2007).

The goal of next experiments was to elucidate whether serotonin reduced the respiratory burst of professional phagocytes via 5-HTR receptors or due to its antioxidative properties. In this case, the effects of serotonin on total human leukocytes were compared with its effects on isolated neutrophils. The ability of cells to produce reactive oxygen species was tested using luminol-enhanced chemiluminescence analysis. In accordance with our hypothesis, it was observed that serotonin and its 5-HTR2 agonist DOI hydrochloride were able to inhibit the production of reactive oxygen species in activated rat and human total leukocytes, but not in isolated human neutrophils. The interpretation of these results could suggest the involvement of 5-HTR2 receptors expressed not on the neutrophils but on the surface of other leukocytes including cells of monocyte/macrophage lineage. Moreover, some of the observed inhibitory effects of serotonin on the respiratory burst of phagocytes were partially caused by its reactive oxygen species scavenging activity (Pracharova et al. 2010).

In recent experiments, we showed that not only serotonin but also its structurally related metabolites such as N-acetyl-serotonin and melatonin were able to reduce oxidative stress

and inhibit production of inflammatory cytokines by murine RAW264.7 macrophages. In a comparison of the tested compounds, serotonin and N-acetylserotonin were more effective reactive oxygen species scavengers than melatonin. Other effects of tested compounds were found to be very similar (Vasicek et al. 2019).

3 EFFECTS OF POLYPHENOLS AND POLYSACCHARIDES OF PLANT ORIGIN ON PROFESSIONAL PHAGOCYTES

A significant attention in medicine is paid to approaches designed either to enhance innate immune mechanisms non-specifically with the aim to increase the defence against microbial infections or to inhibit immune mechanisms involved in chronic or acute inflammation. Thus, the effects of natural substances which can modulate the metabolic activity of professional phagocytes and other immune cells without exerting any unfavourable side effects have recently been investigated. Suitable candidates for this purpose might be compounds contained in root, leaf and/or fruit extracts from herbs, fruits and vegetables. These include especially polyphenols and polysaccharides (Lojek et al. 2014).

3.1 ANTIOXIDANT PROPERTIES OF PLANT POLYPHENOLS

We introduced into our laboratory and optimized several methods such as total peroxyl radical-trapping parameter (TRAP), oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) methods to investigate the antioxidant properties of plant extracts and individual plant polyphenols. Although we found a good correlation among all the methods for assessing antioxidant capacity and a good correlation with polyphenol content, using more than one antioxidant assay was strongly recommended since the combination of individual methods described the antioxidant properties of the sample in more detail. Nevertheless, ORAC assay was found to be the most sensitive method to measure chain-breaking antioxidant activity (hydrogen atom transfer pathway) in plant extracts while HORAC assay measured mainly metal-chelating antioxidant capacity (preventive antioxidants). As a supplement, the Folin–Ciocalteu assay (single electron transfer pathway) should be a part of antioxidant profile measurement (Ciz et al. 2010).

Another parameter which significantly influenced the outcomes of studies investigating the antioxidant properties of plant extracts (extractability of polyphenol compounds and antioxidant activity of samples) was the extraction system used. When the ORAC parameter and total polyphenol content of twenty five Bulgarian medicinal plants were examined, the higher results were obtained for 80 % acetone extractions when compared to water extraction (Kratchanova et al. 2010). Another approach was to use solid-phase extraction to obtain anthocyanin-rich extracts from various berry species and their crude extracts. During solid-phase extraction the majority of impurities (sugars and acids) present in the crude extracts were separated (Denev et al. 2010). It was concluded that the solvent used affected

significantly the polyphenol content and the antioxidant activity of the extracts and therefore it was recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products.

Using the optimized methods for plant polyphenol extraction and antioxidant capacity evaluation we confirmed that many medicinal plants (Kratchanova et al. 2010), vegetables (Ciz et al. 2010) and berries (Denev et al. 2010) tested were rich sources of polyphenol compounds and free radical scavengers. Generally, there was a significant linear correlation between the concentration of total polyphenols and the antioxidant capacity in investigated samples.

In another study the antioxidant properties of four wine polyphenols (flavonoids catechin, epicatechin, and quercetin, and hydroxystilbene resveratrol) were studied. All three flavonoids exerted significant and dose-dependent scavenging effects against peroxyl radical and nitric oxide in chemical systems. The scavenging effect of resveratrol was significantly lower. All polyphenols also decreased production of reactive oxygen species by RAW264.7 macrophages. We concluded that the higher number of hydroxyl substituents is an important structural feature of flavonoids in respect to their scavenging activity against reactive oxygen species and nitric oxide, while C-2,3 double bond (present in quercetin and resveratrol) was important for inhibition of reactive oxygen species and nitric oxide production by RAW 264.7 macrophages. (Ciz et al. 2008) Resveratrol (3,5,4'-trihydroxystilbene) was also shown to possess antioxidant activities in vitro in another study. It dose-dependently inhibited the generation of peroxyl and hydroxyl radicals, peroxides, and lipid peroxidation products in various cell free systems (Nosal et al. 2014).

Small fruits are a rich source of bioactive substances, including polyphenols, and are therefore suitable raw materials for production of functional foods. In next study, we tested the antioxidative properties of six fruits: rosehip (*Rosa canina*), black chokeberry (*Aronia melanocarpa*), hawthorn (*Crataegus monogyna*), blackcurrant (*Ribes nigrum*), blueberry (*Vaccinium myrtillus*) and rowanberry (*Sorbus aucuparia*) using various methods. The antioxidant activity data indicated that all the fruits tested, and particularly rosehip and hawthorn, were very suitable raw materials for the production of foods with strong antioxidant properties (Denev et al. 2014b).

3.2 IMMUNOMODULATORY PROPERTIES OF PLANT POLYPHENOLS

Regardless of their direct scavenging activity, we also studied the immunomodulatory activity of various plant constituents in various biological models. We observed that pinosylvin, a stilbenoid that is synthetized in plants during fungal infections, was able to decrease concentration of reactive oxygen and nitrogen species, along with its capacity to enhance the efficacy of methotrexate in arthritis treatment in neutrophils of rats with adjuvant arthritis and in RAW 264.7 macrophages (Jancinova et al. 2010). Another naturally occurring phenolic derivative of resveratrol, pterostilbene, possessed comparable antioxidant properties as resveratrol in human neutrophils and in cell free system. Reducing the respiratory burst of human neutrophils during their activation in vitro with pterostilbene does not include protein kinase C phosphorylation pathway. Pterostilbene showed dose dependent activation/inhibition of caspase-3 enzyme activity (Perecko et al. 2010). Since little was known about pterostilbene effects on neutrophils during inflammation in vivo, the effect of pterostilbene on neutrophil activity was investigated in experimental arthritis model in following study. Lewis rats were injected by a single intradermal injection of heat-killed *Mycobacterium butyricum* in Freund's adjuvant to develop arthritis. In the pterostilbene treated arthritic rats, the treatment significantly lowered the number of neutrophils in blood without significant downregulation of neutrophil respiratory burst. Pterostilbene only nonsignificantly increased the antioxidant capacity in arthritic animals. These results indicated that pterostilbene affected reactive oxygen species in vitro and in the animal model of inflammation by different mechanisms. It can be concluded that the inhibitory effects of stilbene derivatives and their bioavailability were structure dependent (Perecko et al. 2013). We further demonstrated that resveratrol in a concentration-dependent manner inhibited the respiratory burst of human whole blood professional phagocytes stimulated with various activators. Results from isolated human neutrophils revealed that resveratrol was active extracellularly as well as intracellularly in inhibiting the generation of reactive oxygen species. It was documented that resveratrol significantly decreased phosphorylation of protein kinase C. Inhibition of nitric oxide production and inducible NO synthase protein expression in RAW 264.7 cells indicated possible interference of resveratrol with reactive nitrogen radical generation in professional phagocytes (Nosal et al. 2014).

Another study provided a comprehensive data on the antioxidant, antimicrobial and neutrophil-modulating activities of extracts from six medicinal plants - blackberry (*Rubus*

fruticosus) leaves, black chokeberry (*Aronia melanocarpa*) leaves, hawthorn (*Crataegus monogyna*) leaves, lady's mantle (*Alchemilla glabra*) aerial parts, meadowsweet (*Filipendula ulmaria*) aerial parts and raspberry (*Rubus idaeus*) leaves. Obtained results indicated that all herbs studied were rich sources of chain-breaking, chelating and lipid peroxidation inhibiting antioxidants, rendering high antioxidant activity measured by several methods. Besides that the herb extracts could prevent oxidative stress in the gastrointestinal tract trough modulation of neutrophils and an antimicrobial effect against a broad spectrum of human pathogens (Denev et al. 2014a).

Small fruits are a rich source of several classes of polyphenol compounds and differences in their polyphenol profile led to different biological activities. In next study, we tested the effects of rosehip (*Rosa canina*), black chokeberry (*Aronia melanocarpa*), hawthorn (*Crataegus monogyna*), blackcurrant (*Ribes nigrum*), blueberry (*Vaccinium myrtillus*) and rowanberry (Sorbus aucuparia) on the production of reactive oxygen species by professional phagocytes and their antimicrobial properties against eleven human pathogens. All extracts, especially those from anthocyanin-rich fruits, inhibited reactive oxygen species production in activated phagocytes, indicating that extracts interfered with the signalling cascade of phagocyte activation upstream to the protein kinase C activation. Some of the extracts revealed strong antimicrobial properties against selected human pathogens (Denev et al. 2014b). The recent study reported data on antioxidant, antimicrobial and neutrophil-modulating activities of various polyphenolic preparations from black chokeberry (*Aronia melanocarpa*) fruits: crude extract, purified extract standardized to 20% and 40% anthocyanins, and proanthocyanidins; as well as of pure compounds (chlorogenic acid, cyanidin-3-O-galactoside, epicatechin, rutin and quercetin) present in black chokeberries. The highest antioxidant activity in cell free systems was observed for minor phenolic components such as quercetin and epicatechin. According to the amount of individual phenolics in the fruits, proanthocyanidins were the major contributor to the total antioxidant activity of fresh black chokeberries. Studied polyphenols and preparations had no effect on the spontaneous production of reactive oxygen species in human neutrophils. Greater effects on the production of reactive oxygen species were observed in activated neutrophils, especially for quercetin and rutin. The antimicrobial activity test against ten pathogens showed that black chokeberry proanthocyanidins are the most potent antimicrobial agents in the fruit. The study confirmed our hypothesis that black chokeberry polyphenols differed in their antioxidant, antimicrobial and neutrophil modulating activities (Denev et al. 2019).

3.3 IMMUNOMODULATORY PROPERTIES OF PLANT POLYSACCHARIDES

We observed that leek (*Allium ampeloprasum*) polysaccharides modulated professional phagocytes via different mechanisms. Leek pectic polysaccharides significantly decreased the production of reactive oxygen species by human neutrophils. On the other hand, polysaccharides isolated from alcohol insoluble substances of leek exhibited potent macrophage-activating properties in a concentration-dependent manner resulting in an increased generation of nitric oxide in RAW 264.7 cells via the induction of inducible NO synthase. Moreover, polysaccharides extracted from alcohol insoluble substances with water showed the ability to fix serum complement, especially through the alternative pathway. The polysaccharide with the highest complement-fixing activity was characterized by the highest content of uronic acids and the highest molecular weight. Nevertheless, the molecular weight was not the only parameter that determined the macrophage immunomodulatory activity of leek polysaccharides (Nikolova et al. 2013).

Other polysaccharide complexes containing mainly pectic polysaccharides were isolated from the aerial parts of common purslane (*Portulaca oleracea*), and from the flowers of common lavender (*Lavandula angustifolia*) and silver linden (*Tilia tomentosa*) by boiling water extraction and ethanol precipitation. These samples also exerted stimulating activity on phagocytes of the monocyte/macrophage lineage (CD14+ and CD64+ cells) and induced production of reactive oxygen species. Despite that these extracts stimulated also T-cell populations (CD4+/CD25+ and CD8+/CD25+ cells) and induced IL-6 production in human leukocytes (Georgiev et al. 2017). The polysaccharide extracts with immunomodulatory activity from lavender flowers were further chemically characterised as two pectic and one polyphenolic fractions. One of the pectic polysaccharide fractions (52.4 kDa) contained mainly low-acetylated and high-methoxylated homogalacturonans, and smaller rhamnogalacturonan I backbone fragments rich in 1,3,5-branched arabinan and arabinogalactan II side chains. The other pectic polysaccharide fraction (21.8 kDa) contains predominantly similarly esterified homogalacturonans, followed by rhamnogalacturonan I with arabinogalactan II structures and rhamnogalacturonan II. The prevalence of catechin and epicatechin in polyphenolic polysaccharide fraction indicated that they form weak interactions with pectins. In this set of experiments we confirmed that lavender pectins activated innate and adaptive immune response through the complement system, neutrophils, and macrophages. On the other hand, the observed inhibitory effects of pectins against the

activated reactive oxygen species production and the suppression of nitric oxide generation suggested anti-inflammatory activity of the studied pectins (Georgiev et al. 2017b). Similarly, silver linden pectins with immunomodulation properties were analysed in more details. Linden pectins induced reactive oxygen species and nitric oxide generation in non-stimulated whole blood phagocytes and macrophages, but suppressed activated reactive oxygen species generation, inducible NO synthase expression and nitric oxide production (Georgiev et al. 2017c).

4 SUMMARY

- Our studies bring the evidence that serotonin released from platelets is a very important mediator of professional phagocyte activity. Serotonin inhibitory activity on professional phagocytes is manifested by the decrease in the generation of reactive oxygen species (due to the inhibition of myeloperoxidase activity) and direct scavenging of reactive oxygen species already produced. The effects of serotonin on professional phagocytes are also partially mediated by 5-HTR2 receptor expressed on monocytes/macrophages but not on neutrophils. Thus, serotonin plays a principal role in modulation of inflammation, regulation of function of immune cells and protection of cells against the oxidative stress.
- We introduced and optimized several methods such as total peroxyl radical-trapping parameter (TRAP), oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) methods to investigate the antioxidant properties of plant extracts and individual plant polyphenols. Using the optimized methods for plant polyphenol extraction and antioxidant capacity evaluation we confirmed that many medicinal plants, vegetables and berries tested were rich sources of polyphenol compounds and free radical scavengers.
- We observed the immunomodulatory activity of various plant polyphenols in various biological models. These compounds, namely stilbenes and proanthocyanidins represented effective naturally occurring substances with potent pharmacological effects on respiratory burst of human neutrophils and nitric oxide production by macrophages.
- We demonstrated that various herbal materials contain immunomodulating pectic polysaccharides that could be useful for treatment of compromised immune system and control of inflammation. It was shown for the first time that acetylated and highly glucuronidated rhamnogalacturonans I from Tilia tomentosa blossoms may exhibit immunomodulating properties via neutrophil and monocyte/macrophage activation.

5 FUTURE PERSPECTIVES

5.1 EFFECTS OF HISTONE POST-TRANSLATIONAL MODIFICATIONS ON THE FUNCTIONAL VARIABILITY OF NEUTROPHILS

Post-translational modifications can substantially and rapidly alter the phenotype of neutrophils and influence their function. This suggests that regulation of the phenotype and function of neutrophils represents a common mechanism for modulating innate or adaptive immunity (Galli et al. 2011). Histone proteins are modified in response to various external signals, however, their mechanisms are still not completely elucidated. One such modification is citrullination, which is catalysed by peptidyl arginine deiminases (PADs), a unique family of enzymes that catalyses the hydrolysis of peptidyl-arginine to form peptidyl-citrulline on histones and other biologically relevant proteins. Overexpression and/or increased PAD activity is observed in several diseases, including rheumatoid arthritis (Bicker and Thompson 2013, Neeli et al. 2008).

The role of neutrophil-derived reactive oxygen species in induction of neutrophil extracellular traps (NETs) has been studied and discussed. Generally there are both reactive oxygen species-dependent and reactive oxygen species-independent pathways leading to NET formation. It has been recently reported, that inhibitors of NADPH oxidase and myeloperoxidase disrupt NET formation (Zhou et al. 2018, Tao et al. 2018). Furthermore, it has been shown that PAD4 is physically associated with p47phox and p67phox cytosolic subunits of the neutrophil NADPH oxidase. After activation, PAD4 induced NADPH oxidase subunit citrullination and their dissociation from PAD4, altered NADPH oxidase assembly and production of reactive oxygen species (Hu et al. 2019).

We hypothesise that various activators enhance expression of PAD4, modulates its activity and production of citrullinated histone H3 protein and cytosolic NADPH oxidase subunits in neutrophils. Histone H3 citrullination leads to chromatin decondensation. This is manifested by neutrophil functional variability. Altered reactive oxygen species production also modulates the rate of chromatin decondensation and neutrophil extracellular trap release. A comprehensive survey of the receptors and signalling pathways that regulate PAD4 activation will be important for our understanding of innate immunity. Identification of signalling intermediates in PAD4 activation may also lead to the generation of pharmaceuticals to target histone H3 or NADPH oxidase subunit citrullination-related

pathogenesis. The expected result will be an obtaining of new and original knowledge which will serve as a source information for further research in biology and medicine with possible application in clinical practice.

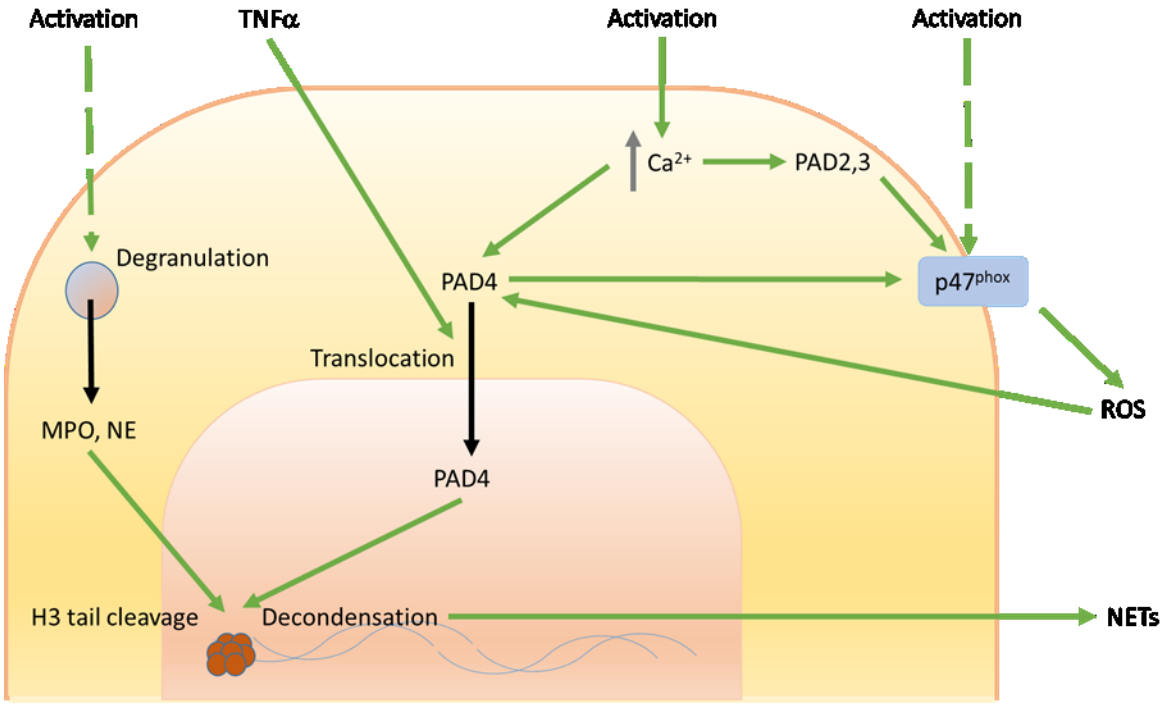

Fig. 3: *Proposal hypothesis scheme*

5.2 PLANT POLYPHENOL EFFECTS ON PROFESSIONAL PHAGOCYTES

Neutrophil lifespan constitutes a sensitive balance between their function and their potential to inflict tissue damage. In the absence of inflammatory stimuli, neutrophils continuously undergo apoptosis. On the other hand, NETs are formed in response to a variety of pro-inflammatory stimuli, microorganisms and pathogens. We hypothesize that plant polyphenols as strong antioxidants and reactive oxygen species scavengers will inhibit neutrophil NETosis and promote their apoptosis. As such, anthocyanins, hydroxycinnamic acids and proanthocyanidins from plants may be a novel class of neutrophil apoptosis-inducing anti-inflammatory compounds for the treatment of human inflammatory diseases. On the other hand, treatment with plant polyphenols may be considered as therapeutic strategy for neutrophil-mediated diseases, in which NETosis play a role. The main aim of the future studies will be to describe the antioxidative and anti-inflammatory effects of plant polyphenols with respect to their role in modulation of neutrophil apoptosis and NETosis and to clarify underlying molecular and signalling mechanisms.

5.3 PLANT AND MUSHROOM POLYSACCHARIDE EFFECTS ON PROFESSIONAL PHAGOCYTES

The search for attractive sources of polysaccharides, the discovery of their new biological effects, and the elucidation of intimate biochemical mechanisms of their action are major scientific problems in the field of carbohydrate chemistry and glycobiology. The general goal of the future project proposal will be to investigate the structure of purified herbal and mushroom polysaccharides and the biochemical mechanisms of immunomodulation of phagocytes by them. Neutrophils and macrophages play a significant role not only in our own immune defence, but also in the development of an immunomodulatory response. Generally, it is hypothesized that immunomodulatory effects of polysaccharides on neutrophils and macrophages may have protective effects in inflammatory, infectious, allergic, tumour, autoimmune and gastrointestinal diseases.

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THROMBOSIS RESEARCH

Regular Article

Inhibition of FMLP-stimulated neutrophil chemiluminescence by blood platelets increased in the presence of the serotonin-liberating drug chloroquine

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Abstract

Introduction: Previously, we reported that human blood platelets significantly decreased the concentration of reactive oxygen species (chemiluminescence) produced by Ca^{2+}-ionophore-stimulated neutrophils and that the reduction was partially mediated by serotonin liberated from platelets during their activation. The aim of the present study was to investigate whether platelet inhibition can occur independently of serotonin liberation and whether it can be pharmacologically enhanced. *Materials and methods*: Chemiluminescence was measured after stimulation of human neutrophils with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) in the presence of luminophore luminol. Concentration of platelet serotonin was estimated fluorometrically. *Results*: Platelets, added to neutrophils in the physiological cell ratio 50:1, decreased neutrophil chemiluminescence by 47%. The inhibition was not accompanied with liberation of platelet serotonin and rose after addition of chloroquine to platelet–neutrophil samples. In the absence of platelets, this drug did not affect neutrophil chemiluminescence. Chloroquine actively liberated serotonin; amine concentrations found in platelet supernatants were sufficient to inhibit neutrophil chemiluminescence. *Conclusions*: The presented results indicate that unstimulated platelets decreased neutrophil chemiluminescence by a serotonin-independent mechanism, yet their inhibitory effect could be enhanced pharmacologically through chloroquine-induced serotonin liberation.

Keywords: Blood platelets; Neutrophils; Chloroquine; Chemiluminescence; Serotonin; FMLP

1. Introduction

New activities of blood platelets are still appearing despite the fact that they have been in the focus of interest for more than 160 years. Of these, inhibition of neutrophil functions seems to be noteworthy since it could indicate platelet involvement in tissue protection against toxic effects of neutrophil products. They include particularly reactive oxygen species and proteolytic enzymes, which if released inappropriately, may induce serious tissue damage, as found in respiratory distress syndrome [1], ischaemia/reperfusion injury [2], renal failure [3], rheumatoid arthritis [4], gastrointestinal inflammation [5], or in cerebral infarction [6].

Blood platelets could represent a unique protective mechanism, active selectively at sites exposed to toxic effects of neutrophil products. As found in patients suffering from myocardial ischaemia/reperfusion injury, binding of platelets to neutrophils decreased production of superoxide anion [7] or conversely, lower platelet reactivity was associated with more severe inflammatory characteristics [8]. Under in vitro conditions, blood platelets diminished formation of reactive oxygen species and/or chemiluminescence of neutrophils [9,10], reduced elastase secretion and

Abbreviations: A23187, Ca^{2+}-ionophore A23187; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylene diamine tetraacetic acid; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PRP, platelet-rich plasma.

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myeloperoxidase activity, and impaired intracellular killing [11,12]. As we reported previously, chemiluminescence (i.e. concentration of reactive oxygen species) produced by Ca^{2+}-ionophore A23187 (A23187)-stimulated neutrophils decreased significantly in the presence of blood platelets, and the reduction was partially mediated by serotonin liberated from platelets during their activation [13,14]. The aim of the present study was to investigate whether platelet inhibition can occur independently of serotonin liberation and whether it can be pharmacologically enhanced.

2. Materials and methods

2.1. Materials

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), dextran (average mol. wt 464,000), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), hypoxanthine, xanthine oxidase, and hydrogen peroxide were from Sigma-Aldrich Chemie (Deisenhofen, Germany); Lymphoprep (density 1.077 g/ml) from Nycomed Pharma (Oslo, Norway); serotonin (5-hydroxytryptamine creatinine sulphate) from Koch Light (Colbrook Bucks, UK); and chloroquine phosphate from ACO (Molndal, Sweden). All other chemicals of analytical grade were from available commercial sources; deionised water was used as a solvent.

2.2. Isolation of blood platelets

Isolation was performed as described previously [15]. Briefly, fresh blood was obtained from healthy male donors (20–50 years) who had not received any medication for at least 7 days. Blood samples were anticoagulated with 3.8% trisodium citrate (blood: citrate ratio = 9:1) and centrifuged at $260 \times g$ for 15 min. Platelet-rich plasma (PRP) was removed, mixed with a solution containing 4.5% citric acid and 6.6% glucose (50 μl/1 ml PRP), and centrifuged at $1070 \times g$ for 10 min. Platelets were resuspended in an equal volume of Tyrode's solution (136.9 mmol/l NaCl, 2.7 mmol/l KCl, 11.9 mmol/l $NaHCO_3$, 0.4 mmol/l $NaH_2PO_4 \cdot 2H_2O$, 1 mmol/l $MgCl_2 \cdot 6H_2O$, 5.6 mmol/l glucose) containing 5.4 mmol/l ethylene diamine tetraacetic acid (EDTA), pH = 6.5. After 10-min stabilisation, the suspension was centrifuged at $1070 \times g$ for 6 min and platelets were resuspended in the same buffer without EDTA (pH = 7.4) to obtain 2×10^5 (chemiluminescence assay) or 5×10^5 (serotonin determination) platelets per 1 μl.

2.3. Isolation of neutrophils

Neutrophils were isolated according to the method described by Drábiková et al. [16]. After removal of PRP (see Isolation of blood platelets), the blood volume was reconstituted with 0.9% NaCl. Then, a 3% dextran solution (1% final concentration) was added and blood was allowed to sediment ($1 \times g$) for 25 min at 22 °C. The neutrophil/dextran mixture was centrifuged at $500 \times g$ for 10 min and the pellet was resuspended in phosphate-buffered saline (PBS: 137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, pH = 7.4). Neutrophil suspension (3 ml) was layered on lymphoprep (3 ml) and centrifuged for 30 min at $500 \times g$. Contaminating red blood cells were removed by hypotonic lysis (3 ml of ice-cold deionised water followed after 45 s by 3 ml of 1.8% NaCl and 4 ml of PBS). After centrifugation ($500 \times g$, 10 min), neutrophils in PBS were stained with Türk solution, counted under light microscope, and adjusted to 10^4 neutrophils per 1 μl. Final suspension contained more than 96% of viable cells, as evaluated by trypan blue exclusion, and was used maximally for 2 h—as long as control chemiluminescence kept constant.

2.4. Chemiluminescence assay

Chemiluminescence of neutrophils was measured in a lumiaggregometer, model 500 (Chrono-log., USA). The reaction mixture consisted of 200 μl of neutrophils (2×10^6), 175 μl PBS, 50 μl 18 mmol/l $CaCl_2$, 25 μl 1 mmol/l $MgCl_2$, and 500 μl platelets (10^8), or Tyrode's solution. After 1 min of incubation at 37 °C and stirring at 1000 rpm, 20 μl of chloroquine (1, 10 and 100 μmol/l final concentration), and 2 min later, 20 μl of luminol (5 μmol/l final concentration) was added. Samples were incubated for further 3 min and stimulated with 10 μl of FMLP (final concentration 0.1 μmol/l). Time-dependent alterations in intensity of the chemiluminescence signal were recorded in the form of curves, the presented results (mV) referring to peak values.

A slightly modified protocol was used when the effect of serotonin was studied. To prevent its degradation, serotonin (20 μl) was added 1 min before FMLP.

Chemiluminescence of cell-free systems was measured in a luminometer Immunotech LM-01T (Immunotech, Czech Republic) at 37 °C for 60 min. Samples for the analysis of hydrogen peroxide chemiluminescence contained 30 μl of serotonin or chloroquine (0.1–100 μmol/l final concentration), 30 μl of luminol (final concentration 1 mmol/l), 60 μl of 0.006% H_2O_2, and 180 μl of deionised water. When chemiluminescence of hydroxyl radical was measured, 30 μl of 10 mmol/l $FeSO_4$ was added to this reaction mixture. Formation of superoxide anion was initiated by the reaction of 210 μl 7.35 mmol/l hypoxanthine with 30 μl of xanthine oxidase (0.084 U/ml), and its chemiluminescence was measured in the presence of 30 μl of luminol and of 30 μl of serotonin (or chloroquine).

2.5. Serotonin determination

A slight modification of the method described by Nosál' et al. [17] was used for the determination of platelet serotonin. Isolated platelets (1100 μl, 5.5×10^8) were treat-

ed with 50 μl of chloroquine (1, 10 and 100 μmol/l final concentration). After 5 min of incubation at 37 °C, 50 μl of FMLP (0.1 μmol/l final concentration) was added. Serotonin liberation was stopped after 5 min by cooling the samples to 4 °C and by immediate centrifugation at 14000 × *g* and 4 °C for 2 min. For determination, 400 μl of supernatant, 2600 μl of 0.02 N HCl, 200 μl of 10% w/w $ZnSO_4$, and 100 μl of 1N NaOH were mixed and centrifuged for 10 min at 1670 × *g*. Native fluorescence of serotonin was measured in a Perkin-Elmer Fluorescence Spectrometer, Model 203, at 300 and 332 nm excitation and emission wavelength, respectively. Concentration of extracellular serotonin was calculated to platelet number (10^8) and sample volume (1 ml) used in the chemiluminescence assay.

When the effect of neutrophils on extracellular serotonin concentration was measured, the reaction mixture consisted of 1000 μl of platelets (4×10^8), 430 μl PBS, 100 μl 18 mmol/l $CaCl_2$, 50 μl 1 mmol/l $MgCl_2$, and 400 μl neutrophils (8×10^6). After 6 min of incubation at 37 °C, samples were stimulated for 5 min with 20 μl FMLP (final concentration of 0.1 μmol/l), and 800 μl of aliquots of supernatants was used for serotonin determination.

2.6. Data analysis

Commercially available computer statistic programmes were used for calculations of means and standard errors of the mean (S.E.M.). Statistical significance of differences between means was established by Student's *t*-test and $p \leq .05$ was taken as statistically significant.

3. Results

Fig. 1 shows platelet-dependent inhibition of FMLP-stimulated neutrophil chemiluminescence and its amplification by chloroquine. Incubation of neutrophils with blood platelets in the physiological cell ratio 1:50 declined their chemiluminescence from 26.86 ± 3.37 to 14.33 ± 2.06 mV.

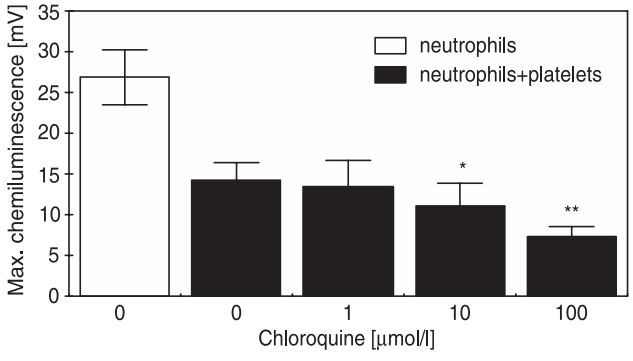

Fig. 1. Inhibition of FMLP-stimulated neutrophil chemiluminescence by blood platelets. Increase of the inhibition by chloroquine. Mean ± S.E.M., $n = 7$, *$p < .05$, **$p < .01$ (vs. chemiluminescence of neutrophils + platelets in the absence of chloroquine).

Table 1
Liberation of platelet serotonin by 1, 10, and 100 μmol/l chloroquine; effect of FMLP

	Extracellular serotonin (μmol/l)	
	No stimulus	FMLP
Platelets	0.0075 ± 0.0040	0.0152 ± 0.0022
Platelets + chloroquine 1	0.0067 ± 0.0031	0.0212 ± 0.0036
Platelets + chloroquine 10	0.0252 ± 0.0064**	0.0511 ± 0.0109*
Platelets + chloroquine 100	0.1075 ± 0.0246**	0.1450 ± 0.0124**

Extracellular serotonin concentration was corrected for 10^8 platelets, and sample volume of 1 ml as used in the chemiluminescence assay. Mean ± S.E.M., $n = 10–12$.
* $p < .05$.
** $p < .01$ (vs. platelets).

Application of 10 or 100 μmol/l chloroquine simultaneously with platelets induced further reduction of the chemiluminescence signal to 11.13 ± 2.73 or 7.40 ± 1.14 mV, respectively.

Co-incubation of platelets with neutrophils and/or with FMLP was not accompanied with liberation of serotonin—a potential platelet-derived chemiluminescence inhibitor. Even in the presence of neutrophils, extracellular serotonin concentration decreased from 0.0079 ± 0.0026 to 0.0008 ± 0.0005 μmol/l (without FMLP) or from 0.0069 ± 0.0028 to 0.0041 ± 0.0011 μmol/l (with FMLP). Significant serotonin discharge resulted however from treatment of platelets with chloroquine (10 and 100 μmol/l), in the absence as well as in the presence of FMLP (Table 1). Maximum extraplatelet (extracellular) serotonin concentrations exceeded 0.1 μmol/l.

The ability of chloroquine to inhibit chemiluminescence depended on the presence of platelets. When platelets were omitted, chloroquine (1–100 μmol/l) did not reduce chemiluminescence of FMLP-stimulated neutrophils and neither did it that of cell-free systems, with the exception of 28% inhibition of hydroxyl radical chemiluminescence observed in the presence of 100 μmol/l concentration (Fig. 2).

On the other hand, serotonin was found to be a potent inhibitor. This amine in the concentration of 0.1 μmol/l (i.e. in the concentration estimated in supernatants of chloro-

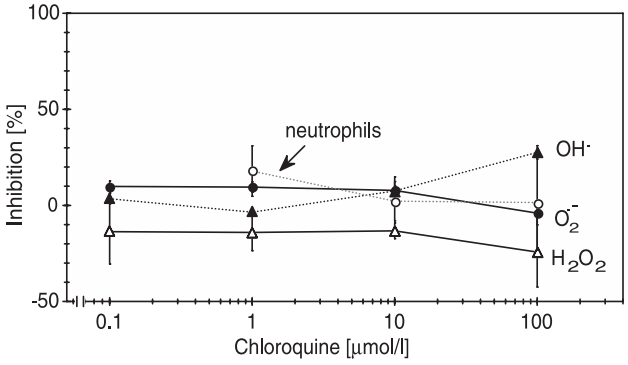

Fig. 2. Effect of chloroquine on chemiluminescence of superoxide anion (O_2^-), hydroxyl radical ($OH^·$), hydrogen peroxide (H_2O_2), and of FMLP-stimulated neutrophils. Mean ± S.E.M., $n = 6$.

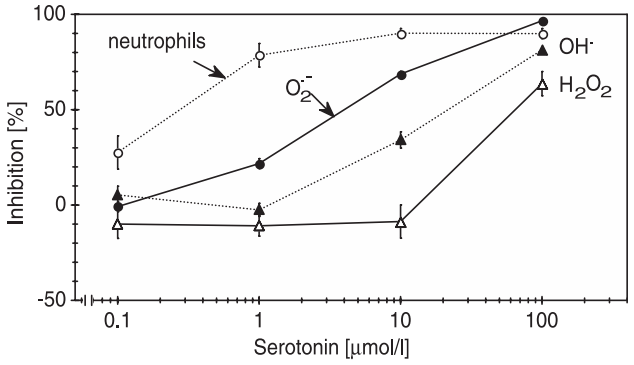

Fig. 3. Chemiluminescence of superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and of FMLP-stimulated neutrophils in the presence of serotonin. Mean ± S.E.M., $n = 6$.

quine-treated platelets) decreased neutrophil chemiluminescence by 28% (Fig. 3). Significant reduction by 77%, 87%, and 86% was observed in the presence of 1, 10, and 100 μmol/l serotonin, respectively. Chemiluminescence of individual oxygen metabolites was inhibited in the following sequence (percentage of inhibition caused by 10 and 100 μmol/l of serotonin is given in parenthesis): superoxide anion (69% and 97%)>hydroxyl radical (34% and 82%)>hydrogen peroxide (0% and 63%). In comparison to chemiluminescence originated from neutrophils, 10 times higher serotonin concentrations were needed to inhibit chemiluminescence of cell-free systems.

4. Discussion

4.1. Platelet inhibition of neutrophil chemiluminescence by a serotonin-independent mechanism

The presented results indicated that blood platelets reduced neutrophil chemiluminescence by a serotonin-independent mechanism. In contrast to platelets activated by A23187 [14], the concentration of serotonin liberated in the presence of FMLP (0.015 μmol/l) was not sufficient to explain the 47% inhibition of chemiluminescence (Figs. 1 and 3, Table 1). Moreover, platelets did not inhibit chemiluminescence of cell-free systems (our preliminary results are not published as of yet), thus involvement of their scavenging effect (mediated by serotonin or other platelet-derived substances) seems to be less likely.

As we found previously, platelet inhibition of FMLP-stimulated chemiluminescence disappeared in the presence of horseradish peroxidase [18], i.e. at increased extracellular concentration of peroxidase. It indicates that decreased chemiluminescence signal could result from inhibition of myeloperoxidase liberation by platelets. This enzyme is essentially involved in luminol excitation [19,20] and its importance was proved by the absence of chemiluminescence in patients with normal radical formation and myeloperoxidase deficiency [21]. Interference of platelets with liberation of myeloperoxidase could be mediated by several mechanisms—action of platelet derived inhibitory protein [12], platelet–neutrophil contact mechanism [11], or platelet induced polymerisation of actin in neutrophils [22,23] could all be taken into account.

Interference of platelets with superoxide anion formation in FMLP-stimulated neutrophils seems to be less likely since it was found to require completely stimulated platelets, releasing adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) from their dense granules, as well as a long-time (30 min) platelet–neutrophil co-incubation [9,24,25]; these requirements were not fulfilled in our experiments. Moreover, platelet inhibition was reversed by increased extracellular peroxidase.

4.2. Amplification of platelet effect by chloroquine; involvement of serotonin

In accordance with previous findings [26–28], chloroquine was not found to considerably inhibit chemiluminescence of FMLP-stimulated neutrophils or of cell-free systems (Fig. 2). The inhibitory effect of this drug appeared in the presence of blood platelets and was dose-dependent; chemiluminescence reduced by platelets declined further by 11%, 26%, and 48% after addition of chloroquine in the concentration of 1, 10, and 100 μmol/l, respectively (Fig. 1). The additional inhibition of neutrophil chemiluminescence may have been mediated by substances released from platelets in the presence of chloroquine. Particular involvement of the antioxidant and scavenger serotonin [29–31] seems to be highly acceptable because this amine was found to be displaced from platelet-dense granules after accumulation of basic drugs including chloroquine [17,32,33]. Moreover, serotonin in the concentration of 0.1 μmol/l, estimated in supernatants of chloroquine-treated platelets, decreased chemiluminescence of neutrophils by 28% (Table 1, Fig. 3).

Serotonin can inhibit neutrophil chemiluminescence by its scavenging effect—i.e. through elimination of existing radicals [34]. The different affinity of serotonin to particular oxygen metabolites, indicated by our measurements (Fig. 3), could explain why chemiluminescence stimulated with FMLP was inhibited by serotonin more effectively than that stimulated with opsonised zymosan [30] or with phorbol 12-myristate 13-acetate (PMA) [29]. Namely, FMLP-induced chemiluminescence was found to be formed by superoxide anion and hydroxyl radical, whereas hydrogen peroxide is prevalent in neutrophils stimulated with opsonised zymosan or with PMA [35]. The interference of serotonin with the formation of reactive oxygen metabolites [36] may represent another mechanism potentially participating in the inhibitory effect of serotonin. This was indicated by the fact that inhibition of neutrophil chemiluminescence by serotonin started at 100 times lower concentrations than the scavenging effect of this amine (Fig. 3).

Reduction of radical concentration, mediated by platelet serotonin, may participate in the anti-inflammatory activity

of chloroquine. This may be operative in patients with rheumatoid arthritis with local chloroquine concentration of 10–100 μmol/l [37]. However, during antimalarial therapy, chloroquine concentrations are substantially lower [38], and thus this chloroquine effect would not impair bactericidal activity of neutrophils.

4.3. Pharmacology of platelet–neutrophil interactions

Inflammation and atherogenesis were found to be multicellular processes, both accompanied with centralisation and activation of blood platelets and neutrophils at the same site [39]. Existing mutual platelet–neutrophil interactions, both stimulatory and inhibitory [40], can weaken the effects of drugs and complicate therapy of these pathological states. On the other hand, pharmacological intervention aimed at inhibition of potentially harmful amplificatory pathways and/or at the enhancement of protective inhibitory mechanisms appears to be more effective, rational, and safe than the current strategy.

Our results are indicative of several facts. Firstly, platelet-mediated reduction of radical concentration (the natural protective mechanism?) could be amplified by serotonin liberating drug(s). Secondly, besides inhibition of platelet functions [17], protection of tissues against aggressive neutrophil products could explain chloroquine benefit in thromboembolic prophylaxis [41]. Finally, effectiveness of chloroquine was found to be altered in the presence of platelets. Lower concentrations and shorter incubation with neutrophils were sufficient to inhibit FMLP chemiluminescence, compared to the chloroquine effect in the absence of platelets [27,28]. This indicated that under in vivo conditions, the effect of a drug on one type of cells could be modified in the presence of cells of another type. Accurate prediction of drug effect in the whole organism does therefore call for the use of multicellular test systems.

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P40
MODULATION OF RAT BLOOD PHAGOCYTE ACTIVITY BY SEROTONIN

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Key words: phagocytes, reactive oxygen species, serotonin, serotonin receptors

Introduction

There is now incontrovertible evidence that the nervous and immune systems interact bidirectionally. One potent mediator which plays a role in regulating both the nervous and immune systems is serotonin[1]. Serotonin acts as a neurotransmitter and a neuromodulator but it appears to also have a considerable influence on constituents of the immune system. During inflammatory processes, large amounts of serotonin are released by local mast cell degranulation and aggregated platelets[2]. Serotonin modulates different aspects of both adaptive and innate immunity; however, the available data are rather controversial[3,4]. The increasing use of serotoninergic agents in therapeutics together with the accumulated evidence for a role of serotonin in the immune system emphasizes the need for immunopharmacological studies. Herein, the effect of serotonin on an oxidative burst of rat blood phagocytes was evaluated and the involvement of different serotonin receptors in the effect of serotonin on phagocytes was tested using receptors agonists and antagonists.

The effects of serotonin (serotonin creatinine sulfate salt monohydrate) in a concentration range of 10^{-7}–10^{-3} M on the CL responses of peripheral rat blood leukocytes were evaluated *in vitro*. Four stimuli of phagocyte oxidative metabolism with different mechanisms of activation – opsonized zymosan (OZP), phorbol myristate acetate (PMA), calcium ionophore A23187 (CaI) and *N*-formyl-methionyl-leucyl-phenylalanin (FMLP) were tested.

Methods

The heparinized rat blood obtained via a heart puncture was layered over the separation solution to remove erythrocytes by 1 h sedimentation at room temperature[5]. The rich plasma (buffy coat) of leukocytes was washed twice and leukocyte counts were adjusted to obtain a final concentration $1 \cdot 10^6$ ml^{-1}. The CL response of leukocytes was measured using the microtitre plate luminometer, LM-01T (Immunotech, Czech Republic) and the microtitre plate luminometr, Orion II with injector (Berthold Detection Systems GmbH, Germany) within 1.5 h after blood collection. The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly, the reaction mixture consisted of $100 \cdot 10^3$ leukocytes, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators. The final concentrations of activators were selected based on our previous results: 62.5 μg ml^{-1} OZP, 9.55 μM Ca-I, 0.81 μM PMA or 2.85 μM FMLP (ref.[5]). The assays were run in duplicates. Spontaneous CL measurements in samples containing leukocytes and all other substances, but none of the activators, were included in each assay. Light emission, expressed as relative light units (RLU), was recorded continuously at 37 °C for 60 min. Each kinetic curve consisted of 100 points. The intensity of the CL reaction was expressed as the integral of the obtained kinetic curves, which corresponds to the total amount of light produced during the measurements. All data are expressed as the mean of n=6.

Results

The resulting data revealed that serotonin inhibited the CL response of rat blood phagocytes activated by OZP in a dose dependent manner (fig. 1). Similarly, serotonin inhibited the CL response of rat blood phagocytes to PMA, FMLP and Ca-I in a dose dependent manner (data not shown).

Further, the involvement of different serotonin receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$ and 5-HT$_7$) was evaluated using various agonists and antagonists of these receptors. None of these agonists and antagonists studied exerted any direct antioxidative properties as we showed previously[6].

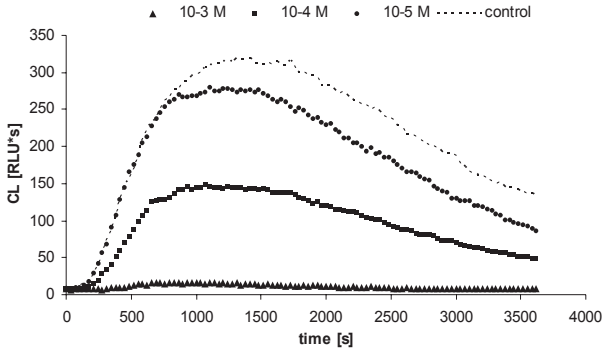

Fig. 1. **The effect of serotonin on the oxidative burst of rat blood phagocytes activated by OZP;** concentrations 10^{-6}–10^{-7} M did not differ from the control

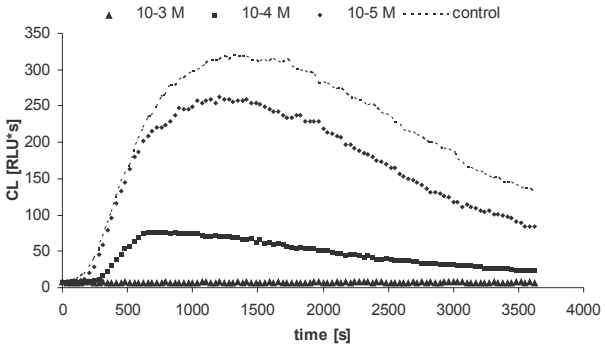

Fig. 2. **The effect of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2--aminopropane hydrochloride on the oxidative burst of rat blood phagocytes activated by OZP;** concentrations 10^{-6}–10^{-7} M did not differ from the control

From all tested agonists, only the selective 5-HT_2 receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI hydrochloride) exerted a similar effect on the respiratory burst as serotonin. In concentrations of 10^{-4} M DOI hydrochloride had an even more potent inhibitory effect on CL response (fig. 2) compared to serotonin despite not having any antioxidative properties[6]. Interestingly, the application of the selective antagonist of this receptor ketanserin did not block effect of serotonin.

Conclusion

The data obtained clearly demonstrated that serotonin was a potent inhibitor of the oxidative burst of rat blood phagocytes. Since the effect of serotonin on phagocytes is complex, our experiments were focused on elucidating the possible individual mechanisms of serotonin activity. It was previously shown that serotonin could act as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes, that it caused aggregation and degranulation of neutrophils, and it inhibited the migration of mononuclear leucocytes[7,8]. Our results suggest that the modulatory effect of serotonin on an oxidative burst of blood phagocytes occurs through the activation of the 5-HT_2 receptor subtype since DOI hydrochloride, a selective 5-HT_2 receptor agonist, had an inhibitory effect on respiratory burst similar to serotonin. However, the application of the antagonist of this receptor did not block the effect of serotonin. Therefore, we could speculate that other mechanisms are involved in the serotonin dependent modulation of rat blood phagocyte activity. Another probable explanation could be the direct scavenging activity of serotonin against free radicals produced during respiratory burst of phagocytes.

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Serotonin modulates the oxidative burst of human phagocytes via various mechanisms

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Abstract

Serotonin, the major secretory product of activated platelets, has been widely reported as regulating various constituents of the immune system and immune functions. This modulation is complex and the data available are rather controversial. The aim of the present study was to clarify the mechanisms of serotonin action on human phagocytes. The effect of serotonin in a concentration range of 10^{-7} M–10^{-3} M on various parameters of oxidative burst of phagocytes was studied using various luminol-enhanced chemiluminescence methods. Serotonin inhibited the chemiluminescence response of the cells in a dose dependent manner. The effect of serotonin on the activity of myeloperoxidase was studied in further experiments. In this case, serotonin again exerted a dose dependent inhibition of the myeloperoxidase activity. The hypothesis that the inhibitory activity of serotonin might be also receptor mediated was evaluated using various serotonin receptor agonists and antagonists. None of the agonists studied exerted any direct antioxidative properties. Only (±)-DOI hydrochloride, a selective 5-HTR_2 agonist, exerted similar effects on phagocytic cells as serotonin. It can be concluded that serotonin could affect the oxidative burst of phagocytes. Responsibility for its inhibitory effects lies with both the decrease in the generation of reactive oxygen species (due to the inhibition of myeloperoxidase activity) and with direct scavenging of reactive oxygen species. The effect of serotonin on phagocytes is also partially mediated by 5-HTR_2 receptor.

Keywords: *Antioxidant, chemiluminescence, phagocytes, serotonin*

Introduction

Oxidative stress and oxidation-induced tissue damage represent a serious problem for medicine. The oxidative injury can potentially occur due to either an excess production of reactive oxygen and nitrogen species or a decrease in antioxidant defences. It is suggested that an excessive production of phagocyte-derived reactive oxygen and nitrogen metabolites plays a role in a number of various diseases [1] connected to oxidative stress. Our previous results showed that the mobilization of neutrophils taking place in an inflammatory response is a very important component in the pathophysiology of ischemia/reperfusion [2–5].

A wide cooperation between platelets and professional phagocytes exists in the sites of inflammation and endothelial cell damage. Cathepsin G [6] and platelet activating factor [7] are among the major neutrophil-derived activators of platelets. On the other hand, vasodilatation produced by activated platelets could be profoundly impaired by activated leukocytes, probably via superoxide anion generated by activated neutrophils [8]. Platelets affect neutrophil activation by releasing serotonin, histamine, thromboxane A2, platelet-derived growth factor, lipoxygenase products, proteases, and adenosine [9–11].

Serotonin (5-hydroxytryptamine, 5-HT) is formed by the hydroxylation and decarboxylation of tryptophan. The greatest concentration of serotonin (90%) is found in the cells of the gastrointestinal tract. Most of the remainder of the body's serotonin is found in platelets and the central nervous system. Vasoconstriction is a classic response to the administration of serotonin. The function of serotonin is exerted upon its interaction with specific receptors [12]. Most of these receptors are coupled to G-proteins that affect the activities of either adenylate cyclase or phospholipase C.

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Specific uptake of serotonin by macrophages may regulate various constituents of the immune system and immune functions [13–14]. Serotonin, the major secretory product of activated platelets, was widely reported to modulate the function of neutrophils. This modulation is complex and the data available is rather controversial [15–17]. Serotonin could act as a true scavenger of the reactive oxygen species that are generated during the respiratory burst of stimulated phagocytes [16], and caused the aggregation and degranulation of neutrophils [18], as well as inhibited the migration of mononuclear leucocytes [19]. It was previously shown that serotonin modulated interferon-gamma-induced phagocytosis in bone marrow macrophages through a 5-HT receptor-mediated mechanism [20]. Taking all these factors into consideration, the accumulating evidence suggests that serotonin is a very important mediator of mutual interactions between platelets and professional phagocytes.

The major aim of the present study was to provide new information on the effects of serotonin on phagocytic cells. The effect of serotonin in a concentration range of 10^{-7} M–10^{-3} M on various parameters of oxidative burst of phagocytes was studied using various luminol-enhanced chemiluminescence (CL) methods.

Materials and methods

The effect of serotonin (serotonin creatinine sulfate salt monohydrate, Sigma, St-Louis, USA) in a concentration range of 10^{-3} M–10^{-7} M on various parameters of oxidative burst of phagocytes was studied using various luminol-enhanced CL methods. Reagents and chemicals were purchased from either Sigma (USA) or local distributors.

Whole blood chemiluminescence

Heparinized (50 IU/ml) blood samples were obtained from healthy male volunteers with informed consent. Sampling procedure was in accordance with the ethical standards of the responsible committee of the Institute of Biophysics on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The number of leukocytes in the blood and their relative differentiation counts were determined using Coulter counter STKS (Coulter Corporation, Mianui, USA). Luminol-enhanced chemiluminescence of human phagocytes in the whole blood was measured using an LM-01 microplate luminometer (Immunotech, Prague, Czech Republic). The principle of the method is based on luminol interaction with the phagocyte-derived oxidizing species, which results in large measurable amounts of light at a peak wavelength of 425 nm. Measurement details have been described previously [21]. Briefly, the reaction mixture consisted of 10 µl whole blood, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators: 62.5 µg/mL opsonized zymosan particles (OZP), 9.55 µM calcium ionophore A23187 (Ca-I), 0.81 µM phorbol-12-myristate-13-acetate (PMA) or 1.14 µM N-formyl-Met-Leu-Phe (fMLP). The total reaction volume of 200 µl was adjusted with Hanks balanced salt solution. The assays were run in duplicates. The CL emission expressed as relative light units (RLU) was recorded continuously for 60 min at 37°C.

Activity of myeloperoxidase

Since luminol-enhanced CL of phagocytes is widely considered to be dependent on a reaction of the myeloperoxidase (MPO) system, the effect of serotonin on the activity of MPO was studied in further experiments. Lysed HL-60 cells were used as a source of MPO and the activity of the enzyme was evaluated using bromide-dependent CL reaction [22].

TRAP (total peroxyl radical-trapping antioxidant parameter) analysis

The luminol-enhanced chemiluminescence was used to follow up peroxyl radical reaction. The chemiluminescence signal is driven by the production of luminol derived radicals from thermal decomposition of 2,2-azo-bis-2-amidinopropane hydrochlorid (ABAP, Polysciences, Inc., Warrington, USA). This method was described previously in Čížová et al. [21]. The TRAP (total peroxyl radical-trapping antioxidant parameter) value is determined from the duration of the period of time during which the serum sample resists lipid peroxidation due to the present antioxidants. A known quantity (8.0 nM) of trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, Sigma-Aldrich Chemie GmbH, Steinheim, USA), a water-soluble analogue of tocopherol was used as a reference inhibitor instead of serum.

Antioxidant properties of serotonin in various chemical systems

Reactive oxygen species (ROS) production was measured by luminol-enhanced chemiluminescence in a microtitre plate computer-driven luminometer LMT-01 (Immunotech) and analysed for light emission expressed in RLU. The inhibition of the CL signal was assessed as a comparison of integral CL signals of samples and control. Samples were not stirred while being measured. The CL assays were performed in a total volume of 300 µl for 30 minutes at 37°C [23]. The hypoxanthine/xanthine oxidase system was used to generate $\cdot O_2^-$. The reaction mixture contained hypoxanthine at a final concentration of 5 mM, xanthine oxidase at

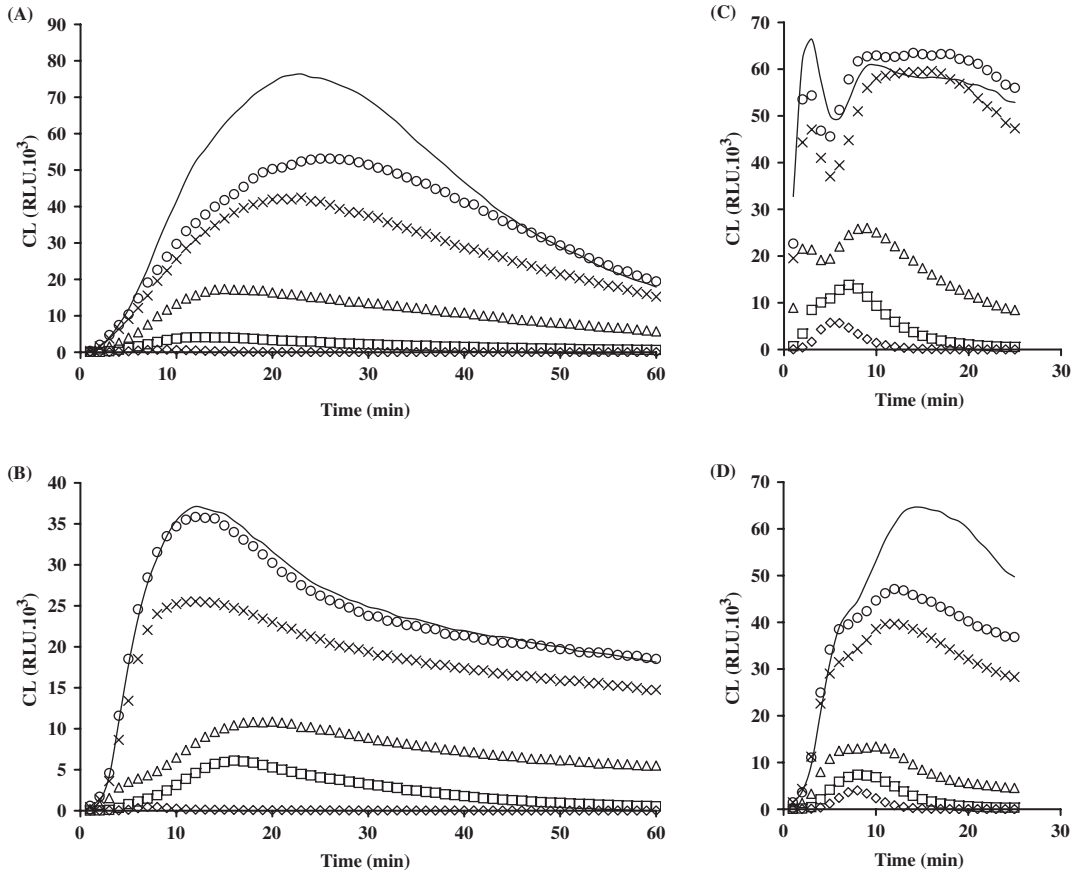

Figure 1. The effect of serotonin in concentrations of 10^{-7} (circle), 10^{-6} (cross), 10^{-5} (triangle), 10^{-4} (square), and 10^{-3} (diamond) on the oxidative burst of human whole blood phagocytes activated by OZP (A), PMA (B), fMLP (C), and Ca-I (D). The smooth line represents control without serotonin.

a final concentration of 7.3 mU/ml and luminol at a final concentration of 0.8 mM. The reaction was initiated by adding xanthine oxidase. The hydrogen peroxide/ferrous sulphate system (H_2O_2/$FeSO_4$) was used for the production of OH. The reaction mixture contained H_2O_2 at a final concentration of 2 mM, $FeSO_4$ at final concentrations of 0.1 and 0.001 mM and luminol at a final concentration of 0.8 mM. Finally, the hydrogen peroxide system (H_2O_2 itself) was employed. The reaction mixture contained H_2O_2 at a final concentration of 2 mM and luminol at a final concentration of 0.8 mM.

Statistical evaluation

All data are expressed as the mean ± standard error of the mean (SEM), n = 8. The data were analysed by one-way analysis of variance (ANOVA) followed by a Students *t*-test with a level of significance of $p < 0.05$.

Results

The effect of serotonin in a concentration range of 10^{-7} M–10^{-3} M on various parameters of oxidative burst of phagocytes was studied using various luminol-enhanced chemiluminescence methods. Serotonin inhibited the CL response of opsonized zymosan particle – OZP (Figure 1A), phorbol myristate acetate – PMA (Figure 1B), formyl-Methionyl-Leucyl-Phenylalanine – fMLP (Figure 1C) and calcium ionophore – Ca-I (Figure 1D) activated phagocytes in human whole blood in a dose dependent manner. The effect of serotonin on the oxidative burst of human whole blood phagocytes expressed as the peak (maximum) of the CL response of phagocytes is shown in Table I.

Since luminol-enhanced CL of phagocytes is widely considered to be dependent on a reaction of the myeloperoxidase system, the effect of serotonin on the activity of MPO was studied in further experiments. Serotonin exerted a dose dependent inhibition of the MPO activity: 17% inhibition (10^{-6} M serotonin), 27% inhibition (10^{-5} M serotonin), 95% inhibition (10^{-4} M serotonin), and 99% inhibition (10^{-3} M serotonin). The lowest concentration of serotonin (10^{-7} M) did not inhibit the activity of MPO at all (Figure 2).

Antioxidant properties of serotonin were studied in various chemical systems producing individual ROS: the thermal decomposition of ABAP (produced peroxyl radical) hypoxanthine/xanthine

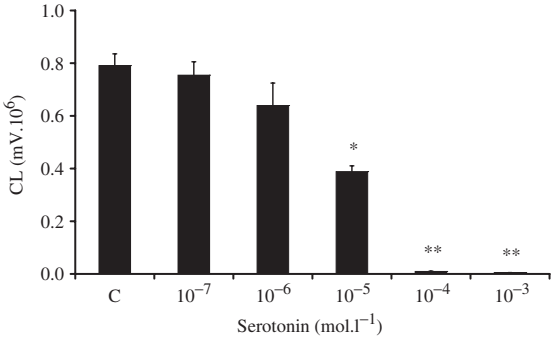

Figure 2. The effect of serotonin in a concentration range of 10^{-7}–10^{-3} on the activity of myeloperoxidase evaluated as a bromide-dependent chemiluminescence. ANOVA: $p<0.01$, *indicates $p<0.05$ from the control and **indicates $p<0.01$ from the control evaluated by *t*-test.

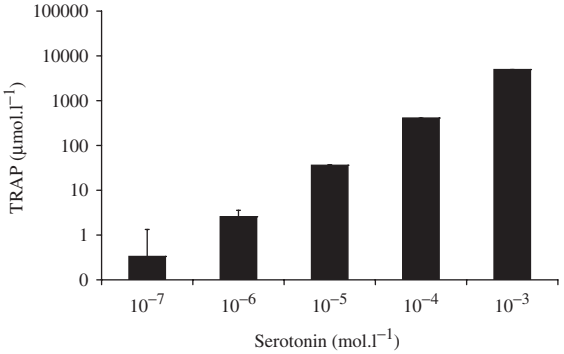

Figure 3. Total peroxyl radical-scavenging antioxidative parameter of serotonin in a concentration range of 10^{-7}–10^{-3}. ANOVA, $p<0.01$.

oxidase system (produced superoxide anion), hydrogen peroxide/ferrous sulphate system (produced hydroxyl radical) and hydrogen peroxide itself. Only the two highest concentrations of serotonin showed a peroxyl radical-scavenging effect evaluated as the TRAP parameter (Figure 3). The TRAP values were 410 μmol/l and 4959 μmol/l at concentrations of 10^{-4} M and 10^{-3} M, respectively. As for the other ROS, serotonin (in a dose-dependent manner) exerted the strongest antioxidant potential against superoxide anion radical (Figure 4A) and hydroxyl radical (Figure 4C) and the weakest antioxidant potential against hydrogen peroxide (Figure 4B). The percentage of inhibition was as follows for individual serotonin concentrations: 10^{-6} M – 22%, 0%, and 0% (CL driven by superoxide anion, hydroxyl radical and hydrogen peroxide, respectively); 10^{-5} M – 69%, 34%, and 0%; 10^{-4} M – 97%, 82%, and 63%; 10^{-3} M – 100%, 98%, and 96%. Serotonin at the concentration of 10^{-7} M did not inhibit CL in any of the chemical systems mentioned above.

The hypothesis that the inhibitory activity of serotonin might also be receptor mediated was evaluated using various serotonin receptor agonists ((±)-8-Hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide; (±)-DOI hydrochloride; 1-(3-Chlorophenyl)biguanide hydrochloride; Cisapride; 5-Carboxamidotryptamine maleate salt; *O*-Methylserotonin hydrochloride) and antagonists. None of the agonists studied exerted any direct antioxidative properties. Only (±)-DOI hydrochloride, a selective 5-HTR_2 serotonin receptor agonist, exerted similar effects on phagocytic cells as serotonin (Figure 5, Table I).

Discussion

Our previous study investigated the effect of activated blood platelets and chloroquine on concentrations of reactive oxygen species produced by polymorphonuclear leukocytes stimulated with calcium ionophore A23187. A significant reduction of chemiluminescence was observed in the presence of platelets (added to polymorphonuclear leukocytes in the physiological cell ratio 50 : 1) and of chloroquine (10 and 100 micromol/L). Although chloroquine decreased effectively both the extra as well as the intracellular part of the chemiluminescence signal, the activity of platelets occurred largely outside polymorphonuclear leukocytes. Serotonin liberated from platelets by calcium ionophore appeared to be involved in the inhibition of chemiluminescence [17]. Moreover, platelet activity on neutrophils might also depend on the extent of platelet activation, as non-activated platelets (in the presence of fMLP) were found to potentiate neutrophil-generated chemiluminescence, while platelets activated with calcium ionophore displayed the opposite effect. The interference of platelets with the formation and liberation of superoxide anion was indicated by platelet-modified isoluminol chemiluminescence. Superoxide dismutase with catalase and sodium azide were used, respectively, to differentiate the intracellular and the extracellular part of the chemiluminescence signal. Platelets were found to be capable of modifying both components of chemiluminescence, i.e. oxygen metabolites produced on the plasma membrane as well as on membranes of intracellular granules [24].

It was clearly demonstrated in our recent experiments that serotonin is a potent inhibitor of the oxidative burst of human phagocytes. Since the effect of serotonin on phagocytes is complex, our experiments were focused to elucidate possible individual mechanisms of serotonin activity. It was previously shown that serotonin could act as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes [16], caused aggregation and degranulation of neutrophils [18], and inhibited the migration of mononuclear leucocytes [19].

We have observed that the effect of serotonin could be at least partially caused by the inhibition of

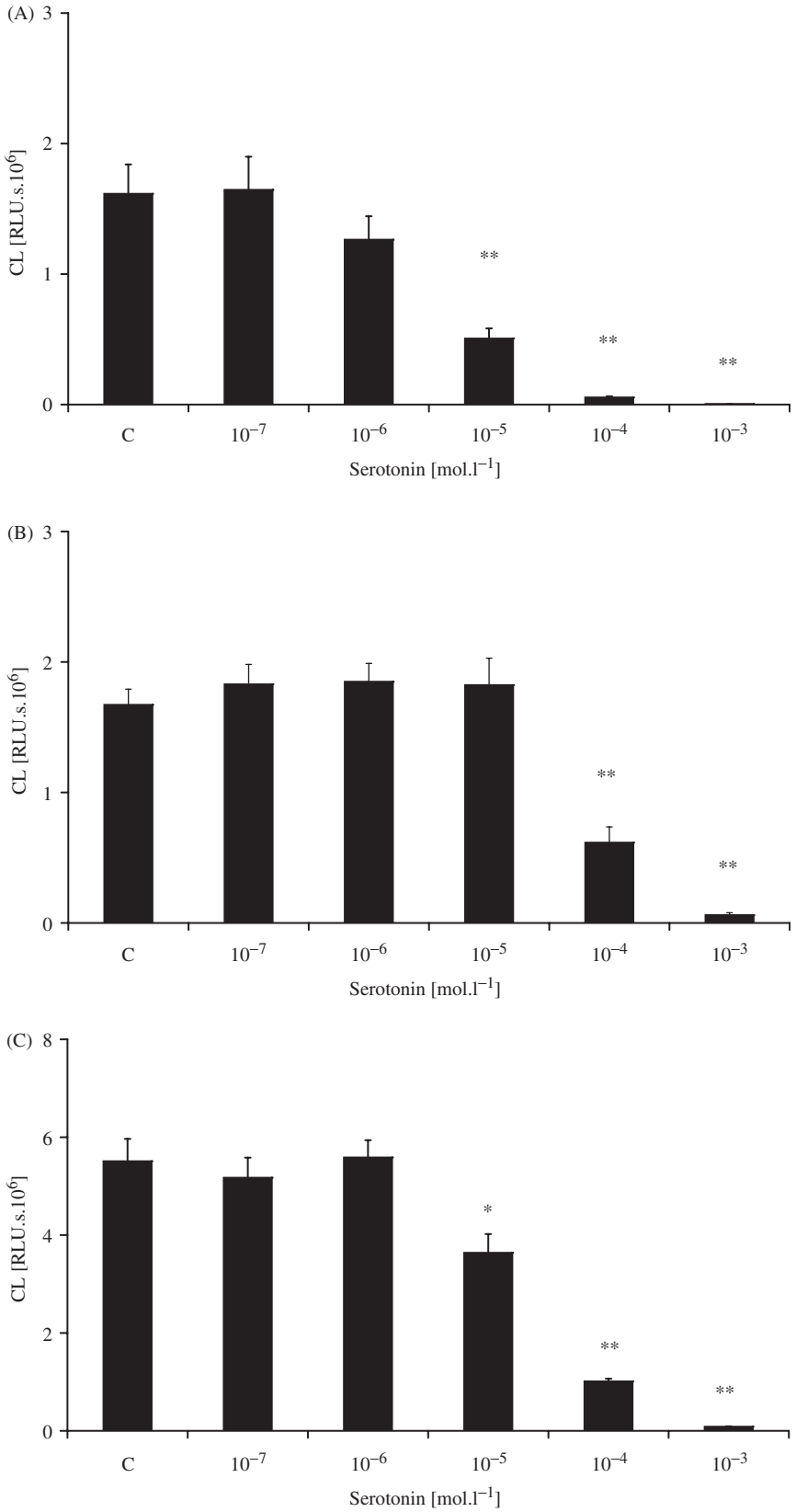

Figure 4. Antioxidative properties of serotonin (10^{-7}–10^{-3}) in various chemical systems producing individual reactive oxygen species: hypoxanthine/xanthine oxidase system (produced superoxide anion (A)), hydrogen peroxide/ferrous sulphate system (produced hydroxyl radical (C)) and hydrogen peroxide itself (B).

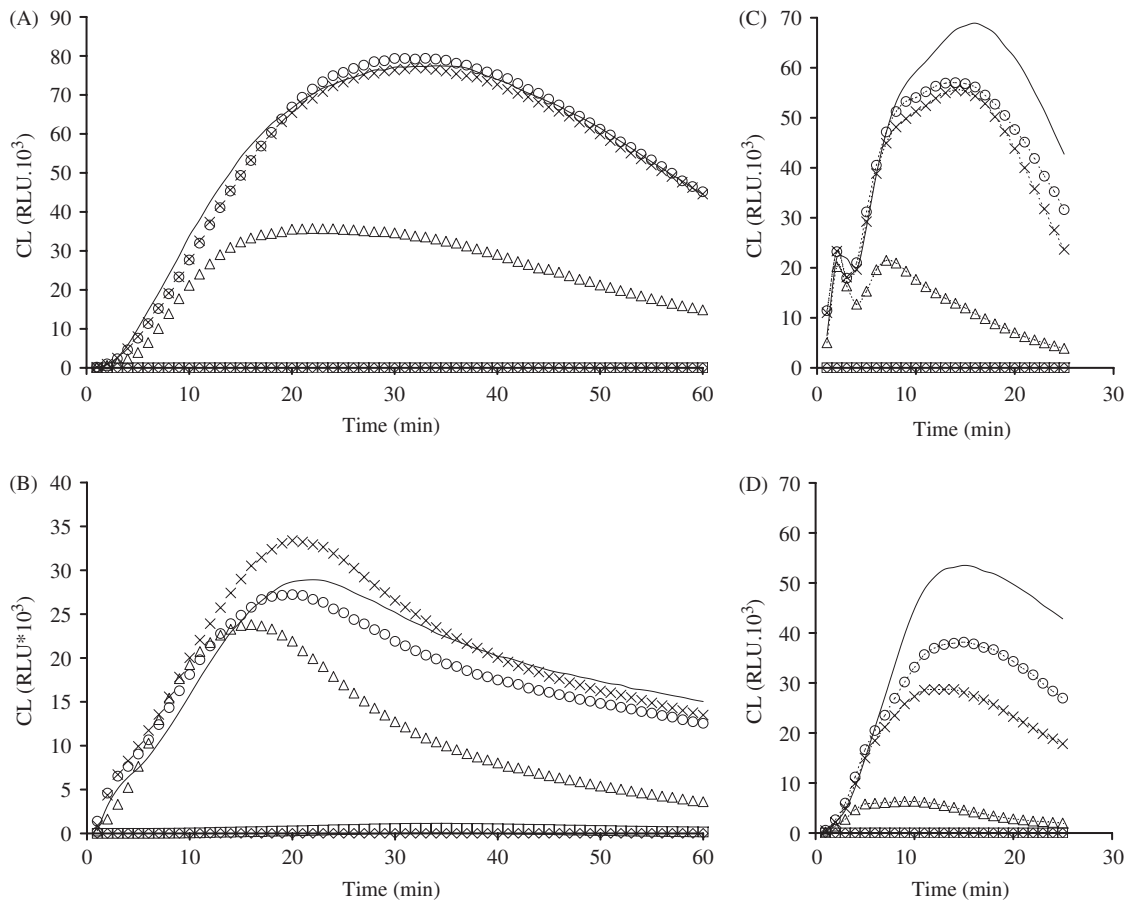

Figure 5. The effect of (±)-DOI hydrochloride in concentrations of 10^{-7} (circle), 10^{-6} (cross), 10^{-5} (triangle), 10^{-4} (square), and 10^{-3} (diamond) on the oxidative burst of human whole blood phagocytes activated by OZP (A), PMA (B), fMLP (C), and Ca-I (D). The smooth line represents control without serotonin.

Table I. The effect of serotonin and (±)-DOI hydrochloride in concentrations of 10^{-7} to 10^{-3} M on the oxidative burst of human whole blood phagocytes activated by OZP, PMA, fMLP, and Ca-I. The data represent the peak (maximum) of the CL response of phagocytes expressed as means±SEM. The asterisks indicate the statistically significant differences from the control at $p < 0.05$.

	Opsonized Zymosan Particles (OZP)	Phorbol-12-Myristate-13-Acetate (PMA)	N-formyl-Met-Leu-Phe (fMLP)	Calcium ionophore A23187 (Ca-I)
Serotonin				
Control	78.92 ± 23.03	37.74 ± 5.61	89.54 ± 14.78	70.17 ± 9.90
10^{-7} M	54.61 ± 8.08	36.41 ± 3.83	77.74 ± 7.56	56.45 ± 10.82
10^{-6} M	43.49 ± 4.91	26.43 ± 3.85	76.84 ± 11.89	47.57 ± 7.48
10^{-5} M	17.96 ± 2.91	11.64 ± 2.19*	26.60 ± 4.05*	14.84 ± 4.68*
10^{-4} M	4.20 ± 0.80*	7.00 ± 1.58*	13.83 ± 4.40*	7.70 ± 3.96*
10^{-3} M	0.72 ± 0.29*	0.52 ± 0.18*	5.95 ± 2.18*	4.09 ± 2.35*
(±)-DOI hydrochloride				
Control	78.00 ± 31.12	28.97 ± 9.10	70.72 ± 19.61	53.73 ± 12.88
10^{-7} M	79.49 ± 37.17	27.29 ± 10.96	58.81 ± 14.57	38.51 ± 14.02
10^{-6} M	77.37 ± 23.46	33.40 ± 8.72	56.90 ± 13.37	30.34 ± 10.35
10^{-5} M	36.27 ± 3.91	34.01 ± 4.15	21.55 ± 2.17	6.46 ± 1.99*
10^{-4} M	0.02 ± 0.00*	0.62 ± 0.10*	0.02 ± 0.00*	0.02 ± 0.00*
10^{-3} M	0.02 ± 0.00*	0.01 ± 0.00*	0.04 ± 0.01*	0.03 ± 0.01*

myeloperoxidase activity. The oxidation of serotonin by the myeloperoxidase intermediates compounds I and II was investigated previously by Dunford and Hsuanyu [25]. Rapid scan spectra demonstrated that both compound I and compound II oxidized serotonin via one-electron processes. A direct competition of serotonin with chloride for myeloperoxidase compound I oxidation was

observed. These findings accord with the results of Salman-Tabcheh et al. [26]. They found that serotonin significantly and dose-dependently suppressed the luminol-enhanced chemiluminescence signal generated by polymorphonuclear leukocytes activated with phorbol myristate acetate, but did not modify either lucigenin-enhanced CL or the reduction of superoxide dismutase-inhibitable cytochrome c. Their results suggested that serotonin metabolism was mediated by hydrogen peroxide and myeloperoxidase which was released by activated polymorphonuclear leukocytes.

In another set of our experiments, we showed that the inhibition of chemiluminescence signal of phagocytes might be caused by the direct scavenging of reactive oxygen species already produced. We have found that serotonin directly scavenged peroxyl radical, superoxide anion radical, hydroxyl radical and to a lesser extent hydrogen peroxide as well. Schuff-Werner et al. [27] and Huether et al. [28] observed that serotonin was oxidized by the reactive oxygen species released by stimulated phagocytes. This oxidation was prevented in the presence of other antioxidants. The major serotonin oxidation product was isolated by gel chromatography and identified by mass-spectrometry as a serotonin dimer, probably 5,5'-dihydroxy-4,4'-bitryptamine. They concluded that serotonin released from activated thrombocytes at the sites of inflammation and endothelial cell damage acts as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes. Schuff-Werner and Splettstoesser found that the modulation of the bactericidal function of neutrophils by serotonin was complex and depended upon the amount of serotonin: at concentrations normally present at sites of tissue injury and consecutive thrombus formation (10^{-6} to 10^{-5} M), bacterial killing increased by about 50%. In contrast, at higher concentrations (10^{-3} to 10^{-2} M) an adverse effect could be observed: the elimination of opsonized *S. aureus* was reduced by 30 to 90% [29]. It is obvious from our results that serotonin rather in concentrations which may be locally reached at the immediated sites of serotonin release by activated platelets, e.g. at sites of inflammation or endothelial damage (10^{-3}–10^{-4} M) than in concentrations found in whole blood (10^{-6} M and lower) [27] could affect the phagocyte derived reactive oxygen species. Thus, serotonin would not interfere with phagocytes in vivo under physiological conditions, but it could control the overproduction of phagocyte-derived reactive oxygen species in limited areas under non-physiological conditions.

The hypothesis that the inhibitory activity of serotonin might also be receptor mediated was evaluated using various serotonin receptor agonists ((±)-8-Hydroxy-2-(di-n-propylamino)-tetralin hydrobromide; (±)-DOI hydrochloride; 1-(3-Chlorophenyl)biguanide hydrochloride; Cisapride; 5-Carboxamidotryptamine maleate salt; O-Methylserotonin hydrochloride) and antagonists. Only (±)-DOI hydrochloride, a selective 5-HTR_2 agonist, exerted similar effects on phagocytic cells as serotonin. None of the agonists studied exerted any direct antioxidative properties.

Conclusion

It can be concluded that serotonin could affect the oxidative burst of phagocytes. Both decreasing the generation of ROS including inhibition of myeloperoxidase activity and direct scavenging of ROS were responsible for its inhibitory effects. The hypothesis that the inhibitory activity of serotonin might also be receptor mediated was evaluated using various serotonin receptor agonists and antagonists. Only (±)-DOI hydrochloride, a selective 5-HTR_2 agonist, exerted similar effects on phagocytic cells as serotonin. Taking all these facts into consideration, the evidence to date suggests that serotonin is a very important mediator of mutual interactions between platelets and phagocytes.

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Serotonin and its 5-HT_2 receptor agonist DOI hydrochloride inhibit the oxidative burst in total leukocytes but not in isolated neutrophils

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ABSTRACT

Aims: Serotonin (5-HT) is capable of reducing the oxidative burst of professional phagocytes. In this study, we investigated whether 5-HT mediates this modulation via 5-HT receptors (5-HTR) or whether this is due instead to 5-HT antioxidative properties.
Main methods: The leukocytes or polymorphonuclear leukocytes (PMNL) were isolated from human blood, and their ability to produce reactive oxygen species (ROS) after 5-HT or its agonist treatment was tested by luminol-enhanced chemiluminescence (CL) analysis.
Key findings: It was found that 5-HTR_2 agonist DOI hydrochloride does not have any antioxidative properties, despite its ability to inhibit the CL response of activated human total leukocytes. On the other hand, DOI hydrochloride was unable to inhibit the CL response of activated human PMNL. It seems that the reduction of the oxidative burst of professional phagocytes was evoked by the activation of 5-HTR not on the neutrophil surface but on the surface of different leukocytes, which produced anti-inflammatory cytokines with NADPH oxidase activity modulating properties.
Significance: Platelets and activated PMNL are in tight contact at sites of inflammation. 5-HT released from platelets might have a protective function against PMNL-derived oxidative stress and oxidative damages.

Introduction

Professional phagocytes are essential components of the innate non-specific immune response both in humans and animals. In blood, they comprise monocytes and the most frequent neutrophils. Neutrophils are accumulated in areas of infection, where they contribute to the killing of invading microorganisms by the generation of reactive oxygen species (ROS) through NADPH oxidase activity (Brechard et al. 2005). The excessive production of phagocyte-derived ROS plays a role in a number of different diseases (Roberts and Sindhu 2009).

Serotonin (5-hydroxytryptamine, 5-HT) is synthesized by the hydroxylation and decarboxylation of the amino acid tryptophan in enterochromaffin cells of the gastrointestinal tract and in the cells of the central nervous system. The highest concentration of 5-HT in the body (about 95%) is found in the cells of the gastrointestinal tract, of which 90% are within enterochromaffin cells and 10% within enteric neurons. The rest of 5-HT is found in the brain. All the serotonin in the blood and platelets is derived from the gastrointestinal tract (Lesurtel et al. 2008).

A wide variety of the physiological effects of 5-HT on cells are mediated by a polymorphic group of specific serotoninergic membrane receptors (5-HTR) classified into 7 families. In humans, these families have been subdivided into 15 distinct receptor subtypes. With the exception of a ligand-gated cation channel (5-HTR_3), most 5-HTR are coupled to G-proteins and linked either to adenylate cyclase (5-HTR_{1A}, 5-HTR_{1B}, 5-HTR_{1D}, 5-HTR_{1E}, 5-HTR_{1F}, 5-HT_4, 5-HT_6, and 5-HT_7) or to phospholipase C/protein kinase C (5-HTR_{2A}, 5-HTR_{2B} and 5-HTR_{2C}) (Nichols and Nichols 2008; Hoyer et al. 2002).

Wide cooperation exists between platelets and professional phagocytes in sites of inflammation and endothelial cell damage. Activation of platelets leads to their aggregation and to a rapid release of 5-HT in micromolar concentrations in proximity to blood cells (Mossner and Lesch 1998). It has been widely reported that 5-HT modulates the function of professional phagocytes. However, this modulation is complex, and the data available are rather controversial (Jancinova et al. 2001; Schuff-Werner and Splettstoesser 1999; Tokmakov et al. 1991). 5-HT could act as a true scavenger of ROS generated during a respiratory burst of stimulated leukocytes (Schuff-Werner and Splettstoesser 1999), could be caused by the aggregation and degranulation of neutrophils (Renesto and Chignard 1994), or by inhibiting the migration of mononuclear leukocytes (Bondesson et al. 1993). It was shown in our previous study that 5-HT inhibits the chemiluminiscence response of neutrophils in human whole blood (Ciz et al. 2007). That inhibition was partially due to the direct quenching activity of 5-HT against ROS, and to the inhibition of myeloperoxidase activity.

Taking all these facts into consideration, accumulating evidence suggests that 5-HT is a very important mediator of mutual interactions between platelets and professional phagocytes. However, additional

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work is needed to clarify the mechanisms of its action on neutrophils. In this study, the hypothesis that the inhibitory activity of 5-HT on the respiratory burst of professional phagocytes might also be receptor mediated was evaluated using various 5-HTR agonists in rat and human total leukocytes and isolated polymorphonuclear leukocytes (PMNL).

Materials and methods

Reagents

5-HT (serotonin creatinine sulfate salt monohydrate), 5-HT agonists: (±)-8-Hydroxy-2-(dipropylamino) tetralin (5-HT_1 receptor agonist), (±)-2,5-dimethoxy-4-iodoamphetamine (DOI) hydrochloride (5-HT_2 receptor agonist), 1-(3-Chlorophenyl) biguanide hydrochloride (5-HT_3 receptor agonist), Cisapride (5-HT_4 receptor agonist), 5-Carboxamidotryptamine maleate salt (5-$HT_{1,5,7}$ receptor agonist), and oxidative burst activators were obtained from Sigma (USA) and luminol from Molecular Probes (USA). A generator of peroxyl radicals 2,2-azo-bis-2-amidinopropane hydrochloride (ABAP) and reference inhibitor 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) from Sigma (USA) were used. Dextran-T500 (Pharmacia, Sweden) and telebrix N 300 (Léčiva, Czech Republic) were used for total leukocyte and PMNL isolation. Anaesthetic for rats was mixed from 10 ml of Narkamon (ketamini hydrochloridum) and 0.5 ml of Rometar (xylazinum hydrochloridum) from Spofa a.s. (Czech Republic). All other chemicals were purchased from local distributors.

Total peroxyl radical-trapping antioxidant parameter analysis (TRAP)

The TRAP analysis is based on the measurement of the luminol-enhanced chemiluminescence (CL) (Cloez-Tayarani et al. 2003) in a chemical system where peroxyl radicals are produced at a constant rate by a thermal decomposition of ABAP. The TRAP value is determined from the duration of the time period during which the sample diminished the CL signal. Trolox, a water-soluble analogue of α-tocopherol, was used as a reference inhibitor (Pavelkova and Kubala 2004).

Isolation of rat total leukocytes

This study was performed in accordance with the National Institute of Health's guidelines for the care and use of laboratory animals. Male Wistar rats were kept under controlled light and temperature conditions; water and food were given ad libitum. The animals were subjected to the experimental procedure at the age of 3 months (body weight 250–300 g). Rats were intraperitoneally anaesthetized with ketamine/xylazine (20/2 mg per 100 g body weight).

Heparinized blood (50 IU/ml) from the anaesthetized rats was obtained via a heart puncture. Erythrocytes were removed after 1 h sedimentation in Dextran separation solution. The leukocyte-rich plasma (buffy coat) was washed twice in HBSS, and total leukocytes were adjusted to 1×10^6 cells$\times$ml^{-1} (Pavelkova and Kubala 2004).

Total leukocyte/PMNL isolation from human whole blood

The heparinized blood (50 IU/ml) from healthy volunteers ($n=5$) was obtained by antecubital venipuncture. Erythrocytes were removed after 1 h sedimentation in Dextran separation solution. For isolation of total leukocytes, the leukocyte-rich plasma (buffy coat) was centrifugated (300 *g*, 20 °C, and 5 min). The pellet was exposed to the hypotonic lysis of contaminating erythrocytes; the rest of the total leukocyte was washed in HBSS, and total leukocytes were adjusted to 1×10^6 cells$\times$ml^{-1}. For isolation of PMNL, Ficoll-Hypaque density gradient centrifugation was used (400 *g*, 20 °C, and 40 min). The pellet composed of erythrocytes and neutrophils (80–93%), eosinophils (1.3–4%), and basophils (<2.7%) was exposed to hypotonic shock to take out the contaminating erythrocytes. The obtained PMNLs were washed in HBSS, and PMNLs were adjusted to 1×10^5 cells$\times$ml^{-1} (Drabikova et al. 2002).

Table 1
The antioxidative properties of 5-HTR agonists expressed as the TRAP mean values ± S.E.M. For comparison, the TRAP value of the reference inhibitor trolox is shown.

5-HT agonists	TRAP (nmol$\times$ml^{-1})
(±)-8-Hydroxy-2-(dipropylamino) tetralin	7.3 ± 7.3
Cisapride	11.0 ± 6.5
1-(3-Chlorophenyl) biguanide hydrochloride	16.8 ± 10.4
(±)-DOI hydrochloride	20.3 ± 18.3
5-Carboxamidotryptamine maleate salt	13.0 ± 10.5
Trolox	800.0 ± 0.0

Total leukocyte/PMNL chemiluminescence

The CL of rat total leukocytes and human total leukocytes and PMNL were measured using the microtitre plate luminometer Orion II (Berthold Detection systems GmbH, Germany). The principle of the method was previously described (Ciz et al. 2007). Each reaction mixture consisted of 1×10^5 total leukocytes or 1×10^4 PMNL, 1 mM luminol (stock solution prepared in borate buffer), one of the activators (opsonized zymosan particles — OZP in a concentration of 62.5 μg$\times$ml^{-1}, phorbol-12-myristate-13-acetate — PMA in a concentration of 0.81 μg$\times$ml^{-1}, calcium ionophore — CaI in a concentration of 9.55 μg$\times$ml^{-1} or N-formyl-methionyl-leucyl-phenylalanin — fMLP in a concentration of 1.14 μg$\times$ml^{-1}) and 5-HT or its agonist in various concentrations between 1×10^{-7} and 1×10^{-4} M. Preparation of the stock solutions of luminol and oxidative burst activators was previously described (Pavelkova and Kubala 2004); 5-HT and its

Table 2
The effect of 5-HT and its 5-HTR agonists on the oxidative burst of rat total leukocytes activated by CaI (A), fMLP (B), PMA (C) and OZP (D). The results are expressed as a percentage of the control ± S.E.M. with level of significance analyzed by ANOVA, $p<0.01$**, $p<0.05$*.

	10^{-6} M	10^{-5} M	10^{-4} M
A)			
5-HT	46 ± 15	26 ± 8	9 ± 4*
(±)-DOI hydrochloride	83 ± 37	39 ± 21	5 ± 5**
(±)-8-Hydroxy-2-(dipropylamino) tetralin	66 ± 20	33 ± 9	7 ± 5
Cisapride	45 ± 8	48 ± 12	7 ± 3
1-(3-Chlorophenyl) biguanide hydrochloride	74 ± 23	73 ± 38	5 ± 2
5-Carboxamidotryptamine maleate salt	91 ± 38	85 ± 41	10 ± 7
B)			
5-HT	99 ± 11	52 ± 11	3 ± 1**
(±)-DOI hydrochloride	93 ± 15	30 ± 10*	0 ± 1**
(±)-8-Hydroxy-2-(dipropylamino) tetralin	131 ± 45	128 ± 36	6 ± 1**
Cisapride	110 ± 12	149 ± 33	81 ± 8
1-(3-Chlorophenyl) biguanide hydrochloride	103 ± 13	99 ± 18	11 ± 3**
5-Carboxamidotryptamine maleate salt	109 ± 13	107 ± 14	25 ± 6*
C)			
5-HT	103 ± 15	76 ± 18	30 ± 7*
(±)-DOI hydrochloride	107 ± 12	86 ± 12	0 ± 1**
(±)-8-Hydroxy-2-(dipropylamino) tetralin	85 ± 21	81 ± 15	49 ± 29
Cisapride	97 ± 13	98 ± 9	79 ± 5
1-(3-Chlorophenyl) biguanide hydrochloride	100 ± 12	108 ± 10	82 ± 13
5-Carboxamidotryptamine maleate salt	109 ± 13	108 ± 10	74 ± 4
D)			
5-HT	88 ± 14	44 ± 9	2 ± 0**
(±)-DOI hydrochloride	84 ± 17	19 ± 4**	0 ± 0**
(±)-8-Hydroxy-2-(dipropylamino) tetralin	95 ± 10	73 ± 12	3 ± 1**
Cisapride	81 ± 9	88 ± 11	70 ± 12
1-(3-Chlorophenyl) biguanide hydrochloride	93 ± 15	77 ± 16	5 ± 1**
5-Carboxamidotryptamine maleate salt	82 ± 8	92 ± 18	45 ± 10

agonists were diluted in HBSS to a concentration of 10 mM. HBSS was used to adjust the total reaction volume to 250 μl.

ATP test of cell viability

Cell viability was determined luminometrically by using the ATP Cell Viability test (BioThema, Sweden). Total leukocytes isolated from human blood were incubated in 96 well plates at 1×10^5 cells/well for 30 min with 5-HT or DOI hydrochloride (10^{-4} M) at 37 °C. After incubation, somatic cell ATP releasing reagent was added. After 15 min shaking, the ATP reagent was added, and the luminescence was immediately measured. The intensity of CL was used for the evaluation of the toxic effects of 5-HT or DOI hydrochloride on total leukocytes (Ambrozova et al. 2009).

Statistical analysis

All samples were measured in duplicate. The results are expressed as the mean + standard error of the mean (S.E.M.), $n=5$. The level of significance was analyzed by one-way analysis of variance (ANOVA), followed by a Student *t*-test, $p<0.01^{**}$, or $p<0.05^{*}$.

Results

Antioxidative properties of 5-HT and its receptor agonists

The antioxidative properties of 5-HT and its agonists in the concentration of 10^{-4} M were tested by TRAP analysis. Our results confirmed the fact that 5-HT is a very strong antioxidant (data not shown). On the other hand, the selected 5-HT agonists (±)-8-Hydroxy-2-(dipropylamino) tetralin, DOI hydrochloride, 1-(3-Chlorophenyl) biguanide hydrochloride, Cisapride, and 5-Carboxamidotryptamine maleate salt, did not exert any antioxidative properties expressed as TRAP values (nmol × ml^{-1}) and shown in Table 1.

Effect of 5-HT and its receptor agonists on rat total leukocytes

The effect of 5-HT and its agonists in a concentration range of 10^{-6} M–10^{-4} M on the oxidative burst of rat total leukocytes was tested. The results showed that the most effective inhibitor of rat total leukocyte CL reaction was DOI hydrochloride (Table 2). This compound exerted the inhibition activity at a concentration of 10^{-4} M for all types of phagocyte activators and also at a concentration of 10^{-5} M when fMLP and OZP were used as phagocyte activators. Based on these results, DOI hydrochloride was chosen for the experiments that followed.

The effect of 5-HT and DOI hydrochloride on cell viability

Cell viability after 5-HT and DOI hydrochloride treatment was tested by ATP test. After 5-HT (10^{-4} M) treatment, the viability of human total leukocytes was 97 ± 0.7% of control (human total leukocytes without treatment); and, after DOI hydrochloride (10^{-4} M) treatment, it was 96 ± 1.7% of control. This means that both 5-HT and DOI hydrochloride at a concentration of 10^{-4} M did not have any toxic effect on the studied cells.

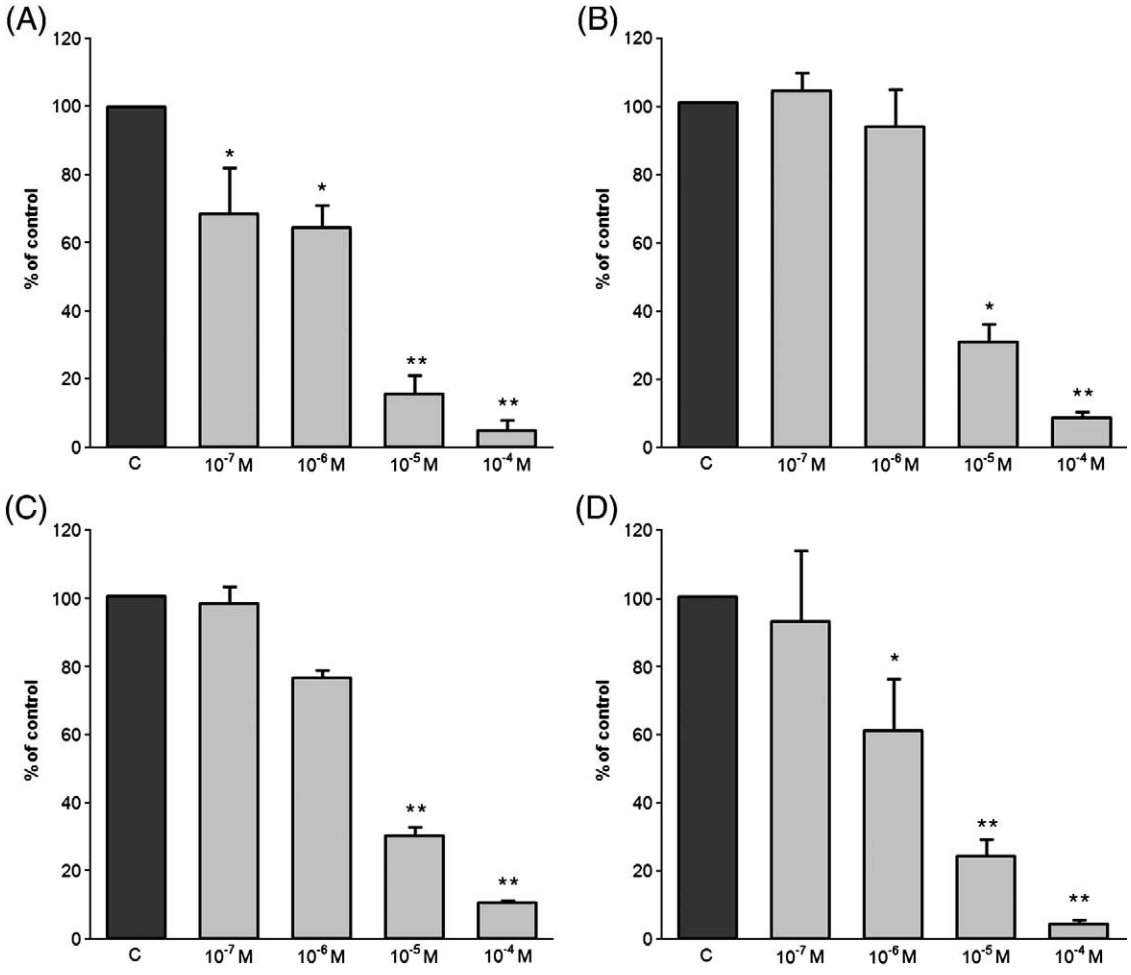

Fig. 1. The effect of 5-HT (10^{-7}–10^{-4} M) on the oxidative burst of human total leukocytes activated by CaI (A), fMLP (B), PMA (C) and OZP (D). The results are expressed as a percentage of the control, with the level of significance analyzed by ANOVA, $p<0.01^{**}$, $p<0.05^{*}$.

Effect of 5-HT and DOI hydrochloride on human total leukocytes

The absolute values of the integrals obtained from the measurements of CL responses of human total leukocytes activated with CaI were 102 ± 12, fMLP 125 ± 20, PMA 168 ± 39, and with OZP were 477 ± 152 RLU × min × 10^6. Then, the oxidative burst of human total leukocytes was measured after incubation (10 min at room temperature) with either 5-HT or DOI hydrochloride. The data showed that 5-HT significantly decreased the CL response of human total leukocytes in a dose-dependent manner for all types of phagocyte activators (CaI, fMLP, PMA, OZP). The CL response was significantly decreased at the concentrations of 10^{-5} M–10^{-4} M for all types of activators. Furthermore, CaI and OZP activated CL responses were significantly inhibited at the concentration of 10^{-6} M and CaI activated CL response even at the concentration of 10^{-7} M (Fig. 1).

The effect of DOI hydrochloride was similar. We observed decreased CL response in a dose-dependent manner. A significant decrease was detected at a concentration of 10^{-4} M for all types of activation and for CaI and fMLP also at a concentration of 10^{-5} M (Fig. 2).

Effect of 5-HT and DOI hydrochloride on human PMNL

The absolute value of the integrals obtained from the measurement of CL response of human PMNL activated with OZP was 449 ± 120 RLU × min × 10^6. The luminol-enhanced CL of isolated human PMNL was not significantly decreased in the presence of either 5-HT or DOI hydrochloride (Fig. 3). The 5-HT exerted a very low inhibition of PMNL chemiluminescence response: 4% inhibition (10^{-6} M 5-HT), 8% inhibition (10^{-5} M 5-HT), and 31% inhibition (10^{-4} M 5-HT). The effect of DOI hydrochloride was similar: 5% inhibition (10^{-5} M DOI hydrochloride) and 28% inhibition (10^{-4} M DOI hydrochloride).

Discussion

It is known from previous studies that 5-HT, liberated from activated platelets, is involved in the reduction of the oxidative burst in activated human professional phagocytes, measured as a luminol-enhanced CL (Jancinova et al. 2001; Ciz et al. 2007). This effect of 5-HT could, at least partially, be caused by its scavenging feature, which has been discussed in several studies (Schuff-Werner et al. 1995; Huether et al. 1997). The aim of this study was to elucidate the effect of 5-HT on the oxidative burst of professional phagocytes and to establish whether 5-HTR is also involved in this process. It is known that 5-HTR is positively coupled through the G_q-protein to phospholipase C and phospholipase A_2, and their activation leads to increased accumulation of inositol phosphates and intracellular Ca^{2+} (Van Oekelen et al. 2003). These events could lead to NADPH oxidase activity modification and subsequent inhibition of ROS production which was observed as the reduction of luminol-enhanced CL response in our previous experiments.

First, the antioxidative properties of selected 5-HT agonists with a different specificity to the 5-HT receptor families were tested by TRAP analysis. None of these studied agonists exerted any direct antioxidative properties. Thus, their potential effect on the oxidative burst of

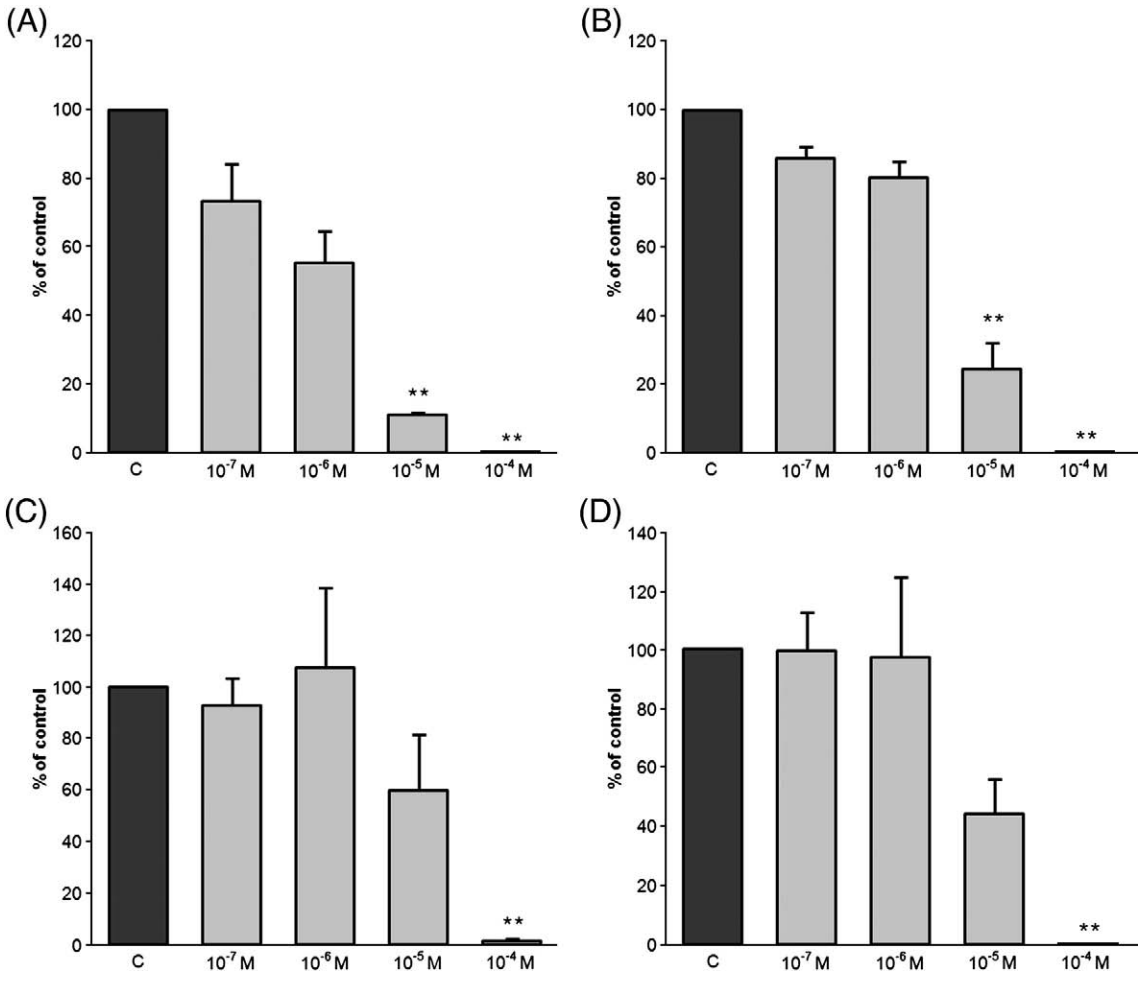

Fig. 2. The effect of DOI hydrochloride (10^{-7}–10^{-4} M) on the oxidative burst of human total leukocytes activated by CaI (A), fMLP (B), PMA (C) and OZP (D). The results are expressed as a percentage of the control, with level of significance analyzed by ANOVA, $p<0.01$**.

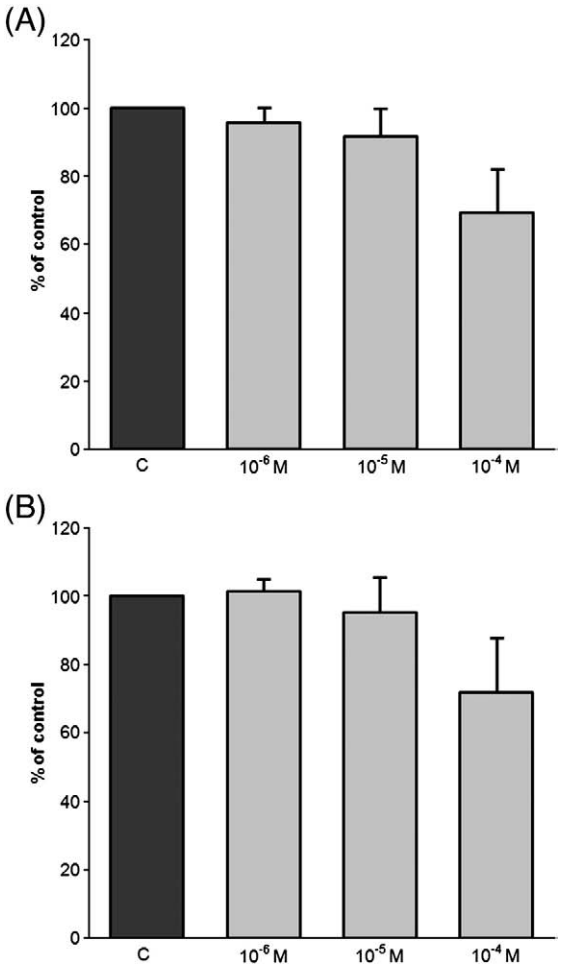

Fig. 3. The effect of 5-HT (A) and DOI (B) hydrochloride (10^{-6}–10^{-4} M) on the oxidative burst of human PMNL activated by OZP. The results are expressed as a percentage of the control.

activated rat and human total leukocytes cannot be caused by their scavenging of produced ROS.

DOI hydrochloride was the most effective 5-HTR agonist which was capable of causing a modification of the oxidative burst of rat total leukocytes in a dose-dependent manner, respectively the reduction of CL response in activated rat total leukocytes, similarly to 5-HT. We suppose that DOI hydrochloride as well as 5-HT influenced the oxidative burst of activated rat total leukocytes via 5-HTR_2.

The following experiments were focused on human immune cells and the effect of 5-HT and its 5-HTR_2 agonists, DOI hydrochloride. First, the influence of 5-HT and DOI hydrochloride on the modification of the oxidative burst, respectively the reduction of CL response in human activated total leukocytes, was tested. Our results showed that 5-HT and DOI hydrochloride were both potent inhibitors of the CL reaction of activated human total leukocytes. These results again indicate the involvement of 5-HTR_2.

Next, the influence of DOI hydrochloride and 5-HT on the CL response of isolated human PMNL was tested. DOI hydrochloride, similarly to 5-HT, in the concentration range of 10^{-7}–10^{-4} M, was unable to significantly decrease the CL response of activated PMNL. The fact that this observation was not caused by overactivation of isolated PMNL was evidenced by the equal absolute values of the integrals obtained from CL measurement of activated human total leukocytes and PMNL. According to these results, we suppose that involvement of 5-HTR_2 during the oxidative burst is not directly through the receptors on the neutrophil surface as we speculated above. But, it seems that 5-HT influenced the oxidative burst indirectly, via activation of 5-HTR_2 on the other leukocytes.

Distribution of 5-HTR on immune cells was described in several studies. While an expression of 5-HTR on the neutrophil surface has not yet been described, it is known that 5-HTR_2 are expressed on the peripheral leukocytes (lymphocytes and monocytes) surface (Mossner and Lesch 1998; Idzko et al. 2004; Durk et al. 2005). Both lymphocytes and monocytes have been described as strong producers of pro-inflammatory and anti-inflammatory cytokines, the production of which could be modulated by 5-HT as has been described by several authors (Idzko et al. 2004; Aune et al. 1994; Arzt et al. 1991; Kubera et al. 2000; Menard et al. 2007). For example, 5-HT and its agonists cause a decreased production of pro-inflammatory cytokines such as interleukin 2 (IL-2) and interferon-γ (INF-γ) by memory T cells (Aune et al. 1994), interferon-γ and tumor necrosis factor (TNF) by human blood leukocytes (Arzt et al. 1991; Kubera et al. 2000), IL-12 and tumor necrosis factor (TNF) by dendritic cells (Idzko et al. 2004) and macrophages. On the other hand, 5-HT can increase anti-inflammatory cytokine IL-10 production by macrophages (Menard et al. 2007), or IL-6 by human vascular smooth muscle cells (Ito et al. 2000). A number of studies exist that explain the mechanisms of 5-HT modulation of cytokine production as a result of 5-HTR_2 activation (Cloez-Tayarani et al. 2003; Kubera et al. 2000; Kubera et al. 2005). According to these, we suppose that 5-HT or DOI hydrochloride can modify macrophage cytokine production towards anti-inflammatory cytokines and that these cytokines exert their anti-inflammatory properties by down-regulation of the oxidative burst, as was described previously (Pilette et al. 2002; Dang et al. 2006).

It seems that 5-HT accumulated at inflammatory sites acts as a protector against oxidative stress-induced tissue damages. In a physiological concentration that is about 10^{-7} M and lower, 5-HT does not have any influence on the oxidative burst of professional phagocytes; but, in a pathophysiological concentration that is about 10^{-5} M (Mossner and Lesch 1998), 5-HT exerts a reduction of the oxidative burst and, in this way, averts the formation of oxidative tissue damages.

Conclusion

In accordance with our hypothesis, it was observed that 5-HT and its 5-HTR_2 agonist DOI hydrochloride were able to inhibit the luminol-enhanced CL response in activated rat and human total leukocytes, but not in isolated human PMNL. The interpretation of these results could suggest the indirect involvement of 5-HTR_2 expressed not on the neutrophil surface but on the surface of other leukocytes. These leukocytes might, after 5-HT or DOI hydrochloride stimulation, be able to produce anti-inflammatory cytokines that cause a down-regulation of the oxidative burst of PMNL. However, more investigations are needed to understand the connection between the activation of 5-HTR_2 and the reduction of the oxidative burst in human professional phagocytes. Moreover, some of the observed inhibitory effects of 5-HT on the oxidative burst of PMNL might be partially caused by other 5-HT receptors, namely 5-HTR_3 and 5-HTR_4.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Title page

Serotonin and its metabolites reduce oxidative stress in murine RAW264.7 macrophages and prevent inflammation

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Serotonin and its metabolites prevent inflammation

Keywords:

Serotonin; N-acetylserotonin; melatonin; RAW264.7 macrophages; reactive oxygen species; nitric oxide; cytokines

Abstract

In this study we focused on comparing the effects of serotonin and its metabolites on the functions of RAW264.7 cells (emphasis on oxidative burst and production of nitric oxide and cytokines), thereby expanding the scope of existing knowledge with advent of novel findings in this field. Changes in production of reactive oxygen species (ROS) by RAW264.7 cells after treatment with serotonin, N-acetylserotonin and melatonin were determined using the chemiluminescence (CL) assay. To exclude the direct scavenging effects of the studied compounds on the CL response, the antioxidant properties of all respective compounds were measured using TRAP and amperometrical method. Nitric Oxide (NO) production was measured by Griess reagent and inducible NO synthase (iNOS) expression by Western blot. Cytokine production was assessed using the Mouse Cytokine Panel A Array kit and ELISA. We showed that all tested compounds were able to reduce oxidative stress, as well as inhibit production of inflammatory cytokines by macrophages. Of the tested compounds, serotonin and N-acetylserotonin were markedly better antioxidants than melatonin. In comparison, other effects of tested compounds were very similar. It can be concluded that antioxidant capacity of tested compounds is a major advantage in the early stages of inflammation. Since plasma concentrations of N-acetylserotonin and melatonin are lower than serotonin, it can be deduced that, serotonin plays a key role in modulation of inflammation and the regulatory functions of immune cells; while also protecting cells against oxidative stress.

Key points:

Serotonin and related compounds reduce oxidative stress in macrophages.

Serotonin modulates inflammation mainly via its antioxidative properties.

Serotonin and N-acetylserotonin are better antioxidants than melatonin.

Introduction

Serotonin, otherwise known as 5-hydroxytryptamine (5-HT), is one of the most extensively studied neurotransmitters of the central nervous system (CNS). However, serotonin is also present in a variety of peripheral tissues, including several constituents of the immune system [50,47,71,31,49,55]. Serotonin, an indolic monoamine compound metabolized from the essential amino acid tryptophan, also functions within the CNS where it is synthesized and stored in presynaptic neurons (serotoninergic neurons, pineal gland and catecholaminergic neurons) [15]. Serotonin in the enteric nervous system (ENS), is synthesized mainly by enterochromaffin (EC) cells lining the lumen of the gastrointestinal tract, subsequently released into the plasma. Directly from the plasma, it is further readily taken up by platelets during their life cycle - thus representing a major storage site for serotonin outside the CNS [59,46,20]. Serotonin is inactivated via combination of oxidative deamination (monoamine oxidase; MAO), conjugation with sulfuric and glucuronic acids, N-acetylation or 5-*O*-methylation processes. Cleavage of the amine group (and adjacent hydrogen atom) by MAO forms the primary metabolic pathway for serotonin in the body. Inactive 5-hydroxyindoleacetic acid (5-HIAA), a major catabolite of MAO metabolism, is then excreted mainly into the urine [45,25]. On the other hand, two highly specific enzymes, serotonin-N-acetyltransferase and hydroxyindol-*O*-methyltransferase, known to catalyze serotonin, are also involved in synthesis of N-acetylserotonin and melatonin, primarily in the pineal gland [36,47]. Melatonin plays an important role in regulation of various physiologic functions, such as circadian rhythms, reproduction, mood, and immune functions. Moreover, exogenously administred melatonin has been demonstrated to express various clinical effects such as sleep modulating, analgesic, anxiolytic, antiinflammatory, and antioxidative activity [43]. It was also shown that circadian secretion of melatonin synchronizes immune cells via melatonin receptors [48]. Several studies have shown that both N-acetylserotonin and

melatonin are also produced at extrapineal sites, such as by immunocompetent cells, whereby local concentrations can reach considerably high levels [9,44,60].

In addition to the neuromodulatory effects, all three tested compounds are able to interact with immune cells, thereby exerting their direct influence on the immune system [50,42,8,10,58]. Evidence suggests that serotonin is capable of regulating immune function through several serotonin receptor subtypes [52,4,53]. Other studies reveal the role of serotonin in upregulating the activity of phagocytes [21], modulating the differentiation of human monocytes [35] and the proliferation of T-cells [3,79,37]. Furthermore, it was concurred that serotonin is involved in modulation of cytokine and chemokine production [19,13,27], migration and recruitment of immune cells [51,18], and modulation of oxidative burst mechanisms [61,12]. Melatonin, as well as serotonin, is recognized for having many effects on immune cells. Carrillo-Vico et al. [10], Calvo et al. [8], and most recently summarized these effects in detailed reviews [24,77,78]. In addition to the expression of melatonin receptors, immune cell are capable to synthetise melatonin and thus induce changes in various immune cell proportions, their viability, trafficking, metabolism and cytokine production in the tumor microenvironment [48]. Huang et al. (2019) found that melatonin treatment significantly decreases the expression of TNF-α, IL-6 and IFN-γ and increases the production of IL-10 and TGF-β in dendritic cells. Melatonin also significantly inhibits the production of TNF-α in CD8 T cells [29]. Meanwhile, further information about the effects of N-acetylserotonin as well as melatonin or serotonin on immune cells largely remains incomplete. Several studies define the capacity of N-acetylserotonin to inhibit NO production and TNF-α synthesis or attenuate the production of superoxide anion radical [58,38]. Oxenkrug [56] also summarized the antioxidant effects, suggesting potential mechanisms of action characteristic of N-acetylserotonin.

Consideration given to these facts and so, this study focused on comparing effects of serotonin and its metabolites on the functions of RAW264.7 cells (especially oxidative burst, production of NO and cytokine production). Therefore, we maintain strong beliefs that our findings significantly enhance current knowledge in the field.

Methods

Serotonin, N-acetylserotonin and melatonin

Serotonin hydrochloride, N-acetylserotonin and melatonin were purchased from Sigma-Aldrich, USA. All compounds were tested according to clinically relevant concentrations of serotonin in different fluid compartments within the body: 10^{-8} M, physiological concentration in plasma; 10^{-6} M, in sites of inflammation (primarily contained in dense granules of activated platelets); and 10^{-3} M, pharmacological concentration of serotonin (the approximate concentration of serotonin inside dense granules of platelets). Melatonin was solubilized in ethanol (final concentration <0,2%). Furthermore, in the concentrations used, all compounds studied were deemed non-toxic to RAW 264.7 cells, as proven by results of the ATP tests (data not shown).

Cell culture

RAW 264.7 cells (American Type Culture Collection, USA), a mouse macrophage cell line, were grown in Dulbecco's Modified Eagle Medium (DMEM; PAN, Germany), supplemented with 10 % of fetal bovine serum (PAN, Germany) and 1 % combination penicillin/streptomycin. Cells were maintained at 37 °C in an atmosphere comprised of 5 % CO_2 and 95 % air.

Chemiluminescence (CL) assay of macrophages

The CL of macrophages was measured using an LM-01 microplate luminometer (Immunotech, Prague, Czech Republic). Protocol was designed based on the method described previously by Vasicek et al. [72]. Briefly: each reaction mixture consisted of $1x10^5$ macrophages, one of the tested compounds (serotonin, N-acetylserotonin or melatonin) in various concentrations (10^{-3} M, 10^{-6} M or 10^{-8} M, respectively) and lipopolysaccharide (LPS)

from *E.coli* (Sigma-Aldrich, USA) in the concentration of 100 ng/ml. 24 h incubation was followed by addition of 1 mM luminol (Sigma-Aldrich, USA, 09253). Hank's Balanced Salt Solution (HBSS) was then used to adjust for the required total reaction volume of 225 µl. The reaction was initiated by adding 25 µl of one of the activators: opsonized zymosan particles (OZP; Sigma-Aldrich, USA, L8274) in a concentration of 62.5 µM, or phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA, P8139) in a concentration of 0.81 µM) and the samples were immediately analyzed. Data acquisition was based on integral values of CL over 90 minutes, whereby they were converted to a percentage of the control as final results.

Total peroxyl radical-trapping antioxidant parameter analysis (TRAP)

TRAP analysis is based on the measurement of luminol-enhanced CL in a chemical system where peroxyl radicals are produced. Published by Denev in 2014 [16], brief outline of the basic method includes the following steps: the reaction mixture contained 160 µl of Phosphate Buffered Saline (PBS; pH 7.4), 16.8 µl of 10 mM luminol, and 6.7 µl of a tested compound. Samples were subsequently incubated at 37 °C in a temperature-controlled carrousel of the luminometer for 10 minutes. 16.8 µl of 400 mM 2,2'-dimethyl-2,2'-azodipropanimidamid-dihydrochlorid (ABAP, Sigma-Aldrich 44,091-4) were added to commence the generation of peroxyl radicals. 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox; Sigma-Aldrich, USA), was used as a reference inhibitor at a concentration of 400 µM. TRAP value was determined by measurement of the time taken for the CL signal to diminish completely by addition of the tested compounds.

Electrochemical detection of nitric oxide scavenging

Evaluation of the scavenging properties and capabilites against nitric oxide (NO) of studied compounds was performed in a chemical system using an amperometrical method as

described previously by Pekarova [57]. The microsensor (AmiNO 700, Innovative Instruments, USA) was connected to the ISO-NO MARK II potentiostat (WPI, USA). Measurement was performed using distilled water saturated with pure NO gas (according to the WPI manual). Temperature of the system was kept at 37 °C. Direct injection of 15 µl of the NO-saturated water into the glass vial containing 5 ml of either tested compounds diluted in PBS; or just PBS (control), caused rapid increase in the detected signal, which was followed by a subsequent gradual decrease of the NO-induced signal until it reached the background level. Index of scavenging (ratio of the signal peak height at time of NO injection, to; time taken for re-entry and the signal to return to baseline) was used for evaluation of radical scavenging properties of the tested compounds.

Measurement of nitrite concentration by Griess reaction

Changes in NO production were measured indirectly, by virtue of the accumulation of nitrites (the end-product of NO metabolism) in a medium using Griess assay, as described previously in various publications [41,2]. Cells were incubated in 6-well plates at concentration of 1×10^6 cells/well; with tested compounds and LPS (100ng/ml) for 24 h at 37°C in 5% CO_2. Positive control cells were treated only by LPS. At the end of the incubation period, culture media was collected from the respective wells and centrifuged (16000*g*/4 °C/5 min). 150 µl of centrifuged supernatant was mixed with an equal volume of Griess reagent (Sigma-Aldrich, USA, G4410) and the mixture was incubated at room temperature (RT) in the dark for 30 min. Absorbance was measured at 546 nm; using sodium nitrite as the standard for the calibration curve.

Western blot analysis of iNOS protein expression

After the treatment procedure, RAW264.7 cells were gently washed with cold PBS and lysed using lysis buffer (1% sodium dodecyl sulfate (SDS); 100 mM Tris, pH 7.4; 10% glycerol) containing phosphatase and protease inhibitor cocktail tablets (Roche, Germany). Protein concentrations were determined using BCA™ protein assay (Thermo Scientific, USA, 23223) and bovine serum albumin (Amresco, 0332, USA) was used as a standard. The same amount of protein (40µg) from each lysate supplemented with Laemli buffer (200mM Tris-HCl, pH 6,8; 3% SDS; 30% glycerol; 0,03 bromphenol blue; 3% ß-mercaptoethanol; 200mM DTT) was subjected to SDS-polyacrylamide gel electrophoresis, using 10 % running gel [22]. Electrophoresis was performed at 60 V for 15 minutes and then at 120V for 1.5 hour. Separated proteins were transferred to PVDF membranes (2 hours at 330 mA) that were blocked in 5% non-fat milk in Tris-buffered saline, 0.1% Tween 20 (TBS-T). Then, the membranes were incubated with monoclonal antibodies against anti-iNOS/NOS Type II mouse monoclonal antibody (BD transduction laboratories, USA, 610431; 1:5000), phospho-protein kinase C (PKC)α/βII and phosphor-PKCδ (Cell signaling technology, USA, 9375, 9374,; 1:2000) and ECL™ Anti-mouse IgG horseradish peroxidase linked whole antibody (Biosciences, USA, 7076; 1:2000) were used. The equal loading of proteins was confirmed by determination of β-actin (Santa Cruz Biotechnology, sc-47778, USA, 1:5000). The immunoreactive bands were detected using an ECL™ detection reagent kit (Pierce, USA) and exposed to radiographic film (AGFA, Belgium). Relative protein levels were quantified by scanning densitometry, using the Image J™ program, and the individual band density value was expressed in arbitrary units.

Cytokine assay

Mouse cytokine array panel A array kit (R&D Systems, Minneapolis, USA) was used for determination of the relative levels of cytokines and chemokines, proceed according to the manufacturer´s instructions. Mouse IL-6 and TNF-α DuoSet ELISA kits (R&D Systems, USA) were used for more accurate measurement of selected cytokines, processed according to the manufacturer´s instructions.

Statistical analysis

Samples were measured in duplicates. Results are reported as mean ± standard error of the mean (SEM). Results were normalized with reference to the control cells in each run to account for variability due to individual cell passages. Data were statistically analyzed using a one-way analysis of variance (ANOVA), which was followed by Dunnett´s multiple comparison test was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. $P < 0.05$ was taken to indicate significant differences between data mean values.

Results

The effect of serotonin, N-acetylserotonin and melatonin on ROS production by macrophages

First, we were able to detect the effect of tested compounds on ROS production by RAW 264.7 cells. When PMA was used as the activator, the tested compounds in concentrations 10^{-6} M and 10^{-8} M did not significantly change the CL signal, however, concentrations of 10^{-3} M dramatically reduced it. Serotonin and N-acetylserotonin reduced the CL signal down to 5 % of control and; melatonin to 52 % of control (Fig. 1, upper panel). Similarly, when OZP was used as the activator, no significant changes in the CL signal were detected for concentrations amounting to 10^{-8} M and 10^{-6} M, but the concentration 10^{-3} M again, reduced the CL signal. Serotonin and N-acetylserotonin reduced the CL signal significantly to 18 % and 20 % of the control, respectively. Less dramatically, melatonin decreased the CL signal to just 90 % of control (Fig. 1, bottom OZP). When LPS was used in combination with PMA and OZP, newly obtained CL signals (Fig. 1, black bars) matched previous results (Fig. 1, white bars). Tested compounds had no significant effects on inhibition of the phosphorylation of PKCα/βII, PKCδ (data not shown).

Serotonin is known for its highly effective antioxidant capacity, therefore, we determined antioxidant capacity of all tested compounds using TRAP method, which largely shares many methodological similarities to CL measurement. Results from TRAP reveal that serotonin (3399±29 mmol/ml) and N-acetylserotonin (2977±306 mmol/ml) are excellent antioxidants. Melatonin, in comparison with the parallel compounds is a markedly weaker antioxidant (760±263 mmol/ml) (Fig. 2).

The effect of serotonin, N-acetylserotonin and melatonin on NO production by macrophages

Here, we focused on NO production, an important signaling molecule of macrophages. The LPS-activated macrophages significantly increase NO production and level of nitrite in

medium. Serotonin, N-acetylserotonin and melatonin decreased the nitrite formation in a concentration-dependent manner (Fig. 3). Concentrations of 10^{-6} M serotonin significantly reduced nitrite formation to 28 % of control; and just 1% of the control at 10^{-3} M. N-acetylserotonin had a similar reduction potential profile to serotonin; in that, the concentration of 10^{-6} M reduced nitrite formation to 30 % of control and to just 3 % of control, at 10^{-3} M. Melatonin also had a similar effects to serotonin and N-acetylserotonin: 10^{-6} M, reduced nitrites formation to 27 % of control. In the case of melatonin concentration of 10^{-3} M reduced the signal to 14 % of control. Lower concentrations of tested compounds did not affect the formation of nitrites in medium (Fig. 3).

Concentration dependent production of nitrites was confirmed by the expression of iNOS protein. To demonstrate presence of iNOS protein expression, corresponding Western blots were chosen (Fig. 4) after treatment with serotonin, N-acetylserotonin or melatonin. Looking at the ratio of optical densities of iNOS and β-actin, it is evident that, all tested compounds in concentration 10^{-3} M and 10^{-6} M, significantly decrease the expression of iNOS to approximately 50 % of control. Compounds tested at 10^{-8} M did not significantly affect the iNOS expression (Fig. 5).

No significant changes in NO scavenging properties of tested compounds were observed using the amperometric method (data not shown).

The effect of serotonin, N-acetylserotonin and melatonin cytokine production by macrophages

The last part of this study was focused on the effects of tested compounds on production of cytokines by RAW264.7 cells. Overall changes in cytokine production were evaluated using Mouse Cytokine Array Kit. Non-stimulated macrophages produced IP-10 (CXCL10/CRG-2), G-CSF, IL-1ra, KC, JE, sICAM-1, MIP-1α, MIP-1β, MIP-2, RANTES and; to a lesser extent also TNF-α. When RAW264.7 cells were stimulated by LPS, there nascent production of IL-

6, M-CSF, TIMP-1, TNF-α and to a lower extent IFN-γ was observed. If RAW264.7 cells were treated with 10^{-3} M serotonin, reduction in IL-6, M-CSF, IFN-γ, TIMP-1 production was also noted. When LPS-stimulated RAW264.7 cells were treated with N-acetylserotonin (10^{-3} M), the results mimicked to some degree, the effect of serotonin. In addition, a reduction in sICAM-1 was visible. When LPS-stimulated RAW264.7 cells were treated with melatonin (10^{-3} M), TIMP-1 and IFN- γ were reduced completely, but IL-6 and M-CSF changed only moderately (Fig. 6).

According to these results, we focused on IL-6 and TNF-α cytokines for further evaluation. For accurate measurement of changes in production of these cytokines, ELISA was used. All tested compounds had notably similar inhibitory effects on production of IL-6. The highest concentration used of tested compounds, significantly decreased IL-6 from 1100 pg/ml to 77 ± 14 pg/ml for serotonin; 100 ± 10 pg/ml for N-acetylserotonin and; 178 ± 4 pg/ml for melatonin. Tested compounds in concentrations 10^{-6} M, decreased IL-6 production to 760 ± 68 pg/ml for serotonin; 830 ± 31 pg/ml for N-acetylserotonin and 772 ± 39 pg/ml for melatonin. No significant changes in IL-6 production were observed when testing 10^{-8} M (Fig. 7). All compounds in concentration 10^{-3} M, significantly decreased TNF-α from 7385 ± 228 pg/ml to 3502 ± 187 pg/ml for serotonin; 5304 ± 187 pg/ml for N-acetylserotonin and 4007 ± 163 pg/ml for melatonin. All compounds in concentration 10^{-6} M, decreased the TNF-α production, however, only N-acetylserotonin and melatonin were remarkable. No significant changes in TNF-α production were observed using 10^{-8} M samples (Fig. 7).

Discussion

Serotonin and its metabolites (N-acetylserotonin and melatonin) play an important role in coordination of the immune system, known for their involvement in regulation of phagocyte responses [8,11,50]. In this study, we compared the effects of serotonin, N-acetylserotonin and melatonin on important biological responses of RAW 264.7 mouse macrophage cell line. Our results show that all tested compounds were able to significantly reduce ROS formation; only at the upper limit of the range of the respective concentrations tested. To distinguish whether tested compounds affect the NADPH oxidase pathway, different types of activators were used. OZP (a typical receptor binding stimulus) triggers the signaling cascade during phagocytosis via complement and/or Fc receptors whereas; PMA (a typical receptor-bypassing stimulus) directly activates PKC. LPS was used as a typical stimulant of macrophages via TLR4 receptors. We found that reduction of CL signal was not caused by direct inhibition of phosphorylation of PKC isoforms, but rather, their ROS-scavenging activity. Our results clearly point to the considerably higher ROS-scavenging activity of serotonin and N-acetylserotonin, compared to melatonin. Moreover, Schuff-Werner et al. (1995), showed that serotonin acts locally, as a releasable antioxidant [67]. Further works reveal a crucial role of serotonin in phagocytosis and its intimate relationship with respiratory burst in human polymorphonuclear phagocytes [66,67,30]. Williams et al. (1998) [74], on the contrary, showed that melatonin did not affect production of ROS and did not have antioxidant properties, although; they used lower concentrations. Serotonin (through serotonin transporter), N-acetylserotonin and melatonin (mainly due to their amphiphilicity) can easily cross cell membranes and may influence for example, intracellular redox processes [76] or serotonylation [64,73].

Another important role of macrophages is undoubtedly NO production. NO plays a pivotal role in the immune system; fundamentally involved in the pathogenesis and control of

infectious diseases, association with tumor formation, autoimmune processes or chronic degenerative diseases [5,7,14,6]. Moreover, NO can react with ROS, most notably superoxide anion, to form peroxynitrite anion, which can potentially affect luminol-enhanced CL, too [62]. Our results showed that all tested compounds were able to inhibit NO production in a concentration-dependent manner. We observed different effects on NO production, only at the highest tested concentrations of respective compounds. Variability, we assume, may be caused by their different scavenging mechanisms [54]. Shimpo et al. (1997) [65], observed similar effect of serotonin on vascular smooth muscle cells. They supposed that the inhibitory effect of serotonin was mediated via the 5-HT_2 receptor subfamily. Klemm et al. (1995) [38], showed that, N-acetylserotonin was able to inhibit nitrite formation in murine macrophages stimulated by a combination of LPS and IFNγ. Several authors have further established the effect of melatonin on NO production in various experimental models. Tamura et al. (2009) [70], demonstrated that melatonin inhibited NO production, induced by LPS in cultured rat endothelial cells and aorta rings. Other authors elucidate the inhibitory effect of melatonin on NO production in murine macrophages [1,17,23,81]. However, none have compared the effects of serotonin and its metabolites on NO production. Recently, Hyeon et al. (2017) [32] indicated that agomelatine, a structural analog of melatonin, suppressed an LPS-induced generation of iNOS-derived NO via the repression of NF-κB, STAT1; STAT3 activation and SOCS1 induction. Similarly, Jeon et al. [34], showed that N-arachidonoyl serotonin (NA-5HT) at non-cytotoxic concentrations suppressed LPS-induced formation of nitric oxide, as well as expression of inducible NO synthase in RAW264.7 murine cells. NA-5HT efficiently reversed LPS-induced phosphorylative activation of nuclear factor-κB pathway, probably through suppression of the mitogen-activated protein kinases (MAPKs) or phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways.

According to the correspondence by the innate immune cells, LPS activates the signaling pathway such as NF-κB via the stimulation of TLR4 which consequently releases not only NO but proinflammatory cytokines including IL-1β, TNF-α, and IL-6 [63] . Based on the results and measurements of cytokine profiling, it is obvious that serotonin, N-acetylserotonin and melatonin possess robust anti-inflammatory properties. Interestingly, tested compounds were able to inhibit early response to cytokines such as TNF-α and IL-6. Conversely, the same compounds were able to partially inhibit production of IL-10, a potent cytokine with reputable anti-inflammatory properties, subsequently repressing expression of inflammatory cytokines, like for example, TNF-α, IL-6 and IL-1 [75]. Kubera et al.(2000) [39], showed in blood samples, that the effect of serotonin on IL-10 production was dependent on the age of volunteers. Several authors found that serotonin, via serotonin receptors (mainly 5-HT_{2A} subtypes) inhibited TNF-α and IL-6; while IL-1β production as a consequence, was increased [40,13,80]. N-acetylserotonin and melatonin were also mentioned in connection with their anti-inflammatory effects [8,11,74,58]. Recently, Jeon et al. (2016) [34], reported that another serotonin metabolite, NA-5HT at non-cytotoxic concentrations, suppressed LPS-induced formation of TNF-α and selected interleukins in RAW264.7 cells.

Latest studies have also addressed the effects of serotonin on phagocytes, despite their rather controversial results [28]. Earlier in 1980s, Sternberg et al. [68,69], showed that serotonin had dose dependent inhibitory or stimulatory effects on murine macrophages. Generally, it seems that, serotonin had suppressive effects at high concentrations and stimulatory effects at lower (physiological) concentrations. As described by Jackson et al. (1988) [33], via expression of serotonergic components like SERT (serotonin transporter) and other serotonin receptors on immune cells, serotonin is able to exert a plethora of complex modulatory effects on monocytes and macrophages. Since, expression of several subtypes of serotonin receptor by

monocytes and macrophages have additionally been reported: 5-HT_2 [68], 5-HT_{1A} [26]; 5-HT_{1E}, 5-HT_{2A}, 5-HT_3, 5-HT_4, and 5-HT_7 [19].

In summary, we showed that all tested compounds were able to reduce oxidative stress and inhibit production of inflammatory cytokines by macrophages. In a comparison of the tested compounds, serotonin and N-acetylserotonin were more effective antioxidants than melatonin. Other effects of tested compounds were found to be very similar. It seems that their antioxidant capacity is a major advantage in the early stages of inflammation. Plasma concentrations of N-acetylserotonin or melatonin are relatively lower than serotonin. It can therefore be deemed, that serotonin plays a principal role in modulation of inflammation, regulation of function of immune cells and protection of cells against the detriment of oxidative stress.

Statement of Contribution

All authors contributed equally to the design and implementation of the research and to the analysis of the results. M.C. supervised the project, O.V. performed the majority of experiments and designed the figures. All authors discussed the results and contributed to the final manuscript.

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Declaration of Interest

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Figures

Figure 1: The effects of serotonin, N-acetylserotonin and melatonin on PMA- and OZP-activated ROS production in murine macrophages of RAW 264.7 cell line. The macrophages were pre-incubated for 24h with tested compounds and addition of 100 ng/ml LPS (black bars) or control medium (white bars) in dark, 37°C and 5% CO_2. After 24 h incubation, luminol was added. HBSS was then used to adjust the total reaction volume to 225 µl. The reaction was initiated by adding 25 µl of one of the activators (OZP or PMA). The results from five independent experiments were integrated, converted to a percentage of the control and expressed as the mean ± SEM. Dashed line indicates 100 % of control. Data were statistically analyzed using ANOVA, which was followed by Dunnett's multiple comparison test (** $p < 0,01$).

Figure 2: The antioxidant properties of serotonin, N-acetylserotonin and melatonin expressed as TRAP mean values ± SEM. Dashed lines indicate the level of negative control. Data were statistically analyzed using ANOVA, which was followed by Dunnett's multiple comparison test (** $p < 0,01$ and * $p < 0,05$).

Figure 3: The effects of serotonin, N-acetylserotonin and melatonin on nitrite production in murine macrophages of RAW264.7 cell line, stimulated by 100 ng/ml LPS. The results from five independent experiments were integrated, converted to a percent of the control and expressed as the mean ± SEM. Dashed lines indicate 100 % of control. Data were statistically analyzed using ANOVA, which was followed by Dunnett's multiple comparison test (** $p < 0,01$).

Figure 4: The effects of serotonin, N-acetylserotonin and melatonin on protein expression of iNOS in murine macrophages of RAW264.7 cell line. Figure showing a representive gel from western blot analysis.

Figure 5: The effects of serotonin, N-acetylserotonin and melatonin on protein expression of iNOS of murine macrophages RAW264.7 cell line stimulated by 100 ng/ml LPS. The results from five independent experiments are expressed as the ratio of optical densities of iNOS and β-actin mean ± SEM. Data were statistically analyzed using ANOVA, which was followed by Dunnett's multiple comparison test (** $p < 0,01$ and * $p < 0.05$).

Figure 6: The effects of serotonin, N-acetylserotonin and melatonin on cytokine production of murine macrophages RAW264.7 cell line stimulated by 100 ng/ml LPS. The results from one experiment (in duplicate) are expressed as the ratio of optical densities of reference spots and cytokine spots mean ± SD calculate from duplicate.

Figure 7: The effect of serotonin, N-acetylserotonin and melatonin on IL-6 and TNF-α production in murine macrophages of RAW264.7 cell line, stimulated by 100 ng/ml LPS. The results from five independent experiments are expressed as the mean ± SEM. Data were statistically analyzed using ANOVA, which was followed by Dunnett's multiple comparison test (** $p < 0,01$ and * $p < 0.05$).

Fig 1

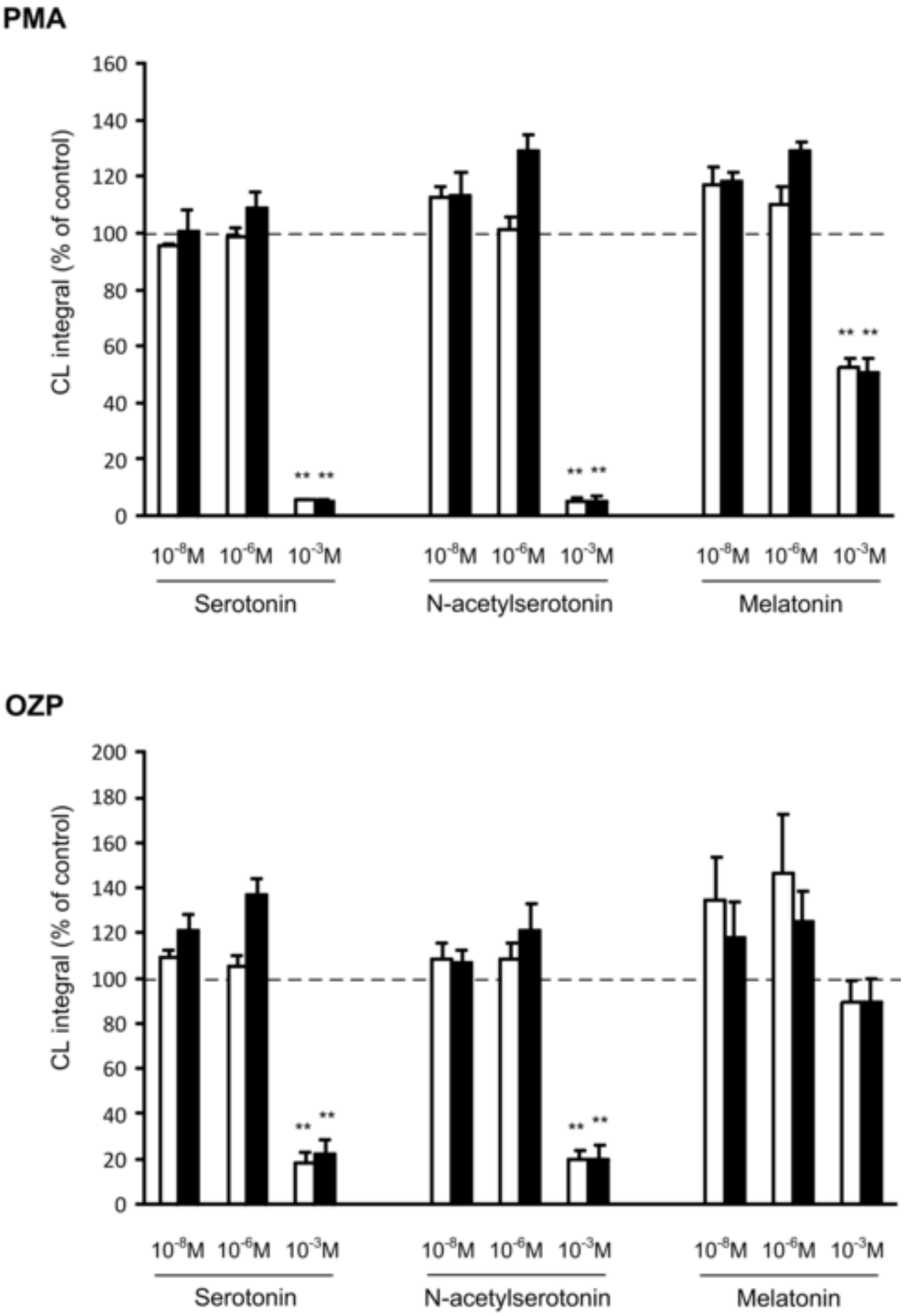
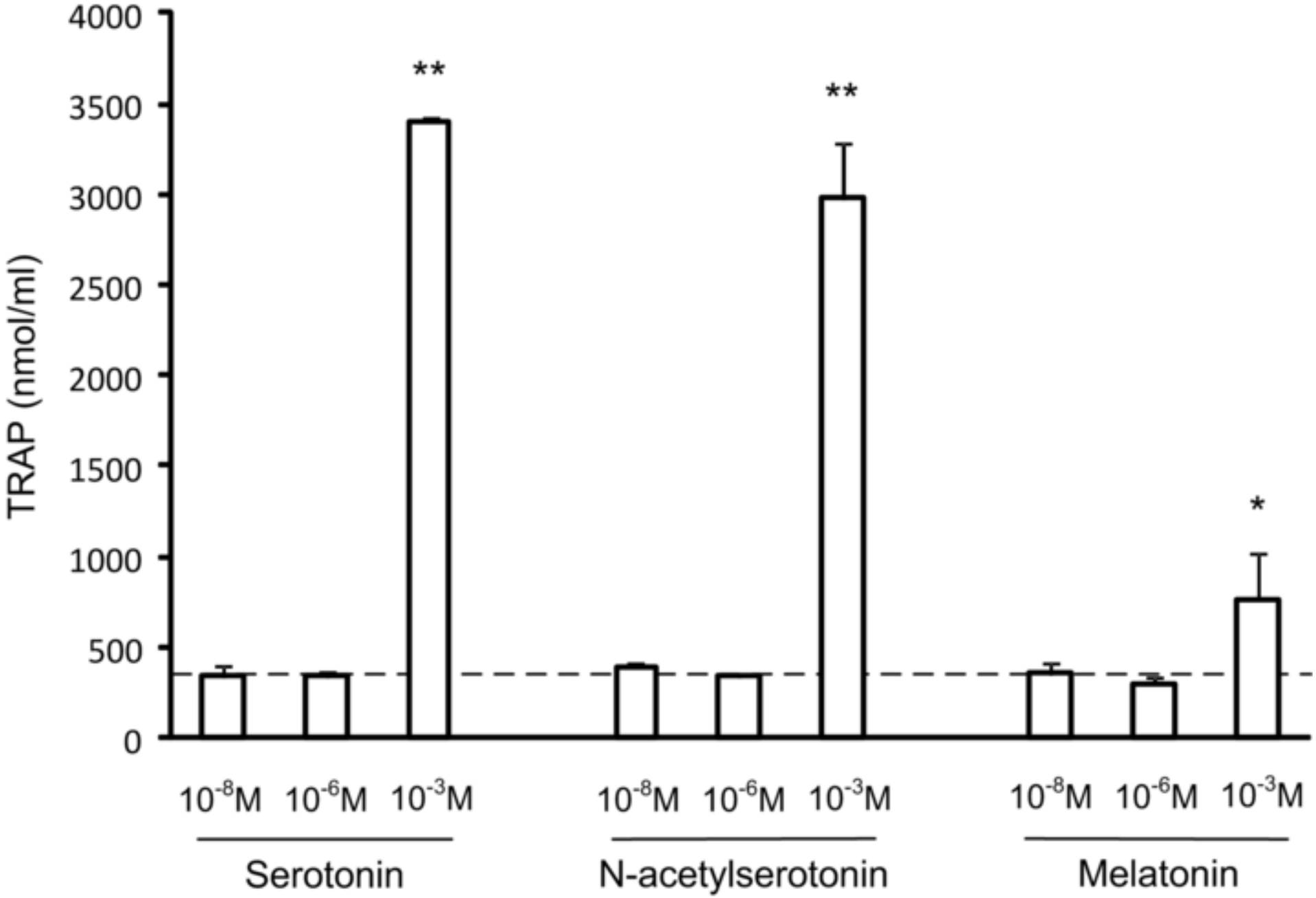


Fig 2



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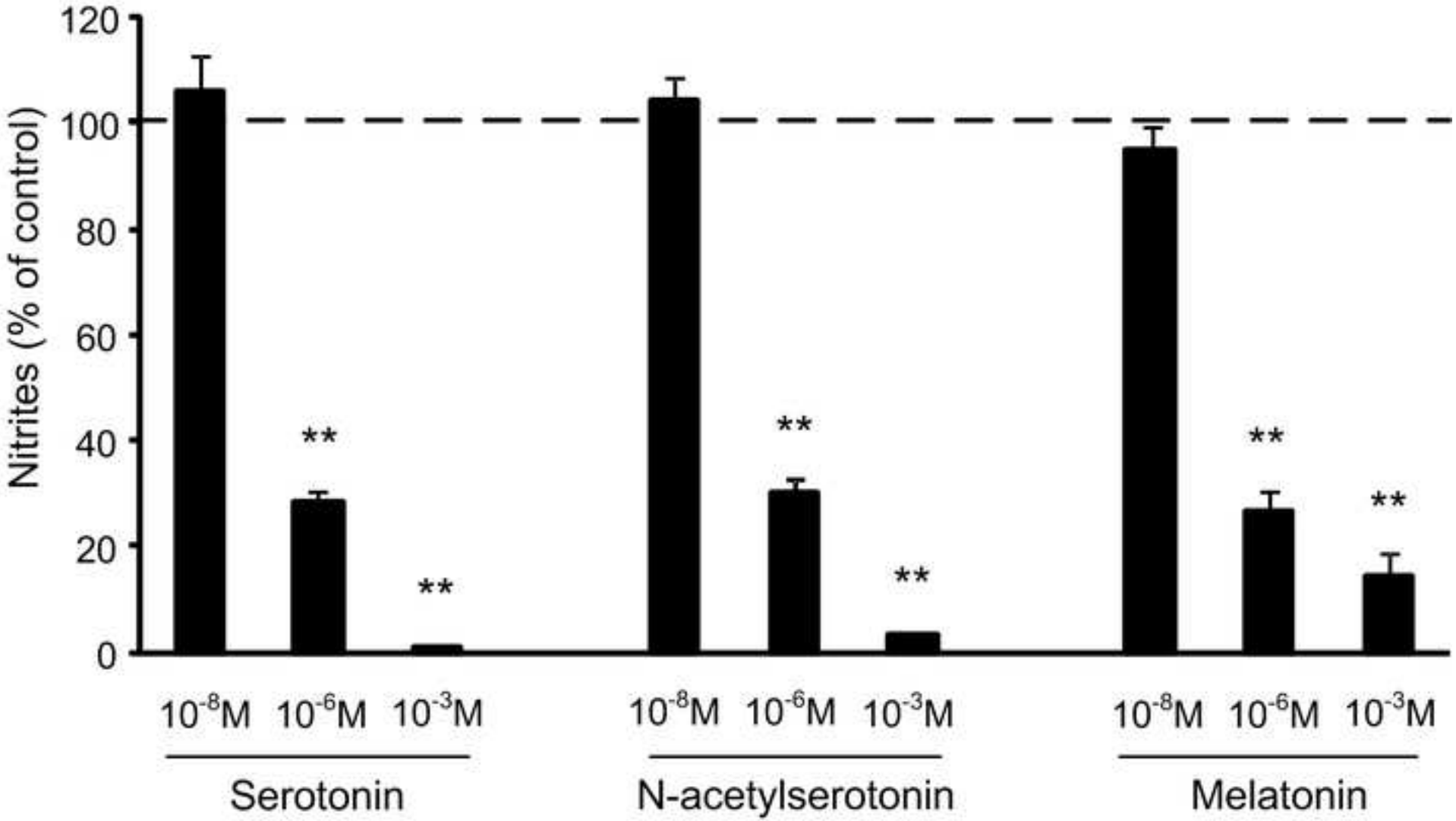

Fig 4

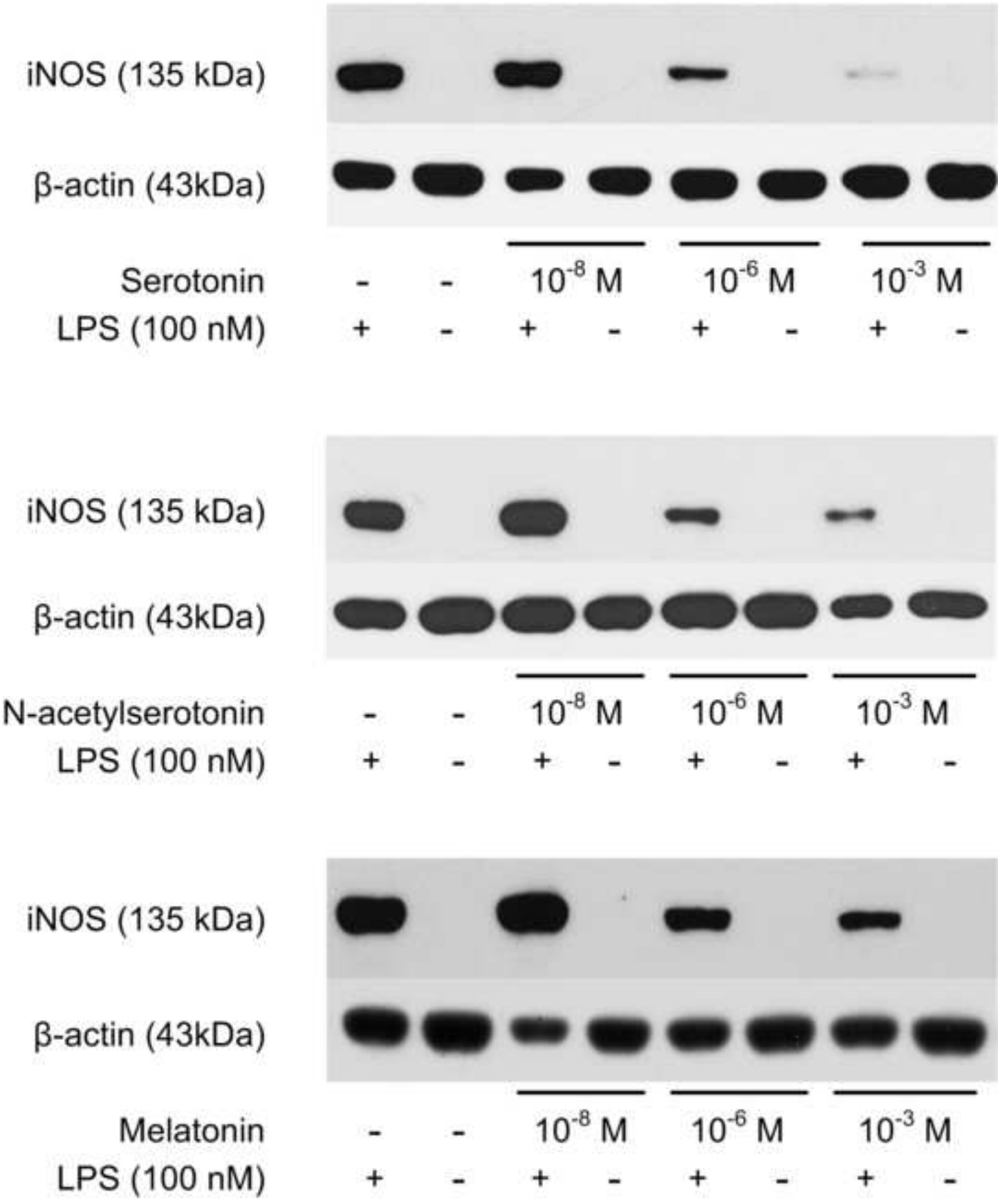

Fig 5

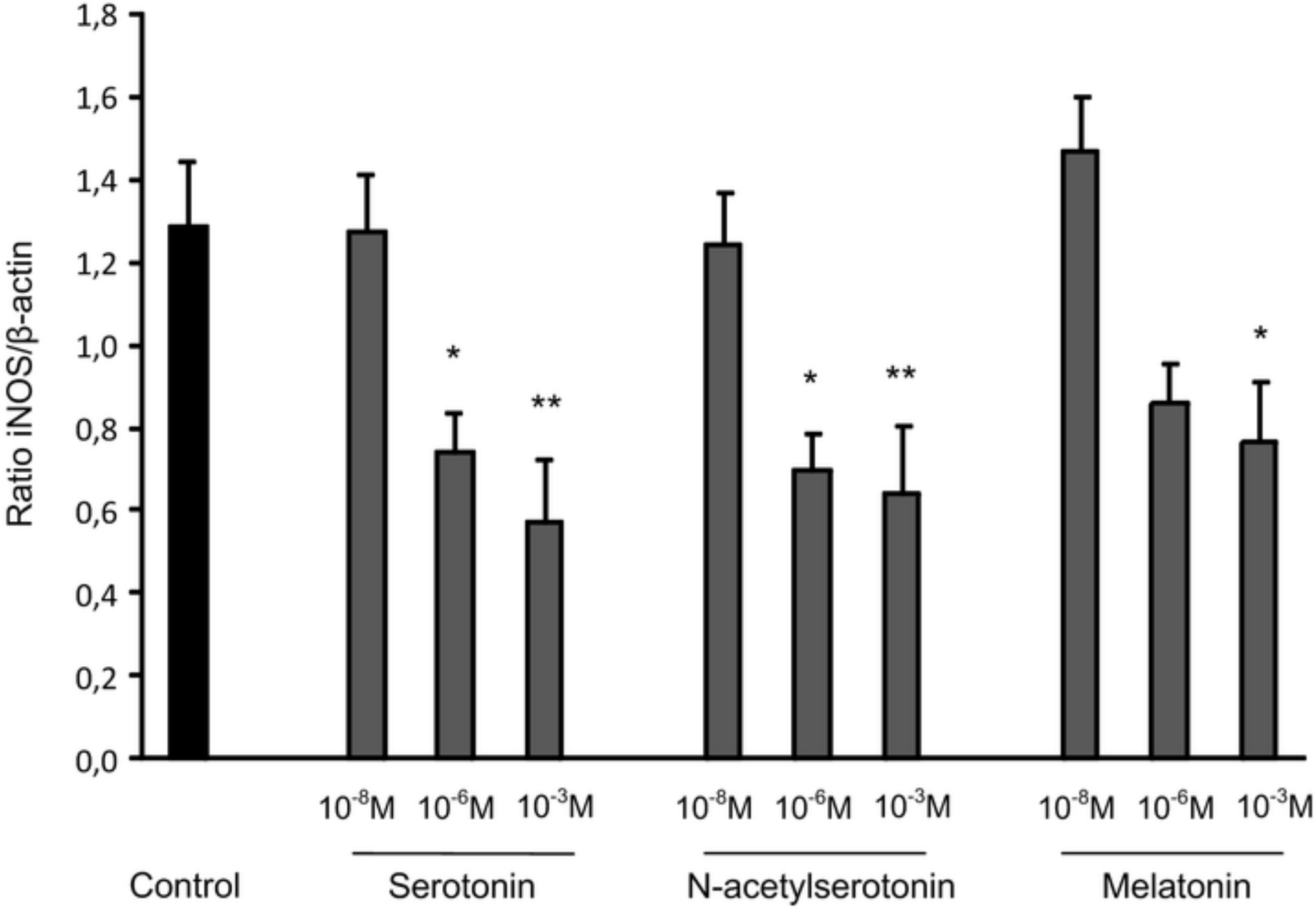

Fig 6

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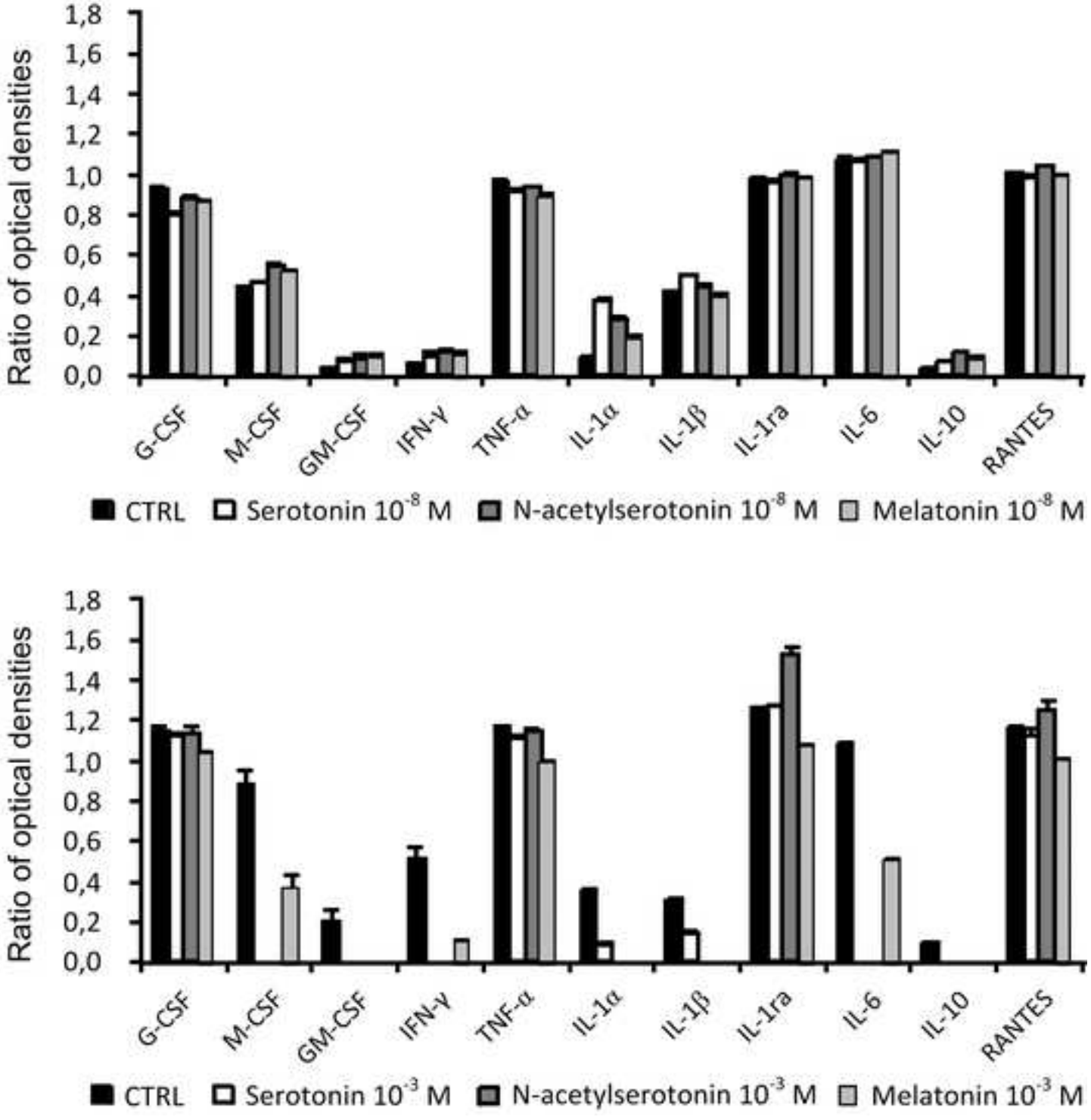

Fig 7

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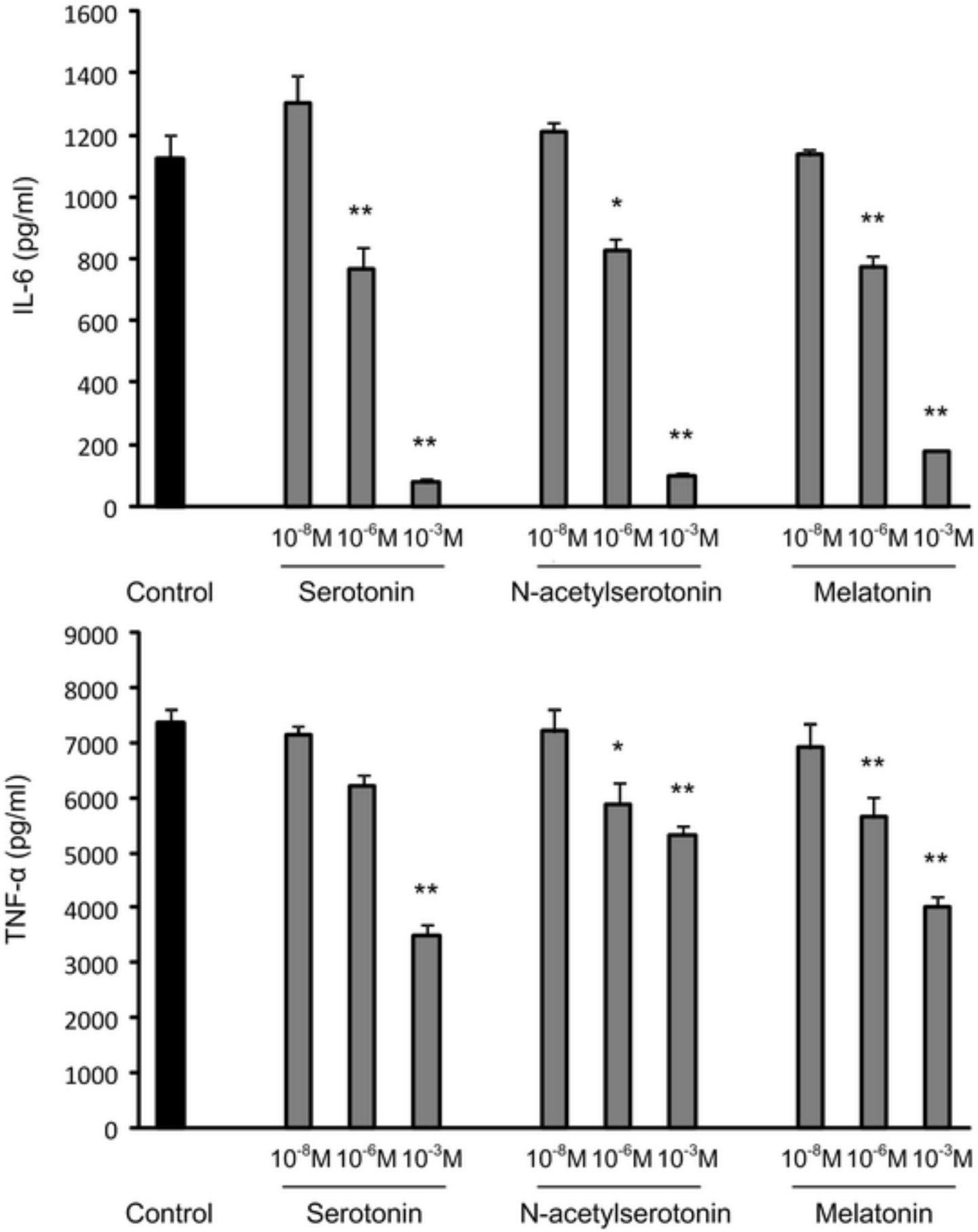

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The Influence of Wine Polyphenols on Reactive Oxygen and Nitrogen Species Production by Murine Macrophages RAW 264.7

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Summary

The aim was to study the antioxidant properties of four wine polyphenols (flavonoids catechin, epicatechin, and quercetin, and hydroxystilbene resveratrol). All three flavonoids exerted significant and dose-dependent scavenging effects against peroxyl radical and nitric oxide in chemical systems. The scavenging effect of resveratrol was significantly lower. All polyphenols decreased production of reactive oxygen species (ROS) by RAW264.7 macrophages. Only quercetin quenched ROS produced by lipopolysaccharide-stimulated RAW264.7 macrophages incubated for 24 h with polyphenols. Quercetin and resveratrol decreased the release of nitric oxide by these cells in a dose-dependent manner which corresponded to a decrease in iNOS expression in the case of quercetin. In conclusion, the higher number of hydroxyl substituents is an important structural feature of flavonoids in respect to their scavenging activity against ROS and nitric oxide, while C-2,3 double bond (present in quercetin and resveratrol) might be important for inhibition of ROS and nitric oxide production by RAW 264.7 macrophages.

Key words

Antioxidants • Polyphenols • Macrophages • Oxidative stress • Nitric oxide

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Introduction

The overproduction of reactive oxygen and nitrogen species (ROS and RNS, respectively) by phagocytes causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins. These reactions have functional consequences, which may be deleterious to cells and tissues. Thus, the inhibition of ROS and RNS production is a popular target for the attenuation of many inflammatory diseases (Shen *et al.* 2002).

Dietary polyphenols with antioxidative effects from fruit and vegetables play an important role in a prevention of the oxidative stress (Mojžišová and Kuchta 2001, Osawa 1999). Another important source of polyphenolic antioxidants is wine, particularly red wine. It has been demonstrated that polyphenols from wine have not only antioxidative but also anti-inflammatory effects (Oak *et al.* 2005) and that they can prevent cardiovascular diseases (Babal *et al.* 2006). It is furthermore suggested that they prevent free radical-mediated lipid peroxidation of low density lipoproteins (LDL), which is associated with cell aging and chronic disesases such as atherosclerosis (Dell'Agli *et al.* 2004, Cook and Samman 1996, Oak *et al.* 2005, Rajdl *et al.* 2006). It is postulated that the antioxidant and free radical scavenging properties of phenolic compounds, present in red wine, may partly explain the ”French paradox”, i.e. the fact that French people have low incidence of coronary heart disease, despite having a diet high in fat and being heavy smokers (Aruoma 1994). The main polyphenolic compounds in red wine belong to two major classes: flavonoids and stilbenes. Of the flavonoids, (+)-catechin, (–)-epicatechin and quercetin and of the stilbenes, trans-resveratrol, are the most abundant polyphenols in wine. Exact mechanisms by which flavonoids protect against oxidative stress-mediated diseases (such as atherosclerosis) are still a matter of debate (Benito *et al.* 2004).

The aim of this experiment was to study the antioxidant properties of four wine polyphenols (catechin

– CAT, epicatechin – EPI, quercetin – QUE, resveratrol – RES) against peroxyl radical and nitric oxide, their immediate and long-term effects on the production of reactive oxygen and nitrogen species by RAW 264.7 macrophages, and their influence on the expression of inducible nitric oxide synthase. Special attention was paid to differentiate between scavenging and inhibiting activity of the studied polyphenols with respect to their chemical structure. As far as we know, there are no similar investigations on the same selection of polyphenols, especially concerning the effects of polyphenols on various parameters linked to the generation of nitric oxide. The advantage of the study is that it took into consideration many factors: kind and dose of polyphenols, time of their action, and different reactive metabolites.

Methods

Materials

Murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were maintained in a Dulbecco`s Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with a 10 % fetal bovine serum, gentamycin (0.045 mg/l), glucose (3.5 g/l) and $NaHCO_3$ (1.5 g/l). The stock solution of lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, St. Louis, Missouri, USA) was made up at 10^{-3} g/l in Hanks' balanced salt solution without phenol red (HBSS, pH 7.4). The final concentration of LPS in a reaction mixture was 10^{-7} g/l. Polyphenols (CAT, EPI, QUE and RES) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Stock solutions ($2x10^{-2}$ mol/l) were always prepared fresh, in 99.8 % ethanol. Then, concentrations of $2x10^{-4}$, $5x10^{-4}$, $1x10^{-3}$, $2x10^{-3}$ mol/l were prepared in RPMI 1640 and added to the reaction mixture to obtain final concentrations of 10^{-5}, $2.5x10^{-5}$, $5x10^{-5}$ and 10^{-4} mol/l, respectively.

ABAP [2,2-azo-bis(2-amidinopropane) hydrochloride] was purchased from Polyscience (Warrington, Pennsylvania, USA) and Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid) from Sigma-Aldrich (St. Louis, Missouri, USA). The stock solution of 10^{-2} mol/l luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Molecular Probes, Eugene, Oregon, USA) was prepared in 0.2 mol/l sodium borate buffer, pH 9.0 (1.24 g of H_3BO_3 and 7.63 g of $Na_2B_4O_7 \cdot 10H_2O$ in one liter of redistilled water). All other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). PMA (phorbol 12-myristate 13-acetate) was dissolved in dimethylsulphoxide to obtain $3x10^{-3}$ mol/l stock solution.

Total radical-trapping antioxidant parameter (TRAP)

The luminol-enhanced chemiluminescence (CL) assay for TRAP was measured using a Luminometer 1251 (BioOrbit, Turku, Finland). The method is based on the measurement of peroxyl radicals produced at a constant rate by a thermal decomposition of ABAP. The TRAP value is determined from the time period during which the CL signal is diminished by antioxidants (Slavíková *et al.* 1998). Trolox, a water-soluble analogue of α-tocopherol, was used as a reference inhibitor for the calculation of the TRAP value.

The reaction mixture contained 475 µl of sodium phosphate buffered saline (PBS 10^{-1} mol/l, pH 7.4), 50 µl of 10^{-2} mol/l luminol in 10^{-1} mol/l borate buffer (pH 10.0), and 20 µl of examined compound. The cuvettes were incubated at 37 °C in the temperature-controlled carousel of the luminometer for 10 min. Then, 50 µl of $4x10^{-1}$ mol/l ABAP (prepared in 10^{-1} mol/l PBS) was added. TRAP value for the sample was obtained from the equation: $TRAP = n\,[Trolox]\tau_{sample} / f\,\tau_{Trolox}$, where n is the stoichiometric factor of Trolox (2.0), τ is the time period of diminished chemiluminescence, and *f* is the dilution factor of the sample.

Scavenging of nitric oxide

Tested compounds were diluted to reach final concentrations of 10^{-5} – 10^{-4} mol/l in a final volume of 10 ml of PBS. 1 µl of saturated nitric oxide solution was added and the concentration of nitric oxide was measured amperometrically using the ISO-NO Mark II isolated nitric oxide meter and ISO-NOP sensor (both from WPI, Sarasota, FL, USA). The method is based on a diffusion of NO through a selective membrane covering the sensor and its oxidation at the working electrode, resulting in an electrical current. The scavenging effect of tested compounds was evaluated based on a decrease in NO concentration in reaction mixture as compared to the control. The data are expressed as a redox current in pA.

Experimental design

When the immediate effect of polyphenols was studied, 2 x 10^5 of RAW 264.7 cells in 100 µl of DMEM were allowed to adhere for 60 min to the edge of each of

the 96-well plate and then all chemicals were added immediately before CL was measured.

When the long-term effect was studied, 96-well cultivation plates ($2x10^5$ cells per well) and 6-well cultivation plates ($2x10^6$ cells per well) were used for CL measurement and Western-blot analysis (and Griess reaction), respectively. Adhered cells (1 h) were preincubated with one of the polyphenols at an indicated concentration for 1 h prior to the 24 h of incubation with LPS (10^{-4} g/l). Adherence as well as incubation steps proceeded at 37 °C with 5 % CO_2. After 24 h the 96-wells were gently washed with HBSS and CL was measured (as described below). The supernatants of the 6-well plates were removed and used for the determination of nitrites by Griess reaction as described below. Cells were used for a Western-blot analysis as described below.

Chemiluminescence assay

The luminol-enhanced CL of RAW 264.7 macrophages was measured using microtitre plate Luminometer LM-01T (Immunotech, Prague, Czech Republic) as described previously (Lojek *et al.* 1997). The principle of the method is based on a luminol interaction with the phagocyte-derived ROS, which results in large measurable amounts of light. Each well (in 96-well culture plates) contained $2x10^5$ RAW 264.7 cells, luminol (at a final concentration of 10^{-3} mol/l) and PMA (at a final concentration of $8x10^{-7}$ mol/l), which was selected on the basis of previous results (Lojek *et al.* 1997) and one of the polyphenols at final concentrations of 0, 10^{-5}, $2.5x10^{-5}$, $5x10^{-5}$ and 10^{-4} mol/l. The total reaction volume of 250 µl was adjusted with HBSS. The assays were run in duplicates. Light emission expressed as relative light units (RLU) was recorded continuously at 37 °C for 60 min. Intensity of the CL reaction is expressed as the integral of the obtained kinetic curves which correspond to the total amount of light produced during the time of measurements.

Determination of nitric oxide

The production of nitric oxide (NO) was estimated indirectly as the accumulation of nitrites (NO_2^-), the metabolic end-product of NO metabolism, in the medium using the Griess reagent as described previously (Migliorini *et al.* 1991). Sodium nitrite was used as a standard. 150 µl of culture supernatant was added to 150 µl of Griess reagent (Sigma-Aldrich, St. Louis, Missouri, USA), then incubated for 15 min in a dark at room temperature and absorbance was measured at 532 nm on a SLT Rainbow spectrophotometer (Tecan, Crailsheim, Germany).

Detection of inducible nitric oxide synthase (iNOS) by Western blot

Cells were washed with cold PBS, scraped and then lysed in the lysis buffer (1 % sodium dodecyl sulphate – SDS, 10^{-1} mol/l Tris pH 7.4, 10 % glycerol, 10^{-3} mol/l sodium ortho-vanadate, 10^{-3} mol/l phenyl-methanesulfonyl fluoride). Protein concentrations were measured with the DC protein assay (Bio-Rad, Hercules, California, USA) using bovine serum albumin as a standard. Equal amounts of proteins (20 µg) were applied on SDS-polyacrylamide gel electrophoresis. Proteins were electrically transferred from the gel to a nitrocelulose membrane and immunoblotted with rabbit antiserum against the murine iNOS (Transduction Lab, Lexington, Kentucky, USA). Horseradish peroxidase-conjugated with anti-rabbit IgG antibody was used as a secondary antibody. The blots were visualized using ECL+ kit (Amersham, Arlington Heights, Illinois, USA) and exposed to CP-B X-ray films (Agfa, Brno, Czech Republic).

Viability

Viability was set using ATP kit SL (BioThema AB, Hanince, Sweden) on microtitre plate Luminometer LM-01T (Immunotech, Prague, Czech Republic).

Statistical analysis

All experiments were done in duplicates and repeated six times. All data are reported as means ± S.E.M. The data were analyzed using the non-parametric Wilcoxon test or one-way analysis of variance (ANOVA) using Statistica for windows 5.0 (Statsoft, USA). $P \leq 0.01$ value was considered to be significant.

Results

Immediate effect of polyphenols

The total antioxidant capacity of four polyphenols studied (CAT, EPI, QUE and RES) measured as the ability to scavenge chemicaly generated peroxyl radical is shown in Figure 1. All polyphenols showed a significant antioxidant capacity, which increased in a dose-dependent manner. Flavonoids (CAT, EPI, QUE) were observed to show an approximately fourfold higher antioxidant capacity than hydroxystilbene RES.

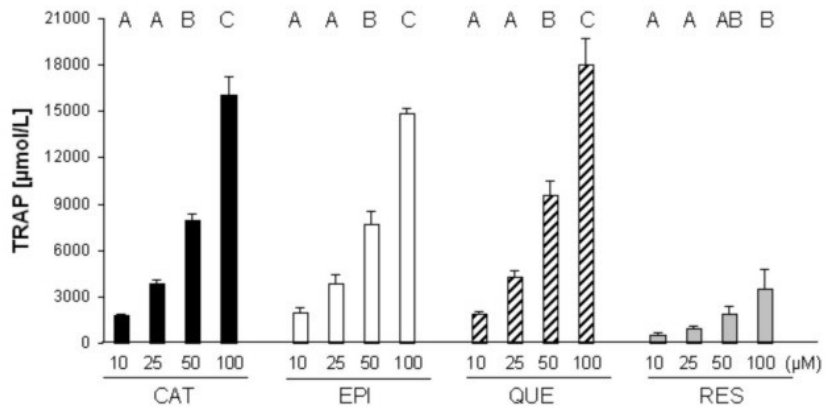

Fig. 1. Total antioxidant capacity of polyphenols. TRAP is expressed as µmol of the peroxyl radical trapped per one liter of polyphenolic solution. The changes of the parameter were found to be significant at the level of p = 0.01 using ANOVA test. Statistically significant contrasts between the values are marked by different capital letters.

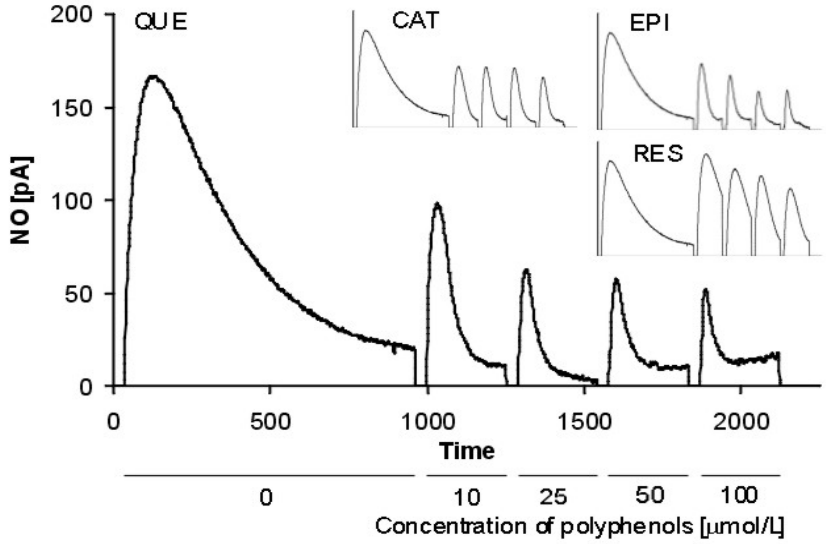

Fig. 2. The ability of polyphenols to scavenge NO determined ampero-metrically in a chemical system. The concentration of NO is expressed as a redox current in picoampers (pA).

Similar results were obtained when the ability of tested compounds to scavenge nitric oxide was evaluated. Flavonoids (CAT, EPI, and especially QUE) were observed to markedly scavenge NO. However, resveratrol scavenged NO only mildly in the highest tested concentration (Fig. 2).

The ability of polyphenols to reduce the oxidative stress caused by biologically generated radicals was studied using murine macrophages RAW 264.7. All polyphenols dose-dependently inhibited the chemiluminescence produced by the PMA-stimulated RAW 264.7 (Fig. 3). The effect of RES was the highest among all compounds tested, because even the lowest concentration markedly inhibited chemiluminescence. Thus, RES seems to be a more potent inhibitor of the oxidative burst than flavonoids.

Long-term effect of polyphenols

LPS (10^{-4} g/l) increased the ROS production by 190 % (51.2 x 10^4 vs. 17.6 x 10^4 RLU in untreated controls), as measured by PMA-activated CL. CAT, EPI and RES did not have any inhibitory effect on ROS production (data not shown). Conversely, QUE significantly decreased the ROS production of LPS-stimulated RAW 264.7 in a dose-dependent manner (Fig. 4A).

LPS evoked a 30-fold induction of nitrite production as opposed to the untreated control ($34.4x10^{-6}$ vs. $1.1x10^{-6}$ mol/l NO_2^-/$2x10^6$ cells, respectively). This induction was inhibited by QUE ($2.5x10^{-5}$-10^{-4} mol/l) treatment in a dose-dependent manner (Fig. 4B). RES at concentrations of $5x10^{-5}$ and 10^{-4} mol/l cut the NO production by 7 and 26 %, respectively. However, no significant inhibition by CAT and EPI was found (data not shown).

The cytotoxicity of polyphenols in RAW 264.7 cells was examined by luminometrical detection of ATP concentration. None of the polyphenols affected the

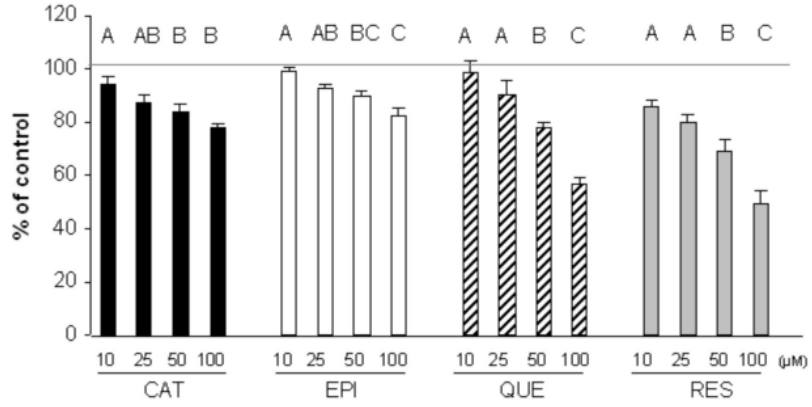

Fig. 3. Activity of polyphenols to scavenge reactive metabolites produced by RAW 264.7 cells (measured by chemiluminiscence). The changes of the parameter were found to be significant at the level of $p \leq 0.01$ using Wilcoxon test. Statistically significant difference between the values are marked by different capital letters.

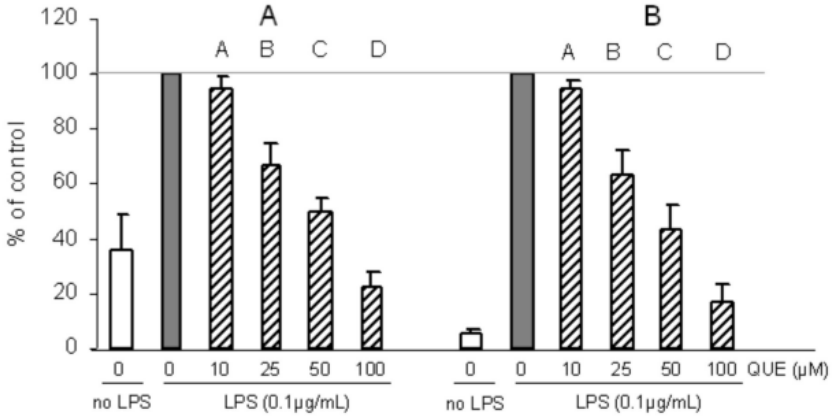

Fig. 4. The effect of quercetin (QUE) on chemiluminescence activity and nitrite production by RAW 264.7 cells. Cells were pretreated with the indicated concentrations of QUE for 1 h before being incubated with LPS (10^{-4} g/l) for 24 h. Control cells were incubated with vehicle alone. The changes of the parameter were found to be significant at the level of $p \leq 0.01$ using Wilcoxon test. Statistically significant contrasts between the values are marked by different capital letters. (A) Inhibition of reactive metabolite production by QUE in LPS-stimulated RAW 264.7 macrophages. Before measurement, supernatant was removed and cells were washed twice with PBS. Phorbol-myristate acetate was used to activate the oxidative burst of RAW 264.7 measured by chemiluminiscence. (B) Inhibition of nitrite production by QUE in LPS-stimulated RAW 264.7 cells. The cultured supernatants were subsequently isolated and analyzed for nitrite production.

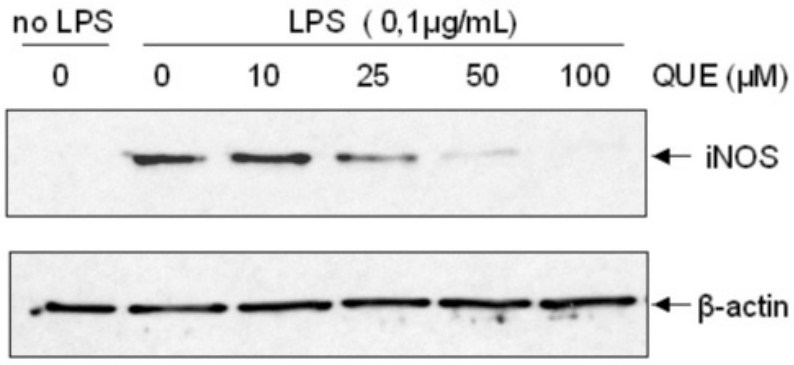

Fig. 5. Inhibition of iNOS protein expression by QUE. Cultures were set up as described in the legend to Fig. 4. Equal loading of proteins was verified by β-actin immunoblotting. One of three representative experiments is shown.

viability of RAW 264.7 cells (data not shown).

In view of the involvement of iNOS in the inflammatory process, we monitored iNOS protein expression by Western blot in RAW 264.7 exposed to polyphenols. As shown in Figure 5, expression of the iNOS protein was not detectable in unstimulated cells, but markedly increased 24 h after LPS (10^{-4} g/l) treatment. Treatment with QUE showed a concentration-dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells. CAT, EPI and RES did not influence iNOS expression in LPS-activated RAW 264.7 cells (data not shown).

Discussion

In our paper, four wine polyphenols (CAT, EPI,

QUE and RES) were studied from the viewpoint of their antioxidant capacity, their ability to scavenge biologically produced ROS and RNS and their influence on nitric oxide production and iNOS expression. Concentrations of polyphenols used in our experiments ranged between 10^{-4} – 10^{-5} M which corresponded to the red wine content of individual compounds: 191 mg/l (6 x 10^{-4} M) CAT, 82 mg/l (3x10^{-4} M) EPI, 8-16 mg/l (2.5-5.0 x 10^{-5} M) QUE, and 1-8 mg/l (0.5-3.5 x 10^{-5} M) RES (Pendurthi and Rao 2002). The knowledge of absorption, biodistribution and metabolism of polyphenols is partial and incomplete. Some polyphenols are bioactive compounds that are absorbed from the gut in their native or modified form. They are subsequently metabolized to products detected in the plasma and then excreted. At concentrations found *in vivo* in human plasma (low nanomolar range), the effect of polyphenols is negligible in comparison with endogenous protection mechanisms against oxidative stress (Huisman *et al*. 2004).

In agreement with other authors (Cuendet *et al.* 2000, Nakao *et al.* 1998, Scott *et al.* 1993) we observed a significant antioxidant capacity against peroxyl radical of all studied polyphenols. Some authors have even found that QUE and CAT showed a greater efficacy to scavenge peroxyl radical on a mole to mole basis than the antioxidant nutrients vitamin C, vitamin E, and beta-carotene (Rice-Evans 1995). In our study, flavonoids (CAT, EPI, QUE) showed an approximately four-fold higher ability to scavenge peroxyl radical than hydroxystilbene RES. The antioxidant potential of polyphenols depends on the number and arrangement of the hydroxyl groups and the extent of structure conjugation (Robak *et al.* 1988). They can donate hydrogen atom from their hydroxyl groups and stabilize the phenoxy radical formed by delocalization of the unpaired electron within the aromatic structure. It is well-known that aromatic compounds containing hydroxyl groups, especially those having an O-dihydroxy group on ring B, appear to be important scavengers as reported for flavonoids (Fauconneau *et al.* 1997). RES, a stilbene, is also known to have a strong antiradical activity, which is due to the presence of a conjugated double bond, which makes the electrons more delocalized (Khanduja and Bhardwaj 2003). The higher TRAP of flavonoids found in our experiments may be caused by the fact that flavonoids contain multiple hydroxyl groups (five OH groups) in comparison to RES (three OH groups), which are able to donate hydrogen atoms to peroxyl radicals and so have a greater potential to act as a scavenger of peroxyl radicals. This is also supported by the findings of López *et al.* (2003), who reported QUE to have higher antioxidant capacity against peroxyl radical than RES. On the other hand, Yilmaz and Toledo (2004) found the RES to be a more potent scavenger of chemically generated peroxide radicals than CAT and EPI.

Since cellular systems generate a variety of radicals including superoxide, hydroxyl and peroxyl radicals, nitric oxide and peroxynitrite, other experiments were performed to demonstrate the ability of flavonoids and RES to scavenge biologically generated radicals.

All tested compounds dose-dependently scavenged the free radicals that were produced by the PMA-stimulated RAW 264.7 cells. RES, interestingly, seems to be a more potent scavenger, although its TRAP was the lowest. The efficiency of individual polyphenols to scavenge peroxyl radical and inhibit the oxidative burst in macrophages was not always the same because of a different specificity of a polyphenol to scavenge peroxyl radical particularly and other free radicals involved in the process of an oxidative burst of macrophages. The TRAP test provides information on the reactivity of phenolic compounds with only one radical in a buffered solution. On the other hand, in the PMA-stimulated macrophage CL assay, a combination of many ROS is present. Moreover, the mechanisms of flavonoid actions may differ from that of stilbenes. In addition to their antioxidative properties, some polyphenols act as metal chelating agents and inhibit the superoxide-derived Fenton reaction, which is an important source of the most reactive hydroxyl radicals. Various authors consider chelation of metal ions as the main mechanism of polyphenolic action (Iwahashi 2000, Morel *et al.* 1994), while others consider ROS scavenging to dominate in the antioxidant effects (Fremont *et al.* 1999). Belguendouz *et al.* (1997) reported that RES protects LDL against peroxidative degradation mainly by chelating copper whereas flavonoids are better scavengers of free radicals. The ability to inhibit the Fenton reaction could explain the effectiveness of RES against biologically generated radicals in contrast to the system, where radicals are chemically produced in a copper-free buffered solution.

All phenomena previously described are responsible for a short-term response mediated by wine polyphenols. Recent studies have pointed out that polyphenolic compounds from several sources may also have long-term effects, as they are able to modulate gene expression in different transformed cell lines and in macrophages (Dell'Agli *et al.* 2004). We used a bacterial

LPS for the induction of inflammation processes in RAW 264.7. LPS, as an outer membrane component of bacteria, triggers the generation of reactive oxygen intermediates as well as the secretion of a variety of inflammatory mediators, such as nitric oxide. In our experiment, a 190 % increase in ROS production was observed when RAW 264.7 cells were incubated with LPS (24 h) in comparison with an untreated control. The incubation of cells with QUE diminished their LPS-activated production of ROS in a concentration-dependent manner (Fig. 4A). We found that QUE, in a concentration of 10^{-4} mol/l, reduced the ROS release even to the level of non-LPS-treated cells. Conversely, CAT, EPI and RES did not decrease the amount of ROS produced by activated macrophages. This indicates that QUE is the most effective modulator of oxidative stress in the long-term.

The large amount of NO produced in response to bacterial lipopolysaccharide plays an important role in endotoxemia and inflammatory conditions (Bellot *et al.* 1996). Therefore, drugs that inhibit NO generation by inhibiting iNOS expression or its enzyme activity may be beneficial in treating diseases caused by an overproduction of NO (Stoclet *et al.* 1998). To investigate the effect of polyphenols on NO production, we measured the accumulation of nitrite, the stable metabolite of NO, in culture media. We found a huge NO release by LPS-stimulated cells as opposed to untreated control. The results are in full agreement with the data of others (Shen *et al.* 2002, Wadsworth and Koop 1999, Wadsworth *et al.* 2001).

When LPS-activated cells were incubated with QUE or RES, significant and dose-dependent decrease of the NO production was observed after 24 h. It seems likely that QUE and RES inhibit NO production by several mechanisms. Suppression of NO release may be attributed to direct NO scavenging activity, which was previously suggested to be due to their ability to scavenge an exogenous NO donor sodium nitropruside (SNP) *in vitro* (Chan *et al.* 2000). Direct NO scavenging activity of studied flavonoids was also proved in our experiments. Another possible mechanism is a modulation of iNOS protein expression. To investigate, whether QUE and RES are able to decrease iNOS protein expression, Western blot analysis of RAW 264.7 exposed to LPS and one of these polyphenols was monitored. As shown in Figure 5, expression of the iNOS protein was not detectable in unstimulated cells, but markedly increased 24 h after LPS treatment. Treatment with QUE showed a concentration-dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells. This finding is in agreement with results of Jung and Sung (2004) who found an inhibitory effect of 5 μmol/l QUE on the expression of iNOS and COX-2 enzymes in lipopolysaccharide-activated RAW 264.7 cells. The results of Kim *et al.* (1999) indicated that the inhibitory activity of studied flavonoids was not due to direct inhibition of iNOS enzyme activity as measured by [^{3}H]citrulline formation from [^{3}H]arginine. The regulation of iNOS expression is complex, but appears to occur primarily at the level of transcription. Activation of mitogen-activated protein kinases (MAPKs) and the redox-sensitive transcription factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are key events in the signal transduction pathways mediating iNOS induction in macrophages exposed to LPS (Sherman *et al.* 1993, Xie *et al.* 1994). Wadsworth *et al.* (2001) have found that QUE inhibited p38 MAPK activity and caused the inhibition of iNOS mRNA expression which resulted in decreasing iNOS protein level and NO release in LPS-stimulated RAW 264.7 macrophages. It is not clear whether QUE acts more by inhibiting iNOS expression or by direct scavenging of NO. Chan *et al.* (2000) speculate that QUE and RES may act more by scavenging of NO radicals than by inhibition of iNOS gene expression. The rationale for this deduction is that these compounds were not very effective in reducing iNOS mRNA, while they readily scavenged NO produced by SNP. In our study, inhibition of iNOS protein expression was in parallel with the comparable inhibition of NO production, thus agreeing with the results obtained by Chen *et al.* (2001), Chan *et al.* (2000) and Wadsworth *et al.* (2001). Therefore we propose that the suppression of NO by QUE was mainly mediated by inhibition of iNOS protein expression. As stated earlier, NO plays an important role in the pathogenesis of various inflammatory diseases. Therefore, the inhibitory effect of QUE on iNOS gene expression suggests that this is one of the mechanisms responsible for the anti-inflammatory action of QUE.

Although RES at higher concentrations decreased NO production, it did not influence iNOS expression in LPS-activated RAW 264.7 cells. Our results agree with those of Cho *et al.* (2002), who found that a higher concentration of RES than needed for the inhibition of NO production was required for the iNOS expression and NF-κB translocation. In contrast, resveratrol in a concentration of 30 μmol/l was found to reduce the amount of cytosolic iNOS protein and steady state mRNA levels (Tsai *et al.* 1999), probably due to

inhibition of phosphorylation as well as degradation of IκBα (inhibitory protein bound to NF-κB in its unstimulated form in the cytosol), and a reduced nuclear content of NF-κB subunits. Thus, resveratrol (and other polyphenolic compounds) may inhibit the enhanced expression of iNOS in inflammation through down-regulation of NF-κB binding activity. Bi *et al.* (2005) reported that despite the inhibition of LPS-induced degradation of IκBα, resveratrol may inhibit iNOS expression also *via* suppression of p38 MAPK in microglial cells. The speculation that RES may act more by scavenging of NO radicals and suppressing the generation of RNS than by the inhibition of iNOS gene expression (Chan *et al.* 2000, Manna *et al.* 2000) was not proved in our experiments since RES exhibited only minor NO-scavenging activity in the highest used concentration. Despite that, resveratrol was previously referred to inhibit neutrophil generation of oxidants like superoxide anion and hypochlorous acid (Cavallaro *et al.* 2003).

In conclusion, this study provides the evidence for *in vitro* antioxidative effects of wine polyphenols (CAT, EPI, QUE and RES). It seems that the higher number of hydroxyl substituents is an important structural feature of flavonoids (QUE, CAT, EPI) when compared to hydroxystilbenes (RES) in respect to their direct scavenging activity against ROS and RNS while C-2,3 double bond (present in QUE and RES) might be important for inhibition of ROS and NO production. Only QUE significantly decreased the ROS and NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner due to its unique chemical structure.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Regular paper

Evaluation of antioxidant activity of medicinal plants containing polyphenol compounds. Comparison of two extraction systems

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This study investigates the influence of extraction system on the extractability of polyphenol compounds and antioxidant activity of various medicinal plants. Oxygen radical absorbance capacity (ORAC) and total polyphenol content of 25 Bulgarian medicinal plants subjected to water or 80% acetone extractions were investigated and compared. The type of extragent significantly influenced the efficiency of the polyphenol extraction and the antioxidant activity. In all cases ORAC results and total polyphenol content were higher for acetone extraction than for water extraction. The acetone extract of peppermint had the highest ORAC value — 2917 μmol Trolox equivalent (TE)/g dry weight (DW) and polyphenol content — 20216 mg/100 g DW. For water extraction thyme exhibited the highest ORAC antioxidant activity — 1434 μmol TE/g DW. There was a significant linear correlation between the concentration of total polyphenols and ORAC in the investigated medicinal plants. It can be concluded that the solvent used affects significantly the polyphenol content and the antioxidant activity of the extract and therefore it is recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products. Several of the investigated herbs contain substantial amounts of free radical scavengers and can serve as a potential source of natural antioxidants for medicinal and commercial uses.

Keywords: medicinal plants, ORAC, polyphenols

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INTRODUCTION

A growing amount of evidence indicates a role of reactive oxygen species (ROS) such as peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) in the pathophysiology of aging and different degenerative diseases such as cancer, cardiovascular diseases, Alzheimer's disease and Parkinson's disease (Davies, 2000; Fenkel & Holbrook, 2000). Living cells possess a protective system of antioxidants which prevents excessive formation and enables the inactivation of ROS. The antioxidants protect from the potentially damaging oxidative stress, which is a result of an imbalance between the formation of ROS and the body antioxidant defense. Antioxidants have also been used in food industry to prevent deterioration, nutritional losses and off-flavoring in various foods, especially those containing polyunsaturated fatty acids. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods because of their potential in health promotion and disease prevention, and their high safety and consumer acceptability (Gorinstein *et al.*, 2003).

In search of novel sources of antioxidants in the last years, medicinal plants have been extensively studied for their antioxidant activity. From ancient times, herbs have been used in many areas, including nutrition, medicine, flavoring, beverages, cosmetics, etc. The ingestion of fresh fruit, vegetables and tea rich in natural antioxidants has been associated with prevention of cancer and cardiovascular diseases (Willcox *et al.*, 2004). The higher intake of plant foods correlates with lower risk of mortality from these diseases (Johnson, 2001). Approximately 60% of the commercially available anti-tumoral and anti-infective agents are of natural origin (Cragg *et al.*, 1997).

Polyphenols are the most significant compounds for the antioxidant properties of plant raw materials. The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin (Rice-Evans *et al.*, 1995; 1997; Prior *et al.*, 2005; Lopez *et al.*, 2007; Ciz *et al.*, 2008; Gebicka & Banasiak, 2009).

Investigation of natural products is a research field with great potential and is especially important in countries possessing great biodiversity, like Bulgaria. About 600 plant species from the Bulgarian flora are recognized as medicinal and are traditionally used in ethnopharmacology and phytotherapy (Dimkov, 1979; Petkov, 1982). There are many reports in the literature about the antioxidant properties of medicinal plants (Zheng & Wang, 2001; Djeridane *et al.*, 2006; Katalinic *et al.*, 2006; Wojdylo *et al.*, 2007), but there are only few papers reporting data about the antioxidant properties of Bulgarian herbs using methods such as DPPH and ABTS (Ivanova *et al.*, 2005; Kiselova *et al.*, 2006). The current study employs the oxygen radical absorbance capacity (ORAC) method, which has been found to be the most relevant one for biologic samples (Wang *et al.*, 2004; Huang *et al.*, 2005; Prior *et al.*, 2005**)**. Different extraction systems were used to extract antioxidant components from the plant material and often it is difficult to compare the results for the antioxidant properties even for the same plant material. Water (Zheng & Wang, 2001; Ivanova *et al.*, 2005; Katalinic *et al.*, 2006; Kiselova *et al.*, 2006), metha-

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Abbreviations: AAPH, 2,2-azobis-(2-amidino-propane)dihydrochloride; ABTS, 2,2'azinobis(3-ethylbenzothiazoline-6-sulfonic acid; AUC, area under the curve; SD, standard deviation; DW, dry weight; FL, fluorescein; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents.

Table 1. Medicinal plants commonly used in traditional medicine

Botanical name	Family	Common name	Part of plant used	Medical use
Achillea millefolium	*Asteraceae*	Yarrow	Flowers	Antiseptic, anti-inflammatory, stomach ulcer, gastrointestinal disorders, liver diseases
Arctium lappa	*Asteraceae*	Greater burdock	Roots	Diuretic, kidney stones, rheumatism, gastritis, stomach ulcer, gout
Betula pendula	*Betulaceae*	Birch	Leaves	Diuretic, kidney disorders, bladder disorders
Calendula officinalis	*Asteraceae*	Marigold	Flowers	Anti-inflammatory, pain-relieving, local treatment of wounds, duodenum and stomach ulcer, gastrointestinal disorders
Cichohrium intybus	*Asteraceae*	Chicory	Aerial parts	Digestive, chologogue, liver diseases
Clinopodium vulgare	*Labiatae*	Wild basil	Leaves	Immunostimulant, cardio-tonic, verruca.
Crataegus monogyna	*Rosaceae*	Hawthorn	Flowers, leaves	Promotes capillary formation and heart microcirculation, cardiovascular diseases, ischemia
Glycyrrhiza glabra	*Fabaceae*	Liquorice	Roots	Adaptogen, anticancer
Humulus lupulus	*Cannabaceae*	Hop	Flowers	Sedative, digestive, menstrual disorders
Hypericum perforatum	*Hypericaceae*	St. John's wort	Aerial parts	Anti-inflammatory, astringent, antibacterial, diuretic, ulcer, colitis, gastritis
Laurus nobilis	*Lauraceae*	Laurel leaves	Leaves	Immunostimulant, antidiabetic, stomatitis, sinusitis
Matricaria chamomilla	*Asteraceae*	Chamomile	Flowers	Anti-inflammatory, antiseptic, sedative, throat and mouth inflammations, gastrointestinal disorders, influenza, pharyngitis, laryngitis
Melissa officinalis	*Labiatae*	Common balm	Leaves	Sedative, gastrointestinal disorders
Mentha piperita	*Labiatae*	Peppermint	Leaves	Spasmolytic, antiseptic, gastric disorders, indigestion, neuralgia, myalgia, antivomiting
Mentha spicata	*Labiatae*	Spearmint	Leaves	Hormone regulating, spasmolytic, antiseptic, gastric disorders, indigestion, neuralgia, myalgia
Ocimum basilicum	*Labiatae*	Basil	Leaves	Antiseptic, spasmolytic, expectorant gastrointestinal diseases, antitussive
Rubus idaeus	*Rosaceae*	Raspberry	Leaves	Anti-inflammatory, antiseptic, antidiarrheic, gastrointestinal disorders
Salvia officinalis	*Labiatae*	Sage	Leaves	Anti-inflammatory, antiseptic, inflammations of throat and mouth
Sideritis scardica	*Labiatae*	Mountain tea	Aerial parts	Expectorant, antitussive, bronchitis, cough
Taraxacum officinale	*Asteraceae*	Dandelion	Aerial parts	Diuretic, cholagogue, appetizer
Thymus vulgaris	*Labiatae*	Thyme	Aerial parts	Expectorant, spasmolytic, antibacterial, antitusive, asthma, emphysema, whooping-cough, diseases of respiratory tract
Tilia cordata	*Tiliaceae*	Lime	Flowers	Anti-inflammatory, expectorant
Tribulus terrestris	*Zygophyllaceae*	Caltrop	Aerial parts	Hormone regulating, sperm promoting, prevents ovary cysts
Trigonella foenum-graecum	*Fabaceae*	Fenugreek	Seeds	Anticancer, metabolic syndrome and diabetes
Urtica dioica	*Urticaceae*	Nettle	Leaves	Hormone regulating, prostate cancer prevention, podagra, diabetes, allergies, anaemia

nol (Shan *et al.*, 2005; Wojdylo *et al.*, 2007) and ethanol (Djeridane *et al.*, 2006) have been widely used. In very few cases only, more than one extragent or sequential multi-solvent extractions were preferred (Su *et al.*, 2007; Wojcikowski *et al.*, 2007). It has been recognized that the extraction solvent may significantly alter the antioxidant activity estimation (Zhou & Yu, 2004). In the present work, two extragents were used for the extraction of plant antioxidants — water and 80% acetone. Plant extracts made with water are nutritionally more relevant, moreover, herbs are traditionally ingested as hot-water infusions. On the other hand, acetone is preferred for more exhaustive extraction of polyphenol compounds and it was of particular interest to compare the polyphenol content and ORAC antioxidant activity in water infusions and acetone extracts.

The objective of the current study was to investigate the influence of the extraction agent on the extractability of polyphenol components and the antioxidant activity of 25 Bulgarian medicinal plants. These two parameters were evaluated in water and 80% acetone extracts of plants. Results from this study will lead to a better characterization of the antioxidant properties of the medici-

nal plants investigated and will reveal which of them are the best sources of dietary antioxidants.

MATERIALS AND METHODS

Chemicals. Fluorescein disodium salt, 2,2-azobis-(2-amidino-propane)dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and gallic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). All other solvents used were of analytical grade and purchased from local distributors.

Plants. All medicinal plants used were either obtained from local pharmacies (Plovdiv, Bulgaria) or collected from nature in 2008. The choice of the plants investigated was based on their use in the traditional medicine. In total, 25 medicinal plants were investigated (Table 1).

Plants were dried, packed in paper bags and stored at ambient temperature prior to the analysis.

Extraction. All plant materials were subjected to extractions with acetone and with water. For the acetone extraction, 10 g of the plant material was powdered in a laboratory mill, then 0.5 g of the powder was transferred into extraction tubes and mixed with 20 ml of the extragent (80% acetone in 0.2% formic acid). Extraction was conducted on an orbital shaker at room temp. for one hour. After that, the samples were centrifuged (6000×*g*) and supernatants were removed. The solid residue was subjected to the second extraction under the same conditions. Both supernatants were combined and analyzed for antioxidant activity and total polyphenol content.

Water infusions were prepared in compliance with the traditional preparation which is close to home conditions. For that purpose 5 g of the herb powder was added to 200 ml water (90°C). Aerial parts of the plants were incubated for 15 min, whereas roots were incubated for 45 min. The slurry was centrifuged (6000×*g*) and supernatants were used for further analysis.

ORAC assay. ORAC was measured according to the method of Ou *et al.* (2001) with some modifications (Ciz *et al.*, 2010). The method measures the antioxidant scavenging activity against peroxyl radical generated by thermal decomposition of AAPH at 37°C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) relative to that of a blank in which no antioxidant has present. Solutions of AAPH, fluorescein and Trolox were prepared in a phosphate buffer (75 mmol/l, pH 7.4). Samples were diluted in the phosphate buffer as well. Reaction mixture (total volume 200 µl) contained FL — (170 µl, final concentration 5.36×10^{-8} mol/l), AAPH — (20 µl, final concentration 51.51 mmol/l), and sample — 10 µl. The FL solution and sample were incubated at 37°C for 20 min directly in a microplate reader, and AAPH (dissolved in buffer at 37°C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 µl of phosphate buffer was used instead of the extract. The antioxidant activity was expressed in micromole Trolox equivalents per gram of dry weight (DW). Trolox solutions (6.25, 12.5, 25, 50 and 100 µmol/l) were used for defining the standard curve.

Table 2. Antioxidant activity of 25 medicinal plants.
Comparison between 80% acetone (ac) and water (w) extraction. Results are presented as mean ± S.D.

Medicinal plant	$ORAC_{ac}$ µmol TE/g	$ORAC_{w}$ µmol TE/g	Ratio $ORAC_{w}/ORAC_{ac}$ %
Peppermint	2917±52	1409±62	48.3
Hawthorn	2163±89	364±28	16.8
Thyme	1637±59	1434±54	87.6
Wild basil	1437±60	844±41	58.7
Birch	1185±73	142±18	12.0
Raspberry	1156±80	608±35	52.6
St. John's wort	1141±93	629±41	55.1
Common balm	1121±60	996±26	88.8
Lime	1020±88	97±11	9.5
Sage	966±69	609±54	63.0
Yarrow	842±80	394±30	46.8
Laurel leaves	837±81	170±12	20.3
Caltrop	819±56	272±16	33.2
Camomile	814±72	469±25	57.6
Mountain tea	778±77	294±21	37.8
Hop	749±62	260±19	34.7
Spearmint	748±57	598±31	79.9
Liquorice	670±48	213±12	31.8
Marigold	407±57	247±17	60.7
Basil	402±40	271±18	67.4
Chicory	398±22	132±14	33.2
Dandelion	381±16	193±16	50.7
Greater burdock	365±31	323±20	88.5
Fenugreek	327±28	320±12	97.9
Nettle	162±11	141±10	87.0

Total polyphenol compounds analysis. Total polyphenols were determined according to the method of Singleton and Rossi (1965) with Folin-Ciocalteu's reagent. Gallic acid was employed as calibration standard and results were expressed as gallic acid equivalents (GAE) per 100 g DW.

RESULTS AND DISCUSSION

It is of particular interest to investigate the antioxidant properties of medicinal plants, especially those traditionally used in folk medicine. More than one extraction system is recommendable for detailed assessment of the antioxidant properties of medicinal plants. It was found in a recent study by Su *et al.* (2007) that the ORAC values of acetone extracts were higher than those for methanolic extracts for several herbs. Therefore, aiming at the maximum extractability of the polyphenol compounds, we chose to extract raw materials with acetone. On the other hand, the traditional ingestion of medicinal plants and their clinical usage usually requires their extraction with water. Table 2 shows the ORAC antioxidant activity of the investigated medicinal plants extracted by acetone and water ($ORAC_{ac}$ and $ORAC_{w}$, respectively).

Table 3. Polyphenol content of 25 medicinal plants.
Comparison between 80% acetone (ac) and water (w) extraction. Results are presented as mean ± S.D.

Medicinal plant	Polyphenols$_{ac}$ mg/100g	Polyphenols$_{w}$ mg/100g	Ratio PF$_{w}$/ PF$_{ac}$ %
Peppermint	20216 ± 359	9356 ± 204	46.3
Hawthorn	7104 ± 111	1903 ± 181	26.8
Thyme	11409 ± 171	8583 ± 241	75.2
Wild basil	9468 ± 128	4645 ± 201	49.1
Birch	5542 ± 201	1197 ± 124	21.6
Raspberry	7759 ± 216	4932 ± 164	63.6
St. John's wort	11283 ± 74	6428 ± 152	57.0
Common balm	11885 ± 109	8240 ± 207	69.3
Lime	9296 ± 427	787 ± 43	8.5
Sage	5295 ± 148	3845 ± 65	72.6
Yarrow	5728 ± 232	1968 ± 84	34.4
Laurel leaves	7081 ± 299	1766 ± 52	24.9
Caltrop	5681 ± 200	2790 ± 101	49.1
Camomile	4665 ± 137	1790 ± 45	38.4
Mountain tea	3984 ± 201	2044 ± 21	51.3
Hop	5728 ± 262	1697 ± 25	29.6
Spearmint	4522 ± 102	3713 ± 46	82.1
Liquorice	3452 ± 98	1548 ± 72	44.8
Marigold	2141 ± 115	1537 ± 33	71.8
Basil	2391 ± 38	1816 ± 52	76.0
Chicory	1821 ± 63	786 ± 46	43.2
Dandelion	2206 ± 58	1577 ± 51	71.5
Greater burdock	2742 ± 112	2531 ± 68	92.3
Fenugreek	1692 ± 105	1445 ± 41	85.4
Nettle	958 ± 43	776 ± 43	81.0

Since polyphenols significantly contribute to the overall antioxidant activity, it was reasonable to determine their total amount in the selected medicinal plants. The total polyphenol content in the medicinal plants is shown in Table 3. It is evident that in all cases the ORAC values and total polyphenol content obtained with acetone extraction were higher than the respective results for water extraction. The observed differences could be explained by the different polarity of the polyphenol compounds present in the investigated medicinal herbs. This observation complies with the findings of Wojcikowski *et al.* (2007) who used sequential three-solvent extraction for herb polyphenols. The antioxidant activity of the samples varied significantly for both acetone and water extracts in our study. The greatest ORAC$_{ac}$ value was found in peppermint, while the highest ORAC$_{w}$ value was found in thyme, followed by peppermint. Since the ORAC method is preferred for the measurement of the antioxidant activity of foods and biological samples, it is surprising that in the literature there are ORAC data just for several of the medicinal plants investigated in the current study. Ninfali *et al.* (2005) performed a comprehensive evaluation of different foods and spices using the ORAC method. On the basis of fresh weight, they reported ORAC values for thyme (274.26 μmol TE/g), sage (320.04 μmol TE/g) and common balm (59.97 μmol TE/g). As the reported data are based on fresh weight, it is difficult to compare them with our results. Zheng and Wang (2001) determined ORAC of five herbs from the current study, but again the results were expressed on the basis of fresh weight. Moreover, they used R-phycoerythrin as a fluorescent agent, which could significantly alter the ORAC results in their study (Ou *et al.*, 2001). In another recent study, Wojcikowski *et al.* (2007) investigated the ORAC antioxidant activity of 55 medicinal plants after sequential three-solvent extraction. Since this presumes very exhaustive extraction, it can explain the higher ORAC values obtained by them for several herbs from our study: liquorice — 1029 μmol TE/g (670 μmol TE/g in our study), basil — 524.7 μmol TE/g compared with 402 μmol TE/g, and nettle — 430.4 μmol TE/g against 162 μmol TE/g. Despite the more exhaustive sequential extraction, three herbs in our work showed ORAC values several times higher

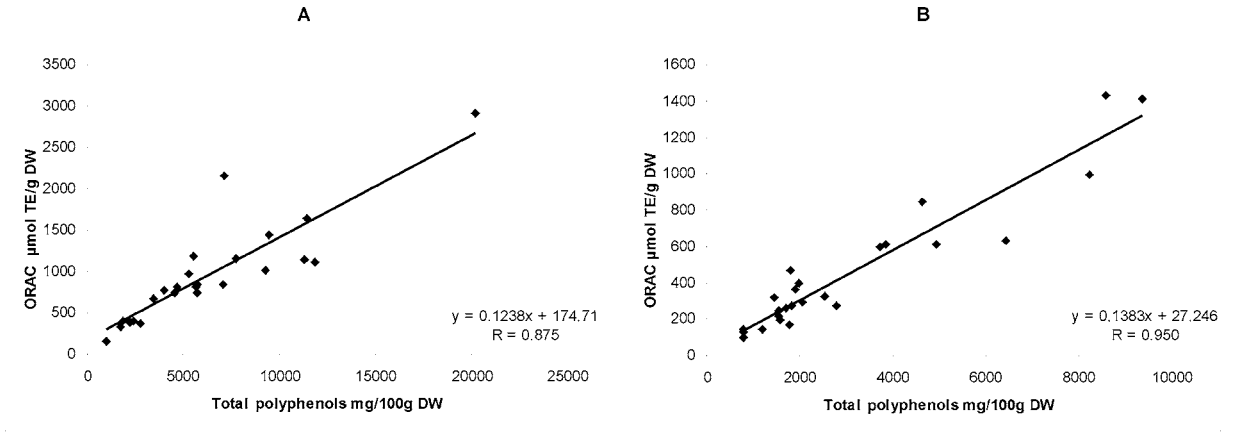

Figure 1. Correlation between total phenolic content and ORAC antioxidant activity in acetone (A) and water (B) extracts obtained from 25 medicinal plants.

than the ones in the Wojcikowski's study. For example, our results for yarrow — 842 μmol TE/g, sage — 966 μmol TE/g and dandelion — 381 μmol TE/g were 3.2-fold, 2.7-fold and 3.9-fold higher, respectively, than the ORAC values for the same herbs in the above-mentioned paper. The differences in the antioxidant activity between the same materials can be attributed to some environmental factors such as climate, location and temperature which can significantly affect the accumulation of the antioxidant components in plant material. From the investigated 55 herbs in the same study, the root of black cohosh (*Cimisifuga racemosa*) showed the highest ORAC value of 1264.9 μmol TE/g. In our study, four medicinal plants (peppermint, hawthorn, thyme and wild basil) revealed higher antioxidant activity, and another four (birch, raspberry, St. John's wort and common balm) showed comparable ORAC values to that result. Our study reports the ORAC values of antioxidant capacity of several plants for the first time — those for wild basil (*Clinopodium vulgare*) leaves, birch (*Betula pendula*) leaves, caltrop (*Tribulus terrestris*) aerial parts, mountain tea (*Sideritis scardica*) aerial parts, hop (*Humulus lupulus*) flowers, marigold (*Calendula officinalis*) flowers and greater burdock (*Arctium lappa*) roots.

Several studies have investigated the relationship between the antioxidant activity and the content of polyphenol compounds in herbs. Some authors have reported good linear correlation between these two parameters (Zheng & Wang 2001; Shan *et al.*, 2005; Djeridane *et al.*, 2006; Katalinic *et al.*, 2006), whereas others have not observed such correlation (Kahkonen *et al.*, 1999). Figure 1 depicts the correlation between the total polyphenols and the ORAC values of the medicinal plants investigated in our study. The correlation coefficient between ORAC and total polyphenol content was $R = 0.875$ for acetone extracts and $R = 0.950$ for water extracts. These correlations suggest that the ORAC antioxidant activity could be attributed to the polyphenol compounds. However, there are several discrepancies in the correlation. Such an example is hawthorn whose high ORAC value does not match its low polyphenol content. Several explanations could be used to account for that. First, it has been reported that polyphenol compounds differ significantly in their antioxidant properties which are determined by several structural features of the polyphenol molecule (Ou *et al.*, 2002). Second, the investigated medicinal plants probably contain other substances with antioxidant effect apart from the polyphenols. Moreover, the amount of polyphenols does not represent the potential synergism or antagonism between the individual compounds in the samples, which depends on their structure and mutual interactions.

A recent study by Prior *et al.* (2007) demonstrated that the consumption of certain foods was associated with increased plasma ORAC in the postprandial state, while the consumption of an energy source of macronutrients containing no antioxidants was associated with a decline in the plasma antioxidant capacity. The authors estimated that according to the energy intake of the diet, 5000–15000 μmol TE are necessary to supply daily human antioxidant needs. The ORAC values reported in the current study are several times higher than the ORAC values of many fruits and vegetables (Ou *et al.*, 2002; Wu *et al.*, 2004; Ciz *et al.*, 2010). This means that the studied medicinal herbs exhibited a higher antioxidant activity and contained more polyphenols than the common vegetables and fruit. In the search for natural antioxidants, herbs turned out to be a suitable source of dietary antioxidants. The differences between the ORAC values and polyphenol content obtained after acetone and water extractions indicate that the traditional way of ingestion of herbs does not fully utilize the available antioxidants in the plant material. These antioxidant compounds could be isolated and then used as antioxidant functional foods (Grajek *et al.*, 2005).

CONCLUSION

It can be concluded that the extracting solvent affects significantly the polyphenol compound content and the antioxidant activity measured and therefore it is recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products. Several of the Bulgarian medicinal plants tested are rich sources of polyphenol compounds and free radical scavengers. Some medicinal plants thus can be considered as promising sources of natural antioxidants for medicinal and commercial uses.

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Different methods for control and comparison of the antioxidant properties of vegetables

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ABSTRACT

The present study investigates the antioxidant properties of selected vegetables, using the total peroxyl radical-trapping parameter (TRAP), oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) methods. ORAC, TRAP and HORAC values well correlated with polyphenol content. A good correlation was found also between the methods for measuring antioxidant capacity. Nevertheless, ORAC has been found to be the most sensitive method to measure chain-breaking antioxidant activity. Although we have found a good correlation between TRAP, ORAC and HORAC, using more than one antioxidant assay is recommended for more detailed understanding the principles of antioxidant properties of samples.

1. Introduction

Reactive oxygen species (ROS) contribute to cellular aging, possibly through the destabilisation of membranes in cells. During evolution, the organisms have developed an antioxidant defence system to cope with oxidative stress. Antioxidants can be divided into two groups – preventive antioxidants and chain-breaking antioxidants. The first group comprises metal chelators such as metallothionein, neuromelanin, transferin and other proteins involved in transition metal transport and storage and antioxidant enzymes such as catalase, superoxide dismutase, glutathione reductase etc. Free-radical scavengers pertain to the second group. They scavenge free radicals and stop the propagation of free radical chain reactions. The most significant chain-breaking antioxidants are vitamins C and E, carotenoids and polyphenols (Davies, 2000; Finkel & Holbrook, 2000; Prior, 2003). Polyphenols are generally divided into hydrolyzable tannins and phenylpropanoids, such as lignins, flavonoids, and condensed tannins. The largest and best studied group of polyphenols are flavonoids. Flavonoids are a class of secondary plant metabolites that are thought to exert beneficial health effects through their antioxidant and chelating properties being the major contributor to the antioxidant capacity of vegetables (Heim, Tagliaferro, & Bobilya, 2002; Williams, Spencer, & Rice-Evans, 2004). In addition to antioxidant function, flavonoids may also modulate cell signalling pathways and could have marked effects on cell function by altering protein and lipid phosphorylation and modulating gene expression (Lotito & Frei, 2006).

It is of a great interest to consumers and nutritionists to quantify the antioxidant properties of various foods because antioxidants are clearly important to human life. Moreover, the attention should be also paid to the processing methods in order to preserve the desirable antioxidant properties of foods (Gorinstein et al., 2009). Antioxidant function can be defined as the ability of a compound to reduce pro-oxidant agents (Prior & Cao, 1999). There are many different methods for determining antioxidant function which rely on different generators of free radicals, acting by different mechanisms (Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005). In the literature, antioxidant properties are denoted as antioxidant capacity (Cao, Alessio, & Cutler, 1993; Pellegrini, Re, Yang, & Rice-Evans, 1999), antioxidant power (Benzie & Strain, 1999) and antioxidant potential (Ghiselli, Serafini, Maiani, Azzini, & FerroLuzzi, 1995). From the current point of view, the mix of methods should be used for assessing antioxidant activities in vitro to cover all the aspects of antioxidant efficacy (Aruoma, 2003; Schlesier, Harwat, Böhm, & Bitsch, 2002).

The present study employs three different methods for assessing antioxidant properties – oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidative parameter (TRAP) and hydroxyl (HO) radical averting capacity (HORAC). The first two methods assess the peroxyl radical chain-breaking ability of antioxidants by hydrogen atom transfer pathway. The third method – HORAC measures their metal-chelating radical prevention activity (Prior et al., 2005). Despite the methods employed in this study there are many other approaches to evaluate antioxidant

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properties of fruits and vegetables such as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical assay, diphenylpicrylhydrazyl (DPPH) radical assay, β-carotene bleaching assay, ferric reducing antioxidant power (FRAP) assay, cupric ion reducing antioxidant capacity (CUPRAC) assay etc. (Ao, Li, Elzaawely, Xuan, & Tawata, 2008; Baydar, Ozkan, & Yasar, 2007; Deba, Xuan, Yasuda, & Tawata, 2008; Gorinstein et al., 2009).

The aim of the study was to compare the antioxidant function of selected vegetables of Bulgarian origin and commonly consumed by the Bulgarian population as an important constituent of their traditional food. Three different antioxidant assays were chosen for the measurement of antioxidant function in vegetable samples and compared. The total polyphenol content of selected vegetables was measured as well, so as to evaluate its contribution to their total antioxidant function. The objective of the study was to supply both new methodological background for the food quality control in food industry and new information on the antioxidant function of selected vegetables for nutritionists and general public.

2. Materials and methods

2.1. Chemicals and instruments

2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium salt, cobalt (II) fluoride tetrahydrate and gallic acid were obtained from Sigma–Aldrich (Steinheim, Germany). Folin–Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). Picollinic acid was purchased from Fluka (Deisenhofen, Germany). All other solvents used were of analytical grade and obtained from local producers.

ORAC and HORAC analyses were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnology, Offenburg, Germany), excitation wavelength = 485 nm and emission wavelength = 520 nm. TRAP assay was conducted on Luminometer Orion II (Berthold Dection System GmbH, Pforzheim, Germany).

2.2. Food sample preparation

Vegetables were purchased from the local supermarkets at the time of peak production, after that immediately frozen and kept at −18 °C. Altogether 22 vegetables of Bulgarian origin were analyzed: celery leaves (*Apium graveolens* var. *dulce*), parsley leaves (*Petroselinum crispum*), chilli pepper (*Capsicum frutescens*), capsicum (*Capsicum annuum* 'Gorogled'), lovage (*Levisticum officinale*), goathorn pepper (*C. annuum*), dill (*Anethum graveolens*), red pepper (*C. annuum*), broccoli (*Brassica oleracea* var. *italica*), eggplant (*Solanum melongena*), green bean (*Phaseolus vulgaris*), green onion (*Allium fistulosum*), radish (*Raphanus sativus*), gumbo (*Abelmoschus esculentus*), red beet (*Beta vulgaris*), green pepper (*C. annuum*), potato (*Solanum tuberosum*), celery root (*A. graveolens* var. *rapaceum*), tomato (*Lycopersicon esculentum*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), and vegetable marrow (*Cucurbita pepo*).

The edible parts of the vegetables (2 g) were unfrozen and homogenized in a laboratory blender before the analyses. Samples were transferred into extraction tubes and mixed with 20 ml of the extragent (80% acetone in 0.2% formic acid). Extraction was conducted on an orbital shaker at room temperature for 1 h. After that the sample was centrifuged at 6240 g and supernatant was removed. The solid residue was subjected to a second extraction under the same conditions. Both supernatants were combined and further used for total polyphenol and antioxidant function determinations after a dilution with a phosphate buffer.

2.3. TRAP assay

The luminol-enhanced chemiluminescence (CL) was used to follow up the peroxyl radical reaction and the principle was described previously in Čížová, Lojek, Kubala, and Číž. (2004) and Uotila, Kirkkola, Rorarius, Tuimala, and Metsä-Ketelä. (1994). The CL signal is driven by the production of luminol derived radicals from thermal decomposition of AAPH. The TRAP value is determined from the duration of the time period (T_{sample}) during which the sample quenched the CL signal due to the present antioxidants. A known quantity (8.0 nM) of trolox, a water-soluble analogue of tocopherol was used as a reference inhibitor (T_{trolox}) instead of the sample. The calculation of the TRAP value is represented by the equation:

$$\mathrm{TRAP} = 2.0[\mathrm{Trolox}]T_{\mathrm{sample}}/f \cdot T_{\mathrm{Trolox}},$$

where 2.0 is the stoichiometric factor of trolox (the number of peroxyl radicals trapped per one molecule of trolox) and f is the dilution of the sample.

2.4. ORAC assay

The ORAC assay measures the antioxidant scavenging function against peroxyl radical induced by AAPH at 37 °C. Fluorescein is used as a fluorescent probe. The loss of fluorescence of fluorescein is an indication of the extent of damage from its reaction with the peroxyl radical (Gomes, Fernandes, & Lima, 2005; Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002; Ou, Hampsch-Woodill, & Prior, 2001). Working solution of fluorescein (70 nM) was prepared by dissolving fluorescein disodium salt in phosphate buffer (75 mM, pH = 7.4).

The total reaction mixture volume was 200 μl and all solutions were prepared in a phosphate buffer (75 mM, pH = 7.4). One-hundred and seventy micro-litres of fluorescein solution (60 nM final concentration) and 10 μl of the sample were placed in the well of the microplate and incubated at 37 °C directly in the FLUOstar plate reader for 10 min. After the incubation 20 μl of AAPH (51.5 mM final concentration) was added rapidly using a multichannel pipette to start the reaction. The fluorescence was recorded every minute and the microplate was automatically shaken prior to each reading. A blank using phosphate buffer instead of the antioxidant and calibration solutions of Trolox (12.5, 20, 50, and 100 μM) as antioxidant were also carried out in each assay. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve (AUC). The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. ORAC values were expressed as μmol Trolox equivalents per gram of fresh weight (FW) of the samples.

2.5. HORAC assay

The HORAC assay developed by Ou et al. (2002) measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical.

Hydrogen peroxide solution of 0.55 M was prepared in distilled water. 4.6 mM Co(II) was prepared as follows: 15.7 mg of $CoF_2{\cdot}4H_2O$ and 20 mg of picolinic acid were dissolved in 20 ml of distilled water.

Fluorescein – 170 μl (60 nM, final concentration) and 10 μl of sample were incubated in 37 °C for 10 min. directly in the FLUOstar plate reader. After incubation 10 μl H_2O_2 (27.5 mM, final concentration) and 10 μl of Co(II) (230 μM final concentration) solutions were added subsequently. The initial fluorescence was measured

after which the readings were taken every minute after shaking. For the blank sample, phosphate buffer solution was used. 100, 200, 600, 800 and 1000 μM gallic acid solutions (in phosphate buffer 75 mM, pH = 7.4) were used for building the standard curve. The AUC were calculated as they were for the ORAC assay. The final HORAC values were calculated using a regression equation between the gallic acid concentration and the net area under the curve. One HORAC unit is assigned to the net protection area provided by 1 μM gallic acid and the activity of the sample is expressed as μmol gallic acid equivalents (GAE) per gram of fresh weight of the samples.

2.6. Total polyphenol content analysis

The total polyphenol content was determined by the Folin–Ciocalteu method. Gallic acid was employed as calibration standard and results were expressed as gallic acid equivalents. Food extracts (1 ml) or gallic acid standard solutions were mixed with 10 ml deionized water and 1.0 ml of Folin–Ciocalteu phenol reagents. After 5 min, 2.0 ml of 20% sodium carbonate was added to the mixture. After 1 h in darkness the absorbance at 750 nm was measured. The concentration of total polyphenols was expressed as GAE per 100 g of fresh weight (Stoilova, Krastanov, Stoyanova, Denev, & Gargova, 2007).

3. Statistical analysis

All experiments were repeated six times. The results are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Student's *t*-test were used to evaluate the differences of the mean between groups, and Newman–Keuls test was used to analyse the contrasts. Pearson correlation coefficient (r) was used to express correlations and linear regressions were calculated. A p value of <0.05 was taken to be significant.

4. Results

The antioxidant capacity expressed as the TRAP, ORAC and HORAC values of selected vegetables as well as the amount of total polyphenols of these vegetables in rank order are shown in Table 1. Using the ORAC assay we obtained a hierarchy of antioxidant capacity ranging from 113.0 to 1.2 μmol TE/g of fresh weight. Interestingly, the highest antioxidant function was found in the samples of green parts of vegetables belonging to the *Apiaceae* family with the exception of dill. On the other hand, the antioxidant function of the root vegetables of the *Apiaceae* family was significantly lower. The most widely used vegetables in Bulgarian cuisine (peppers, tomato, and cucumber) belonged to the weakest ones from the point of view of their antioxidant function.

The TRAP values ranged from 68.1 to 0.0 μmol TE/g of fresh weight with hierarchy similar to ORAC on the first five positions and the last three positions. The range of HORAC values was from 82.9 to 0.0 GAE/g fresh weight.

The total polyphenol content in the vegetable extracts analyzed was in the range of 605.6–20.0 mg GAE/100 g fresh weight. The highest content of total polyphenols was in accordance with the results of antioxidant function determination found in celery leaves and parsley leaves, followed by various types of peppers (*Capsicum* sp., excepting green pepper), lovage and dill. The lowest total polyphenol content was recorded in tomato, carrot, cucumber and vegetable marrow corresponding to their low antioxidant function.

There was a direct relationship between the total polyphenol content and antioxidant function in the extracts of various vegetables. ORAC, TRAP and HORAC values were significantly linearly correlated to the total polyphenol content: $r = 0.95$, $r = 0.97$ and $r = 0.94$, respectively (see Fig. 1).

In addition, there was a good correlation between the methods for measuring peroxyl radical-trapping antioxidant function of vegetables – TRAP vs. ORAC, $r = 0.96$. A good correlation was also found between methods for measuring the peroxyl radical-trapping capacity and hydroxyl radical averting capacity – TRAP vs. HORAC, $r = 0.94$ and ORAC vs. HORAC, $r = 0.94$ (see Fig. 2).

5. Discussion

Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites, and are an integral part of the human diet. Vegetables are known to possess a variety of antioxidant effects and properties. Flavonols (such as quercetin, myricetin,

Table 1
The rank order of antioxidant activity expressed as ORAC (μmol TE/g FW), TRAP (μmol TE/g FW) and HORAC (μmol GAE/g FW) of vegetable extracts and the rank order of total polyphenol content (mg GAE/100 g FW) in vegetable extracts. There are no significant differences among values marked with the same superscript letters in individual columns.

Vegetables	ORAC		TRAP		HORAC		Total polyphenols	
	Value	Rank	Value	Rank	Value	Rank	Value	Rank
Celery leaves	$113.5^{a} \pm 6.1$	1	$68.1^{a} \pm 4.8$	1	$55.0^{b} \pm 2.9$	2	$605.6^{a} \pm 9.4$	1
Parsley leaves	$108.6^{b} \pm 13.1$	2	$67.2^{a} \pm 8.9$	2	$82.9^{a} \pm 1.2$	1	$599.7^{b} \pm 0.4$	2
Lovage	$57.3^{c} \pm 5.0$	3	$28.4^{b} \pm 1.3$	3	$19.2^{d} \pm 2.1$	4	$267.0^{e} \pm 2.1$	5
Chilli pepper	$36.1^{d} \pm 6.5$	4	$23.7^{cd} \pm 3.1$	5	$17.7^{de} \pm 0.4$	5	$298.6^{c} \pm 1.8$	3
Goathorn pepper	$30.6^{e} \pm 1.4$	5	$24.8^{c} \pm 1.2$	4	$13.7^{f} \pm 1.8$	7	$260.5^{f} \pm 5.0$	6
Radish	$23.6^{f} \pm 1.7$	6	$11.4^{h} \pm 2.2$	11	$15.9^{e} \pm 2.0$	6	$89.9^{j} \pm 1.7$	13
Capsicum	$19.9^{fg} \pm 1.4$	7	$20.9^{de} \pm 1.2$	6	$21.3^{c} \pm 5.0$	3	$286.7^{d} \pm 5.1$	4
Eggplant	$16.2^{gh} \pm 2.0$	8	$13.4^{gh} \pm 1.5$	10	$5.5^{h} \pm 0.7$	15	$102.9^{i} \pm 4.7$	9
Broccoli	$16.1^{gh} \pm 1.2$	9	$3.4^{ijk} \pm 0.1$	17	$7.2^{h} \pm 1.6$	10	$102.1^{i} \pm 5.9$	10
Celery root	$15.3^{ghi} \pm 1.2$	10	$1.0^{k} \pm 0.4$	19	$7.5^{h} \pm 0.6$	9	$43.8^{m} \pm 2.3$	18
Green onion	$14.7^{ghi} \pm 1.5$	11	$3.5^{ijk} \pm 0.5$	16	$5.7^{h} \pm 0.6$	13	$92.7^{j} \pm 0.9$	12
Gumbo	$14.6^{ghi} \pm 0.8$	12	$7.4^{i} \pm 0.8$	12	$5.7^{h} \pm 0.5$	14	$89.8^{j} \pm 4.0$	14
Green bean	$14.5^{ghi} \pm 1.2$	13	$16.9^{fg} \pm 0.5$	8	$5.7^{h} \pm 0.5$	12	$101.5^{i} \pm 3.3$	11
Red beet	$12.6^{hi} \pm 1.6$	14	$17.9^{ef} \pm 0.3$	7	$6.1^{h} \pm 1.0$	11	$81.5^{k} \pm 1.6$	15
Dill	$10.5^{hij} \pm 1.1$	15	$13.7^{h} \pm 1.5$	9	$11.3^{g} \pm 0.8$	8	$150.4^{g} \pm 0.9$	7
Potato	$10.3^{hij} \pm 1.3$	16	$3.8^{ijk} \pm 0.1$	15	$2.9^{i} \pm 0.2$	17	$65.5^{l} \pm 1.1$	17
Red pepper	$9.3^{ij} \pm 0.9$	17	$5.9^{ij} \pm 0.1$	13	$5.3^{h} \pm 0.3$	16	$115.7^{h} \pm 1.5$	8
Green pepper	$5.6^{jk} \pm 0.3$	18	$2.8^{jk} \pm 1.9$	18	$0.9^{i} \pm 0.3$	20	$80.7^{k} \pm 3.6$	16
Tomato	$5.4^{jk} \pm 0.3$	19	$4.4^{ijk} \pm 0.5$	14	$1.9^{i} \pm 0.3$	18	$41.3^{m} \pm 2.2$	19
Carrot	$4.8^{jk} \pm 1.1$	20	n.d.	20	n.d.	21	$35.2^{n} \pm 2.0$	20
Vegetable marrow	$2.9^{k} \pm 0.3$	21	n.d.	21	$1.8^{i} \pm 0.1$	19	$20.0^{p} \pm 1.7$	22
Cucumber	$1.2^{k} \pm 0.2$	22	n.d.	22	n.d.	22	$24.2^{o} \pm 2.4$	21

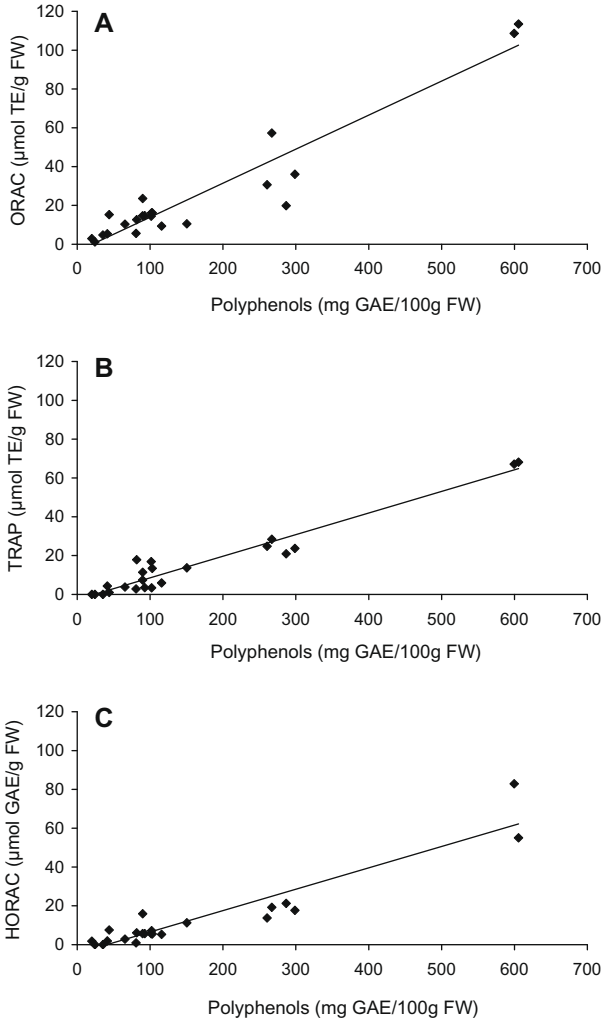

Fig. 1. Correlations among total polyphenol content expressed as mg GAE/100 g FW of vegetable extracts and various measurements of antioxidant activity: (A) ORAC expressed as μmol TE/g FW of vegetable extracts ($y = 0.1754x - 3.6334$; $r = 0.950$), (B) TRAP expressed as μmol TE/g FW of vegetable extracts ($y = 0.1113x - 2.6021$; $r = 0.969$) and (C) HORAC expressed as μmol GAE/g FW of vegetable extracts ($y = 0{,}1101x - 4.4667$; $r = 0.935$).

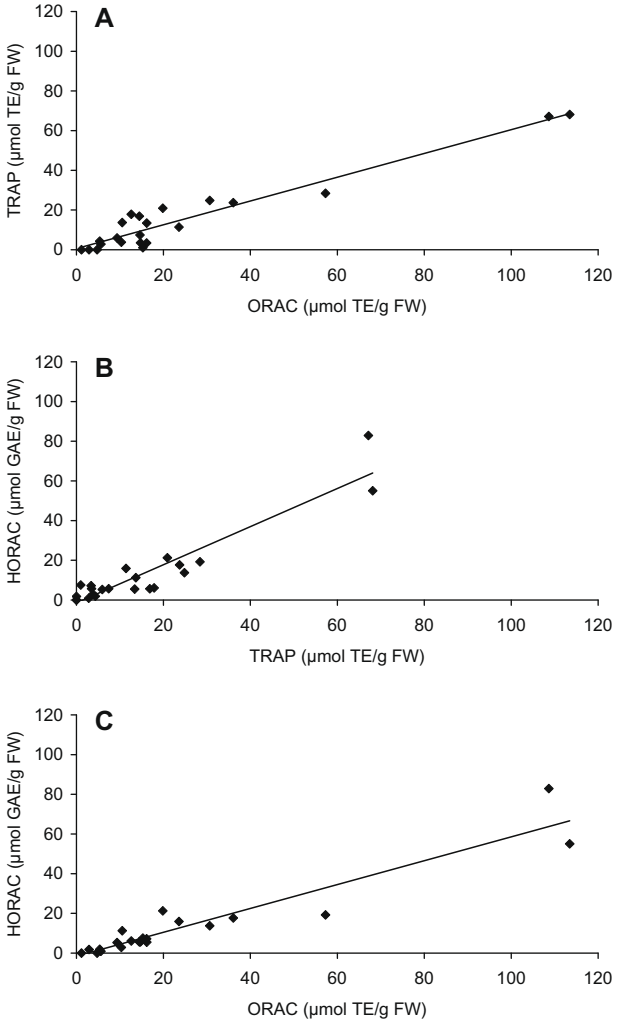

Fig. 2. Correlations among various types of measurements of antioxidant activity of vegetable extracts: (A) TRAP vs. ORAC ($y = 0.5991x + 0.5859$; $r = 0.962$), (B) HORAC vs. TRAP ($y = 0.9596x - 1.4438$; $r = 0.936$), and (C) HORAC vs. ORAC ($y = 0.6012x - 1.5316$; $r = 0.942$).

kaempherol) and flavones (e.g. apigenin, luteolin) in plant materials are closely associated with their antioxidant function mainly due to their redox properties exerted by various possible mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity (Lotito & Frei, 2006; Shan, Cai, Sun, & Corke, 2005). There are many papers attempting to rank the antioxidant properties of different plant materials using different methods (Pellegrini et al., 2003; Proteggente et al., 2002) including ORAC (Ou, Hampsch-Woodill, et al., 2002; Velioglu, Mazza, Gao, & Oomah, 1998; Wang, Cao, & Prior, 1996; Wu et al., 2004). The antioxidant capacity assays measure the combined effect of many antioxidants present in the sample, which are able to scavenge free radicals generated in the assays. Interactions between antioxidants are also reflected in the assay value. Both ORAC and TRAP are indicators of the free-radical scavenging ability of antioxidants against peroxyl radical, using the same mechanism of hydrogen atom transfer. On the other hand, HORAC measures the metal-chelating properties of antioxidants thereby expressing a radical prevention ability of the sample. According to Ou, Hampsch-Woodill, et al. (2002) and Ou, Huang, Hampsch-Woodill, Flanagan, and Deemer (2002) pure flavonoids can differ significantly in their ORAC and HORAC values. Nevertheless in contrast to Ou, Hampsch-Woodill, et al. (2002) and Ou, Huang, et al. (2002), who did not find any general agreement among the antioxidant methods used, our data correlated well (see Fig. 2). The data obtained using the ORAC and TRAP assays (Fig. 2A) had the best correlation coefficient ($r = 0.96$). Also the TRAP and HORAC values ($r = 0.94$, Fig. 2B) and ORAC and HORAC values ($r = 0.94$, Fig. 2C) correlated strongly. A slight variation among the results obtained by the ORAC, HORAC and TRAP assays might relate to the different conditions of measurement and the sensitivity of the assays. Therefore several methods should be used in parallel to elucidate the complex field of antioxidants and oxidation (Ou, Huang, et al., 2002).

In such complex samples as vegetable extracts where many individual polyphenols occur, some discrepancies between polyphenol content and antioxidant properties could be expected. Nevertheless in the work reported here, the data unequivocally show that ORAC, TRAP and HORAC values (see Table 1) are strictly dependent on polyphenol content ($r = 0.95$, $r = 0.97$ and $r = 0.94$, respectively, see Fig. 1). These data are in accordance with that of other authors, who have shown that a high total polyphenol content increases antioxidant function and that there is a linear correlation between polyphenol content and antioxidant function (Gorinstein, Martin-Belloso, et al., 2003; Gorinstein, Yamamoto, et al., 2003).

In our experiments, it was impossible to detect the antioxidant capacity using TRAP method in samples where the quantity of polyphenols was equal to or less than 35 mg per 100 g of fresh weight. The ORAC assay is more sensitive and reflects antioxidant properties even of such a low quantity of polyphenols. The advantage of the ORAC assay is that it combines both the inhibition time and inhibition degree of the radical generation, as it takes the oxidation reaction to completion and uses the area under the curve to quantify the antioxidant capacity (Prior et al., 2003).

The antioxidant properties depend on several structural features of the molecule of polyphenols in its base structure and are primarily attributed to the high reactivity of hydroxyl substituents. The B-ring hydroxyl configuration is the most significant determinant of scavenging of ROS. 3′,4′-di OH (catechol) structure in the B-ring strongly enhances lipid peroxidation inhibition. A free 3-hydroxyl group and 3′,4′-catechol (dihydroxy) structure, a C_2=C_3 double bond, and a 4-oxo group on the C ring endow the flavonoid with potent antioxidant function. The superiority of quercetin in inhibiting both metal and nonmetal-induced oxidative damage is partially ascribed to its free 3-OH substituent which is thought to increase the stability of the flavonoid radical (Davies, 2000). Fenton-induced oxidation is strongly inhibited by flavonoids with catechol, 4-oxo, and 5′-OH arrangements (Cao, Sofic, & Prior, 1997; Cheng & Breen, 2000; Wang et al., 2006) which is superior to isoforms that lack these features.

Because fruit and vegetables are the major antioxidant sources in our daily diet, an estimation of daily antioxidant capacity intake from these foods is beneficial (Davies, 2000). Although the poor bioavailability of flavonoids is widely discussed (Prior et al., 2005) the availability of databases of the flavonoid content is important to identify the major contributors to the antioxidant activities of the flavonoid-rich dietary components (Proteggente et al., 2002). As we have shown here, aromatic vegetable such as parsley, dill and lovage with high polyphenol content and high ORAC, HORAC and TRAP values could be excellent sources of antioxidant and should be a part of everyday diet.

Although we have found a good correlation among all the methods used here for assessing antioxidant capacity, using more than one antioxidant assay is strongly recommended – a single method will provide basic information about antioxidant properties, but a combination of methods describes the antioxidant properties of the sample in more detail.

Although these individual assays can be used for screening the antioxidant properties of the food sample with similar results, we recommend using the ORAC assay combined with HORAC for evaluating overall antioxidant capacity. The ORAC has been found to be the most sensitive method to measure chain-breaking antioxidant function (hydrogen atom transfer pathway) while the HORAC measures metal-chelating antioxidant capacity (preventive antioxidant). As a supplement, the Folin–Ciocalteu assay (single electron transfer pathway) should be a part of antioxidant profile measurement.

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Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties

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ABSTRACT

Solid-phase extraction (SPE) was used to obtain anthocyanin-rich extracts from five berry species: chokeberry, elderberry, black currant, blackberry and blueberry. During SPE more than 94.4% of the sugars and more than 88.5% of the acids present in the crude extracts were separated. The SPE resulted in 90–95.6% anthocyanins recovery. The antioxidative properties of the anthocyanin-rich extracts were tested by measuring their oxygen radical absorption capacity (ORAC), hydroxyl radical averting capacity (HORAC), total peroxyl radical trapping antioxidant parameter (TRAP), scavenging of nitric oxide and inhibition of lipid peroxidation. Elderberry extract revealed the highest ORAC value of 5783 μmol TE/g. Chokeberry extract was the most potent inhibitor of lipid peroxidation and had the highest TRAP value of 4051 μmol TE/g. Blueberry extract had the highest HORAC result – 1293 μmol GAE/g and was the most powerful scavenger of NO. The high antioxidant activity according to all antioxidant assays revealed opportunities to apply these preparations as antioxidants.

1. Introduction

Anthocyanins comprise of a large group of water-soluble pigments which pertain to the flavonoid class. They give red, blue, purple and black colours to many fruits, vegetables and flowers. There is an increasing interest in anthocyanins not only as natural food colourants (Bridle & Timberlake, 1997) but also as pharmaceutical products because of various therapeutic effects. Anthocyanins reveal potent P-vitamin activity, reduce capillary fragility and increase their permeability (Wagner, 1985). They take part in collagen reticulation and inhibit the enzyme degradation of collagen during inflammation (Ronziere, Herbage, Garrone, & Frey, 1981). Anthocyanins are applied in prevention and treatment of glaucoma and other eyesight disorders (Ghosh & Konishi, 2007). In model systems, anthocyanin-rich extracts reveal cardio-protective effect (Bell & Gochenaur, 2006). Inhibition of cancer-cells growth by such extracts has also been reported (Zhao, Giusti, Malik, Moyer, & Magnuson, 2004). These biological effects are in part due to their antioxidant capacity (Kahkonen & Heinonen, 2003). Anthocyanins are potent radical scavengers and are very effective in the inhibition of low density lipoprotein oxidation, which is a key step for atherosclerosis development (Satue-Gracia, Heinonen, & Frankel, 1997). Among all common fruits and vegetables in the diet, berries, especially those with dark blue or red colours, have the highest content of anthocyanins and antioxidant capacities (Wu et al., 2004) which makes them a suitable raw material for anthocyanin extraction especially in a large scale.

The use of anthocyanins for pharmaceutical purposes requires their separation and purification. Their polarity makes them soluble in several types of polar solvents, such as methanol, ethanol, acetone, and water. Solvent extraction of anthocyanins is the initial step prior to quantification, purification, separation, and characterisation and generally involves the use of an acidified extragent. Common extraction procedures are non-selective and yield pigment solutions with large amounts of by-products, such as sugars, sugar alcohols, pectin, organic acids, amino acids and proteins. Some of these impurities may accelerate anthocyanin degradation or cause problems in further processing steps, such as freeze drying or spray drying. Furthermore, the low content of anthocyanins in raw materials hinders the commercial large scale production of these pigments. Purification by solid-phase extraction (SPE) is a relatively simple method allowing the elimination of non-phenolic impurities. Currently SPE gains wider application as a rapid and economical process and because different cartridges with a great variety of sorbents can be used. In addition, it can also be automated reducing the processing time. Different SPE conditions have

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already been used successfully for purification of anthocyanins (Kraemer-Schafhalter, Fuchs, & Pfannhauser, 1998; Nicoue, Savard, & Belkacemi, 2007). The absorbent Amberlite XAD7 has proved to be very suitable for chokeberry anthocyanin purification. The SPE process includes application of the berry extracts into the SPE column, elution of the impurities and finally, elution of the anthocyanin fractions.

The aim of the current work was to further extend the application of SPE for the purification of anthocyanins from different berries and to investigate the antioxidant properties of the anthocyanin-rich extracts. The antioxidant activity of the extracts obtained was evaluated by determining their oxygen radical absorption capacity (ORAC), hydroxyl radical averting capacity (HORAC), total peroxyl radical trapping antioxidant parameter (TRAP), scavenging of nitric oxide (NO) and inhibition of lipid peroxidation. The methods chosen embrace different aspects of the antioxidant action and give a comprehensive picture about the antioxidant potential of the extracts produced.

2. Materials and methods

2.1. Chemicals

Fluorescein disodium salt, 2,2-azobis-(2-amidino-propane)-dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, thiobarbituric acid and Amberlite XAD7 were obtained from Sigma–Aldrich (Steinheim, Germany). Folin–Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). All other solvents used were of analytical grade and purchased from local distributors.

2.2. Raw materials

Chokeberry (*Aronia melanocarpa*), elderberry (*Sambucus nigra*), black currants (*Ribes nigrum*), blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium myrtillus*) were supplied at the time of peak production from the region of Rodopi Mountains, Bulgaria. Chokeberry and black currant were cultivated whereas elderberry, blackberry and blueberry were wild-grown. Fruits were immediately frozen and kept at −18 °C until analysed.

2.3. Extraction

For the extraction of anthocyanins, different extraction systems have been used (Nicoue et al., 2007). Due to their polarity, anthocyanins are usually extracted by methanol, ethanol, acetone and water. Although methanol and acetone are quite effective extragents, their application in foods is limited due to their toxicity. Ethanol is more suitable for food application but at the same time is more difficult to eliminate in the purification process. Using the absorbent Amberlite XAD7 Kraemer-Schafhalter et al. (1998) observed that the application of ethanolic chokeberry extract to SPE resulted in incomplete anthocyanin retention and moreover colour pigments evolved in two separated peaks, the first one overlapping with that of the sugars. In our study we used the same filling material which excluded the possibility to use ethanol as extragent. Generally, the presence of acid is required for improved anthocyanin extractability. The use of acid stabilizes anthocyanins in the flavylium cation form, which is red at low pH. However, a solvent, acidified with hydrochloric acid, may hydrolyse acylated anthocyanins. To avoid, or at least, minimise the breakdown of acylated anthocyanins, organic acids, such as acetic, citric, or tartaric acids, which are easier to eliminate during anthocyanin concentration, have been preferred (Perez-Magarino, Ortega-Heras, & Cano-Mozo, 2008). Bearing in mind that these extracts are intended for food and pharmaceutical uses we chose 1% citric acid solution in water as an extraction agent. For the extraction 50 g of the fruits were weighed accurately, defrosted and homogenised in a laboratory blender. Samples were transferred into extraction tubes and mixed with 150 ml of 1% citric acid solution in water. Extraction was conducted on an orbital shaker at 60 °C for 1 h. In a preliminary study we found out that these conditions provide maximum extractability of anthocyanins and these remain stable after 1 h extraction (unpublished results). After that, samples were centrifuged (20 min, 6200*g*) and the obtained extracts were used for SPE.

2.4. HPLC

High performance liquid chromatography (HPLC) of the sugars and citric acid was performed on Waters 484 system, connected to a refractometric Waters R401 detector and Aminex HPX – 87H column (300 × 7.8 mm, BioRad), eluent 0.004 mol/l H_2SO_4, flow 0.5 ml/min, temperature 23 °C. The standard compounds were purchased from Sigma–Aldrich (Steinheim, Germany).

2.5. Total phenolic compound analysis

Total phenolics were determined according to the method of Singleton and Rossi (1965) with Folin–Ciocalteu's reagent. Gallic acid was employed as calibration standard and results were expressed as gallic acid equivalents (GAE) per gram dry weight.

2.6. Anthocyanins determination

Anthocyanins were determined by the pH-differential method (Lee, 2005). Anthocyanin pigments change colour with pH. The absorption of the sample was measured at pH 1.0 and pH 4.5. The difference in absorbance is proportional to the anthocyanin content. The total anthocyanin content was expressed as cyanidin-3-glucoside equivalents and calculated via the following formula:

$$\text{Anthocyanin content (mg/l)} = \frac{A \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times L}$$

where $A = (\text{A510 nm pH 1.0} - \text{A700 nm pH 1.0}) - (\text{A510 nm pH 4.5} - \text{A700 nm pH 4.5})$, MW = cyanidin-3-glucoside molecular weight (449.2); DF = dilution factor; ε = cyanidin-3-glucoside molar absorbtivity (26,900); L = cell pathlength (usually 1 cm).

2.7. Solid-phase extraction

The SPE material (Amberlite XAD7) was soaked with bidistillated water and then loaded into the column. After that, the column was rinsed with bidistillated water and the crude extract was applied. The impurities were eluted with bidistillated water. Ten millilitres of fractions of the eluate were collected until no soluble dry solids were detected. After that, anthocyanins were eluted with 96% ethanol. To represent the elution profile of anthocyanins, 10 ml fractions were collected and analysed. The dense part of the anthocyanin-containing ethanol was concentrated via rotary evaporation at 50 °C. After that, concentrated extracts were freeze dried to obtain a powdered dry product. SPE was performed on SUPELCO VISIPER system, connected to a vacuum pump (KNF lab) with 50 ml glass cartridges. Dry extracts obtained after SPE were kept at −18 °C until analysed. Before the analyses, extracts were dissolved in phosphate buffered saline pH 7.4 (PBS) in concentration 2 mg sample/1 ml PBS. The SPE procedure was repeated three times for all berry extracts.

2.8. ORAC assay

ORAC was measured according to the method of Ou, Hampsch-Woodill, and Prior (2001) with some modifications. The method measures the antioxidant scavenging activity against peroxyl radical induced by 2,2′-azobis-(2-amidino-propane)dihydrochloride (AAPH) at 37 °C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) as compared to that of blank in which no antioxidant is present. Solutions of AAPH, fluorescein and Trolox were prepared in a phosphate buffer (75 mmol/l, pH 7.4). Samples were diluted in phosphate buffer as well. Reaction mixture (total volume 200 μl) contained FL – (170 μl, final concentration 5.36×10^{-8} mol/l), AAPH – (20 μl, final concentration 51.5 mmol/l), and sample – 10 μl. FL solution and sample were incubated at 37 °C for 20 min, and AAPH (dissolved in 37 °C buffer) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle after shaking. For the blank, 10 μl of phosphate buffer was used instead of a sample. Antioxidant activity was expressed in Trolox equivalents. Trolox solutions (6.25; 12.5; 25; 50 and 100 μmol/l) were used for defining the standard curve. One ORAC unit is assigned to the net protection area, provided by a Trolox solution with concentration of 1 μmol/l. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve. Results were expressed as micromole Trolox equivalents per gram of dry extract.

2.9. HORAC assay

HORAC measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical (Ou et al., 2002). Hydrogen peroxide solution of 0.55 mol/l was prepared in distilled water. Co(II) (4.6 mmol/l) was prepared as follows: 15.7 mg of $CoF_2{\cdot}4H_2O$ and 20 mg of picolinic acid were dissolved in 20 ml of distilled water. Fluorescein – 170 μl (60 nmol/l, final concentration) and 10 μl of sample were incubated at 37 °C for 20 min directly in the FLUOstar plate reader. After incubation, 10 μl H_2O_2 (27.5 mmol/l, final concentration) and 10 μl of Co(II) (230 μmol/l, final concentration) solutions were added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. For the blank sample, a phosphate buffer solution was used. Gallic acid solutions of 100, 200, 400, 500 and 600 μmol/l (in phosphate buffer 75 mmol/l, pH 7.4) were used for building the standard curve. The final HORAC values were calculated using a regression equation between gallic acid concentration and the net area under the curve. One HORAC unit was assigned to the net protection area provided by 1 μmol/l gallic acid and the activity of the sample is expressed as μmol gallic acid equivalents (GAE) per gram of dry extract. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany), excitation wavelength of 485 nm and emission wavelength of 520 nm were used.

2.10. TRAP assay

The luminol-enhanced chemiluminescence (CL) was used to monitor the peroxyl radical reaction as described previously (Čížová, Lojek, Kubala, & Číž, 2004). The CL signal is driven by the production of luminol derived radicals from thermal decomposition of AAPH. The TRAP value was determined from the duration of the time period (T_{sample}) during which the sample quenched the CL signal due to the present antioxidants. A known quantity (8.0 nmol) of trolox, a water-soluble analogue of tocopherol was used as a reference inhibitor (T_{trolox}). The calculation of the TRAP value is represented by the equation:

$$\mathrm{TRAP} = 2.0[\mathrm{Trolox}]T_{\mathrm{sample}}/f\ T_{\mathrm{Trolox}},$$

where 2.0 is the stoichiometric factor of trolox (the number of peroxyl radicals trapped per one molecule of trolox) and f is the dilution of the sample. The activity of the sample is expressed as Trolox equivalents per gramme dry extract. TRAP assay was conducted on Luminometer Orion II (Berthold Dection System GmbH, Pforzheim, Germany).

2.11. NO-scavenging activity

The potential ability of extracts to scavenge NO in chemical systems was tested by the electrochemical measurement of NO. This chemical system consisted of 1 ml of PBS (control sample) or 1 ml of PBS with one of tested extracts (permanent mixing), and the temperature was kept at 37 °C. NO was measured using three electrode systems as described by Hrbac et al. (2007). A porphyrinic microsensor working electrode, counter electrode and a reference electrode were connected to the ISO-NO MARK II potentiostat (WPI, USA). The injection of the 2 μl NO-saturated water into the measurement glass vial (final concentration of NO = 2.38 μmol/l) caused the rapid increase with a subsequent gradual decrease of the NO induced signal until it reached the background current. In our experiments, the electrochemical signal was followed up for 1000 s to obtain kinetic curves. The scavenging properties of the tested extract were represented as a very rapid decrease of the NO induced signal. The scavenging properties of tested extracts were evaluated as the time needed for reaching again the background current.

2.12. Lipid peroxidation inhibition

0.9 ml of 0.5 mmol/l α-linolenic acid (Sigma–Aldrich, Steinheim, Germany) was mixed with 0.1 ml sample. Then, a system generating hydroxyl radical (0.1 ml Co(II) and 0.1 ml hydrogen peroxide – for detailed description see section HORAC) was added for the induction of lipid peroxidation and the mixture was incubated for 2 h in 37 °C. After the end of incubation, the concentration of thiobarbituric acid-reactive substances (TBARS) was measured as the index of lipid peroxidation as described previously (Slavíková et al., 1998) with some modifications. Briefly, 0.5 ml of the analysed mixture was added to 1 ml of the mixture consisting of 0.6% thiobarbituric acid, 0.25 N HCl and 15% trichloracetic acid at a ratio of 1:1:1 (v/v). Then the samples were incubated in a water bath (100 °C) for 45 min. After cooling to ambient temperature, 1.5 ml of *n*-butanol were added and the mixture was shaken vigorously. The samples were centrifuged (5 min, 1500*g*), and the absorbance of the upper layer was measured at 532 nm. 1,1,3,3-Tetraethoxypropane (Sigma–Aldrich, Steinheim, Germany) at a final concentration of 0.1 μmol/l was used as a standard. Lipid peroxidation was expressed in nmol of TBARS per 1 ml of the mixture α-linolenic acid/analysed sample.

2.13. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Newman–Keuls test were used to evaluate the differences of the mean between groups. A *P*-value of 0.05 was considered significant. Pearson correlation

coefficient (R) was used to show correlations and linear regressions were calculated.

3. Results and discussion

3.1. Extraction of berries and solid-phase extraction of the crude extracts

Among most fruits of common use, the chosen berries are distinctive with their anthocyanin content (Wu et al., 2006). This determined our choice in chokeberry, elderberry, black currant, blackberry and blueberry as anthocyanin sources. The characteristics of berry extracts are shown in Table 1. The obtained extracts differed significantly in their sugar composition and anthocyanins content. The amount of anthocyanins varied from 0.45 g/l for blackberry to 1.69 g/l for elderberry crude extract. It is evident from the data in Table 1 that anthocyanins comprised only a small part of the soluble dry solids of the extracts – about 0.98% in blackberry, 1.26% in chokeberry, 1.67% in black currant, 1.77% in blueberry and 3.52% in elderberry crude extracts. All extracts contained fructose, glucose and sucrose (except blackberry). Chokeberry and blueberry extracts contained sorbitol as well. Citric acid and other organic acids were also present in the crude extracts. The majority of citric acid in the crude extracts comes from the extraction agent, since citric acid is used to improve anthocyanins extractability. After extraction the obtained crude extracts

Table 1
Characteristics of berry crude extracts obtained after extraction of 50 g fruits with 150 ml 1% solution of citric acid in water.

Fruit	Volume (ml)	Dry solids (%)	Polyphenols (g/l)	Anthocyanins (g/l)	Sugars (g/l)				Citric acid (g/l)	Total acidity (g/l)	Ratio anthocyanins/dry solids (%)
					Sucrose	Fructose	Glucose	Sorbitol			
Chokeberry	175	5.7	2.60	0.72	0.16	9.58	6.29	20.1	10.5	13.2	1.26
Elderberry	178	4.8	2.66	1.69	0.28	7.12	8.31	–	13.2	14.2	3.52
Black currant	168	4.9	2.02	0.82	0.95	10.7	12.7	–	19.0	19.3	1.67
Blackberry	170	4.6	1.57	0.45	–	14.0	14.3	–	11.9	13.3	0.98
Blueberry	164	3.5	1.37	0.62	0.04	6.64	7.90	1.94	10.5	12.4	1.77

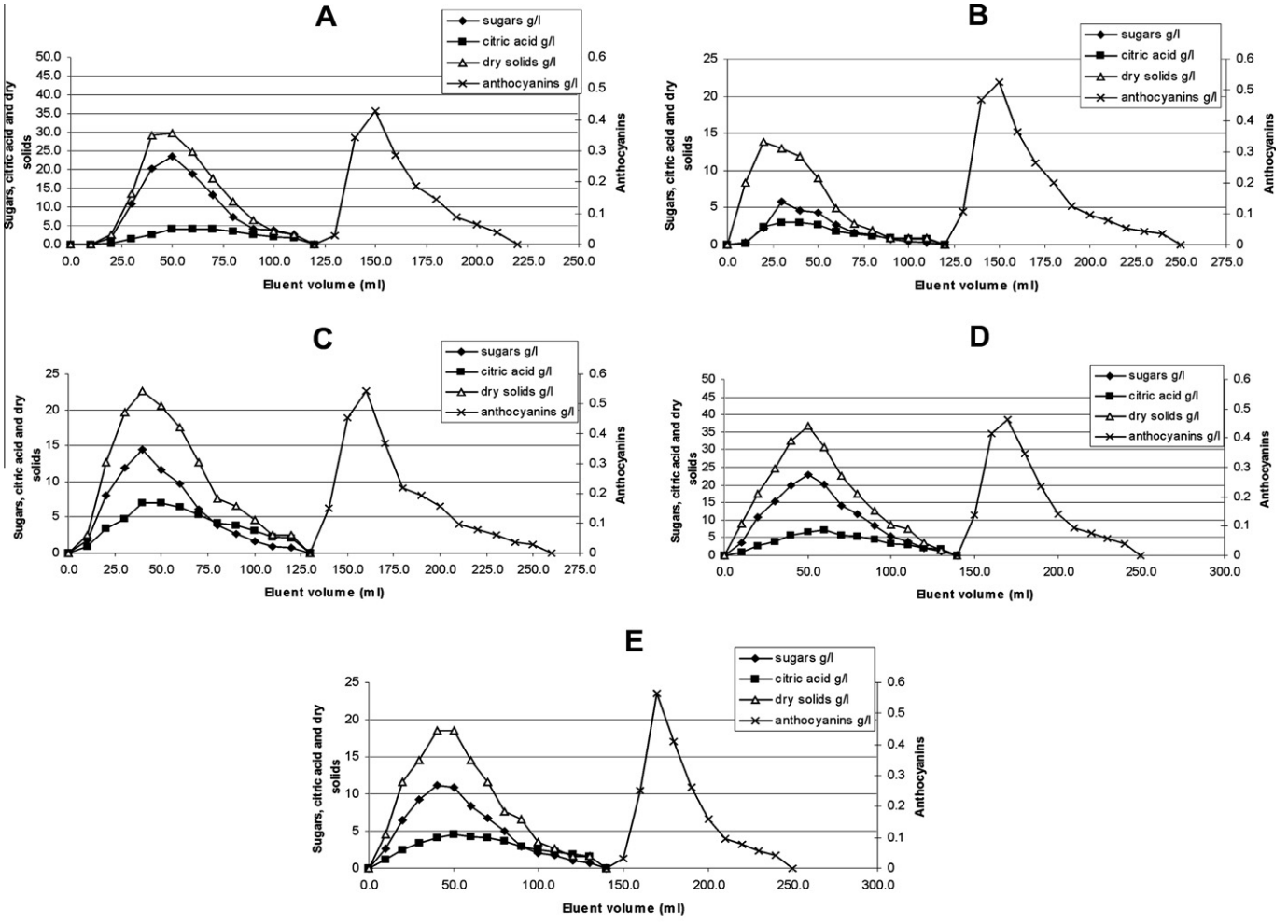

Fig. 1. SPE of berry extracts. Column volume – 50 ml, SPE material Amberlite XAD7. (A) Chokeberry extract: volume 30 ml, dry solids – 57 g/l, sugars – 36.1 g/l, citric acid – 10.5 g/l. (B) Elderberry extract: volume 15 ml, dry solids – 48.3 g/l, sugars – 15.7 g/l, citric acid – 13.2 g/1. (C) Black currant extract: volume 30 ml, dry solids – 49.6 g/l, sugars 24.3 g /l, citric acid – 19.0 g/l. (D) Blackberry extract: volume 50 ml, dry solids – 45.9 g/l, sugars – 28.3 g /l, citric acid – 11.9 g/l. (E) Blueberry extract: volume 30 ml, dry solids – 35.6 g/l, sugars – 16.5 g /l, citric acid – 10.5 g/l .

Table 2
Characteristics of anthocyanin-rich berry extracts after SPE.

Fruit	Yield of dry extract[a] (g)	Polyphenols (mg/g)	Anthocyanins[a] (mg/g)	Anthocyanin recovery[a] (%)	Sugars (mg/g)	Citric acid (mg/g)
Chokeberry	0.92 ± 0.12	352	115 ± 8.2	91.2 ± 1.02	119	229
Elderberry	1.17 ± 0.09	283	246 ± 12.3	95.6 ± 2.10	124	289
Black currant	0.81 ± 0.10	239	158 ± 6.4	93.1 ± 1.82	87.2	421
Blackberry	0.72 ± 0.13	245	95.8 ± 11.2	90.2 ± 4.06	206	310
Blueberry	0.66 ± 0.04	267	139 ± 10.8	90.0 ± 0.80	80.2	303

[a] Data are presented as means from three independent SPE ± standard deviation.

were subjected to SPE. The choice of SPE material is crucial for the good separation of anthocyanins. Various SPE materials have been applied for that purpose (Kraemer-Schafhalter et al., 1998; Nicoue et al., 2007). Kraemer-Schafhalter et al. (1998) performed a comparison of 16 different SPE materials for purification of chokeberry anthocyanins. These included reversed phase silica gels and macroreticular non-ionic acrylic polymer absorbents. The absorbent Amberlite XAD7 was found to be very suitable for chokeberry anthocyanin purification. The applicability of Amberlite XAD 7 for SPE of different berry extracts was extended in our study. The volume of extract applied to the SPE column depended on the loading capacity of the SPE material and the amount of anthocyanins present in the sample. It was found that for the column used and packing material, quantities of anthocyanins higher than 25 mg led to column overloading. This amount determined the volume of each extract to be applied for SPE. Elution profiles of the soluble dry solids, sugars and anthocyanins during SPE are shown in Fig 1. The quantity of sugars is given as the sum of glucose, fructose, sucrose and sorbitol in the sample. Depending on the volume of the applied extract, the elution profile was different. In all cases, there was good separation of sugar and acids from anthocyanins despite the different anthocyanin profile of the investigated raw materials. Anthocyanins differ in their polarity and often a gradient elution is performed in order to separate the anthocyanins. This leads to several anthocyanin fractions of a different profile. In the current work, the separation of the anthocyanins in the column was excellent in all extracts studied. Under the SPE conditions used, the elution was faster, anthocyanin fractions were more concentrated, no gradient elution was required and only a single eluent was used. By measuring the presence of soluble dry solids in the rinsing waters, the purification process could be controlled in an easy manner. This makes the SPE process simple to perform which is of a great importance especially in cases of enlarged scale. The anthocyanin fractions were concentrated by evaporation of the alcohol and then subjected to freeze drying to obtain powdered products. The characteristics of the powdered extracts obtained are shown on Table 2. The content of anthocyanins in the dry extracts varied from 9.6% for blackberry to 24.6% for elderberry extracts. Very good purification from sugars was achieved. The amount of removed sugars varied from 94.4% of elderberry extract to 98.1% of total amount of sugars of blackcurrant and blueberry extracts. The same observation was made for the removal of citric acid – over 88.5%. The residual citric acid in the dry extracts is not undesirable. Anthocyanins are more stable in the presence of acids, moreover citric acid acts as a metal ion chelator and may show a protective effect on anthocyanins during storage and processing (Timberlake & Bridle, 1980, chap. 5). Very good recovery of anthocyanins 90–95.6% was achieved during the SPE process. This shows that the chosen SPE material is suitable for all anthocyanins regardless of the aglycone type.

3.2. Antioxidant activity

Many methods for determination of antioxidant activity have been developed and reviewed. Various methods rely on the generation of different radicals acting by different mechanisms (Huang, Ou, & Prior, 2005) and usually it is recommendable to evaluate the antioxidant properties of the investigated materials using several assays (Ciz et al., 2010). In our study, the antioxidant properties of the purified extracts were analysed via five different methods – ORAC, TRAP, HORAC, NO-scavenging and lipid peroxidation inhibition. The chosen methods embrace different aspects of the antioxidant action and give a comprehensive view on the antioxidant potential of the investigated extracts. The first two methods assess the radical scavenging activity of the sample against peroxyl radicals, whereas HORAC method measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions and hence the protecting ability against formation of hydroxyl radical. Since lipids are very susceptible to lipid peroxidation, we also tested the ability of the samples to prevent the peroxidation of polyunsaturated fatty acids induced by hydroxyl radical. In addition to reactive oxygen species, reactive nitrogen species (produced mainly by macrophages) are one of the important microbicidal tools in the process of inflammation during the fight against pathogenic microorganisms, bacteria and tumour cells. Nitric oxide, a member of reactive nitrogen species, is an important molecule involved in the regulation of many physiological and microbicidal processes. However, the overproduction of NO occurs in several chronic inflammatory diseases, such as bronchitis, osteoarthritis and rheumatoid arthritis (Pekarova et al., 2009). For this reason the elimination of NO from the inflammatory site is expected to exhibit a very beneficial therapeutic effect. The scavenging properties of our samples were tested using electrochemical analysis, which is considered to be a reliable method for verifying NO-scavenging.

All five berry extracts exhibited strong radical scavenging activities measured by ORAC and TRAP methods. ORAC values were quite high from 3949 to 5783 μmol TE/g (Table 3). Elderberry and blueberry extracts showed the highest levels of peroxyl radical scavenging via the ORAC method, while chokeberry extract was the most potent antioxidant measured by the TRAP method. There are several papers reporting antioxidant properties of anthocyanin-rich extracts. Espin, Soler-Rivas, Wichers, and Garcia-Viguera (2000) reported that a chokeberry extract, measured by the DPPH· method, showed higher radical scavenging activity compared to that of BHA-butylated hydroxyanisole and BHT-butylated hydroxytoluene. Using gel filtration as a method for anthocyanin

Table 3
ORAC, TRAP and HORAC antioxidant activity of anthocyanin-rich berry extracts. Data are presented as means from six independent measurements ± standard deviation. There are no significant differences among values marked with the same superscript letters in individual columns.

Extract	ORAC (μmol TE/g)	TRAP (μmol TE/g)	HORAC (μmol GAE/g)
Chokeberry	5165 ± 223^{B}	4051 ± 82^{D}	1265 ± 50^{B}
Elderberry	5783 ± 202^{C}	3230 ± 62^{C}	1264 ± 74^{B}
Black currant	3949 ± 121^{A}	2132 ± 28^{A}	874 ± 50^{A}
Blackberry	4042 ± 88^{A}	2771 ± 42^{B}	834 ± 50^{A}
Blueberry	5646 ± 102^{C}	2860 ± 76^{B}	1293 ± 55^{B}

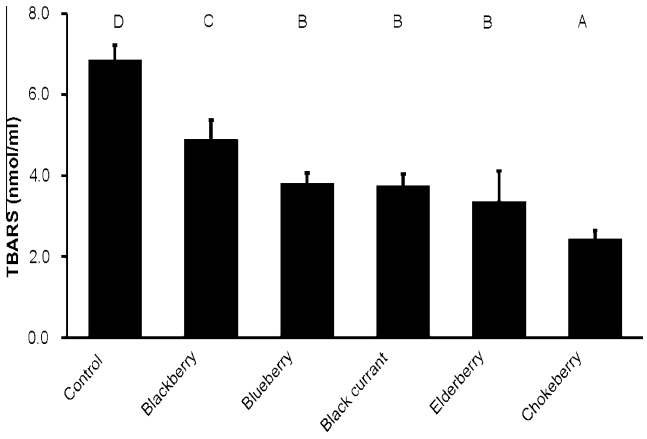

Fig. 2. Inhibition of induced lipid peroxidation by analysed extracts. Data are presented as means from at least three independent measurements ± standard deviation. There are no significant differences among values marked with the same superscript letters.

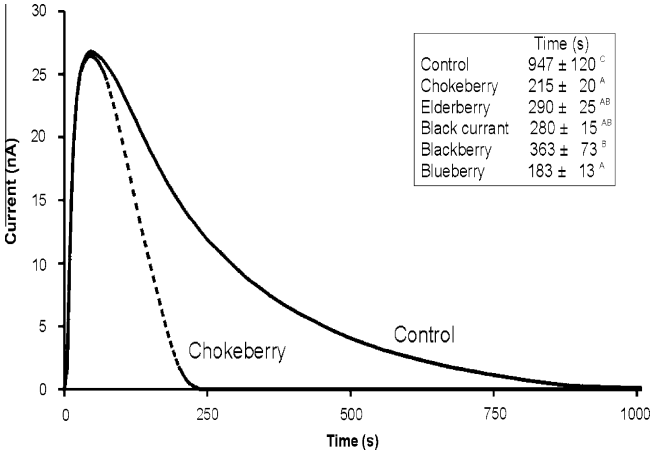

Fig. 3. Scavenging of nitric oxide by analysed samples. The representative curve shows the scavenging effect of chokeberry extract in comparison with control amperometrical signal. Inserted table shows values obtained for all analysed samples. Data are presented as means from at least three independent measurements ± standard deviation. There are no significant differences among values marked with the same superscript letters.

purification Elisia, Hu, Popovich, and Kitts (2007) obtained an anthocyanin-enriched blackberry extract which showed a slightly higher ORAC value – 4885 μmol TE/g than the blackberry extract reported in our study. In one of the first attempts to quantify dietary antioxidant needs of the body Prior et al. (2007) demonstrated that consumption of certain berries and fruits such as blueberries, mixed grape and kiwifruit was associated with increased ORAC plasma antioxidant capacity in the postprandial state and consumption of an energy source of macronutrients containing no antioxidants was associated with a decline in plasma antioxidant capacity. They estimated that according to the energy intake of the diet, 5000–15,000 μmol TE/g are necessary to cover human daily antioxidant needs. However, further long-term clinical studies are needed to clarify the connection between the increment of plasma antioxidant capacity and the potential decreased risk of chronic degenerative disease. The ORAC values of the anthocyanin-rich extracts studied are proof that these extracts present a good source of dietary antioxidants. In the literature scarce data are available about the HORAC values of foods (Ou et al., 2002). To our knowledge, such data for anthocyanin-rich extracts is lacking. Hydroxyl radicals ($HO^{\cdot}$) are highly reactive and can be generated via the Fenton reaction. Antioxidants, which are able to act as metal chelators, may be able to prevent the formation of hydroxyl radical, thus acting as a preventive antioxidant. Since dry extracts contain some citric acid, which can act as metal chelator it was reasonable to check whether citric acid contributes to the HORAC antioxidant activity of the extracts. In model system, pure citric acid did not show HORAC antioxidant activity even in concentration 50 times higher that the concentration of citric acid in the dried anthocyanin extracts. This means that in the conditions of HORAC assay citric acid does not show metal-chelating properties. The elderberry and blueberry extracts obtained in our study revealed four and eight times higher HORAC values, respectively, than the data for elderberry and blueberry fruits (Ou et al., 2002). All the investigated extracts showed several times higher HORAC activity than apple, peach, sour cherry, grapes, cranberry, raspberry, strawberry (Ou et al., 2002) and many vegetables (Ciz et al., 2010). Similar results were obtained in the experiments further carried where all investigated extracts showed high potential to prevent lipid peroxidation and the following rank order of potency was obtained: chokeberry > elderberry > black currant > blueberry > blackberry (Fig 2). Very high scavenging activity against NO was exhibited by all investigated extracts (Fig. 3). These results are original and of particular interest because, according to our knowledge, the direct measurement of the NO-scavenging activity of anthocyanin-enriched extracts has not been performed as yet and some authors only speculated that the NO-scavenging activity of vegetable extracts could be caused by a high content of total phenolic compounds and anthocyanins (Bor, Chen, & Yen, 2006).

Generally, antioxidant activity correlated better with the polyphenol content than to the anthocyanin content. The correlation between polyphenols and antioxidant activity was as follows: TRAP ($r = 0.95$); lipid peroxidation inhibition ($r = 0.86$); HORAC ($r = 0.68$); ORAC and NO-scavenging activity ($r = 0.51$). The highest correlation between antioxidant activity and anthocyanin content was found for ORAC ($r = 0.52$) and HORAC ($r = 0.38$). Taking in mind that these were partially purified extracts of natural colourants, the existence of several radical scavenger compounds in these extracts was possible. It is well known that berries contain a large amount of phenolic compounds that act as antioxidants besides anthocyanins (Prior et al., 1998). Other possible reason for this observation could be the different antioxidant potential of different anthocyanins which is determined by their structural characteristics (Zheng & Wang, 2003).

4. Conclusions

Solid-phase extraction (SPE) using the absorbent Amberlite XAD7 is a fast, simple and reliable method for purification of anthocyanins from complex extracts, containing impurities such as sugars, acids, proteins, pectin, etc. The amount of the anthocyanins in the dried extracts after SPE was 9.6–24.6%. During SPE, more than 94% of the sugars present in the extracts were eluted. A good anthocyanin recovery was observed in the purification process as well. The obtained anthocyanin-rich extracts showed high antioxidant efficacy in all five assays of antioxidant activity determination: ORAC, TRAP, HORAC, scavenging of nitric oxide and inhibition of lipid peroxidation. This reveals the possibilities for application of these preparations as effective antioxidants.

Acknowledgements

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Formation of reactive oxygen and nitrogen species in the presence of pinosylvin – an analogue of resveratrol

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Abstract

OBJECTIVE: Formation of reactive oxygen species in neutrophils of rats with adjuvant arthritis and generation of nitric oxide in RAW 264.7 macrophages were analysed in the presence of pinosylvin.

METHODS AND RESULTS: The method of chemiluminescence was used for the detection of reactive oxygen species in blood of rats with adjuvant arthritis. Pinosylvin (50 mg/kg, daily, p.o.) and methotrexate (0.4 mg/kg, twice a week, p.o.) were applied separately or in a combination over a period of 28 days from the day of immunisation. Adjuvant arthritis was accompanied by a significantly increased number of neutrophils, by elevated concentration of oxidants in blood and by excessive responsiveness of neutrophils to stimulation with PMA. In rats treated with methotrexate, all these changes were significantly reduced and the inhibition became more pronounced when methotrexate was applied in the combination with pinosylvin; the monotherapy with pinosylvin did not induce any detectable changes in the parameters tested. Under *in vitro* conditions, pinosylvin inhibited formation of nitric oxide (NO) in macrophages, as demostrated by the decreased concentration of nitrite – the end-product of NO metabolism (assessed by Griess' method), by the reduced expression of inducible NO synthase (detected by Western blot), and by the failure of pinosylvin to scavenge nitric oxide (measured amperometrically in cell-free system).

CONCLUSION: The observed ability of pinosylvin to decrease concentration of reactive oxygen and nitrogen species, along with its capacity to enhance the efficacy of methotrexate in arthritis treatment may shed more light into the pharmacological potential of this prospective natural substance.

ORIGINAL ARTICLE

Abbreviations:

NO	- nitric oxide
iNOS	- inducible NO synthase
NFκB	- nuclear factor κB
PMA	- 4β-phorbol-12β-myristate-α13-acetate
RLU	- relative luminescence units

INTRODUCTION

Reactive oxygen and nitrogen species, produced by neutrophils and other inflammatory cells, participate actively in the initiation and development of many pathological states (Witko-Sarsat *et al.* 2000; Cascao *et al.* 2010). In rheumatoid arthritis, the oxidants can induce cartilage degradation, depolymerise hyaluronan and decrease its lubricative properties, they can reduce the protective antioxidant and antiproteinase capacity of synovial fluid and participate in this way in joint erosion (Edwards & Hallett 1997; Cross *et al.* 2006; Cascao *et al.* 2009). These facts focussed attention on antioxidative and neutrophil targeting substances as they may increase the effectiveness and minimise unwanted side effects of disease-modifying antirheumatic therapy. Several compounds of plant or microbial origin were found to be promising from this perspective (Drábiková *et al.* 2009; Jančinová *et al.* 2009a; Rovenský *et al.* 2009).

Pinosylvin (3,5-dihydroxystilbene), one of the naturally occurring resveratrol (3,4',5-trihydroxystilbene) analogues, is formed constitutively and after UV irradiation or microbial attack in the wood and needles of *Pinus* species. The majority of the available data characterises antifungal, antibacterial and anticancer activities of pinosylvin (Lee *et al.* 2005; Roupe *et al.* 2006a; Simard *et al.* 2008), yet little is known about its antioxidant and antiinflammatory effects (Park *et al.* 2004; Adams *et al.* 2005; Lee *et al.* 2006). Previously, we found that incubation of human neutrophils with this stilbene reduced production of reactive oxygen species and this effect involved the inhibition of protein kinase C isoforms α and βII (Perečko *et al.* 2008; Jančinová *et al.* 2009b). In the present study, its interference with neutrophils was further tested under *in vivo* conditions – in rats with adjuvant arthritis, and formation of the inflammatory mediator nitric oxide in macrophages was also analysed.

MATERIALS AND METHODS

Materials

Pinosylvin was synthesised at the Institute of Organic Chemistry and Biochemistry AS CR (Praha, Czech Republic), methotrexate was from Pharmachemie (Haarlem, Netherlands), Griess' reagent, lipopolysaccharide from *Escherichia coli*, luminol, and PMA from Sigma-Aldrich Chemie (Deisenhofen, Germany), murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Formation of reactive oxygen species – effects of pinosylvin in arthritis

Adjuvant arthritis was induced in male Lewis rats by a single intradermal injection of heat-killed *Mycobacterium butyricum* (Poništ *et al.* 2010). The study was performed in compliance with Principles of Laboratory Animal Care and was approved by the local Ethics Committee and by the State Veterinary and Food Administration of the Slovak Republic. Pinosylvin (50 mg/kg, daily, p.o.) and methotrexate (0.4 mg/kg, twice a week, p.o.) were applied separately or in a combination over a period of 28 days after arthritis induction. Then neutrophil count and concentration of reactive oxygen species in blood were assessed. The production of oxidants (spontaneous or stimulated with 0.05 μmol/l PMA) was estimated on the basis of luminol-enhanced chemiluminescence and presented as the mean integral values over 3600 s (Nosáľ *et al.* 2007; Jančinová *et al.* 2009a).

Formation of nitric oxide – effects of pinosylvin in vitro

Generation of reactive nitrogen species was determined indirectly as the accumulation of nitrites – end products of nitric oxide (NO) metabolism. The detection was performed spectrophotometrically by Griess' method in the supernatant of murine macrophages RAW 264.7, which were pre-incubated for 1 h with pinosylvin and after that stimulated for 24 h with lipopolysaccharide (LPS). The concentrations of nitrites were derived by regression analysis using serial dilutions of sodium nitrite as a standard (Králová *et al.* 2008). The cell fractions of these samples were used for the detection of inducible NO synthase expression by Western blot. Relative protein levels were quantified by scanning densitometry using the Image J programme (Ambrožová *et al.* 2010). The NO scavenging activity of pinosylvin was measured amperometrically using an ISO-NO Mark II NO meter and NO standard solutions. Changes in electrical current were recorded and the index of scavenging was calculated by dividing the maximum height and width of the obtained curves (Číž *et al.* 2008).

Statistical analysis

Statistical significance of differences between means was established by one-way analysis of variance (ANOVA); *p*-values below 0.05 were considered statistically significant.

RESULTS

Adjuvant arthritis was accompanied by a significantly increased number of neutrophils and by massive formation of oxidants, which was manifested by elevated chemiluminescence of blood (Table 1). Stimulated chemiluminescence was increased seven times and it rose more rapidly than the other parameters tested. This indicates that neutrophils of arthritic rats responded to PMA by an excessive production of radicals, similarly as found by hyper-reactive „primed" neutrophils

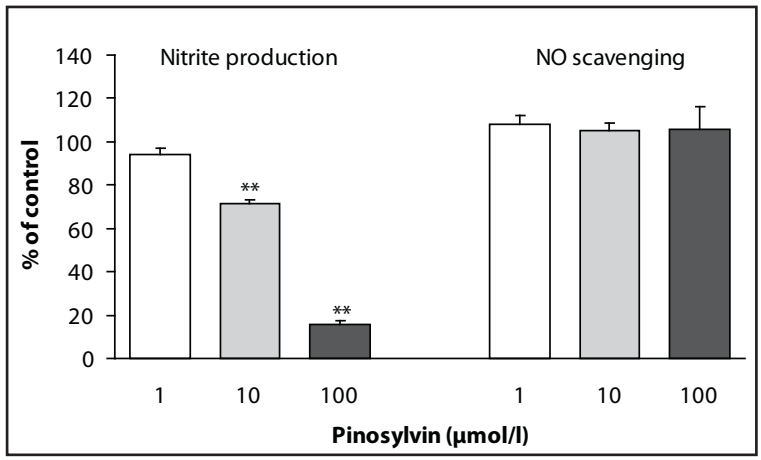

Fig. 1. Nitrite production and nitric oxide (NO) scavenging assessed in the presence of pinosylvin.
Concentration of nitrites was measured spectrophotometrically in supernatants of RAW 264.7 cells stimulated with LPS; the NO scavenging effect of pinosylvin was determined amperometrically in a cell-free chemical system. Pinosylvin-induced changes in nitrite concentration or in the scavenging index were expressed as percentage of controls (i.e. samples incubated in the absence of pinosylvin). Mean ± SEM, n = 3, ** p<0.01 (vs Control).

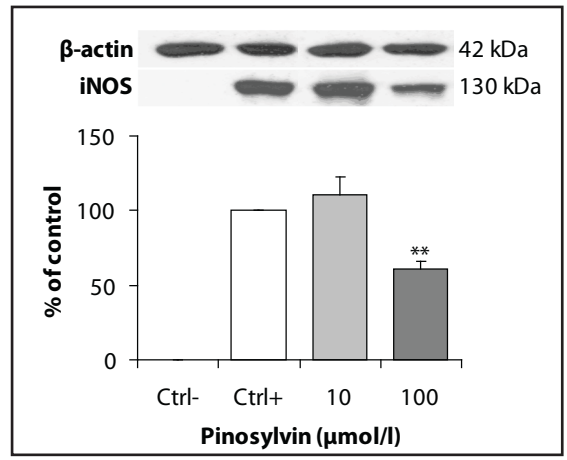

Fig. 2. Effect of pinosylvin on the expression of inducible nitric oxide synthase (iNOS).
Densitometric analysis and representative Western blot of iNOS protein expression in RAW 264.7 cells treated with 10 and 100 µmol/l pinosylvin and stimulated with LPS. Unstimulated (Ctrl–) and LPS-stimulated (Ctrl+) control cells were used to express effects of the stimulus and of pinosylvin, respectively. The immunoblotting of β-actin verified the equal loading of proteins. Mean ± SEM, n = 3, ** p<0.01 (vs Ctrl+).

of patients with rheumatoid arthritis (Fairhurst *et al.* 2007). In rats treated with methotrexate, all the arthritis-induced changes were significantly reduced and this inhibition became more pronounced when methotrexate was applied along with pinosylvin. Methotrexate alone decreased neutrophil count, spontaneous and stimulated chemiluminescence by 28%, 41% and 43%, respectively, whereas in combination with pinosylvin, it inhibited these parameters by 59%, 69% and 63%. The monotherapy with pinosylvin failed to induce any detectable changes either in the number of neutrophils or in oxidant concentration.

Considering the fact that overproduction of nitric oxide is involved in rheumatoid arthritis, and with the aim to find further mechanism(s) potentially involved in the antiinflammatory effect of pinosylvin, the interference of this stilbene with macrophage activity and with NO formation was analysed (Figures 1 and 2). Pinosylvin significantly reduced the quantity of NO produced by stimulated RAW 264.7 macrophages, as evidenced by the decreased accumulation of nitrites in supernatants of these cells. The reduced expression of inducible NO synthase and the absence of NO scavenging indicated that this inhibition resulted from diminished generation of nitric oxide.

Tab. 1. Effect of pinosylvin (PIN) and methotrexate (MTX), applied separately or in combination, on spontaneous and stimulated chemiluminescence and neutrophil count in blood of arthritic rats.

	Chemiluminescence (RLU*s)		Neutrophil count
	Spontaneous	PMA stimulated	in 1 µl of blood
Healthy control	41 802±2 452	150 789±9 159	12 174±747
Arthritis	168 203±12 815	1 165 603±94 470	40 260±3 325
Arthritis + PIN	190 157±32 580	1 132 747±104 750	44 002±4 398
Arthritis + MTX	98 806±8 136**	664 618±68 695**	29 180±1 903*
Arthritis + PIN + MTX	51 739±3 437++	429 813±45 494++	16 635±2 157++

Mean ± SEM, n = 8-10, *p<0.05, **p<0.01 (vs Arthritis) ++p<0.01 (vs Arthritis + MTX), PMA – 4β-phorbol-12β-myristate-α13-acetate; RLU – relative luminescence units

DISCUSSION

The recently confirmed active involvement of neutrophils in the initiation and development of rheumatoid arthritis (Cascao *et al.* 2009 and 2010; Wright *et al.* 2010) focussed attention on substances which are able to repress the activity of these cells both *in vitro* (Adams *et al.* 2005; Perečko *et al.* 2008; Jančinová *et al.* 2009b) and *in vivo* (Nosáľ *et al.* 2007; Drábiková *et al.* 2009; Jančinová *et al.* 2009a; Rovenský *et al.* 2009; Poništ *et al.* 2010). Elimination of the inflammatory reaction caused by neutrophils and resulting in tissue damage may significantly potentiate the effectiveness of antirheumatic therapy. Moreover, co-application of natural medicines which are able to reduce harmful effects of neutrophils could lower the dosage of antirheumatic drugs and thus decrease their toxicity.

The presented results indicate that pinosylvin may be considered a candidate for the combined therapy of rheumatoid arthritis. This stilbene potentiated the suppressive effect of methotrexate on neutrophil activity and concentration of oxidants in blood. Moreover, the rats treated with the combined therapy showed reduced hind paw swelling in comparison with those treated with methotrexate alone (Bauerová *et al.* 2010). The antiinflammatory activity of methotrexate arises from its ability to inhibit T cell proliferation and cytotoxicity, to decrease recruitment of monocytes and other cells to the inflamed joint and from the increased release of the endogenous anti-inflammatory mediator adenosine (Cronstein 2005). All these alterations could lead to a decreased number and activity of neutrophils and to reduced whole blood chemiluminescence. The interference of pinosylvin with inflammation seems to be related to other mechanisms. As found for resveratrol – a compound structurally similar to pinosylvin, the stilbene-induced downregulation of the inflammatory response involves reduced synthesis and release of pro-inflammatory mediators, modified eicosanoid synthesis, decreased activity of immune cells and suppressed activation of nuclear factor κB – NFκB (Alarcón de la Lastra & Villegas 2005; Khanna *et al.* 2007). Moreover, resveratrol or its oligomeric derivative α-viniferin induced apoptosis of human rheumatoid arthritis synovial cells (Nakayama *et al.* 2010) and suppressed tissue destruction in model arthritis (Lee *et al.* 2004; Elmali *et al.* 2005). Some of these effects have been already observed in the presence of pinosylvin – e.g. reduced production of pro-inflammatory mediators (tumour necrosis factor α, interleukin-8, prostaglandin E_2 and leukotriene B_4) in neutrophils and macrophages as well as the suppressed cyclooxygenase-2 protein and gene expression resulting from inhibition of NFκB activation (Park *et al.* 2004 and 2005; Adams *et al.* 2005; Lee *et al.* 2006). Contrary to *in vitro* experiments (Perečko *et al.* 2008; Jančinová *et al.* 2009b), orally administered pinosylvin did not alter the activity of neutrophils when applied in monotherapy. All the beneficial activities of pinosylvin might intensify the anti-inflammatory activity of methotrexate, although by itself it was not sufficient to induce any detectable changes. This might be due to the lower oral bioavailability of pinosylvin (Roupe *et al.* 2006b) and/or to the period of administration – not sufficient to achieve an effective concentration in neutrophils.

Besides neutrophils and reactive oxygen species, the benefit of pinosylvin applied in combined therapy might involve interference of this stilbene with other inflammatory cells and mediators. Under *in vitro* conditions, pinosylvin decreased the activity of stimulated RAW 264.7 macrophages and reduced the concentration of nitric oxide in the supernatant of these cells. Šmidrkal *et al.* (2010) observed similar effects in murine peritoneal macrophages. In the present study, this inhibition was in parallel with the decreased expression of inducible NO synthase protein, indicating an interference of pinosylvin with nitric oxide formation. The mechanism of this effect may be related to the ability of this stilbene to suppress NFκB activation and iNOS gene expression, documented in cancer cells (Park *et al.* 2005). In contrast to flavonoids (Číž *et al.* 2008), pinosylvin did not possess any NO scavenging activity, as confirmed by amperometrical measurement in cell-free system. Considering the fact that in the presence of neutrophils and superoxide, nitric oxide is transformed into highly reactive peroxynitrite which activates pro-inflammatory signalling (Gao 2010) and contributes to the pathogenesis of arthritis (Abramson 2008), the ability of pinosylvin to decrease the concentration of reactive oxygen and nitrogen species can be considered beneficial and may be involved in the antiinflammatory activity of this prospective natural substance.

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ORIGINAL ARTICLE

Molecular targets of the natural antioxidant pterostilbene: effect on protein kinase C, caspase-3 and apoptosis in human neutrophils *in vitro*

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Abstract

OBJECTIVE: Pterostilbene, a naturally occurring phenolic derivative, exhibits various pharmacological effects, e.g. anti-cancerous, antioxidant, anti-inflammatory and anti-diabetic. Based on our previous study, we assessed the cellular and molecular effects of pterostilbene on human neutrophils and in cell free systems. Experimental and theoretical molecular descriptors of stilbene derivatives were also determined.

METHODS: We assessed the antioxidant properties of pterostilbene using cell free system and computational methods. The effect of pterostilbene on protein kinase C activation/phosphorylation was detected by special anti-phospho protein kinase C antibodies. Membrane associated changes determining the life span of neutrophils and human recombinant caspase-3 assay were examined.

RESULTS: Pterostilbene possessed comparable antioxidant properties as resveratrol in cell free system. Computational methods were used to establish the molecular characteristics of stilbene derivatives. The values of electronic parameters suggest a slight enhancement of electron donor properties of pterostilbene compared to resveratrol. Phosphorylation and thus activation of protein kinase C alpha/beta II in activated neutrophils was not decreased by pterostilbene. Pterostilbene in concentrations of 10–100 μM was found to inhibit the activity of human caspase-3 purified enzyme and did not influence cell viability significantly.

CONCLUSION: Pterostilbene, an analog of resveratrol, was identified as a good natural antioxidant compound. However, reducing the oxidative burst of human neutrophils during their activation *in vitro* with pterostilbene does not include protein kinase C phosphorylation pathway. Pterostilbene showed dose dependent activation/inhibition of caspase-3 enzyme activity.

INTRODUCTION

Knowledge that consumption of red wine leads to lower cardiovascular risk opened the scientific scene for resveratrol from stilbene type polyphenols (Fauconneau *et al.* 1997; Šmidrkal *et al.* 2001). Chemical similarity in the stilbene group made us switch from resveratrol (trans-3,4´,5–trihydroxy-stilbene) to pterostilbene, i.e. trans-3,5-dimethoxy-4´-hydroxy-stilbene (Figure 1.A). Both chemical entities exist in cis/trans conformation, but naturally they occur mostly in the trans form, which is also more effective as to antioxidant properties than the cis form (Šmidrkal *et al.* 2001).

Pterostilbene is of natural origin, contained in leaves and grapes of Vitis vinifera, berries of Vaccinium spp., Pterocarpus marsupianum, etc. (Grover *et al.* 2005; Paul *et al.* 1999). Pterostilbene was reported to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and to inhibit lipid peroxidation in rat liver microsomes and there are reports about growth inhibition, antiproliferative effects and induction of apoptosis by cell cycle arrest induced by derivatives of stilbene in a number of normal and cancer cell lines (Pan *et al.* 2008; Rimando and Suh 2008; Stivala *et al.* 2001). Pterostilbene is assumed to possess anti-inflammatory, antioxidant, anti-diabetic, antifungal and anti-cancerous effects (Remsberg *et al.* 2008; Roupe *et al.* 2006).

The physiological activity of human neutrophils plays an important role in fighting intruding microorganisms or foreign particles (Bissonnette *et al.* 2008). Neutrophils may be called "first line defense system". In the process of oxidative burst, neutrophils by using the enzyme NADPH-oxidase produce a variety of cytotoxic products, e.g. superoxide, hydrogen peroxide and hypochloric acid (El-Benna *et al.* 2008). All three substances are known as reactive oxygen species (ROS) and are effective in killing microorganisms. The whole process of NADPH-oxidase activation is strictly controlled by various regulating/phosphorylating enzymes, such as p21-activated kinase, phosphatidyl inositol 3-kinase and nuclear factor κB (El-Benna *et al.* 1996; Myhre *et al.* 2009; Sheppard *et al.* 2001). A key enzyme in NADPH-oxidase activation is protein kinase C (PKC) (Dekker *et al.* 2000). Increased production of ROS during inflammation could be harmful to surrounding tissues. This has been observed in chronic inflammatory diseases, e.g. rheumatoid arthritis (Cross *et al.* 2006). The importance of the regulation of activated human neutrophils is the subject of our study. Activated neutrophils represent a good model for testing inflammation.

The present study is based on our previous research on the generation of ROS by activated neutrophils by using chemiluminescence assay (Perečko *et al.* 2008). To evaluate the antioxidant capacity of pterostilbene, we used oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) assays (Číž *et al.* 2010). Moreover, we examined the effect of pterostilbene on the life-span of isolated human neutrophils. Delayed apoptosis of neutrophils may play a role in the injury of surrounding tissue in chronic inflammatory diseases, e.g. rheumatoid arthritis (Wong *et al.* 2009). Finally, we investigated the effect of pterostilbene on phosphorylation of PKC of neutrophils pre-activated with phorbol-myristate-acetate, which activates cells via PKC (Klink *et al.* 2009). For better understanding the biological effects of pterostilbene, its molecular descriptors were estimated.

MATERIALS AND METHODS

Chemicals

Pterostilbene and resveratrol were prepared by targeted regioselective synthesis, both compounds purely as trans isomers (Šmidrkal *et al.* 2010). Phorbol-myristate-acetate (PMA) was purchased from Sigma (Steinheim, Germany). Polyclonal antibody against phosphorylated-PKC-alpha/betaII (Thr638/641) was purchased from Cell Signaling (Danvers, MA, USA), secondary anti-rabbit antibody and Lumigen Detection Reagent were supplied by GE Healthcare Life Sciences (former Amersham), Little Chalfont, UK. Human AnnexinV/

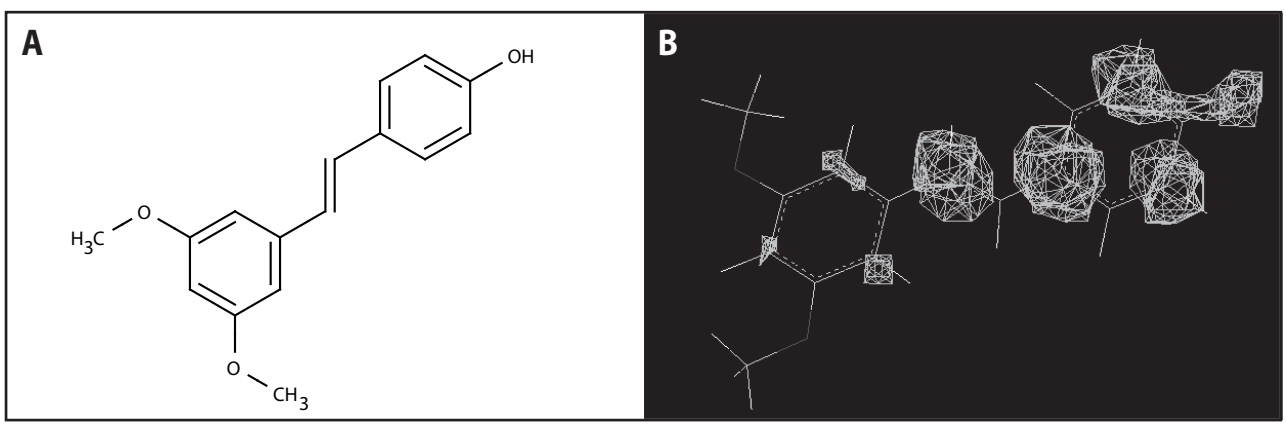

Fig. 1. Pterostilbene. (**A**) Structure of pterostilbene. (**B**) Spin density isosurface map with a contour value of 0.002 of the optimal conformer of pterostilbene in the form of radical derived from the only position (ΔH = 37.2 kcal/mol).

FITC Kit was purchased from Bender MedSystems, Vienna, Austria. Caspase-Glo 3/7 Assay was from Promega (Madison, WI, USA), human purified caspase-3 was from Enzo Life Sciences, Lausen, Switzerland. All other products are available commercially or their origin is mentioned in the text.

Computational methods for pterostilbene and resveratrol

The lowest energy molecular conformations of the compounds tested were calculated using Conformational Search module in HyperChem molecular modeling software (Račková *et al.* 2005) using Austin model 1, and Polak-Ribiere conjugate gradient algorithm with 0.01 convergence limit in vacuum. For optimal conformers of antioxidants, the heat of formation was calculated. The ΔH values were calculated as described previously (Račková *et al.* 2005). Further calculated parameters of the compounds tested included energy values of the highest occupied molecular orbital (HOMO), energy of the lowest unoccupied molecular orbital (LUMO), HOMO–LUMO energy gap (Δε) and spin density (S_D) belonging to the oxygen radical derived from OH groups, which characterizes the distribution of the electron spin and therefore decides the stability of radicals (Cao *et al.* 2003).

Determination of R_m values

The lipophilicity parameters represented by R_m values were measured by reversed-phase thin layer chromatography. The mobile phase consisted of diluted acetic acid (pH 2.5) mixture with acetonitril (20:80, v:v) (Király-Véghely *et al.* 2004). The stationary phase was obtained by impregnation of the layer of Silica gel G F254 plates with 5% solution of liquid paraffin in ether. The compounds were dissolved in methanol and about 1 μg of the compound was spotted onto the plates. A migration of 10 cm was obtained by spotting the compound on a line 2 cm from the lower edge of the plate. The developed plates were dried and the compounds were detected in UV light at 254 nm. The R_m values were calculated by the formula: $R_m=\log(1/R_F-1)$.

Antioxidant assays

The protective effects of pterostilbene and resveratrol (0.01–100 μM) were measured by comparing the area under curve of the sample to that of a known antioxidant, trolox for ORAC assay and gallic acid for HORAC assay. The loss of fluorescein fluorescence indicated the formation of peroxyl radicals (ORAC) or hydroxyl radicals (HORAC) in the sample. Production of peroxyl radicals was induced by 2, 2´-azobis-(2-amidino-propane)-dihydrochloride (AAPH) at 37 °C. The HORAC assay reveals the metal-chelating activity of polyphenols, and thus the protective action against hydroxyl radical formation. Complex Co (II) was used as an initiator. High ORAC and HORAC values indicated that the sample tested possessed a high potency of antioxidant activity (Číž *et al.* 2010). The values of ORAC are expressed as relative trolox equivalent (TE), HORAC as relative gallic acid equivalent (GAE). Results are expressed as means of triplicates in three independent measurements.

PKC blotting

Human neutrophils were isolated as described previously (Jančinová *et al.* 2009). Neutrophil suspension (100 μl) containing 5×10^6 cells was preincubated at 37°C for 60 seconds with different concentrations of pterostilbene (10 or 100 μM) prior to addition of PMA (0.15 μM). Incubation with PMA (60 s) was stopped by using a lysing medium containing protease and phosphatase inhibitors (Na_3VO_4 and NaF). The suspension was sonicated at 4°C for 20 minutes and centrifuged at 18 625×g at 4°C for 5 min. Finally, the supernatant was taken for blotting assay. Total protein was measured using Bradford Dye Reagent detection kit from Bio-Rad.

For the assay, the supernatant was boiled in sample buffer (0.05 M Tris, 2% SDS, 2.5% mercaptoethanol) containing 0.01% bromphenol-blue. The samples (20 μl with 20 μg of protein fraction) were loaded on 10% polyacrylamide gel. Separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore). The blot containing the transferred proteins was blocked in 1% BSA (Sigma-Aldrich) buffer followed by incubation with primary anti-phospho-PKC-alpha/beta II (Thr638/641) antibody (1:8 000) and secondary anti-rabbit antibody (1:10 000). After washing in TBS, the proteins were detected with Lumigen Detection-Reagent kit, scanned and measured densitometrically using free ImageJ program.

Analysis of apoptosis

Citrated whole human blood was collected from healthy male volunteers. Dextrane (3%) was added (blood 2:1 dextrane) and centrifugated at 10×g at room temperature. One ml of buffy coat containing mainly leukocytes was collected and stored on ice before use. The cells were counted on hemocytometer (Coulter Counter), focusing on granulocytes. The cell suspension was then dissolved to reach 200 000 neutrophils per sample. Three different concentrations of pterostilbene (1, 10 and 100 μM) and a control sample were incubated at 37°C for 10 min. The cells were stained with Annexin-V conjugated with FITC (fluorescein isothiocyanate) (BenderMedSystems) in dark at 4°C for 10 min, followed by staining with propidium iodide (1μg/ml) and analyzed immediately by Beckman Coulter Cytomics FC500 cytometer. All samples were analyzed under the same conditions (gains, volts). From the granulocytic area, 5 000 cells were gated and analyzed.

Caspase-3 activity

Following caspase cleavage of the Z-DEVD-amino-luciferin substrate, resulting in the reaction of luciferase with amino-luciferin, the measurement of light production was made on the Luminometer Immunotech

Tab. 1. Electronic and physicochemical characteristics of pterostilbene and resveratrol.

	Pterostilbene	Resveratrol
ΔH (kcal/mol)	37.17	37.39
εHOMO (eV)	–8.45	–8.53
εLUMO (eV)	–0.417	–0.517
Δe (eV)	8.033	8.013
R_m	1.45	0.21

ΔH – parameter of 4´-OH bond strength (corresponding to minimum value of DH with regard to values of 3 and 5 –OH bonds in resveratrol), εHOMO – energy of highest occupied molecular orbital, εLUMO – energy of lowest unoccupied molecular orbital, Δe - energy difference between the HOMO and LUMO, R_m – lipophilicity index.

Tab. 2. Antioxidant profile of pterostilbene and resveratrol.

Pterostilbene (μM)	ORAC μM TE	HORAC μM GAE
100	14.75 ± 2.58	85.48 ± 15.65
10	5.45 ± 2.16	20.58 ± 1.30
1	0.94 ± 0.62	n.d.
Resveratrol (μM)	**ORAC μM TE**	**HORAC μM GAE**
100	17.98 ± 0.36	113.92 ± 23.18
10	10.71 ± 2.63	88.37 ± 13.66
1	2.84 ± 1.36	2.41 ± 1.50

ORAC (peroxyl radical quenching) is measured as trolox equivalent (TE), HORAC (hydroxyl radical quenching) as gallic acid equivalent (GAE). Results are expressed as Mean±SEM of triplicates in three independent measurements. n.d. for not detectable.

LM-01T. According to the manufacturers´ instructions, 10 μl of 0.1 IU caspase was added to 20 μl aliquots of pterostilbene and buffer solution, finally 50 μl of Caspase-Glo 3/7 Reagent was added, the mixture was measured for 60 minutes and the activity of caspase-3 (EC 3.4.22.56) was identified. The vehiculum solution for pterostilbene i.e. competent concentrations of NaOH, were also tested.

Statistics

Data are expressed as mean±SEM, unless stated otherwise. Statistical analysis was performed using Student´s *t*-test to examine differences between treatments and control. Differences were considered to be statistically significant when $p<0.05$ (*) or $p<0.01$ (**).

RESULTS

Computer assisted evaluation of pterostilbene and resveratrol properties and determination of R_m

With regard to its relation to the potential of radical scavenging effect, we computed the parameter energy of HOMO for pterostilbene and resveratrol. The εHOMO expresses the easiness of abstraction of an electron from the neutral molecule, which may be involved in the mechanism of reaction with free radicals (Cao *et al.* 2003; Velkov *et al.* 2007). The value of εHOMO for pterostilbene was –8.45 eV. Compared to resveratrol, a typical representative of natural stilbene derivatives (εHOMO = –8.53 eV), pterostilbene is more effective in electron-donating processes. Furthermore, the value of parameter of 4´–OH bond strength, ΔH (Korzekwa *et al.* 1990), was slightly lower for pterostilbene than for resveratrol molecule (37.17 kcal/mol vs 37.39 kcal/mol, respectively) (Table 1).

However, the values of HOMO-LUMO energy difference (a characteristic of the molecule excitability) for both molecules differed just by 0.02 eV (8.033 eV for pterostilbene vs 8.013 eV for resveratrol), indicating similar chemical reactivity of both compounds (Table 1).

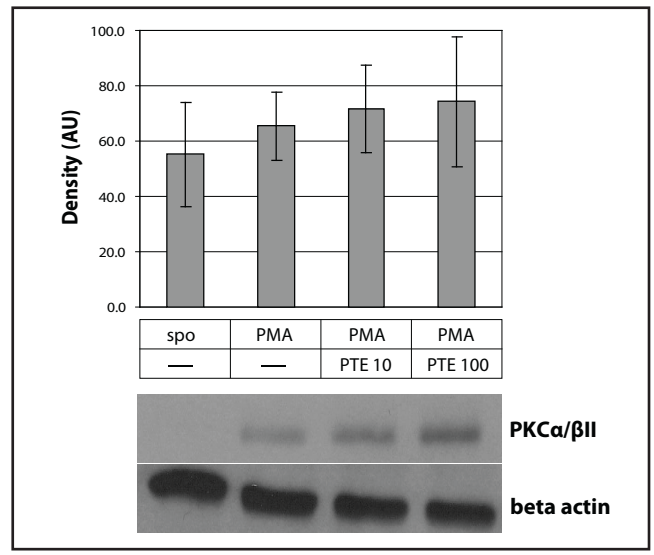

Fig. 2. Effect of pterostilbene on PKC phosphorylation. Densitometric evaluation of protein kinase C phosphorylation from control, PMA (0.15 μM) and pterostilbene (10 and 100 μM) treated isolated human neutrophils. (Mean±SEM, n=4) (spo – control, PTE – pterostilbene, PMA – phorbol-myristate-acetate, AU – Arbitrary Units).

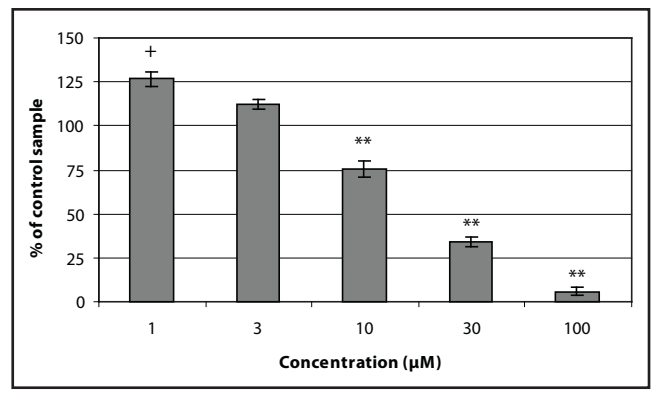

Fig. 3. Effect of pterostilbene on human recombinant caspase-3 activity. Effect of different concentrations of pterostilbene (1–100 μM) on human recombinant caspase-3 activity shown as percentage of control sample. (Mean±SEM, n=5). +$p<0.05$ for activation, *$p<0.05$ and ** $p<0.01$ for inhibition (versus vehiculum).

The spin density isosurface map shows the efficient delocalization of the radical resulting from the only 4´-OH group through the extended conjugation system of the stilbenoid structure (Figure 1.B). Correspondingly spin density (S_D) belonging to the oxygen radical derived from 4`-OH groups showed the same values for the molecules of both resveratrol and pterostilbene (0.101).

Due to substitution by two methyl-groups, pterostilbene showed the higher value of lipophilicity parameter (R_m = 1.45) than resveratrol (R_m = 0.21) (Table 1).

Antioxidant activity assays

The ORAC assay provides data to measure the quenching of peroxyl radicals expressed as trolox equivalent. HORAC measures quenching of hydroxyl radicals (due to metal-chelating activity) expressed as gallic acid equivalent. Results on pterostilbene and resveratrol antioxidant properties are expressed in Table 2. The antioxidant capacity of 10 μM pterostilbene is equal to that of 5.45 μM trolox and 20.58 μM gallic acid standards. Pterostilbene of 100 μM concentration possessed less antioxidant capacity in comparisson with trolox (ORAC), but using HORAC method pterostilbene was as equal as gallic acid standard (85.48 μM). However, resveratrol in concentration of 1, 10 and 100 μM is better antioxidant than gallic acid (HORAC), using ORAC, the antioxidant effect diminished.

PKC response to incubation with pterostilbene

To get further insight into molecular mechanisms underlying inhibition of ROS generation in whole human blood and isolated neutrophils by pterostilbene, we examined the effect of pterostilbene on the phosphorylation of PKC α/β II. No significant changes in PKC α/β II (Thr638/641) phosphorylation after PMA stimulus were achieved by pterostilbene in either concentration (10 and 100 μM) (Figure 2).

Effect of pterostilbene on life-span of human neutrophils

The percentage of viable and apoptotic cells was calculated by flow cytometer measurement after staining the cells with FITC conjugated Annexin-V plus propidium iodide (PI). Only Annexin positive cells were considered for apoptotic (also mentioned as pre-apoptotic) cells and double positive cells (Annexin+/PI+) were considered for late-apoptotic or dead cells. Pterostilbene in any concentration tested (1, 10 and 100 μM) did not change neutrophil viability significantly (data not shown).

The role of pterostilbene on caspase-3 activity

Figure 3 shows biphasic concentration-dependent effect of pterostilbene on the human recombinant caspase-3 activity: in the concentration of 1 μM, pterostilbene induces the activity of caspase-3 significantly, in concentrations of 10, 30 and 100 μM, pterostilbene inhibits the activity of the enzyme. The effect of appropriate vehiculum concentrations (4–400 μM NaOH) did not significantly influence the activity of caspase-3.

DISCUSSION

The antioxidant properties of pterostilbene have, over the years, attracted the interest of many researcher groups however the effect of pterostilbene on human neutrophils has not yet been fully investigated. Pterostilbene is a dimethylether analogue of the well-known stilbene derivative resveratrol. These compounds are mainly synthetized in plants after stress stimuli (e.g. irradiation, microorganism infection) (Keller *et al.* 2003) and act as phytoalexins (Breuil *et al.* 1999). But there are few reports on pterostilbene effects on human cells. Pterostilbene has been widely discussed because of its anti-cancer and chemopreventive properties in different *in vivo* and *in vitro* cancer models and cultured cell lines (Cichocki *et al.* 2008; Rimando and Suh 2008; Tolomeo *et al.* 2005). Pterostilbene exhibits also immunomodulatory (Šmidrkal *et al.* 2010) and anti-inflammatory effects (Cichocki *et al.* 2008). Based on this information and our previous studies, we investigated antioxidant effects of pterostilbene in cell free systems and focused also on the effects of pterostilbene on activated human neutrophils *in vitro*. Electronic parameters for the minimum energy conformers of pterostilbene in comparison to resveratrol were calculated. Lipophilicity parameters for the compounds tested were evaluated as well.

The polyphenolic structure of stilbenes, and thus of pterostilbene, justifies its antioxidant testing. Pterostilbene has been suggested to be a good antioxidant in different cell free system methods, e.g. based on formation of DPPH. In the study of Stivala *et al.* (2001) pterostilbene gave a value comparable to trans-resveratrol in antioxidant testing, using DPPH assay. Our experiments with the ORAC and HORAC method describe the antioxidant effects against peroxyl (ORAC) and hydroxyl (HORAC) radicals in cell free system. In the concentrations of 1, 10 and 100 μM, pterostilbene was less effective in comparison to trolox standard. Remsberg *et al.* (2008) showed better prevention of oxidation in the lower concentration of pterostilbene. This may be due to the pro-oxidant activity in higher concentrations that is in agreement with its higher value of εLUMO (–0.417 eV) compared to resveratrol (–0.517 eV) (Table 1). Our study suggests that substitution of both 3 and 5 hydroxy-groups with methoxy-groups does not decrease the antioxidant properties of pterostilbene in cell free system dramatically (compared to resveratrol), though resveratrol is showing better antioxidant properties in HORAC assay (Table 2). The 4´-hydroxy group in pterostilbene is able to assure sufficient antioxidant activity. However 4´-methoxy derivative was less effective in the study of Stivala *et al.* (2001). We may hypothesize that the 4´-hydroxy group may play a crucial role in antioxidant effects.

For better understanding of the antioxidant effects of pterostilbene, we calculated the molecule parameters for the minimum energy conformers of this molecule

in comparison to the well-known stilbene representative resveratrol. The structure of pterostilbene (Figure 1.A) enables only one proton subtraction in 4´-position, thus forming a phenoxy-radical. The distribution of spin density can have a decisive effect on the stability of the phenoxyl radicals derived from stilbenoid structure (Cao *et al.* 2003). Comparably to resveratrol, the abstraction of proton from OH in para-position to the double-bond of ethene group allows efficient electron delocalization of the generated radical through the pterostilbene molecule, as shown in the spin density map (Figure 1.B). Moreover, the spin densities (S_D) belonging to the oxygen radical derived from 4´-OH group for both stilbene analogues showed the same value. Thus, the methylation of the free OH groups at meta-position of ring A in pterostilbene can not significantly reduce the scavenging activity compared to resveratrol. In general, both pterostilbene and resveratrol showed comparable values of parameters εHOMO, ΔH and Δε (HOMO-LUMO energy gap), suggesting similar free radical scavenging properties of both stilbene derivatives. This is in accordance with the results of Stivala *et al.* (2001), showing comparable DPPH radical scavenging activity of both compounds in a chemical system.

As discussed in the two paragraphs above, the physicochemical characteristics of both derivatives tested are conformable, but according to our results with chemiluminescence assay (Perečko *et al.* 2008), the stilbenes tested vary in biological medium. Substitution of two hydroxyl groups with methoxy-groups increased lipophilicity parameter R_m of resveratrol from 0.21 to 1.45 for pterostilbene. This may enhance the bioavailability of pterostilbene in contrast to the low bioavailability of resveratrol (Cichocki *et al.* 2008). On the other side lipophilicity may influence the transition of substances through cell membrane into cytosol. Also the number and position of hydroxyl groups play a role in the antioxidant effects of polyphenols in different cell assays. Taking into consideration the effects of different resveratrol derivatives on the production of thiobarbituric acid reactive substances (TBARS) in normal human fibroblasts (Stivala *et al.* 2001), pterostilbene was as good as resveratrol. Its 4´-methoxy derivative as well as 3, 4´, 5-trimethoxystilbene did not exert a significant inhibition of TBARS production (Stivala *et al.* 2001). These results support our findings with chemiluminescence assay in whole blood. The 3, 5-methoxy groups increased the antioxidant properties of pterostilbene compared to resveratrol in whole human blood. However, inhibition of extracellular and intracellular chemiluminescence with pterostilbene was lower than with resveratrol and the difference was more significant for intracellular compartment (Perečko *et al.* 2008). In agreement with the biological activity-lipophilicity parabolic findings (Hansch and Clayton 1973) during transportation through the membrane system, the entrapment of more hydrophobic pterostilbene in the lipid phase of outer membrane could diminish its inhibitory effect on intracellular ROS production. Thus, free hydroxy-groups of resveratrol may be appropriate for antioxidant activity in respect of its intracellular availability directed by lipophilicity parameter.

In the attempt to elucidate the molecular mechanism of reducing the production of reactive oxygen species, we examined the effect of pterostilbene on activation/phosphorylation of PKC. Phosphorylation of PKC is an activation marker and antecedent step of phosphorylation of NADPH-oxidase subunits. Pterostilbene is known to affect e.g. the p38-phosphorylation pathway in breast cancer cell line (Pan *et al.* 2009), but the effect on human neutrophil PKC phosphorylation has not been established. In our study, pterostilbene did not change the phosphorylation of PKC α/β II of isolated human neutrophils activated by PMA, which is known to activate cells just via activation of PKC (Klink *et al.* 2009). We are suggesting that other mechanisms in reducing of ROS production may be involved, e. g. inhibition of NADPH-oxidase.

The *in vitro* Annexin-V/propidium iodide staining assay revealed no significant effects of pterostilbene in concentrations tested on human neutrophil cell death. This is in agreement with other studies (Stivala *et al.* 2001). Remsberg *et al.* (2008) showed apoptotic effect of pterostilbene on different cancer cell lines, suggested also by other studies (Tolomeo *et al.* 2005), yet pointing out that pterostilbene, and thus other stilbene derivatives, was not cytotoxic against e.g. normal human umbilical vein epithelial cells. Generally, pterostilbene, comparable to resveratrol, was found to have antiproliferative, cytostatic activity on different cell lines, but it did not exhibit cytotoxic activity in the concentrations tested (Stivala *et al.* 2001). This was acknowledged by our study using ATP-cytotoxicity test (Perečko *et al.* 2008). According to caspase-3 activity assay, Pan *et al.* (2007) showed activation of caspase-2,-3,-8 and -9 by pterostilbene in human gastric carcinoma AGC cells. In our experiment, pterostilbene in concentration of 1 μM showed significant increase of activity of caspase-3, while in concentrations of 10, 30 and 100 μM it inhibited caspase-3 activity. This result is in agreement with no apoptotic changes caused by pterostilbene in the concentrations tested on human neutrophil viability studied by flow cytometry. Nevertheless caspase transduction is not the only apoptotic signaling pathway.

Taking into account the above presented results and discussion, pterostilbene represents a derivative with significant antioxidant effect. The structure-activity relationship analysis may be used to advantage in rational design and synthesis of derivatives with desired antioxidant, anti-inflammatory and anti-proliferative properties.

ACKNOWLEDGEMENTS

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Research Article

The Effects of Pterostilbene on Neutrophil Activity in Experimental Model of Arthritis

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It has been demonstrated that pterostilbene inhibits reactive oxygen species production in neutrophils *in vitro*. However, little is known about its effects on neutrophils during inflammation *in vivo*. In this study, the effect of pterostilbene on neutrophil activity was investigated in experimental arthritis model. Lewis rats were injected by a single intradermal injection of heat-killed *Mycobacterium butyricum* in Freund's adjuvant to develop arthritis. Another group of arthritic animals received pterostilbene 30 mg/kg, daily, p.o. The number and activity of neutrophils in blood were measured on a weekly basis during the whole experiment. Moreover, the total radical trapping potential in plasma was measured at the end of the experiment. In the pterostilbene treated arthritic group, the treatment significantly lowered the number of neutrophils in blood on days 14 and 21 without significant downregulation of neutrophil oxidative burst. Pterostilbene nonsignificantly increased total radical trapping potential in arthritic animals. These results indicate that the promising effects of pterostilbene on reactive oxygen species operate by different mechanisms *in vitro* and in the animal model of inflammation. In conclusion, the positive effects of pterostilbene in the model of arthritis may be attributed to regulation of neutrophil number.

1. Introduction

Neutrophils are a crucial part of the innate immune system. Once activated, they adhere to and migrate through the endothelium to get to the inflamed tissue. Neutrophils contain NADPH-oxidase which generates superoxide anion [1]. In response to infection or foreign particles, neutrophils produce reactive oxygen species (ROS) to destroy the invading pathogens, which is known as oxidative burst of neutrophils [2]. The dark side of neutrophil activity is its contribution to tissue damage. This is due to overproduction of ROS seen in many inflammatory diseases, for example, rheumatoid arthritis [3–5].

Rheumatoid arthritis is a chronic autoimmune inflammatory disease characterised by bone erosion and cartilage damage with synovial hyperplasia and pain [6]. Neutrophils are present in the synovial fluid and on the pannus-cartilage interface in arthritis [7, 8]. In addition, isolated neutrophils from arthritic patients showed preactivation (priming) which may be due to the presence of different cytokines in the synovial fluid [3]. Thus by producing ROS, activated neutrophils could contribute to joint destruction (Figure 1). This highlights the importance of searching for therapeutic agents capable of controlling the oxidative burst of neutrophils in neutrophil-dominant inflammatory diseases. Recently, significant research interest has been focused on resolution as a means to treat inflammatory diseases [9]. Resolution was thought to be a passive process caused by reduction of proinflammatory chemokines. Now, however, it is believed to be an active process with significant impact on neutrophil functionality [10, 11]. During resolution, in particular, the activity of neutrophils is downregulated [12]. To study the influence

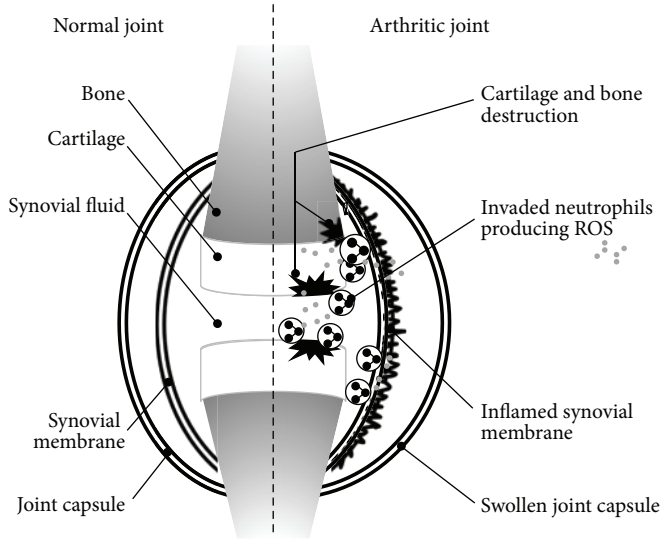

Figure 1: The role of neutrophils in pathogenesis of arthritis.

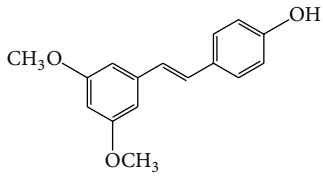

Figure 2: Structure of pterostilbene.

of neutrophils on pathogenesis of rheumatoid arthritis and to investigate the effects of new compounds on this process, different animal models of arthritis are used. One of them is the model of adjuvant arthritis induced by heat-inactivated *Mycobacterium butyricum* in Freund's adjuvant [13]. Mohr et al. showed that under normal conditions, there were no neutrophils in the articular capsule of nonarthritic animals, while the inflamed synovial and capsular tissue of adjuvant injected animals is heavily infiltrated with neutrophils [7]. We used this *in vivo* model of rat adjuvant arthritis to study whether pterostilbene—a stilbene type polyphenol—was capable of downregulating the activity of neutrophils.

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene, Figure 2) is a dimethyl derivative of resveratrol (*trans*-3,5,4′-trihydroxystilbene). The natural sources of pterostilbene are various herbal drugs, for example, leaves and grapes of *Vitis vinifera*, *Vaccinium* spp., and *Pterocarpus marsupium* [14–16]. Pterostilbene was found to have anti-inflammatory, antidiabetic, antifungal, and anticancerous effects [17, 18]. It was reported to inhibit lipopolysaccharide induced expression of COX-2 [19]. In our previous experiments, pterostilbene was the most effective resveratrol derivative tested in inhibiting ROS production by human neutrophils *in vitro*. Moreover, pterostilbene decreased the production of extracellular ROS, similarly as did resveratrol. Yet on intracellular ROS production, the effect of pterostilbene was lower in comparison with resveratrol [20]. Extracellular ROS are responsible for the tissue destruction, whereas intracellular ROS are involved in killing pathogens and in intracellular signalling [21].

In this study we investigated the effects of pterostilbene on the oxidative burst of neutrophils in arthritic rats. According to our knowledge, no papers have been published on the effects of pterostilbene on neutrophil activity in an *in vivo* model of chronic inflammation.

2. Materials and Method

Pterostilbene (precisely specified as *trans*-pterostilbene in this paper) was synthesised at the Institute of Chemical Technology, Prague, Czech Republic, and structurally characterised as (E)-4′-hydroxy-3,5-dimethoxystilbene in the Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic [22]. TLC, RP-HPLC, and ^{1}H-NMR spectroscopy were used for standardisation before and during the experiments.

2,2-Azo-bis-2-amidinopropane dihydrochloride (AAPH), horseradish peroxidase (HRP), luminol, phorbol myristate acetate (PMA), trichloroacetic acid, and Trolox were purchased from Sigma, Germany. *Mycobacterium butyricum* obtained from Difco Laboratories (Detroit, MI, USA) was suspended in Freund's adjuvant. All other products are available commercially or their origin is mentioned in the text.

2.1. Model of Adjuvant Arthritis in Rats. The study was performed in compliance with Principles of Laboratory Animal Care and was approved by the institutional Ethics Committee and by the State Veterinary and Food Administration of the Slovak Republic (Ro-1668/09-221). Animals were kept in an air-conditioned room with 12 hours day/night mode and drinking water *ad libitum*.

Male Lewis rats (Dobra Voda, Slovaki) were injected by a single intradermal injection of heat-killed *Mycobacterium*

butyricum in Freund's adjuvant to develop arthritis [23]. Healthy control group and arthritic control group were treated with the solvent agent-sunflower oil. The doses of pterostilbene used in *in vivo* studies with rats range from 10 to 40 mg/kg [24–26]. In our experiment, pterostilbene 30 mg/kg, daily, p.o. in sunflower oil, was applied over a period of 21 days after arthritis induction. There were 10 animals in each group. On days 0, 7, 14, and 21, whole blood (10 μL) from the tip of the tail was taken to citrated pipette tip and immediately diluted in Tyrode solution. The procedure was performed under local anaesthesia. The blood was subjected to further analysis of chemiluminescence and neutrophil numbers. After the experiment, the animals were sacrificed by overdosing with ketamine/xylazine anaesthesia. The blood was taken by cardiac puncture and plasma was obtained by 2000 g/15 min centrifugation for further analysis of total peroxyl radical trapping capacity (TRAP).

2.2. Evaluation of Neutrophil Number and ROS Production in Rats. The number of neutrophils in rat blood was calculated with a haemocytometer (Beckman Coulter). Spontaneous and PMA-stimulated oxidative burst of neutrophils in rat blood were determined by using chemiluminescence. Briefly chemiluminescence of neutrophils in blood was measured in a 96-well microplate luminometer (LM-01T Immunotech) at 37°C. Aliquots of Tyrode buffer and luminol (250 μmol/L) were added. To ensure sufficient concentration of extracellular peroxidase in stimulated cells, we added horseradish peroxidase (HRP) to the final concentration 8 U/mL. Finally, 50-times diluted blood was added and the reaction was started by adding phorbol myristate acetate (PMA) to the final concentrations of 0.005–0.5 μmol/L [27]. The chemiluminescence of the samples was recorded for 1 hour and area under the curve was examined. Normalisation of the oxidative burst of neutrophils in rat blood to the number of neutrophils in rat blood was calculated by using

$$\text{neutrophil activity} = \frac{\text{CL of sample}}{\text{number of neutrophils in the sample}} \quad (1)$$

(CL: chemiluminescence).

2.3. Total Peroxyl Radical Trapping Capacity (TRAP) of Rat Plasma. By using thermal decomposition of 2,2-azo-bis-2-amidinopropane dihydrochloride (AAPH), peroxyl radicals were monitored by luminol-enhanced chemiluminescence [28]. The reaction mixture consisted of 480 μL of PBS and 50 μL of 10 mmol/L luminol. Then 20 μL of plasma samples or Trolox was added directly into the cuvette and the samples were preincubated for 10 min/37°C. Finally, 50 μL of AAPH was added directly into the cuvette. Time needed for a 50% recovery of the original steady-state signal (so-called half peak time) was identified for each sample. Trolox was used as a standard inhibitor. The results obtained were expressed as μmol of peroxyl radical trapped by one litre of plasma.

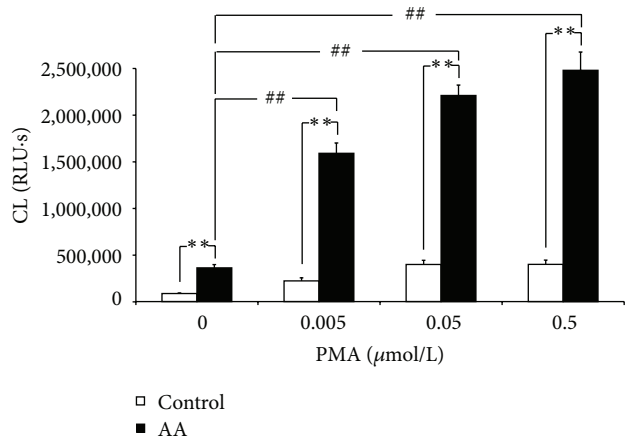

Figure 3: Priming of neutrophils in arthritic rats. Arthritic animals (AA) showed significantly higher spontaneous (0) and PMA-stimulated ROS production ($^{**}P < 0.01$ AA versus control). PMA caused dose-dependent increase in ROS production which was significantly higher in comparison with spontaneous ROS production in arthritic rats ($^{\#\#}P < 0.01$ AA-stimulated versus AA-spontaneous). Values are mean ± SEM, $n = 10$. RLU∗s: relative light units multiplied by time (seconds).

2.4. Statistics. Data were examined using the Student's t-test, and P values below 0.05 and 0.01 were considered statistically significant.

3. Results

3.1. Arthritis Caused Priming of Neutrophils. Arthritic animals (rats with induced arthritis with solvent treatment only) showed significantly ($^{**}P < 0.01$) higher production of ROS in blood in nonstimulated (spontaneous) and PMA-stimulated conditions in comparison with healthy controls (rats with solvent treatment only) (Figure 3). In arthritic animals the use of PMA increased significantly ($^{\#\#}P < 0.01$) the production of ROS in comparison with spontaneous ROS production. The effect of PMA stimulation was concentration dependent. The highest concentration of PMA used (0.5 μmol/L) did not induce much higher ROS production in comparison with 0.05 μmol/L PMA. In the further analysis, we therefore discuss spontaneous and 0.05 μmol/L PMA-stimulated samples.

3.2. Effect of Pterostilbene on Neutrophil ROS Production in Arthritic Rats. Nonstimulated (spontaneous) neutrophil ROS production in arthritic rats is evident already on day 7 and was significantly ($P < 0.01$) higher in comparison with healthy controls during the whole experiment, reaching its maximum on day 21 (Figure 4(a)). Although the arthritic rats treated with pterostilbene exhibited lower values of spontaneous ROS production, these changes were not significant compared to the nontreated arthritic group.

The profile of PMA-stimulated neutrophil ROS production was similar to the spontaneous one (Figure 4(b)), with a

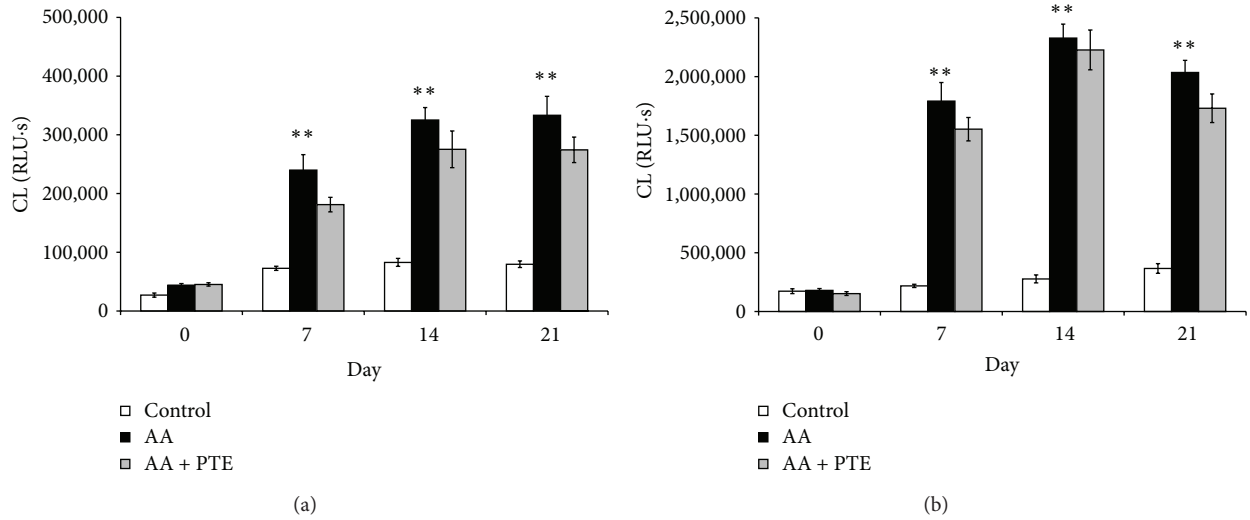

Figure 4: Effect of pterostilbene on ROS production in arthritic rats. (a) Spontaneous and (b) PMA-stimulated (0.05 μmol/L) whole blood chemiluminescence (CL) of healthy (control), arthritic (AA), and pterostilbene treated arthritic rats (AA + PTE). Chemiluminescence was measured at the beginning and every 7 days for 21 days of the experiment. Values are mean ± SEM, $n = 10$, $^{**}P < 0.01$ AA versus control. RLU∗s: relative light units multiplied by time (seconds).

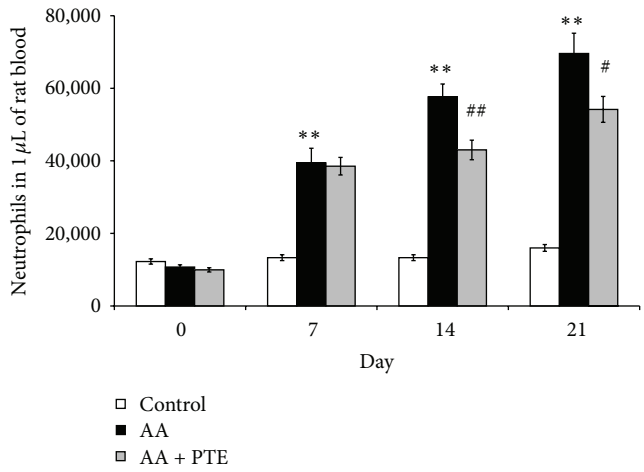

Figure 5: Pterostilbene downregulated the number of neutrophils in arthritic rats. Number of neutrophils in 1 μL of rat blood in healthy (control), arthritic (AA), and pterostilbene treated arthritic rats (AA + PTE) measured at the beginning and every 7 days for 21 days of the experiment. Values are mean ± SEM, $n = 10$, $^{**}P < 0.01$ AA versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ AA + PTE versus AA.

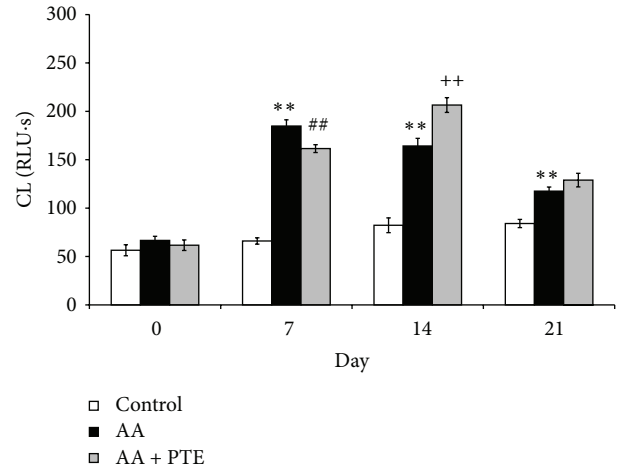

Figure 6: Effect of pterostilbene on neutrophil activity in arthritic rats. PMA (0.05 μmol/L) stimulated ROS production shown as chemiluminescence (CL) per 1 neutrophil in whole blood of healthy (control), arthritic (AA), and pterostilbene treated arthritic rats (AA + PTE) measured at the beginning and every 7 days for 21 days of the experiment. Values are mean ± SEM, $n = 10$, $^{**}P < 0.01$ AA versus control, $^{\#\#}P < 0.01$ AA + PTE versus AA for inhibition, $^{++}P < 0.01$ AA + PTE versus AA for elevation. RLU∗s: relative light units multiplied by time (seconds).

maximum value on day 14 and with no significant reduction caused by pterostilbene.

3.3. Effect of Pterostilbene on Number of Neutrophils and Neutrophil Activity in Arthritic Rats. Figure 5 shows the change in the number of neutrophils in healthy, arthritic, and in pterostilbene treated arthritic animals. Arthritic animals demonstrated a significant increase ($P < 0.01$) in neutrophil numbers from day 7 till the end of the experiment, with a maximum value on day 21. A significant decrease was observed within the arthritic group treated with pterostilbene compared to the arthritic group on days 14 and 21 ($P < 0.01$ and $P < 0.05$, resp.).

By normalisation of the oxidative burst to the number of neutrophils (neutrophil activity), we found a significant ($P < 0.01$) increase in PMA-stimulated neutrophil activity in arthritic rats compared to healthy controls, with a maximum value on day 7 (Figure 6). Neutrophils from

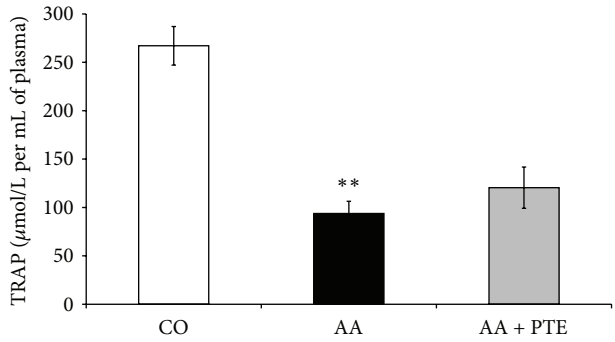

Figure 7: Effect of pterostilbene on TRAP in arthritic rats. Total peroxyl radical trapping capacity (TRAP) in plasma of healthy (CO: control), arthritic (AA), and pterostilbene treated arthritic rats (AA + PTE) measured at the end of the experiment. Values are mean ± SEM, $n = 10$, $^{**}P < 0.01$ AA versus control. The values are expressed as μmol of peroxyl radical trapped by one litre of rat plasma.

pterostilbene treated arthritic animals showed significantly decreased activity on day 7 compared to arthritic controls. After day 14, however, neutrophils from pterostilbene treated arthritic animals showed higher activity compared to arthritic animals. No significant differences were seen in spontaneous neutrophil activity in healthy, arthritic, or pterostilbene treated arthritic rats (data not shown).

3.4. Effect of Pterostilbene Treatment on TRAP Levels in Rat Plasma. The total peroxyl radical trapping capacity of plasma (TRAP) was determined in the control, arthritic, and pterostilbene treated arthritic groups. The arthritic rats showed a significant ($P < 0.01$) decrease in TRAP (94 μmol/L) compared to the healthy control group (267 μmol/L). Pterostilbene treated arthritic rats showed nonsignificant increase in the total peroxyl radical trapping capacity of plasma (120 μmol/L) compared to the arthritic control group (Figure 7).

4. Discussion

The formation of reactive oxygen species (ROS) in neutrophils is one of the essential microbicidal mechanisms in an organism. When produced in high amounts, these highly reactive substances may contribute to tissue injury. This is seen in chronic inflammatory diseases (e.g., rheumatoid arthritis) or ischaemic-reperfusion injury [3, 4, 29]. In this work we examined the potential protective effects of pterostilbene on neutrophil ROS production in adjuvant arthritis—a model of neutrophil-dominant chronic autoinflammatory disease. Pterostilbene was chosen because of its inhibitory effects on neutrophil ROS production *in vitro* [20].

Adjuvant induced inflammation significantly increased the production of ROS and the number of neutrophils in arthritic animals. However, the increased spontaneous (nonstimulated) ROS production in arthritic animals is due to the increase of blood neutrophils rather than the increase in neutrophil activity. This is because proinflammatory cytokines involved in the development of rheumatoid arthritis cause only weak ROS production in neutrophils [30]. Different cytokines present in the synovial fluid induce priming of the neutrophils [3]. By normalisation of ROS production to the number of neutrophils (neutrophil activity), there was no difference between the activities of nonstimulated neutrophils from arthritic and healthy rats. But primed neutrophils have upregulated ROS production when exposed to a secondary stimulus such as PMA [30]. By using PMA, the ROS production in whole blood of arthritic rats was approximately 7 times higher than that in control animals, and the neutrophil activity was significantly increased. Thus, the increase in PMA-stimulated ROS production in arthritic rats is due to an increase in neutrophil number and to an increase in activity of primed neutrophils.

Administration of pterostilbene (30 mg/kg, daily, p.o.) decreased significantly the number of neutrophils in arthritic rats but pterostilbene had only limited effect on ROS production and neutrophil activity. However, the effect of pterostilbene on decreasing the number of neutrophils seems to be more important than the effect on neutrophil oxidative burst. This is because we found that the ROS production in arthritic animals is due to the increase of blood neutrophils rather than the increase of neutrophil activity. The higher neutrophil activity in arthritic rats treated with pterostilbene in comparison with nontreated arthritic rats on day 14 could be explained by the fact that pterostilbene on day 14 significantly decreased the number of neutrophils but the ROS production was nearly the same as in arthritic rats. The lower effect of pterostilbene *in vivo* is seen also with TRAP assay, where pterostilbene did not increase significantly the antioxidative capacity of arthritic rat plasma. These results are in contrast to our findings with pterostilbene effects on neutrophil activity *in vitro* and in cell free assays ([20] and unpublished data), suggesting that the mild decrease in ROS production in arthritic animals receiving pterostilbene treatment may be attributed to pterostilbene downregulation of neutrophilia in arthritic rats.

One of the limits of stilbene derivatives and polyphenols is their low bioavailability. The bioavailability of stilbene derivatives depends on the substitution of the hydroxyl groups. Pterostilbene has a higher bioavailability in comparison with resveratrol [31]. The doses of pterostilbene used in *in vivo* studies with rats range from 10 to 40 mg/kg [24–26]. It is questionable whether increasing the pterostilbene dose would increase its bioavailability and thus the effects *in vivo*.

Another stilbene derivative, pinosylvin (*trans*-3,5-dihydroxy stilbene), was more effective in the inhibition of both spontaneous and PMA-stimulated ROS production in arthritic rat blood and significantly increased TRAP in plasma of arthritic animals [32]. Pinosylvin (30 mg/kg, p.o., daily), though not pterostilbene, decreased the hind paw volume (clinical symptom of adjuvant arthritis) and myeloperoxidase (MPO) activity in hind paw joint homogenates of arthritic rats [23, 33]. MPO may be used to assess the infiltration of neutrophils in the joints [33]. Downregulation of the number of neutrophils in arthritic rats was seen also with pinosylvin [32]. We suggest that stilbene derivatives may interfere with cytokine signalling leading

to a decrease of neutrophils in arthritic rats. Resveratrol was found to regulate different cytokines and intracellular messengers involved in inflammation [34, 35]. Paul et al. reported that pterostilbene decreased the levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-4 [36].

Protein kinase C (PKC) is important in the activation of neutrophil NADPH-oxidase and thus in the production of ROS [37]. Inhibition of PKC could be used as a strategy for regulating various diseases involving PKC [38]. Derivatives of resveratrol are able to attenuate the activity or activation of PKC. Pinosylvin, but not pterostilbene, decreased the activation of PKC in neutrophils *in vitro* [32, 39].

In conclusion, stilbene derivatives are effective in the inhibition of neutrophil ROS production but this is structure dependent. The structure influences also the bioavailability of stilbene derivatives. Despite higher activity against neutrophil ROS production *in vitro*, pterostilbene did not decrease significantly the oxidative burst of neutrophils *in vivo*. On the other hand, pterostilbene decreased the number of neutrophils in arthritic rats. Our results contribute to the knowledge of structure-dependent benefits of stilbene derivatives in the management of chronic inflammatory diseases where neutrophils play a role.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Regular paper

Antioxidant, antimicrobial and neutrophil-modulating activities of herb extracts

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The present study provides a comprehensive data on the antioxidant, antimicrobial and neutrophil-modulating activities of extracts from six medicinal plants — blackberry (*Rubus fruticosus*) leaves, chokeberry (*Aronia melanocarpa*) leaves, hawthorn (*Crataegus monogyna*) leaves, lady's mantle (*Alchemilla glabra*) aerial parts, meadowsweet (*Filipendula ulmaria*) aerial parts and raspberry (*Rubus idaeus*) leaves. In order to analyze the antioxidant activity of the herbs, several methods (ORAC, TRAP, HORAC and inhibition of lipid peroxidation) were used. Blackberry leaves and meadowsweet extracts revealed the highest antioxidant activities *via* all methods. All extracts studied blocked almost completely the opsonized zymosan particle-activated ROS production by neutrophils from human whole blood. On the other hand, the effect of extracts on phorbol myristate acetate-activated ROS production was much milder and even nonsignificant in the case of chokeberry leaves. This latter result suggests that extracts (apart from their antioxidative activity) interfere with the signaling cascade of phagocyte activation upstream of the protein kinase C activation. The antimicrobial activity of the investigated extracts against 11 human pathogens was investigated using three different methods. Meadowsweet and blackberry leaves extracts had the highest antimicrobial effect and the lowest minimal inhibiting concentrations (MICs) against the microorganisms tested.

Key words: herbs, polyphenols, antioxidant activity, antimicrobial activity, phagocytes, reactive oxygen species

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INTRODUCTION

Human gastrointestinal tract (GIT) is constantly exposed to oxidizing agents of diverse origins. The diet contains various prooxidants, including metals (copper and free or heme-bound iron), lipid hydroperoxides, aldehydes and nitrite, and consequently elevated levels of lipid peroxides have been observed in the postprandial state (Kanner & Lapidot, 2001). These prooxidants may induce oxidative stress in the GIT, resulting in stomach ulcer and stomach, colon, and rectal cancers. Besides that, oxidative stress induced by microbial infections is another major contributor to the development of gastro-intestinal diseases. There is increasing evidence that microbial pathogens induce oxidative stress in host cells, resulting in considerable accumulation of inflammatory cell, which could be related to the development of gastric mucosal as well as neuromuscular disorders (Suzuki *et al*., 2012). Recent studies have shown that many bacterial pathogens can trigger apoptosis of infected host cells (Krzyminska *et al*., 2011). It has been shown that phagocyte-derived reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important factors inducing apoptosis (Circu & Aw, 2010). The activation of phagocytes in the gut may also increase the ROS and RNS production, and gastric juice may promote lipid peroxidation (Kanner & Lapidot, 2001). Phagocytic cells such as macrophages and neutrophils play a key role in innate immunity owing to their ability to recognize, ingest, and destroy pathogens by oxidative and nonoxidative mechanisms. In response to a variety of stimuli, NADPH oxidase present in neutrophils is activated in a phenomenon described as the respiratory burst, characterized by the production of the superoxide anion which gives rise to other forms of ROS. In addition nitric oxide (NO) and other RNS produced mainly by macrophages are among the major microbicidal agents of inflammation during the fight against pathogenic microorganisms and tumor cells. However, excessive or inappropriate ROS and RNS production by phagocytes is associated with oxidative damage to membrane lipids, DNA, proteins, and lipoproteins, resulting in various autoimmune and inflammatory diseases. Thus, the modulation of inflammation and oxidative stress by natural substances can be beneficial. Therefore, the role of antioxidants in the GIT may be very important and antioxidants contained in foods could suppress the oxidative stress and related diseases in the gastrointestinal tract before being absorbed (Halliwell *et al*., 2000) and thus could modulate the level of oxidative stress by enhancing anti-inflammatory or antioxidant capacity (Suzuki *et al*., 2012). In the recent search for novel sources of anti-inflammatory and antioxidant agents medicinal plants have attracted particular attention. Since ancient times herbs have been used in

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Abbreviations: AAPH, 2,2´-azobis (2-amidino-propane) dihydrochloride; AUC, area under the curve; CL, chemiluminescence; DW, dry weight; GAE, gallic acid equivalents; GIT, gastrointestinal tract; HBSS, Hank's balanced salt solution; HORAC, hydroxyl radical averting capacity; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; MIC, minimal inhibiting concentration; ORAC, oxygen radical absorbance capacity; OZP, opsonized zymosan particles; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol myristate acetate; RLU, relative light units; RNS, reactive nitrogen species; ROS, reactive oxygen species; S.D., standard deviation; TE, trolox equivalents; TRAP, total peroxyl-radical antioxidant parameter

numerous areas, including nutrition, healing, cosmetics, etc., without the exact mechanisms of their action being known. In our recent study we observed that herbs are a rich source of polyphenol compounds (Kratchanova *et al*., 2010) and their antioxidant activities are several times higher than those of vegetables (Ciz *et al*., 2010) and fruits (Denev *et al*., 2013). There was also a good correlation between the polyphenol content and the ORAC antioxidant activity of the herbs investigated, indicating that the polyphenol compounds are responsible for the free-radical scavenging capacity. Bearing in mind the great variety of medicinal plants and their potent antioxidant properties, herbs appear to be a valuable but still poorly-explored source of natural antioxidant and antimicrobial agents. Therefore, the aim of the present study was to investigate the polyphenol composition and content of six less-investigated herbs and to determine their antioxidant and antimicrobial activities, and their effect on the production of ROS by activated neutrophils. In order to obtain a broader view on their antioxidant properties we used several antioxidant activity assays (ORAC, TRAP, HORAC and inhibition of lipid peroxidation) relying on different mechanisms. The antimicrobial properties and the minimal inhibiting concentration of the herb extracts were investigated against 11 human pathogens and a genetically modified luminescent *E. coli* was used for the first time to determine the antimicrobial activity of natural products.

MATERIALS AND METHODS

Chemicals. Fluorescein disodium salt, 2,2-azobis-(2-amidino-propane)dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), $CoF_2 \times 4H_2O$, picolinic acid, linoleic acid and HPLC reference compounds (gallic acid, 3,4-dihydroxybenzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, rutin, naringin, myrecetin, quercetin, naringenin and kaempherol) were purchased from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany) and luminol from Molecular Probes, (Eugene, Oregon, USA). All other reagents used were of analytical grade and purchased from local distributors.

Herb samples. All medicinal plants used were either obtained from local pharmacies (Plovdiv, Bulgaria) or collected from nature in 2012. The choice of the plants investigated was based on the available data on their biological activities. The following medicinal plants were investigated: blackberry (*Rubus fruticosus*) — leaves, chokeberry (*Aronia melanocarpa*) — leaves, hawthorn (*Crataegus monogyna*) — leaves, lady's mantle (*Alchemilla glabra*) — aerial parts, meadowsweet (*Filipendula ulmaria*) — aerial parts and raspberry (*Rubus idaeus*) — leaves. Freshly collected plants were dried in the shade at room temperature and all plant materials were inspected for contaminants. 1 kg of each herb was packed in paper bags and stored at ambient temperature prior to extraction and analysis.

Extraction. All plant materials were subjected to extraction under the following conditions: 50 g of the dried plant material was powdered in a laboratory mill. Then 5 g of the powder was transferred into extraction tubes and mixed with 100 ml of the extragent (80% acetone in 0.2% formic acid). Extraction was conducted on an orbital shaker at room temperature for one hour. After that, the samples were centrifuged (6000×*g*) and supernatants collected. Extracts were concentrated via rotary evaporation to a volume of 15 ml in order to remove the acetone. Then the volume was adjusted to 50 ml with ultra clean water and the extracts were centrifuged. Clear supernatants were used for all further analysis after proper dilutions.

HPLC analysis of phenolic compounds. High Performance Liquid Chromatography (HPLC) analysis of phenolic components was performed on an Agilent 1220 HPLC system (Agilent Technology, Palo Alto, USA) equipped with a binary pump and UV-Vis detector (Agilent Technology) set at 280 nm. TC-C18 column (5 μm, 4.6 mm×250 mm) was used at 25ºC. The mobile phases were 0.5% acetic acid and 100% acetonitrile at a flow rate of 0.8 ml/min. The eluent gradient started with 14% acetonitrile for 6 min, and linearly increased to 25% acetonitrile in 24 minutes and to 50% acetonitrile in 10 min. Neochlorogenic acid content was calculated as chlorogenic acid equivalents. Results are given as mean values from three measurements of the same extract and expressed as mg per 100 grams of dry weight (DW).

Total polyphenol compounds analysis. Total polyphenols were determined according to the method of Singleton & Rossi (1965) with Folin-Ciocalteu's reagent. Gallic acid was employed as a calibration standard and the results were calculated as mean values from three measurements of the same extract and expressed as gallic acid equivalents (GAE) per 100 grams of dry weight.

ORAC assay. ORAC was measured according to the method of Ou *et al*. (2001) with some modifications (Ciz *et al*., 2010). The method measures the antioxidant scavenging activity against peroxyl radical generated by thermal decomposition of 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) at 37°C. Fluorescein was used as the fluorescent probe. The loss of fluorescence of fluorescein was an indication of the extent of its oxidation through reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the fluorescence area under the curve (AUC) plot relative to that of a blank in which no antioxidant was present. Solutions of AAPH, fluorescein and trolox were prepared in a Na phosphate buffer (75 mmol/l, pH 7.4). Samples were diluted in the phosphate buffer as well. Reaction mixture (total volume 200 μl) contained fluorescein (170 μl, final concentration 5.36×10^{-8} mol/l), AAPH (20 μl, final concentration 51.51 mmol/l), and sample — 10 μl. The fluorescein solution and sample were incubated at 37°C for 20 min directly in a microplate reader, and AAPH (dissolved in buffer at 37°C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 μl of phosphate buffer was used instead of the extract. Each herb extract was analyzed in two runs with four replicates and results are presented as mean value ± standard deviation from the eight values. The antioxidant activity is expressed in micromole trolox equivalents (TE) per gram of dry weight (DW). Trolox solutions (3.125, 6.25; 12.5; 25 and 50 μmol/l) were used for defining the standard curve.

TRAP assay. Luminol-enhanced chemiluminescence (CL) was used to follow the peroxyl radical reaction and the principle was described previously in Cizova and coworkers (2004). The CL signal is driven by the production of luminal-derived radicals from thermal decomposition of AAPH. The TRAP value is determined from the duration of the time period (T_{sample}) during which the

sample quenched the CL signal due to the present antioxidants. Trolox solution (8.0 nM) was used as a reference inhibitor (T_{trolox}) instead of the sample. The calculation of the TRAP value followed the equation:

$$TRAP = 2.0\ [trolox]T_{sample} / f\,T_{trolox},$$

where 2.0 is the stoichiometric factor of trolox (the number of peroxyl radicals trapped per one molecule of trolox) and *f* is the dilution of the sample. All herb extracts were analyzed in two runs with three replicates each, and results are presented as mean ± standard deviation from all six values.

HORAC assay. The HORAC assay developed by Ou and coworkers (2002) measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical. Hydrogen peroxide solution of 0.55 M was prepared in distilled water, and 4.6 mM Co(II) was prepared as follows: 15.7mg of $CoF_2{\times}4H_2O$ and 20mg of picolinic acid were dissolved in 20 ml of distilled water. Fluorescein (170 µl, final concentration 60 nM) and 10 µl of sample were incubated at 37°C for 10 min directly in a FLUOstar plate reader, then 10 µl of H_2O_2 (final concentration 27.5 mM) and 10 µl of Co(II) (final concentration 230 µM) solutions were added. Initial fluorescence was measured immediately, then readings were taken every minute after shaking. For the blank sample, phosphate buffer (75 mM, pH=7.4) was used. Gallic acid solutions (100, 200, 600, 800 and 1000 µM in phosphate buffer) were used for standard curve. The AUC values were calculated as they were for the ORAC assay. Each herb extract was analyzed in two runs with four replicates and results are presented as mean value ± standard deviation from the eight values. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) with excitation wavelength of 485 nm and emission wavelength of 520 nm.

Inhibition of lipid peroxidation assay. A modified assay described by Anthon & Barrett (2001) and Kikuzaki and coworkers (2002) was used. A mixture of 1 ml sample, 1 ml of 83.5 mM linoleic acid in 99.5% ethanol, 2 ml of 0.05 M phosphate buffer (pH 7.0), and 1 mL of water in a glass tube with a plastic stopper was incubated at 40°C in the dark. Absorbance at 234 nm was measured after one, three and six days of incubation on Infinite M200 microplate reader (Tecan, Austria). Initial values of absorbance (day zero before incubation) were subtracted from these values. For conversion of absorbance to the concentration of hydroperoxide, the extinction coefficient 23 000 $M^{-1}cm^{-1}$ was used. Results are presented as mean values from three independent measurements each in duplicate.

Oxidative burst. Heparinized blood (50 IU/ml) from healthy volunteers (n=7) was obtained by antecubital venipuncture. The oxidative burst of phagocytes in whole blood was measured chemiluminometrically using an LM-01 microplate luminometer (Immunotech, Czech Republic). The principle of the method was described previously (Ciz *et al*., 2007). Each reaction mixture consisted of 25 µl of whole blood diluted 10× in Hank's balanced salt solution (HBSS) pH 7.4, 2.5 µl of plant extract, 25 µl of 1 mM luminol, and 25 µl of one of the activators — 62.5 µg/ml of opsonized zymosan particles (OZP; Sigma, USA) or 0.81 µg/ml of phorbol-12-myristate-13-acetate (PMA; Sigma, USA). HBSS was used to adjust the total reaction volume to 250 µl. The assays were run in duplicates. Measurements of spontaneous CL in samples containing blood and other substances except any activator were included in each assay. The CL activity of the samples was measured immediately at 37°C. Light emission expressed as relative light units (RLU) was recorded continuously for 60 minutes to obtain kinetic curves. Integrals of CL signal over 60 min were also calculated. Results are presented as means ± standard error of the mean from six measurements.

Determination of antimicrobial activity by agar diffusion method. The agar diffusion method was used according to Denev and coworkers (2014). Eleven human pathogens (*Escherichia coli* ATCC 8739; *Escherichia coli* ATCC 25922; *Salmonella* sp.*; Salmonella enterica* ssp. *enterica* ATCC BAA-2162; *Staphylococcus aureus* ATCC 6538P; *Staphylococcus aureus* ATCC 25923; *Listeria monocytogenes*; *Listeria monocytogenes* I; *Proteus vulgaris* G; *Pseudomonas aeruginosa* ATCC 9027; *Klebsiella pneumoniae*) were used. All experiments were performed in duplicates and results are expressed as mean values.

Determination of minimal inhibiting concentration (MIC). The antimicrobial effects of the extracts were determined by analyzing the MIC using a 96 well microtiter plate method (broth dilution) according to the modified protocol described by Gutierrez and coworkers (2008). The first column of each plate contained 300 µl of nutrient broth (tryptone — 5.0 g/l, yeast extract — 2.5 g/l, glucose — 1 g/l, pH 7.0) and 100 µl of the tested extract. From the second to the twelfth column, 200 µl of broth were added and 200 µl of the mixture from the first well was added to the second one and so on (serially diluted two-fold) along each row, until the eleventh column. The twelfth column was used for growth control (positive control) and a row was used for negative control at each dilution. Finally, 30 µl of working culture of each test microorganism ($1{\times}10^7$ cfu/ml) was added to all the wells except the negative controls. The positive control contained distilled water instead of extract. Plates were incubated at 37ºC for 24 h and then turbidity was determined at 600 nm in a microplate spectrophotometer (SPECTROstar^{Nano}, BMG Labtech, Germany). The lowest concentration of the tested extract required to reduce the growth of the tested microorganism to 50% of the positive control was designated as the MIC. Results are presented as mean values from three independent measurements.

Antimicrobial activity against genetically modified *Escherichia coli*. In the following experiments transformed *E. coli* K12 resistant to ampicillin carrying the luxABCDEamp gene was used, expressing bacterial luciferase and its substrate (Atosuo *et al*., 2013). Bacteria were cultivated in LB medium with 100 µg/ml ampicillin, washed twice in phosphate buffer (pH 7.0, 133 mM) and adjusted with the phosphate buffer to the final concentration of approximately 330 000 cells per 100 µl before each measurement. The bioluminescence signal of bacterial suspensions after exposition to herb extracts was measured in relative light units (RLU) for one hour on LM-01T plate luminometer (Immunotech, Czech Republic) at 37°C. The wells of microplates contained 180 µl of bacterial suspension and 10 µl (5% concentration) of extracts, buffer was used as a control. The light emission during reaction is positively correlated with bacteria viability (Atosuo *et al*., 2013). To determine antibacterial properties of extracts, integrals (RLU*s) under kinetics curves were calculated and expressed as percentage of control. All samples were prepared in two independent repetitions and each repetition was analysed three times. Results are presented as mean values from the six measurements.

Table 1. Content of phenolic acids and flavonoids in herbs

Herb	gallic acid, mg/100g	neochlorogenic acid, mg/100g	3,4-dihydroxy-benzoic acid, mg/100g	chlorogenic acid, mg/100g	caffeic acid, mg/100g	p-coumaric acid mg/100g	ferulic acid, mg/100g	ellagic acid, mg/100g	total phenolic acids, mg/100g*
Blackberry leaves	–	–	208	–	–	–	–	19	227
Chokeberry leaves	–	1350	–	2014	–	21	–	24	3409
Hawthorn leaves	–	426	–	654	–	–	–	–	1080
Lady's mantle	63	–	135	80	151	–	–	–	429
Meadowsweet	261	–	–	232	107	728	122	81	1531
Raspberry leaves	28	–	58	–	40	–	–	37	163
	catechin, mg/100g	epicatechin, mg/100g	rutin, mg/100g	naringin, mg/100g	myrecetin, mg/100g	quercetin, mg/100g	naringenin, mg/100g	total flavonoids, mg/100g*	total polyphenols, mg/100g
Blackberry leaves	–	–	–	–	466	–	–	466	12678
Chokeberry leaves	–	–	22	–	–	–	–	22	6111
Hawthorn leaves	53	–	843	–	–	68	–	964	5283
Lady's mantle	250	524	1057	–	–	–	–	1831	8377
Meadowsweet	–	–	1374	–	–	62	11	1447	14596
Raspberry leaves	–	283	118	–	–	–	–	401	3976

*Calculated as sum of individual representatives

RESULTS AND DISCUSSION

Polyphenol composition and antioxidant activity of herb extracts

Results of the HPLC analysis of herb extracts are shown in Table 1. The analyzed extracts showed substantial differences in their polyphenol composition and content. Aronia leaves are distinctive with a high content of hydroxycinnamic acids (chlorogenic and neochlorogenic) among polyphenol acids with a total content of 3409 mg/100 g. Lady's mantle contains 3,4-hydroxybenzoic acid, chlorogenic acid and p-coumaric acid totaling 429 mg/100 g. Among the investigated herbs, lady's mantle is the richest source of flavonoids, containing catechin (250 mg/100 g), epicatechin (524 mg/100 g) and a significant amount of rutin (1057 mg/100 g). The meadowsweet has the highest content of total plyphenols (14596.2 mg/100 g) and is a balanced source of phenolic acids (total content — 1531 mg/100 g) and flavonoids — 1447 mg/100 g. Hawthorn leaves contain 1080 mg/100 g of phenolic acids and 1831 mg/100 g of flavonoids. Blackberry leaves are a rich source of polyphenol compounds — 12678 mg/100 g. They contain 3,4-dihydroxybenzoic acid (208 mg/100 g), ellagic acid (19 mg/100g) and myrecetin (466 mg/100 g). Raspberry leaves contain gallic, 3,4-dihydroxybenzoic, caffeic and ellagic acids. Epicatechin and rutin are the most abundant flavonoids in that herb. It is obvious from the results in Table 1 that the sum of the investigated phenolic acids and flavonoid contents is less than the total polyphenol content of the investigated herbs. We analyzed 16 of the most common polyphenol constituents in plants but it could be expected that other phenolic acids and flavonoids, not analyzed in this work were also present. Smolyakova *et al.* (2012) reported that lady's mantle polyphenol content is up to 9.6% of the dry herb weight, which is closed to our result – 8.4%, but luteolin and apigenin, and their glycosides not analyzed in our study are the main flavonoids in that herb. Besides flavonoids and phenolic acids, medicinal plants are known to be a rich source of tannins (condensed and hydrolysable), which were not analyzed in the current study either. For example, Duckstein and coworkers (2013) identified 24 phenolic compounds in lady's mantle including pedunculagin and agrimoniin, and other monomeric and oligomeric ellagitannins, which constituted the major phenolic fraction. Gudej and Tomczyk (2004) reported that tannins are the main polyphenol constituents in raspberry and blackberry leaves. Fecka (2009) found 27 polyphenol compounds in meadowsweet, including substantial amounts of ellagitannins (up to 12500 mg/100 g DW) and rugosins A, B and E were identified for the first time as meadowsweet constituents. According to Fecka, the total polyphenol content of meadowsweet reached 16568 mg/100 g, which is similar to our result — 14596 mg/100 g. The high con-

Table 2. ORAC, TRAP and HORAC antioxidant activity of herbs

	ORAC, μmol TE/g	TRAP, μmol TE/g	HORAC, μmol GAE/g
Blackberry leaves	1806±105	2937±139	2076±113
Chokeberry leaves	1363±69	1263±56	1120±53
Hawthorn leaves	1405±66	1301±27	882±203
Lady's mantle	1337±68	1815±38	1999±70
Meadowsweet	1555±85	1993±102	1640±223
Raspberry leaves	1349±119	647±50	1257±82

tent of hydrolysable tannins in that herb was confirmed by other studies (Barros *et al.*, 2013). Chokeberry leaves' polyphenols have not been sufficiently investigated and data regarding their polyphenol composition and content are scarce. A recent study found 12 polyphenol compounds in chokeberry leaves and confirmed our results that chlorogenic and neochlorogenic acids are the main phenolic constituents (Lee *et al.*, 2014). Beside that, dicaffeoylquinic acid was detected in high amounts, along with other minor components. Bearing in mind that chokeberry fruits contain substantial quantity of condensed tannins (Denev *et al.*, 2012), we could expect that their leaves also accumulates significant amounts of these compounds.

The differences in the polyphenol content and composition of the investigated herbs are reflected in their biological activities and particularly antioxidant activity. When investigating antioxidant properties of natural antioxidants it is recommended to use more than one antioxidant assay for better understanding the principles of their antioxidant action (Ciz *et al.*, 2010). Therefore, we used several assays addressing various aspects of the antioxidant action of polyphenols in our study – ORAC, TRAP, HORAC and inhibition of lipid peroxidation. The methods used embrace different aspects of the antioxidant action and give a broader view on the antioxidant potential of the herb extracts. The ORAC and TRAP methods measure the ability of antioxidants to scavenge peroxyl radicals via hydrogen atom transfer. These radicals are physiologically the most important ones and the hydrogen atom transfer is the most physiologically relevant mechanism of antioxidant action. The HORAC method measures the metal-chelating activity of antioxidants under conditions of Fenton-like reactions and hence indicates the protecting ability against formation of hydroxyl radical. Results for the ORAC, TRAP and HORAC antioxidant activities are shown in Table 2. In our previous study, we reported ORAC antioxidant activity of 25 herbs used traditionally in the Bulgarian folk medicine (Kratchanova *et al.*, 2010). Compared to those results all the herbs investigated in the current study showed relatively high ORAC antioxidant activities, ranking among top ten herbs with the highest ORAC values. Blackberry leaves revealed the highest antioxidant activity using the three methods (ORAC — 1805.8 μmol TE/g; TRAP — 2936.6 μmol TE/g; and HORAC — 2075.6 μmol GAE/g). Meadowsweet showed the second highest ORAC and TRAP values — 1555.0 μmol TE/g and 1992.7 μmol TE/g, respectively, indicating that this herb is a rich source of chain-braking polyphenolic antioxidants. Interestingly, lady's mantle extract revealed the second highest chelating ability expressed as a HORAC value of 1999.4 μmol GAE/g.

The effect of the extracts on lipid peroxidation was evaluated by assaying the autoxidation of linoleic acid

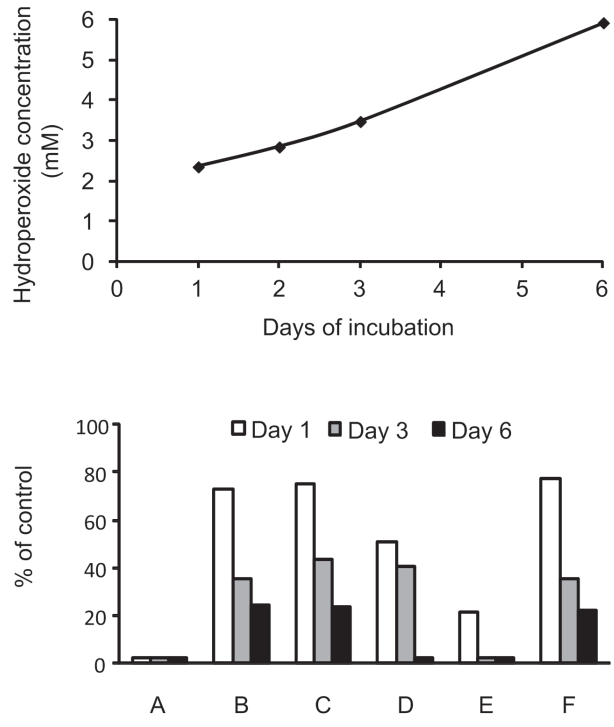

Figure 1. Inhibition of lipid peroxidation by herb extracts. Kinetics of hydroperoxide concentration in control samples without herb extracts during six days of autoxidation of linoleic acid (upper panel). Eeffect of herb extracts on hydroperoxide accumulation at the end of the 1st, 3rd and 6th days of autoxidation of linoleic acid (lower panel). Results are expressed as percentage of control. A — blackberry leaves; B — chokeberry leaves; C — hawthorn leaves; D — lady's mantle; E — meadowsweet; F — raspberry leaves.

in ethanol-buffer system by measuring the formation of linoleic acid hydroperoxides. This is a simple and reliable method for evaluation of the effects of potential antioxidants. As shown in Fig. 1A, the concentration of hydroperoxides rises gradually during the six days of linoleic acid autoxidation at 40°C. The formation of hydroperoxides in the presence of the herb extracts is shown in Fig. 1B. It is obvious that the autoxidation of linoleic acid was very effectively inhibited, especially by the extract from blackberry leaves. In this case, the concentration of hydropexides was lowered to 2% of the control of all time points. The second most effective extract was that from meadowsweet. It inhibited the generation of hydroperoxides to 22% of control at day 1 and to 2% at days 3 and 6. The third most effective was the extract from lady's mantle, followed by extracts from chokeberry, hawthorn and raspberry leaves. For the latter four extracts the inhibition of lipid peroxidation increased from day 1 to day 6.

Effect of herb extracts on ROS production by phagocytes

The effects of studied extracts on the production of ROS by human whole blood phagocytes were studied using luminol-enhanced CL. The results are shown in Fig. 2 and expressed as kinetic curves of the CL response after OZP activation (upper panel), PMA activation (lower panel) and the integral values of the CL response. It is obvious that all the extracts studied significantly inhibited the ROS production, to the background value in the case of OZP-activated phagocytes. On the

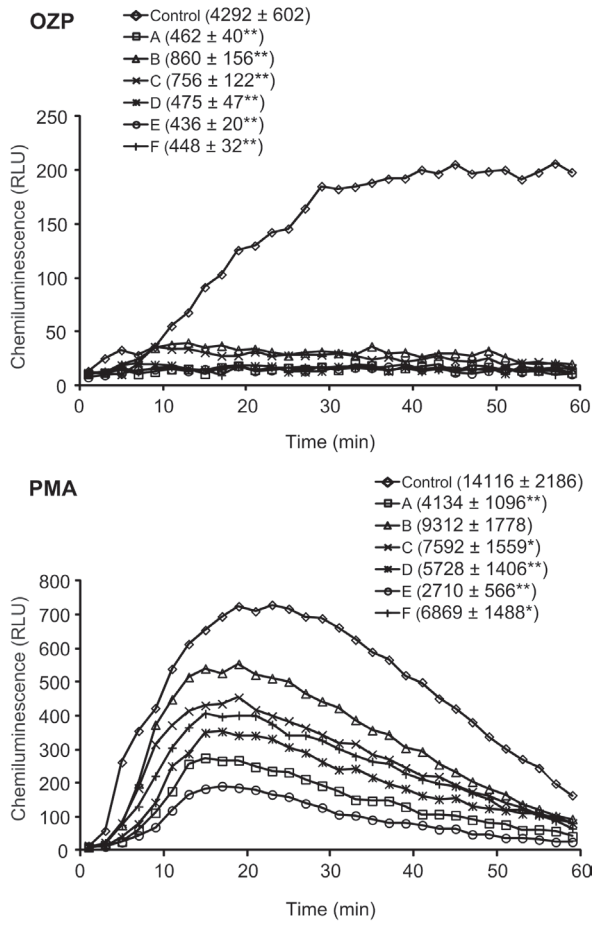

Figure 2. Effect of herb extracts on the production of reactive oxygen species by phagocytes.
Kinetic curves of the CL response after OZP activation (upper panel) and PMA activation (lower panel) are shown. A — blackberry leaves; B — chokeberry leaves; C — hawthorn leaves; D — lady's mantle; E — meadowsweet; F — raspberry leaves. Integral values of CL response (mean ±S.E.M.) are also included. Statistically significant differences against control are shown by $^{**}p<0.01$ and $^{*}p<0.05$.

other hand, some differences were observed when the effects were studied using PMA-activated human whole blood phagocytes. The most effective was the extract of meadowsweet ($p\leq0.01$) followed by blackberry leaves ($p\leq0.01$), lady's mantle ($p\leq0.01$), raspberry leaves ($p\leq0.05$), hawthorn leaves ($p\leq0.05$), chokeberry leaves. The least effective extract of chokeberry leaves still inhibited the PMA-activated CL response, but the effect was statistically non-significant. The inhibitory effects of the herb extracts on the PMA-activated CL response of phagocytes and partially also on the OZP-activated CL response of phagocytes could be caused by a direct ROS scavenging activity of the extracts due to their total polyphenol content. There was a strong negative correlation between the PMA-activated CL response and the total polyphenol content and a strong positive correlation between the TRAP antioxidative parameter and total polyphenols (not shown).

The effects of polyphenols as the basic constituents of various plant extracts on the respiratory burst of neutrophils have already been demonstrated. For example, cocoa flavonoids have been found to moderate a subset of signaling pathways derived from LPS stimulation of neutrophils, mainly the neutrophil oxidative burst and activation markers (Kenny *et al.*, 2009). Those authors hypothesized that flavonoids could decrease the impact of LPS on the fMLP-primed neutrophil ability to generate ROS by partially interfering in the activation of the MAPK pathway. In another study, *Ginkgo biloba* extract containing flavonoids slowed down the respiratory burst of stimulated human neutrophils (Pincemail *et al.*, 1987). An inhibitory effect of pure flavonoids on the respiratory burst of neutrophils has also been demonstrated (Pagonis *et al.*, 1986; Zielinska *et al.*, 2000). Wang and coworkers (2002) observed that cirsimaritin inhibited superoxide anion radical generation and oxygen consumption of neutrophils. On the other hand, cirsimaritin slightly enhanced the superoxide anion radical generation by PMA-activated NADPH oxidase. The results of those authors indicate that it is likely that the inhibition of the fMLP-induced respiratory burst by cirsimaritin in rat neutrophils is mainly through the blockade of the phospholipase D (PLD) signaling pathway. Selloum and coworkers (2001) observed that myricetin, quercetin, kaempferol and rutin inhibited the pholasin luminescence of fMLP-stimulated neutrophils. Lee and coworkers (2010) revealed that luteolin attenuated neutrophil respiratory burst but had a negligible effect on superoxide anion generation during PMA stimulation. Furthermore, luteolin effectively blocked MAPK/ERK kinase 1/2 and Akt phosphorylation in fMLP-stimulated neutrophils. Thus, an inhibition of enzymes involved in signaling, rather than scavenging of superoxide anion radicals dominated in fMLP-stimulated neutrophils exposed to flavonoids in those studies. fMLP used in the cited studies is a soluble activator of neutrophils which binds to specific membrane receptors. Similarly, OZP binds to surface opsonin (either complement or Fc) receptors on phagocytes and triggers a signaling cascade leading finally to the activation of protein kinase C (PKC) with subsequent activation of NADPH oxidase, producing the superoxide anion — the first of the ROS produced by phagocytes. In contrast, PMA activates directly PKC, the step before NADPH oxidase activation. Our present results indicate in accordance with the literature that the herb extracts studied do not inhibit PKC or NADPH oxidase itself, but interfere with the signaling cascade of phagocyte activation upstream of the PKC activation.

Antimicrobial activity of herbs

The investigated plants are used in traditional medicine in the treatment of different infections (Shmid & Gorris, 2007), but their antimicrobial properties are poorly investigated. Only few studies on antimicrobial activities of their extracts have been published to date. Gniewosz and coworkers (2014) determined the influence of meadowsweet flower extract on three pathogens and four saprophytes of apples, using fruit covered with a pullulan film containing the extract. Martini and coworkers (2009) analyzed the activity of blackberry leaves extract against *Helicobacter pylori*. In our study initial screening of the antimicrobial activity of the tested extracts was performed against eleven foodborne pathogens using the agar diffusion method. The antimicrobial activity was classified according to Rota and coworkers (2008) as weak (inhibition zone <12 mm), medium (inhibition zone between 12 and 20 mm), and strong (inhibition zone above 20 mm). The results are presented in Table 3. The blackberry and raspberry leaves extracts exhibited antimicrobial activity against all test microorganisms. In some cases single colonies were present in the inhibition zones (marked with an asterisk in Table 3). Some of the cells of *E. coli* ATCC 25922, both *Sal-*

Table 3. Antimicrobial activity of herb extracts against human pathogens measured by agar diffusion method

Test-microorganisms	Concentration of viable cells x10^7, cfu/cm^3	Blackberry leaves			Chokeberry leaves			Hawthorn leaves			Lady's mantle			Meadowsweet			Raspberry leaves		
		Extract concentration, %																	
		100	10	2	100	10	2	100	10	2	100	10	2	100	10	2	100	10	2
		Zone of inhibition, mm																	
Escherichia coli ATCC 8739	4.8	8	8	–	–	–	–	–	–	–	15	15	–	–	–	–	15	12	–
Escherichia coli ATCC 25922	7.7	17*	12*	–	–	–	–	–	–	–	18	13	–	–	–	–	15	11	–
Salmonella sp.	3.2	16*	12*	8*	–	–	–	–	–	–	–	–	–	–	–	–	16	11	–
Salmonella enterica ssp. *enterica* ATCC BAA–2162	1.2	16*	14*	11*	–	–	–	–	–	–	–	–	–	–	–	–	16*	11*	–
Staphylococcus aureus ATCC 6538P	1.9	17	11	9	13	–	–	9	8	–	19	12	9	21	14	10	15	11	–
Staphylococcus aureus ATCC 25923	2.7	18	15	8	15	–	–	9	8	–	22	14	–	24	15	12	16	12	–
Listeria monocytogenes	4.1	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	16	11	–
Listeria monocytogenes I	2.5	24*	18*	11*	–	–	–	–	–	–	–	–	–	–	–	–	17	11	–
Proteus vulgaris G	2.6	20	16	8	13	–	–	12	–	–	22	17	11	25	19	15	19	12	–
Pseudomonas aeruginosa ATCC 9027	4.9	24	22	11	–	–	–	–	–	–	–	–	–	–	–	–	15*	11*	–
Klebsiella pneumoniae	2.9	17	12	8	–	–	–	–	–	–	15	13	9	20	11	–	16	11	–
Candida albicans ATCC 10231	1.3	24*	14*	10*	–	–	–	–	–	–	16	14	11	25	19	13	18*	10*	–

*single colonies present in the inhibition zone

monella strains, *L. monocytogenes* I and *C. albicans* were less susceptible to the effect of the blackberry leaves extract and were able to grow in its presence. A number of *S. enterica* ssp. *enterica* ATCC BAA-2162, *P. aeruginosa* and *C. albicans* cells were also more resistant to the activity of the raspberry leaves extract than others. The highest activity of the blackberry extract was observed against *P. aeruginosa* and *P. vulgaris,* where inhibition was detected even for 2% extract. The raspberry leaves extract produced the largest inhibition zones (19 mm) against *P. vulgaris*. Chokeberry and hawthorn leave extracts had medium and weak antimicrobial activites, respectively, against three of the test strains — the two *S. aureus* strains and *P. vulgaris*. The activity was observed only for undiluted extracts. Lady's mantle extract did not inhibit the growth of *Salmonella, L. monocytogenes* or *P. aeroginosa*, but antimicrobial activity against *S. aureus* ATCC 6538P, *P. vulgaris*, *K. pneumoniae* and *C. albicans* was detected even when the lowest extract concentration was used. The activity against *S. aureus* ATCC 25923 and *E. coli* was weaker — an inhibitory effect was observed only for 10% solutions. Meadowsweet extract also demonstrated antimicrobial activity against *S. aureus* strains, *P. vulgaris, K. pneumoniae* and *C. albicans* even in a 2% solution. Based on the obtained results four extracts (blackberry, lady's mantle, meadowsweet and raspberry leaves) were selected for further experiments.

The minimal inhibitory concentration (MIC_{50}) of the extracts was determined in order to quantify their antimicrobial activity (Table 4). The lowest MIC_{50} values were determined for the raspberry leaves extract against *S. aureus* ATCC 25923 — 0.031% and the meadowsweet extract against *P. vulgaris* and *K. pneumoniae* – 0.08%. *S. aureus* ATCC 25923 was the most susceptible to the activity of the herb extracts. Its growth was inhibited at concentrations lower than 0.3% of the extracts in the medium. MIC_{50} values below 1% were found for: blackberry leaves against *Salmonellas* sp., *S. aureus* ATCC 6538P, *P. vulgaris* and *K. pneumoniae;* Lady's mantle against *P. vulgaris*; meadowsweet against *P. vulgaris* and *K. pneumoniae*; raspberry leaves against *Salmonella* sp. and *Proteus vulgaris*. For lady's mantle and raspberry leaves extracts the MICs were 2.5% against *S. aureus* ATCC 6538P. None of the extracts reduced the growth of the *L. monocytogenes* strains below 50% of their initial viable cell counts. They also had low (MIC_{50}=25%) or no activity against *S. enterica* ssp. *enterica* ATCC BAA-2162, *P. aeruginosa* or *E. coli* strains.

All six extracts showed an activity against genetically modified *E. coli*. The bioluminescence signal decreased within few minutes after exposition of the bac-

Table 4. Minimal inhibiting concentration (MIC_{50}) of chosen herb extracts against human pathogens

Test microorganisms	Blackberry leaves	Lady's mantle	Meadowsweet	Raspberry leaves
	$MIC_{50,}$ %			
Escherichia coli ATCC 8739	25	–	25	–
Escherichia coli ATCC 25922	–	–	25	–
Salmonella sp.	0.19	–	–	0.78
Salmonella enterica ssp. *enterica* ATCC BAA-2162	25	–	–	–
Staphylococcus aureus ATCC 6538P	0.63	2.5	0.63	2.5
Staphylococcus aureus ATCC 25923	0.13	0.25	0.25	0.031
Listeria monocytogenes	–	–	–	–
Listeria monocytogenes I	–	–	–	–
Proteus vulgaris G	0.16	0.1	0.08	0.78
Pseudomonas aeruginosa ATCC 9027	25	–	–	–
Klebsiella pneumoniae	0.63	25	0.08	25

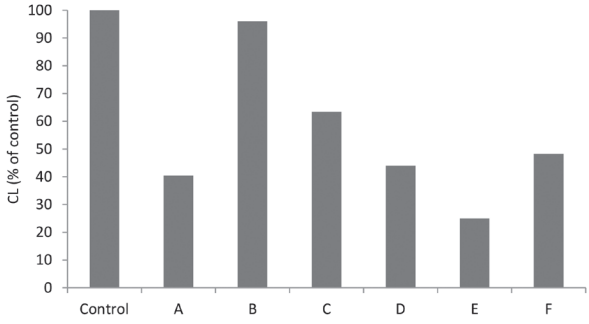

Figure 3. Luminometric measurement of antibacterial effect of herb extracts on *E. coli* viability.
Results are expressed as percentage of control samples without herb extracts. A — blackberry leaves; B — chokeberry leaves; C — hawthorn leaves; D — lady's mantle; E — meadowsweet; F — raspberry leaves.

teria to herb extracts and stayed low during the entire experiment. Meadowsweet showed the highest antibacterial activity (75% inhibition of control, Fig. 3) followed by blackberry leaves (60%), lady's mantle (56%), raspberry leaves (52%) and hawthorn leaves (27%). A very low activity was found for chokeberry leaves with only 4% growth inhibition. The antibacterial activity of all the extracts supports the lipid peroxidation inhibition results and those regarding production of ROS by phagocytes.

CONCLUSION

Results obtained in the present study indicate that all herbs studied, especially blackberry leaves and meadowsweet, are rich sources of chain-breaking, chelating and lipid peroxidation inhibiting antioxidants, rendering high antioxidant activity measured by several methods. Besides that the herb extracts could prevent oxidative stress in the gastrointestinal tract trough modulation of neutrophils and an antimicrobial effect against a broad spectrum of human pathogens.

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Biological activities of selected polyphenol-rich fruits related to immunity and gastrointestinal health

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ABSTRACT

Small fruits are a rich source of bioactive substances, including polyphenols, and are therefore suitable raw materials for the production of functional foods. In the current work, we studied the antioxidative properties of six fruits: rosehip, chokeberry, hawthorn, blackcurrant, blueberry and rowanberry via different methods (ORAC, TRAP, HORAC and inhibition of lipid peroxidation). Their effect on the production of reactive oxygen species (ROS) by phagocytes, antimicrobial properties against 11 human pathogens, and mitogenic effect on hamster spleen lymphocytes were also tested. Rosehip extract showed the highest antioxidant activity via ORAC, TRAP and HORAC assays, whereas blueberry extract was the most potent inhibitor of lipid peroxidation. All extracts inhibited ROS production of opsonized zymosan-activated phagocytes, indicating that extracts interfere with the signaling cascade of phagocyte activation upstream to the protein kinase C activation. Chokeberry, blackcurrant and rowanberry extracts revealed strong antimicrobial properties against a broad spectrum of microorganisms and also had the highest mitogenic activity.

1. Introduction

There are numerous studies demonstrating a correlation between the consumption of fresh fruits and vegetables with the prevention of socially significant diseases including cancer, cardiovascular diseases and Alzheimer disease (Heber, 2004; Hertog et al., 1996). A considerable amount of evidence indicates that increased oxidative damage contributes to the development of these diseases and several epidemiologic studies point out that the consumption of polyphenol-rich foods and beverages is associated with a lower risk of oxidative stress-related diseases (Ames, Shigenaga, & Hagen, 1993; Hertog et al., 1996). Polyphenols are a class of secondary plant metabolites that are thought to exert beneficial health effects through their antioxidant properties (Halliwell, Zhao, & Whiteman, 2000). Although there is a considerable volume of scientific data regarding flavonoid health benefits, the majority of the mechanisms of their bioactivity still remain unclear. Many of the polyphenol compounds are barely, if not at all, absorbed in the gastrointestinal tract (GIT). For example, anthocyanins which are broadly distributed in many plants, appear to be poorly absorbed in the small intestine. They are recovered in blood and urine in nanomolar concentrations, so significant amounts probably pass into the large intestine where bacterial degradation occurs. Similarly, proanthocyanidins, which are referred to as polymeric flavonoids and are strong antioxidants *in vitro*, have very limited absorption in the GIT; oligomers larger than trimers are unlikely to be absorbed in the small intestine in their native forms (Denev, Kratchanov, Ciz, Lojek, & Kratchanova, 2012). Although some flavonoids can be absorbed through the GIT, maximal plasma concentrations achieved are very low, in part because of rapid metabolism by human tissues and colonic bacteria (Halliwell, Rafter, & Jenner, 2005). Since absorption of phenolic compounds is incomplete, the majority passes through the GIT and enters the colon. Their local action may nevertheless be important because the intestine is particularly exposed to oxidising agents and may be affected by

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inflammation and numerous diseases, such as cancer. The human diet contains various pro-oxidants, including metals such as iron, copper, lipid hydroperoxides, aldehydes and nitrite, and elevated levels of lipid peroxides have been observed in the postprandial state. These pro-oxidants may induce oxidative stress in the GIT, induce stomach ulcers and the development of stomach, colon and rectal cancers. In addition, there are vast numbers of phagocytic cells that normally reside in the GIT. Phagocytic cells, such as macrophages and neutrophils, play a key role in innate immunity because of their ability to recognise, ingest and destroy pathogens by oxidative and nonoxidative mechanisms. In response to a variety of stimuli, NADPH oxidase present in neutrophils is activated in a phenomenon described as "the respiratory burst," characterised by the production of superoxide anion, which gives rise to other forms of reactive oxygen species (ROS). In addition to ROS, nitric oxide (NO) and other reactive nitrogen species (RNS) produced mainly by macrophages are one of the important microbicidal tools in the process of inflammation during the fight against pathogenic microorganisms, bacteria and tumor cells (Pekarova et al., 2009). However, excessive or inappropriate ROS and RNS production by phagocytes is associated with oxidative damage to membrane lipids, DNA, proteins and lipoproteins, resulting in various autoimmune and inflammatory diseases. Thus, the modulation of inflammation and oxidative stress by natural substances can be beneficial. The antioxidants contained in foods may suppress such oxidative stress and related diseases in the gastrointestinal tract before they are absorbed (Halliwell et al., 2000). Many of the phenolic metabolites in the colon still have free –OH groups and maintain antioxidant activity. Thus, in addition to the original phenolic compounds, their metabolites also have to be considered to understand the biological and antioxidant functions of foods. Polyphenol concentrations in the colon can reach several hundred micromoles per litre (Scalbert & Williamson, 2000) and together with a few carotenoids, they constitute the only dietary antioxidants present in the colon, because vitamins C and E are absorbed in the upper segments of the intestine. Therefore, foods rich in flavonoids and other polyphenols may protect against gastric, and possibly colon cancers.

In a recent comparative study, we observed that fruits such as rosehip (*Rosa canina*), chokeberry (*Aronia melanocarpa*), hawthorn (*Crataegus monogyna*), blackcurrant (*Ribes nigrum*), blueberry (*Vaccinium myrtillus*) and rowanberry (*Sorbus aucuparia*) possess the highest antioxidant activity measured by the ORAC method among 26 kinds of fruits (Denev, Lojek, Ciz, & Kratchanova, 2013). Based on these results we decided to perform more detailed studies on the antioxidative properties of these fruits, their effect on the production of ROS by phagocytes and their antimicrobial properties. Therefore, the aim of the current study was to determine the antioxidant properties of six fruit extracts with various methods (ORAC, TRAP, HORAC and inhibition of lipid peroxidation), to investigate their effect on the production of ROS from activated macrophages and to determine their antimicrobial properties and the minimal inhibiting concentration against 11 human pathogens. The major polyphenol constituents of the extracts were also identified, and their mitogenic effect on hamster spleen lymphocytes was tested. Since some of the fruits such as chokeberry, rosehip, rowanberry and hawthorn are not widely consumed fresh, the results of the currents study will be used for the development of functional foods directed to maintain immunity and gastrointestinal health.

2. Materials and methods

2.1. Plant materials

All fruit samples were collected from the region of Rhodopi Mountain in the stage of full maturity in 2012. The following 6 kinds of fruits were investigated: rowanberry (*S. aucuparia*), hawthorn (*C. monogyna*), rosehip (*R. canina*), chokeberry (*A. melanocarpa*), blackcurrant (*R. nigrum*) and blueberry (*V. myrtillus*). Chokeberries were cultivated, while all other fruits were widely collected in nature. Fresh fruits were frozen immediately and freeze dried in a Cryodos-50 laboratory freeze drier (Telstar Industrial, Spain).

2.2. Extraction

All plant materials were subjected to extractions under the following conditions: 50 g of the freeze dried fruits (edible part) were powdered in a laboratory mill. Then 5 g of the powder was transferred into extraction tubes and mixed with 100 ml of the extragent (80% acetone in 0.2% formic acid). Extraction was conducted on an orbital shaker at room temperature for one hour. After that, the samples were centrifuged (6000*g*) and supernatants were removed. Extracts were concentrated via rotary evaporation to a volume of 15 ml in order to fully remove the acetone. Then the volume was adjusted to 50 ml with ultra clean water and the extracts were centrifuged. Clear supernatants were used for all further analysis after proper dilutions.

2.3. HPLC analysis of phenolic compounds

High Performance Liquid Chromatography (HPLC) analyses of phenolic components was performed on an Agilent 1220 HPLC system (Agilent Technology, USA), equipped with a binary pump and UV–vis detector. A wavelength of $\lambda = 280$ nm was used. Separation of phenolic compounds was performed using an Agilent TC-C18 column (5 μm, 4.6 × 250 mm) at 25 °C. Mobile phases constituted of 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 ml/min. A gradient was used with 14% B, between 6 min and 30 linearly increased to 25% B and then 50% B at 40 min. The standard compounds (gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, rutin, naringin, myrecetin, quercetin, naringenin and kaempherol) were purchased from Sigma–Aldrich (Steinheim, Germany).

2.4. Total polyphenol compound analysis

Total polyphenols were determined according to the method of Singleton and Rossi (1965) with Folin–Ciocalteu's reagent. Gallic acid was employed as a calibration standard and results were expressed as gallic acid equivalents (GAE) per litre of extract.

2.5. Total anthocyanins determination

Anthocyanins were determined by the pH-differential method (Lee, 2005). The absorption of the sample was measured at pH 1.0 and pH 4.5 and the difference in absorbance is proportional to the anthocyanin content. The total anthocyanin content was expressed as mg cyanidin-3-glucoside equivalents per litre of extract and calculated via the following formula:

$$\text{Anthocyanin content (mg/l)} = \frac{A \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times L},$$

where $A = (A_{510\text{nm}}\ \text{pH}\ 1.0 - A_{700\text{nm}}\ \text{pH}\ 1.0) - (A_{510\text{nm}}\ \text{pH}\ 4.5 - A_{700\text{nm}}\ \text{pH}\ 4.5)$, MW = cyanidin-3-glucoside molecular weight (449.2); DF = dilution factor; ε = cyanidin-3-glucoside molar absorbtivity (26,900); L = cell pathlength (usually 1 cm).

2.6. Antioxidant activity assays

2.6.1. Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC was measured according to the method of Ou, Hampsch-Woodill, and Prior (2001) with some modifications described in details by Denev et al. (2010). The method measures the antioxidant scavenging activity against peroxyl radical generated by the thermal decomposition of 2,2′-azobis[2-methyl-propionamidine] dihydrochloride (AAPH) at 37 °C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) relative to that of a blank, in which no antioxidant has been present. Solutions of AAPH, fluorescein and trolox were prepared in a phosphate buffer (75 mmol/l, pH 7.4). Samples were also diluted in the phosphate buffer. The reaction mixtures (total volume 200 μl) contained FL – (170 μl, final concentration 5.36×10^{-8} mol/l), AAPH – (20 μl, final concentration 51.51 mmol/l), and sample – (10 μl). The FL solution and sample were incubated at 37 °C for 20 min directly in a microplate reader and AAPH (dissolved in buffer at 37 °C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 μl of phosphate buffer was used instead of the extract. The antioxidant activity was expressed in micromole trolox equivalents (μmol TE) per litre of extract. Trolox solutions (6.25; 12.5; 25 and 50 μmol/l) were used for defining the standard curve. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany), with an excitation wavelength of 485 nm and emission wavelength of 520 nm.

2.6.2. Total Peroxyl Radical Trapping Parameter (TRAP) assay

The luminol-enhanced chemiluminescence (CL) was used to follow the peroxyl radical reaction and the principle of this method is described by Cizova, Lojek, Kubala, and Ciz (2004). The CL signal is driven by the production of luminol derived radicals from thermal decomposition of AAPH. The TRAP value is determined from the duration of the time period (T_{sample}) during which the sample quenched the CL signal due to the present antioxidants. A known quantity (8.0 nM) of trolox (a water-soluble analogue of tocopherol) was used as a reference inhibitor (T_{trolox}) instead of the sample. The calculation of the TRAP value is represented by the equation:

$$\mathrm{TRAP} = 2.0\ [\mathrm{Trolox}]\ T_{sample}/f\ T_{trolox},$$

where 2.0 is the stoichiometric factor of trolox (the number of peroxyl radicals trapped per one molecule of trolox) and f is the dilution of the sample. The activity of the sample is expressed as trolox equivalents (TE) per litre of extract. TRAP assay was conducted on a Luminometer Orion II (Berthold Dection System GmbH, Germany).

2.6.3. Hydroxyl Radical Averting Capacity (HORAC) assay

The HORAC assay developed by Ou et al. (2002) measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co(II) complex and, hence, measures the protecting ability against formation of hydroxyl radical. Hydrogen peroxide solution of 0.55 M was prepared in distilled water. 4.6 mM Co(II) was prepared as follows: 15.7 mg of $CoF_2{\cdot}4H_2O$ and 20 mg of picolinic acid were dissolved in 20 ml of distilled water. Fluorescein (170 μl, 60 nM final concentration) and 10 μl of sample were incubated in 37 °C for 10 min directly in the FLUOstar plate reader. After incubation, 10 μl of H_2O_2 (27.5 mM final concentration) and 10 μl of Co(II) (230 μM final concentration) solutions were added subsequently. The initial fluorescence was measured after which the readings were taken every minute after shaking. For the blank sample, phosphate buffer solution was used. Gallic acid solutions (100, 200, 600, 800 and 1000 μM, in phosphate buffer 75 mM, pH = 7.4) were used for building the standard curve. The AUC were calculated the same way as the ORAC. The results were expressed in micromole galic acid equivalents (μmol GAE) per litre of extract.

2.6.4. Inhibition of lipid peroxidation assay

A modified assay described by Anthon and Barrett (2001) and Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi (2002) was used in the study. A mixture of 1 ml of a weighed test sample in 99.5% ethanol, 1 ml of 83.5 mM linoleic acid in 99.5% ethanol, 2 ml of 0.05 M phosphate buffer (pH 7.0), and 1 ml of water in glass tube with a plastic stopper was placed in a thermostat and incubated at 40 °C in the dark. All samples were run in duplicates and the absorbance at 234 nm was measured after 1, 3 and 6 days of incubation using a microplate reader Infinite M200 (Tecan, Austria). For conversion of absorbance to the concentration of hydroperoxide, the extinction coefficient 23,000 M^{-1} cm^{-1} was used.

2.7. Oxidative burst

Heparinised blood (50 IU/ml) from healthy volunteers ($n = 7$) was obtained by antecubital venipuncture. The oxidative burst of phagocytes in whole blood was measured chemiluminometrically using an LM-01 microplate luminometer (Immunotech, Czech Republic). The principle of the method was described previously (Ciz et al., 2007). Each reaction mixture consisted of 25 μl of whole blood diluted 10× in Hank's balanced salt solution (HBSS), 2.5 μl of one of the tested samples, 25 μl of 1 mM luminol and 25 μl of one of the activators –62.5 μg/ml of opsonized zymosan particles (OZP; Sigma, USA) or 0.81 μg/ml of phorbol-12-myristate-13-acetate (PMA; Sigma, USA). HBSS was used to adjust the total reaction volume to 250 μl. The assays were run in duplicates. Spontaneous CL measurements in samples containing blood and other substances except any activator were included in each assay. The chemiluminescence (CL) activity of the samples was immediately measured at 37 °C. Light emission expressed as relative light units (RLU) was recorded continuously for 60 min to obtain kinetic curves. Integrals of CL signal over 60 min were also calculated.

2.8. Mitogenic activity

The spleens from healthy hamsters were isolated and mechanically dispersed, filtered over a fine mesh and layered on Ficoll-Paque at a ratio of 2:1. The tubes with spleen cell suspensions were centrifuged at 1800 rpm for 30 min at room temperature. Cells at interface-splenic lymphocytes (splenocytes) were collected and washed three times with RPMI-1640 medium (without FCS). Spleen cells were finally suspended in complete RPMI-1640 medium (supplemented with 100 μg/ml penicillin–streptomycin and 10% FCS). Hamsters' splenocytes were counted in a haemocytometer and cell viability was determined using 0.2% trypan blue. Cell concentration was adjusted to 2×10^6 cells/ml in complete RPMI-1640 medium.

The effect of fruit extracts on normal hamsters' spleen lymphocytes was assessed colorimetrically by the MTT assay as described by Mossmann (1983). Cell number and viability were evaluated by measuring the mitochondrial-dependent conversion of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals by metabolically active cells. Cells were seeded in a 96-well microtiter plate at a concentration of 2×10^4 cells per well. After incubation overnight at 37 °C in humidified air with 5% CO_2 to allow cell

attachment, the medium was changed and cells were treated with different concentrations of the tested compounds. Cells cultured in only medium were used as negative control. After treatment for 24 and 48 h, the cells were washed twice with PBS (pH 7.4) and further incubated with 100 μl of MTT working solution (Sigma–Aldrich, Steinheim, Germany) at 37 °C for 3 h. Supernatants were aspirated and 100 μl of lysing solution (dimethylsulfoxide:ethanol = 1:1, v/v) an added to each well to dissolve the resulting formazan. MTT assay was performed using an ELISA plate reader (Tecan, Austria). The percentage of viable cells was calculated as follows:

$$\text{Cell viability } (\%) = \text{OD}_{570} \text{ (experimental)}/\text{OD}_{570} \text{ (control)} \times 100.$$

2.9. Ethical aspects

The animal tests were conducted in accordance with the principles for laboratory animal use and care as found in the European Community guidelines, Committee on Care of Laboratory Animal resources, Commission on Life Sciences, National Research Council. Hamsters were housed in the animal care facilities of the IEMPAM Institute, which are fully accredited for Laboratory Animal Care.

2.10. Determination of the antimicrobial activity through agar diffusion method

A suspension of each test microorganism (concentration $\sim 1.10^7$ cfu/cm^3) was prepared with saline solution. Melted PCA (Scharlau) nutrient medium was inoculated through the addition of 1 cm^3 of microbial suspension and was poured in Petri dishes (15 cm^3 in each dish). Wells with 7 mm diameter were made in the solidified and cooled agar medium. Tested extracts (50 μl) were pipetted in the wells. The Petri dishes were incubated at 37 °C for 24–48 h. The inhibition zone was measured. Zones with a diameter greater than 7 mm were considered zones of inhibition.

2.11. Determination of minimal inhibiting concentration (MIC)

The antimicrobial effects of the extracts were determined by analysing the MIC using a 96 well microtiter plate method (broth dilution) according to the modified protocol described by Gutierrez, Barry-Ryan, and Bourke (2008). The first row of each plate contained 300 μl of nutrient broth (tripton – 5.0 g/dm^3, yeast extract – 2.5 g/dm^3, glucose – 1 g/dm^3, pH 7.0) and 100 μl of the tested extract. From the second to the twelfth row, 200 μl of broth was added and wells containing each extract in the first row were then diluted twofold along each column, up to the eleventh column. The twelfth column was used for growth control (positive control) and a row was used for control sample (negative control) for each dilution. These additional controls contained distilled water and growth media inoculated with each microorganism under investigation. Finally, 30 μl of each working culture of the test microorganism (1×10^7 cfu/cm^3) was added to all the wells except the negative controls. Plates were then incubated at 37 °C and after 24 h of incubation, they were placed in a microplate spectrophotometer (absorbance at 600 nm; SPECTROstar^{Nano}, BMG Labtech, Germany) to determine the presence or absence of turbidity. For each treatment, the absence of turbidity in the wells compared to the control was considered as an inhibitory effect. The lowest concentration of the tested extract required to reduce the growth of the tested microorganism to 50% of the positive control was designated as the MIC.

2.12. Statistical analysis

The results are expressed as the means from at least three independent experiments ± standard error of the mean (SEM). Experiments were conducted in duplicates or triplicates. Comparisons with the control were performed by analysis of variance (ANOVA), followed by the Newman–Keuls Post-hoc test. *P* values less than 0.05 were considered significant.

3. Results and discussion

3.1. Major polyphenol constituents of fruit extracts

The results from the HPLC analysis of the fruit extracts are shown in Table 1. The analysed extracts showed big differences in the polyphenol composition and content. The total amount of polyphenol compounds was the highest in chokeberry extract – 6274.8 mg/l, whereas blueberry extract was the richest source of anthocyanins – 1242.4 mg/l. Rowanberry and chokeberry had the highest content of phenolic acids. In rowanberry extract, the hydroxycinnamic acids, chlorogenic and neochlorogenic acids, were the only detected polyphenol compounds and their total amount was 1133.1 mg/l. The total amount of these two acids in aronia extract was 1112.8 mg/l. In hawthorn, rosehip and blackcurrant flavonol and flavan-3-ol representatives were predominant over the phenolic acids. From the investigated extracts, rosehip had the highest rutin content at 107.3 mg/l, hawthorn the highest epicatechin content – 119.8 mg/l and blackcurrant extract had the highest amount of catechin – 144.5 mg/l. The differences in the polyphenol profile of the investigated extracts are prerequisite for different biological activity.

3.2. Antioxidant activity of fruit extracts

When assaying the antioxidant activity of natural antioxidants, it is recommended to use more than one antioxidant assay for a detailed understanding of the antioxidant properties of substances (Ciz et al., 2010). Therefore, in our study we used several assays expressing various aspects of the antioxidant action of polyphenols – ORAC, TRAP, HORAC and inhibition of lipid peroxidation. The methods used embrace different aspects of the antioxidant action and give a broader view on the antioxidant potential of fruit extracts. ORAC and TRAP methods measure the ability of the antioxidant to scavenge peroxyl radicals via hydrogen atom transfer. These radicals are physiologically the most important ones and the hydrogen atom transfer is the most physiologically relevant mechanism of antioxidant action. The HORAC method measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions and hence indicates the compounds protecting ability against formation of hydroxyl radical. Using the ORAC, TRAP and HORAC assays rosehip extract had the highest antioxidant activity (Fig. 1, ORAC – 93677.6 μmol TE/l; TRAP – 87109.4 μmol TE/l and HORAC – 76069.4 μmol GAE/l). Hawthorn extract revealed the second highest antioxidant activity with these three methods. The results obtained for the antioxidant activities indicate that all fruits tested and particularly rosehip and hawthorn, are suitable raw materials for the production of functional foods with antioxidant activity. These results are interesting because, with the exception of blackcurrant and blueberry, all other fruits used are rarely consumed in fresh form. The elaboration of antioxidant functional foods from them will make them accessible for more people who will benefit from their high antioxidant activity.

The effect of the tested samples on lipid peroxidation was assayed using the autoxidation of linoleic acid in an ethanol-buffer system and measuring the formation of linoleic acid hydroperoxides. This is a simple and reliable method for the evaluation of potential antioxidants (Kikuzaki et al., 2002). As shown in Fig. 2A, the concentration of hydroperoxides rises gradually during the six days of linoleic acid autoxidation at 40 °C. The formation of

Table 1
Major polyphenol and anthocyanin contents of fruit extracts.

Extract	Gallic acid (mg/l)	Neochlorogenic acid (mg/l)	Chlorogenic acid (mg/l)	*p*-Coumaric acid (mg/l)	Ferulic acid (mg/l)	Ellagic acid (mg/l)	Catechin (mg/l)	Epicatechin (mg/l)	Rutin (mg/l)	Quercetin (mg/l)	Total polyphenols (mg/l)	Total anthocyanins (mg/l)
Rowanberry		427.4	705.7	–	–	–	–	–	–	–	2148.2	5.3
Hawthorn	–	34.2	60.6	–	2.0	16.9	45.9	119.8	14.4	–	4025.8	13.0
Rosehip	–	–	44.4	–	–	–	–	–	107.3	14.7	5609.9	–
Chokeberry	–	650.9	461.9	–	–	–	72.5	–	–	14.8	6274.8	875.0
Blackcurrant	15.1	29.5	–	1.8	–	–	144.5	–	25.8	6.9	4038.2	654.6
Blueberry	–	–	201.9	–	11.2	–	–	–	93.3	–	6012.5	1242.4

hydroperoxides in the presence of the fruit extracts is shown in Fig. 2B. It is obvious that the autoxidation of linoleic acid was very effectively inhibited by extracts from blueberry, rowanberry and blackcurrants at the end of the 1st day of linoleic acid autoxidation (concentration of hydroperoxides 40%, 30%, and 27% of control, resp.). The inhibition of hydroperoxide formation by these fruits and, in addition by chokeberry, was observed also after the 3rd day of linoleic acid autoxidation (less than 50% of control). And finally, hydroperoxide formation was inhibited to more than 50% of the control value by all extracts analysed after 6 days of linoleic acid autoxidation. Extract from blueberry was the most effective since it diminished the concentration of hydroperoxides to 8% of the control value.

3.3. Effect on the production of ROS from phagocytes

The effects of studied extracts on the production of ROS in human whole blood phagocytes was studied using luminol-enhanced CL. The results, depicted as kinetic curves and integral values of the CL response, are shown on Fig. 3. It is obvious that all studied extracts significantly inhibited ROS production in the case of OZP-activated phagocytes. The most effective was the extract of blackcurrants fruits and the effectiveness of all extracts was in the following order: blackcurrants > blueberry > chokeberry > rosehip > hawthorn > rowanberry. The weakest extract of rowanberry still inhibited the OZP-activated CL response by more than 50%. On the other hand, none of the studied extracts inhibited the PMA-activated CL response of human whole blood phagocytes.

The inhibitory effect of flavonoids (kaempferol, morin, quercetin and fisetin) on the respiratory burst of neutrophils was observed by Pagonis, Tauber, Pavlotsky, and Simons (1986). Similarly, Zielinska, Kostrzewa, and Ignatowicz (2000) approved the inhibitory effects of natural flavonoids (quercetin, kaempferol and isorhamnetin) against the respiratory burst of neutrophils from healthy human donors *in vitro*, as measured by flow cytometry using dichlorofluorescein diacetate and luminol-dependent chemiluminescence. Wang, Chang, Hsu, Chen, and Kuo (2002) investigated the cellular localisation of the inhibitory effect of a natural flavonoid cirsimaritin against a formyl-methionyl-leucyl-phenylalanine (fMLP)-induced respiratory burst in rat neutrophils. Cirsimaritin inhibited the superoxide anion radical generation and the oxygen consumption of neutrophils. On the other hand, cirsimaritin did not reduce, but slightly enhanced, the superoxide anion radical generation in PMA-activated NADPH oxidase. The results from these authors indicate that it is likely that the inhibition of the fMLP-induced respiratory burst by cirsimaritin in rat neutrophils is mainly through the blockade of the phospholipase D (PLD) signaling pathway.

Selloum, Reichl, Muller, Sebihi, and Arnhold (2001) compared the effects of three aglycone flavonols (myricetin, quercetin and kaempferol) and the natural glycoside rutin on superoxide anion radical generation. All flavonols tested inhibited the pholasin luminescence of fMLP-stimulated neutrophils. Lee et al. (2010)

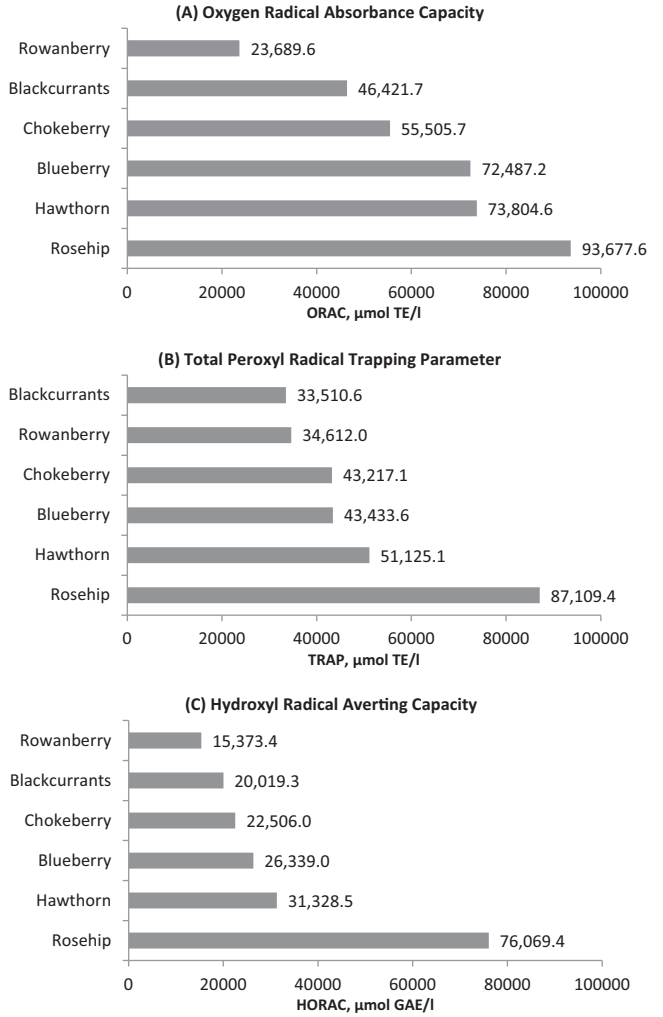

Fig. 1. Antioxidant activity of fruit extracts measured by different assays: (A) Oxygen Radical Absorbance Capacity (ORAC); (B) Total Peroxyl Radical Trapping Parameter (TRAP); (C) Hydroxyl Radical Averting Capacity (HORAC).

observed that luteolin attenuated neutrophil respiratory burst but had a negligible effect on superoxide anion generation during PMA stimulation. Furthermore, luteolin effectively blocked MAPK/ERK kinase 1/2 and Akt phosphorylation in fMLP-stimulated neutrophils. Thus, an inhibition of enzymes involved in signaling, rather than a scavenging of superoxide anion radicals dominates in fMLP-stimulated neutrophils exposed to flavonoids in these particular studies.

The effects of flavonoids from various plant extracts on the respiratory burst of neutrophils were also studied. For example, Kenny, Shu, Moritoki, Keen, and Gershwin (2009) demonstrated that flavonoids isolated from cocoa could moderate a subset of signaling pathways derived from the LPS stimulation of neutrophils,

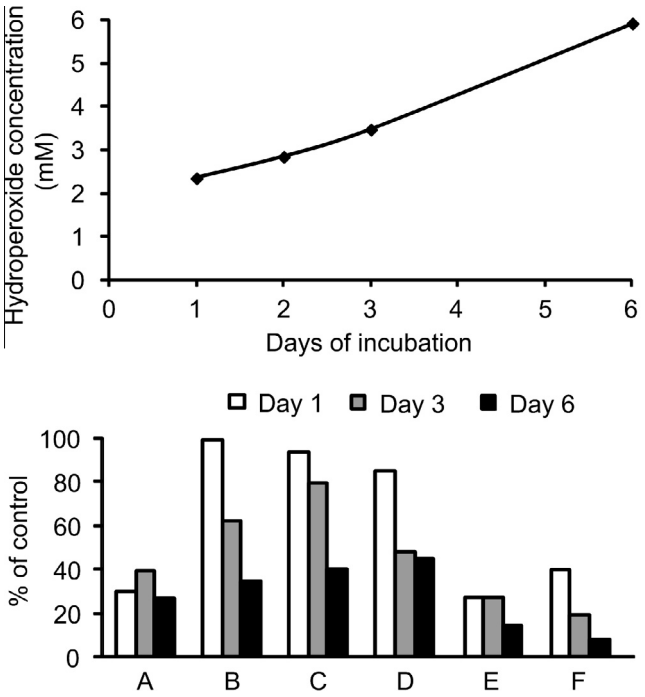

Fig. 2. Inhibition of lipid peroxidation by fruit extracts. (A) The kinetics of hydroperoxide concentration in the control samples without fruit extracts during the 6 days of autoxidation of linoleic acid. (B) The effect of fruit extracts on hydroperoxide accumulation at the end of 1, 3 and 6 days of autoxidation of linoleic acid. The results are expressed as percentage of control samples without fruit extracts. A – rowanberry; B – hawthorn; C – rosehip; D – chokeberry; E – blackcurrants; F – blueberry.

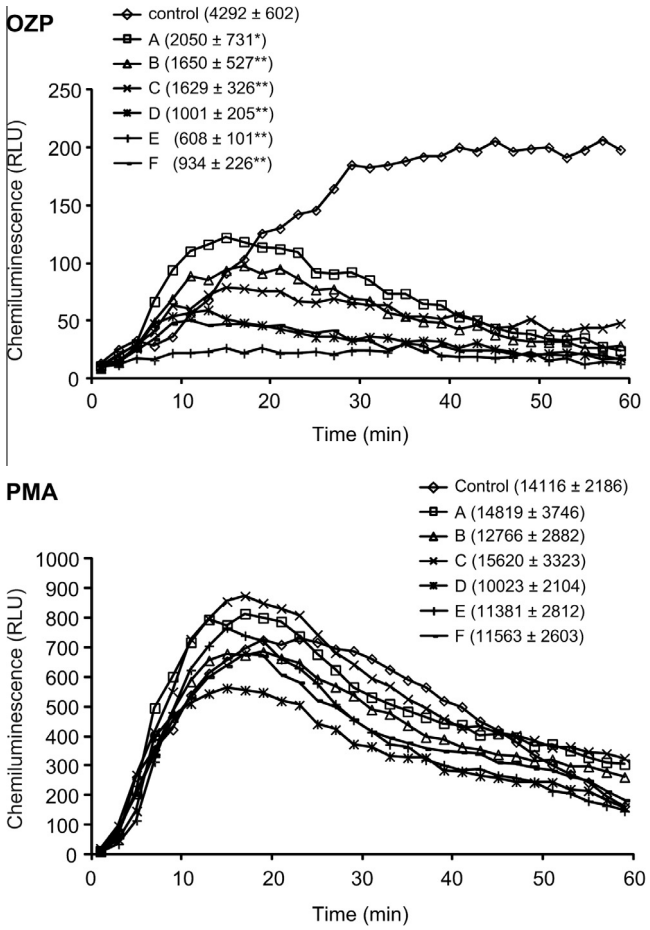

Fig. 3. Effect of fruit extracts on the production of reactive oxygen species by phagocytes expressed as the kinetic curves of the CL response after OZP activation (upper panel) and PMA activation (lower panel). A – rowanberry; B – hawthorn; C – rosehip; D – chokeberry; E – blackcurrants; F – blueberry. Integral values of the CL response (RLU * min) are shown in the legend.

mainly neutrophil oxidative bursts and activation markers. They hypothesised that flavonoids could decrease the impact of LPS on the fMLP-primed neutrophil ability to generate ROS by partially interfering in the activation of the MAPK pathway. A *Ginkgo biloba* extract containing flavonoids was tested by Pincemail et al. (1987) for its effect on the release of ROS during the stimulation of human neutrophils by a soluble agonist. The extract slowed down the oxygen consumption (respiratory burst) of the stimulated cells. fMLP used in the cited studies is a soluble activator of neutrophils which binds to specific membrane receptors. Similarly, OZP binds to the surface opsonin (either complement or Fc) receptors on phagocytes and triggers the signaling cascade leading finally to the activation of protein kinase C (PKC) with subsequent activation of NADPH oxidase, the enzyme producing superoxide anion – the first of ROS produced by phagocytes. On the contrary, PMA directly activates the PKC, the step before NADPH oxidase activation. Our results obtained are in accordance with the literature indicating that all fruit extracts used do not inhibit either PKC or NADPH oxidase itself, but interfere with the signaling cascade of phagocyte activation upstream to the PKC activation.

3.4. Effects of fruit extracts on lymphocytes proliferation

Proliferation of hamsters' lymphocytes, evaluated by MTT test, was significantly stimulated by berry extracts in a dose-dependent manner ($p < 0.01$) after a 48 h incubation period when compared to the control group (Fig. 4). Mitogenic activity of fruit extracts on lymphocyte cell proliferation was in the decreasing order: blackcurrants > chokeberry > rowanberry > rosehip > hawthorn > blueberry, and was several fold higher than the control, untreated cells. The extracts of blackcurrants exhibited the highest proliferation activity at a dose-dependent manner, followed by the extracts of chokeberry and the higher used dose of rowanberry and rosehip after 24 h incubation, but these extracts had a lower stimulatory effect on lymphocytes than those observed after 48 h incubation. In contrast, the extracts of blueberry, hawthorn and the lower used dose of rowanberry and rosehip had no stimulatory effect on lymphocytes. Many of the health benefits derived from fruit consumption are due to their wide range of bioactive phytochemicals, the most prominent of which are polyphenols, which are responsible for many of their biological activities. The immunomodulatory role of antioxidants has been shown in immune cell functions in ageing and in an oxidative stress experimental model, namely endotoxic shock, in which immune functions are altered in a way similar to ageing (De la Fuente, 2002). Thus, since the immune system is a health indicator and longevity predictor, the protection of this system by an antioxidant diet supplementation may be useful for health preservation.

3.5. Antimicrobial activity

The antimicrobial activity of the fruit extract was tested via two assays and eleven foodborne pathogens were used as test microorganisms. The initial screening of the antimicrobial activity was performed using the agar diffusion method. The antimicrobial activity was classified as weak (inhibition zone < 12 mm), medium (inhibition zone between 12 and 20 mm) and strong (inhibition zone above 20 mm) (Rota, Herrera, Martinez, Sotomayor, & Jordan,

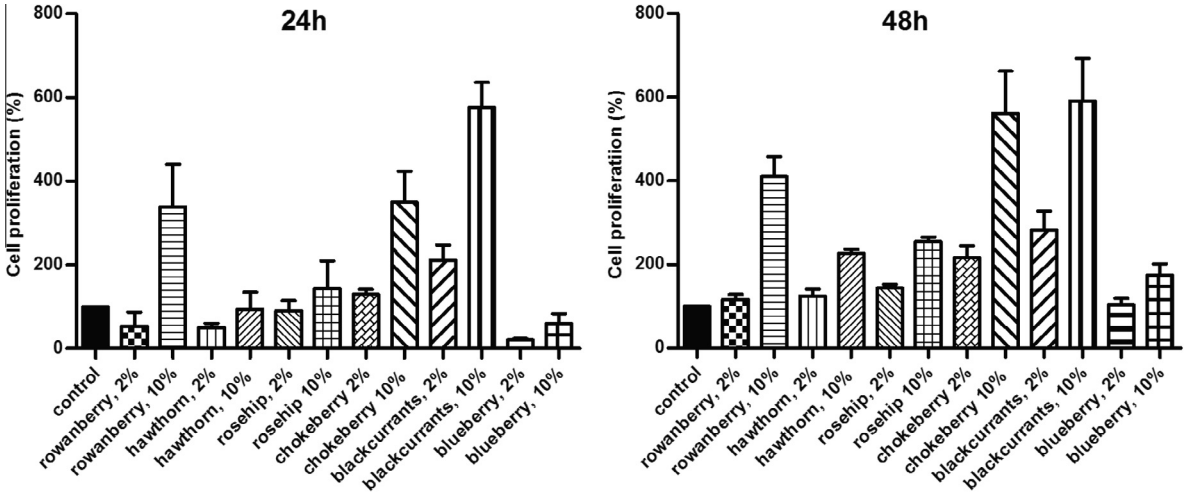

Fig. 4. Effects of fruit extracts on the proliferation activity of lymphocytes from healthy hamsters.

Table 2
Antimicrobial activity of fruit extracts against human pathogens measured as zones of inhibition and minimal inhibiting concentrations.

Microorganism	Rowanberry extract			Chokeberry extract			Blackcurrant extract		
	100%	10%	MIC_{50} %	100%	10%	MIC_{50} %	100%	10%	MIC_{50} %
	Zone of inhibition, mm			Zone of inhibition, mm			Zone of inhibition, mm		
Escherichia coli ATCC 8739	12	–	12.5	11	–	3.125	11	–	3.125
Escherichia coli ATCC 25922	13	–	–	30	–	1.56	13	–	1.56
Salmonella sp.	12	–	1.56	10	–	1.56	11	–	1.56
Salmonella enterica ssp. *enterica* ATCC BAA-2162	15	–	6.25	10	–	0.39	13	–	12.5
Staphylococcus aureus ATCC 6538P	9	–	6.25	13	–	6.25	15	9	3.125
Staphylococcus aureus ATCC 25923	11	–	0.1	13	–	0.02	12	–	0.02
Listeria monocytogenes	13	–	12.5	11	–	12.5	12	–	12.5
Listeria monocytogenes I	14	–	–	11	–	6.25	13	–	3.125
Proteus vulgaris G	14	–	12.5	11	–	–	14	–	–
Pseudomonas aeruginosa ATCC 9027	15	–	–	12	–	12.5	13	–	25
Klebsiella pneumoniae	12	–	25	10	–	–	–	–	–

2008). The hawthorn and rosehip extracts displayed weak antimicrobial activities, and the best results were obtained against *Staphylococcus aureus* ATCC 6538P and *Proteus vulgaris* G (data not shown). Only two *Staphylococcus* strains were sensitive against the antimicrobial effect of the blueberry extract (data not shown). Via this method, three extracts (rowanberry, chokeberry and blackcurrant) showed the most significant antimicrobial activities against broad spectrum of microorganisms. The rowanberry extract exhibited highest activity against *Salmonella enterica* ssp. *enterica* ATCC BAA-2162 and *Pseudomonas aeruginosa* ATCC 9027. It also showed moderate activity against the two *Listeria monocytogenes* strains and *P. vulgaris* G. The strongest antimicrobial activity in the experiment was shown by the chokeberry extract against *Escherichia coli* ATCC 25922, where an inhibition zone of 30 mm was detected. The extract was moderately active against the *Staphylococcus* strains. The blackcurrant extract exhibited medium antibacterial activity against all test microorganisms, except *Klebsiella pneumonia*. The highest activity was determined against *S. enterica* ssp. *enterica* ATCC BAA-2162, where even a 10% concentration of the extract caused inhibition of bacterial growth. Based upon the obtained results rowanberry, chokeberry and blackcurrant extracts were selected for further experiments and determination of minimal inhibiting concentration (MIC) (Table 2).

The lowest MICs were determined for chokeberry extracts. It presented antibacterial activity even at 0.02% concentration in the medium. The most sensitive strains against its presence were *S. aureus* ATCC 25923 and *S. enterica* ssp. *enterica* ATCC BAA-2162. Two more strains (*E. coli* ATCC 25922 and *Salmonella* sp.) were inhibited by the extract at concentrations below 2%. The rest of the microorganisms were also inhibited but at concentration higher than 3%. The strains *E. coli* ATCC 25922, *Salmonella* sp. and *S. aureus* ATCC 25923 were the most susceptible to the antibacterial action of chokeberry, rowanberry and blackcurrant. The latter had very similar antimicrobial activity to chokeberry with the same bacteria inhibited. Rowanberry, however, showed the lowest activity compared to the other extracts. Only two pathogens from the microbes tested were inhibited at concentrations <2% in the medium, namely *Salmonella* sp. and *S. aureus* ATCC 25923. There were strains (rowanberry against *L. monocytogenes* I; chokeberry and blackcurrant against *P. vulgaris* G and *K. pneumoniae*) for which MIC were not determined because reduction in bacterial growth was less than 50%. In other cases, MIC were determined for extract concentrations above 10%. These included: rowanberry against *E. coli* ATCC 8739, *L. monocytogenes*, *P. vulgaris* G and *K. pneumoniae*; chokeberry against *L. monocytogenes* and *P. aeruginosa* ATCC 9027; blackcurrant against *S. enterica* ssp. *enterica* ATCC BAA-2162, *L. monocytogenes* and *P. aeruginosa* ATCC 9027.

4. Conclusion

The current study assayed the potential of six edible fruits as raw materials for the production of functional foods with antioxidant, antimicrobial and mitogenic activities. The fruits investigated

are rich sources of several classes of polyphenol compounds and differences in their polyphenol profile led to different biological activities. The antioxidant activity data indicates that all the fruits tested, and particularly rosehip and hawthorn, are very suitable raw materials for the production of antioxidant foods. All extracts studied and especially those from anthocyanin-rich fruits (blackcurrants, chokeberry and blueberry) significantly inhibited the ROS production of OZP-activated phagocytes, indicating that these extracts interfere with the signaling cascade of phagocyte activation upstream to the protein kinase C activation. Chokeberry, blackcurrant and rowanberry extracts revealed strong antimicrobial properties against a broad spectrum of microorganisms. These extracts also had the highest mitogenic activity expressed as the stimulating effect on hamster lymphocyte proliferation. These results are interesting because with exception to blackcurrant and blueberry, all the other fruits used are rarely consumed in the fresh form. The elaboration of functional foods will make the investigated fruits accessible for more people who will benefit from their biological activities related to immunity and gastrointestinal health.

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Research Article

On the Molecular Pharmacology of Resveratrol on Oxidative Burst Inhibition in Professional Phagocytes

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Resveratrol—3,5,4′-trihydroxystilbene—possesses antioxidant activities *in vitro*. It dose-dependently inhibited the generation of peroxyl, hydroxyl, peroxides, and lipid peroxidation products in cell free systems. Oxidative burst of whole human blood stimulated with PMA, fMLP, OpZ, and A23187 was inhibited in a concentration-dependent way, indicating suppression of both receptor and nonreceptor activated chemiluminescence by resveratrol. Results from isolated human neutrophils revealed that resveratrol was active extracellularly as well as intracellularly in inhibiting the generation of reactive oxygen species. Liberation of ATP and analysis of apoptosis showed that in the concentration of 100 μM, resveratrol did not change the viability and integrity of isolated neutrophils. Western blot analysis documented that resveratrol in concentrations of 10 and 100 μM significantly decreased PMA-induced phosphorylation of PKC α/βII. Dose-dependent inhibition of nitrite production and iNOS protein expression in RAW 264.7 cells indicated possible interference of resveratrol with reactive nitrogen radical generation in professional phagocytes. The results suggest that resveratrol represents an effective naturally occurring substance with potent pharmacological effect on oxidative burst of human neutrophils and nitric oxide production by macrophages. It should be further investigated for its pharmacological activity against oxidative stress in ischaemia reperfusion, inflammation, and other pathological conditions, particularly neoplasia.

1. Introduction

Neutrophils are present in high numbers in areas of inflammation, where they constitute an important source of reactive oxygen species (ROS). The massive production of antimicrobial and tumoricidal ROS in an inflammatory environment is called "oxidative burst" and plays an important role as the first line of defense against environmental pathogens. Paradoxically, however, neutrophils are also implicated in tissue-damaging inflammatory reactions that underlie the pathogenesis and exacerbation of many inflammatory diseases [1, 2]. There are at least two signalling pathways responsible for induction of neutrophil activation: one is the protein kinase C (PKC) mediated pathway, which can be driven by stimulation with phorbol-4β-12β-myristate-13α-acetate (PMA), and the other is the *Src* family protein tyrosine kinase mediated pathway [3].

Apoptosis is critical for the regulation of life span of circulating as well as emigrated neutrophils. Accumulating evidence indicates that neutrophil apoptosis is one of the critical determinants of the outcome of the inflammatory response and is a potential target for therapeutic interventions. A delay of neutrophil apoptosis exacerbates and prolongs inflammation or even prevents spontaneous resolution of inflammation [4]. The apoptotic neutrophil and the process of cell death exert anti-inflammatory effects that have been shown to be of therapeutic value in inflammatory diseases [5, 6].

A wide array of phenolic substances, particularly those present in edible and medicinal plants, have been reported to possess substantial antioxidative, anticarcinogenic, and antimutagenic activities by modulating important cellular signalling processes [7–10]. Natural polyphenols suppressed oxidative burst of stimulated human neutrophils by enhancing their apoptosis and decreasing protein kinase C activation [11–17].

Resveratrol (RES), a polyphenolic phytoalexin, is one of the most extensively studied natural products, with wide-ranging biological activities and tremendous clinical potential [18]. RES has been shown to have antioxidant, antiinflammatory, antiproliferative, and anti-angiogenic effects, while those on oxidative stress are presumably the most important [19].

In spite of the fact that almost 5000 papers are evidenced in PubMed database, there is a lack of evidence about the mechanism of the effect of resveratrol on oxidative burst in human professional phagocytes at molecular level. In this study, we investigated the effect of RES on the mechanism of oxidative burst in human whole blood, isolated neutrophils at extra- and intracellular level, activation of protein kinase C, caspase-3 activity and cellular viability and on free radical scavenging activity in cell free systems (oxygen radical absorption capacity—ORAC, hydroxyl radical averting capacity—HORAC, scavenging of ROS generation, nitric oxide production, and inhibition of lipid peroxidation). Moreover, we studied the effect of resveratrol on nitrite production and iNOS protein expression in murine RAW 264.7 macrophage cell line.

2. Methods and Materials

2.1. Chemicals. Luminol, isoluminol, PMA (phorbol-4β-12β-myristate-13α-acetate), Ca^{2+}-ionophore A23187, superoxide dismutase, dextran (average MW 464,000), zymosan A (from *Saccharomyces cerevisiae*), luciferase (from firefly *Photinus pyralis*), and D-luciferin sodium salt from Sigma-Aldrich Chemie (Deisenhofen, Germany). HRP (horseradish peroxidase), catalase, and Folin-Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany) and Lymphoprep (density 1.077 g/mL) from Nycomed Pharma AS (Oslo Norway). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium salt, cobalt(II) fluoride tetrahydrate, and gallic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Picolinic acid was purchased from Fluka (Deisenhofen, Germany), and human purified caspase-3 was from Enzo Life Sciences, Lausen, Switzerland. All other chemicals used were of analytical grade and obtained from commercial sources.

ORAC and HORAC analyses were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnology, Offenburg, Germany).

The phosphate buffered saline solution (PBS) used in this study contained 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 0.5 mM $MgCl_2 \times 6H_2O$ and had a pH of 7.4. Tyrode's solution used in this study consisted of 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM NaH_2CO_3, 0.4 mM $NaH_2PO_4 \times 2H_2O$, 1 mM $MgCl_2 \times 6H_2O$, and 5.6 mM glucose, pH of 7.4.

Resveratrol (RES) was prepared by targeted regioselective synthesis purely as transisomer [20] and was diluted in 1 : 50 (v/v) of 1 M NaOH in water.

2.2. Blood Collection and Neutrophil Separation. Fresh human blood was obtained at the blood bank by venipuncture from healthy male volunteers (20–50 years) who had not received any medication for at least 7 days. It was anticoagulated with 3.8% trisodium citrate (blood : citrate ratio = 9 : 1). The Ethical Committee license for blood sampling at the National Transfusion Service NTS-KRA/2012/SVI was registered. Human neutrophils were isolated from whole blood, as described previously [21, 22]. The final suspension of neutrophils contained more than 96% of viable cells, as evaluated by trypan blue exclusion and was used within 2 h, as long as the control chemiluminescence remained constant.

2.3. Chemiluminescence (CL) Assay of Whole Blood and Isolated Neutrophils. The oxidative burst in whole blood was stimulated with phorbol myristate acetate (PMA 0.05 μM), opsonized zymosan (OpZ; 0.5 mg/mL), fMLP (1 μM), or Ca ionophore A23187 (1 μM). CL was measured in 250 μL samples consisting of 50 μL aliquots that contained blood (50× diluted), luminol (200 μM), RES (0.01–100 μM), and phosphate buffer [11]. The effect of RES on extra- and intracellular ROS production was measured in unstimulated and PMA (0.05 μM) stimulated neutrophils (5×10^5 per sample) by isoluminol/luminol-enhanced CL. The CL of both whole blood and isolated neutrophils was evaluated and measured in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37°C [23].

2.4. Analysis of Apoptosis. Citrated whole human blood was collected as described above. Dextran (3%) was added (blood : dextran = 2 : 1) and centrifuged at 10 ×g at room temperature [16]. Before use, 1 mL of buffy coat that contained leukocytes was collected and stored on ice. The cells were counted on the hemocytometer (Coulter Counter), which focused on granulocytes. The cell suspension was adjusted to get 2×10^5 neutrophils per sample. Three different concentrations of RES (1, 10, and 100 μM) were applied and incubated with a control sample at 37°C for 10 min. The cells were stained with Annexin V, conjugated with FITC (BenderMedSystems) in the dark at 4°C for 10 min, followed by staining with propidium iodide (1 μg/mL), and then analysed immediately by the Beckman Coulter Cytomics FC500 cytometer (for details, see Perečko et al. [16]).

2.5. Neutrophil Integrity. The cytotoxic effect of RES was evaluated by means of ATP liberation by luciferin-luciferase chemiluminescence [11]. The neutrophil suspension (30 μL; 30 000 cells/sample) and 20 μL of Tyrode's solution were incubated with 50 μL of RES (1 to 100 μM) for 15 min at

37°C. The total ATP content was assessed immediately after sonication of neutrophils for 10 s.

2.6. Recombinant Caspase-3 Activity. To determine the caspase-3 activity, a modified method was applied [16]. The final reaction with luciferase was detected by CL. The light production was measured in the Luminometer Immunotech LM-01T. The reagent was added and the mixture was measured for 60 min to determine caspase-3 activity. The solvent for RES, containing NaOH, was also evaluated.

2.7. Protein Kinase C Activation. Phosphorylation of protein kinase (PKC) isoenzymes α and βII was detected [11]. Isolated human neutrophils (5×10^6) were incubated at 37°C with RES for 1 min, stimulated with PMA (0.15 μM, 1 min), and lysed by the addition of solubilisation buffer. After sonication on ice, samples were centrifuged to remove unbroken cells, the supernatant was boiled for 5 min with sample buffer, and samples were loaded on 9.8% SDS polyacrylamide gels. Membrane strips were blocked for 60 min with 1% bovine serum albumin in Tris buffered saline. This was followed by 60 min incubation in the presence of the phospho-PKC α and βII (Thr638/641) antibody (rabbit anti-human, 1 : 8000, Cell Signaling Technology) or β-actin antibody (rabbit anti-human, 1 : 4000, Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently washed six times with TBS and incubated 60 min with the secondary antibody conjugated to horseradish peroxidase (anti-rabbit from donkey, 1 : 10,000, Amersham, UK). The optical density of each PKC band was corrected by the optical density of the corresponding β-actin band.

2.8. Cell Culture. Murine peritoneal macrophage cell line RAW 264.7 (American Type Culture Collection, USA) was cultivated in Dulbecco's Modified Eagle Medium (PAN, Germany) supplemented with 10% foetal bovine serum (PAN, Germany) and 1% gentamycin (Sigma, USA). Cells were maintained at 37°C and 5% CO_2. After reaching confluence, cells were harvested and washed. Cell numbers and viability were determined by ATP test [24].

2.9. Formation of Nitric Oxide. Generation of reactive nitrogen species was determined indirectly as the accumulation of nitrites in the supernatant of murine macrophages RAW 264.7 [12]. Control cells were incubated with LPS without RES treatment. At the end of the incubation period, culture media were collected from wells and centrifuged at 5000 ×g and 4°C for 5 min. Then 150 μL of supernatant was mixed with equal volume of Griess reagent (Sigma, USA) in a 96-well plate, and the mixture was incubated at room temperature and in the dark for 30 min. The cell fractions of these samples were used for the detection of inducible NO synthase expression by Western blot.

2.10. Western Blot Analysis of iNOS Expression. After removing the supernatant for nitrite measurement, the remaining cells were washed with cold phosphate buffered saline (PBS) and lysed in the lysis buffer (1% sodium dodecyl sulphate—SDS, 10^{-1} M Tris pH 7.4, 10% glycerol, 10^{-3} M sodium orthovanadate, 10^{-3} M phenylmethanesulfonyl fluoride). Protein concentrations were determined by using BCATM protein assay (Pierce, USA), with bovine serum albumin as standard. Equal amounts of protein were then subjected to SDS-polyacrylamide gel electrophoresis using 7.5% running gel. The expression of iNOS protein was quantified by Western blot analysis [25]. Relative protein levels were quantified by scanning densitometry using the Image JTM programme, and the individual band density value was expressed in arbitrary units.

2.11. Antioxidant Activity Assays (Free Radical Scavenging Activity in Cell Free Systems)

2.11.1. ORAC Assay. The ORAC method measures the antioxidant scavenging activity against peroxyl radical induced by 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) at 37°C [26, 27]. Fluorescein (FL) was used as the fluorescent probe. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC). The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve.

2.11.2. HORAC Assay. HORAC measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical [26, 27]. The initial fluorescence was measured, after which the readings were taken every minute after shaking in the presence of 100, 200, 400, 500, and 600 μM gallic acid solutions (in phosphate buffer 75 mM, pH 7.4). The final HORAC values were calculated using a regression equation between gallic acid concentration and the net area under the curve.

2.12. ROS Scavenging in Luminol-Horseradish Peroxidase (HRP)-H_2O_2 Cell Free System. Aliquots of 50 μL of RES solutions, HRP (2 U/mL), and luminol (10 μM) were mixed in a 96-well luminescence plate to yield final concentrations of RES 1, 10, and 100 μM. The reaction was started by adding hydrogen peroxide at the final concentration of 100 μM (final volume of the sample was 200 μL). Chemiluminescence was measured for 10 minutes at 37°C with Luminometer Immunotech LM-01T (Beckman Coulter).

2.13. NO Scavenging Activity. The potential ability of extracts to scavenge NO in chemical systems was tested by electrochemical measurement of NO [28]. NO was measured using three electrode systems: a porphyrinic microsensor working electrode, counter electrode, and a reference electrode were connected to the ISO-NO MARK II potentiostat (WPI, USA) [28]. The injection of the 2 μL NO-saturated water into the measurement glass vial (final concentration of NO = 2.38 μM) caused a rapid increase with subsequent gradual decrease of the NO induced signal until it reached the background current [29]. The scavenging properties of the

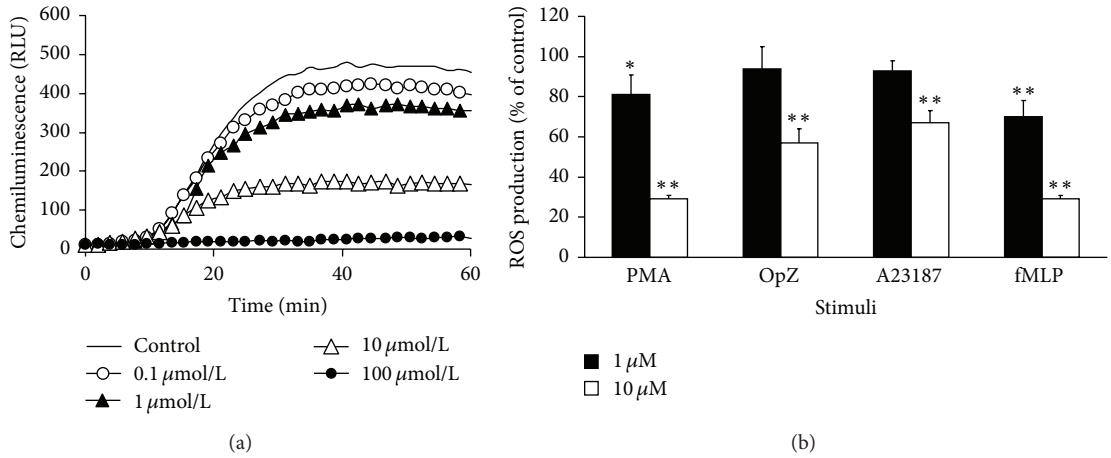

Figure 1: (a) Resveratrol dose-dependently decreased luminol-enhanced representative chemiluminescence curves of human whole blood stimulated with phorbol myristate acetate (PMA = 0.05 μM) at 37°C. (b) Effect of resveratrol in 1 and 10 μM concentration on PMA (0.05 μM), OpZ (0.5 mg/mL), fMLP (1 μM), and A23187 (1 μM) stimulated chemiluminescence. n = 6–8, Mean ± SEM, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

extracts tested were evaluated as the time needed for reaching again the background current.

2.14. Lipid Peroxidation. The amount of 0.9 mL of 0.5 mM α-linolenic acid (Sigma-Aldrich, Steinheim, Germany) was mixed with 0.1 mL sample. Then, a system generating hydroxyl radical (0.1 mL Co(II) and 0.1 mL hydrogen peroxide for details, see section HORAC) was added for the induction of lipid peroxidation and the mixture was incubated for 2 h in 37°C. The concentration of thiobarbituric acid-reactive substances (TBARS) was measured as the index of lipid peroxidation [30]. The absorbance of the upper layer was measured at 532 nm. 1,1,3,3-Tetraethoxypropane (Sigma-Aldrich, Steinheim, Germany) in the final concentration of 0.1 μM was used as standard. Lipid peroxidation was expressed in nM of TBARS per 1 mL of the mixture α-linolenic acid/analysed sample.

2.15. Statistical Analysis. Data represent the mean ± SEM, unless stated otherwise. Statistical analysis was performed using the ANOVA paired test to examine differences between the treatments and control. Differences were considered to be statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

3. Results

Figure 1(a) demonstrates representative dose-dependent CL curves of whole blood treated with RES and stimulated with PMA (0.05 μM). Figure 1(b) shows the effect of RES in 1 and 10 μM concentration on whole human blood CL stimulated with PMA (0.05 μM), OpZ (0.5 mg/mL), A23187 (1 μM), and fMLP (1 μM). In 1 μM concentration, RES significantly decreased CL for PMA and fMLP stimuli to 19 and 30 per cent of control value (=100%), respectively. RES in 10 μM concentration significantly inhibited CL with all stimuli applied in the rank order of potency PMA = fMLP > OPZ > A23187 demonstrating an evident difference in resveratrol activity to decrease stimulated chemiluminescence of whole blood.

Table 1: Effect of resveratrol in concentrations of 1 to 100 μM on viability of isolated neutrophils. Cells were incubated with resveratrol at 37°C for 10 min, stained with Annexin V, subsequently conjugated with FITC in the dark at 4°C for 10 min, followed by staining with propidium iodide (1 μg/mL), and then analysed immediately by the Beckman Coulter Cy. n = 4–6, mean ± SEM.

Resveratrol (μM)	Live cells	Apoptotic cells	Dead cells
0	91.90 ± 1.02	7.90 ± 1.00	0.20 ± 0.04
1	92.20 ± 1.31	7.60 ± 1.30	0.20 ± 0.05
10	92.10 ± 1.16	7.80 ± 1.60	0.10 ± 0.03
100	81.20 ± 2.73**	18.60 ± 2.70**	0.20 ± 0.03

$^{**}P \leq 0.01$.

The effect of RES on extra- and intracellular CL of isolated neutrophils stimulated with PMA is demonstrated in Figure 2. It is evident that RES dose-dependently decreased extracellular CL, significantly starting at 0.1 μM concentration. At 100 μM concentration (Figure 2(a)), there was complete inhibition of stimulated CL due to PMA. At intracellular level (Figure 2(b)), RES significantly decreased CL at 10 and 100 μM concentrations to 26 and 0.33 percent of the control value, respectively (Figure 2(c)).

Isolated intact neutrophils liberate 18 ± 3.8 nM ATP, which represents 3.2% from the total ATP amount (548 ± 112 nM/3 × 10^4 cells). RES in any concentration used did not liberate ATP from isolated neutrophils (results not shown), indicating that RES did not disintegrate isolated neutrophils in any concentration used.

Table 1 demonstrates the effect of RES in concentrations of 1 to 100 μM on viability of isolated neutrophils. In concentrations of 1 and 10 μM, RES did not change significantly the amount of dead cells as compared with control cells. In 100 μM, concentration RES increased the number of apoptotic cells from 7.9% (controls) to 18.6% of the total viable

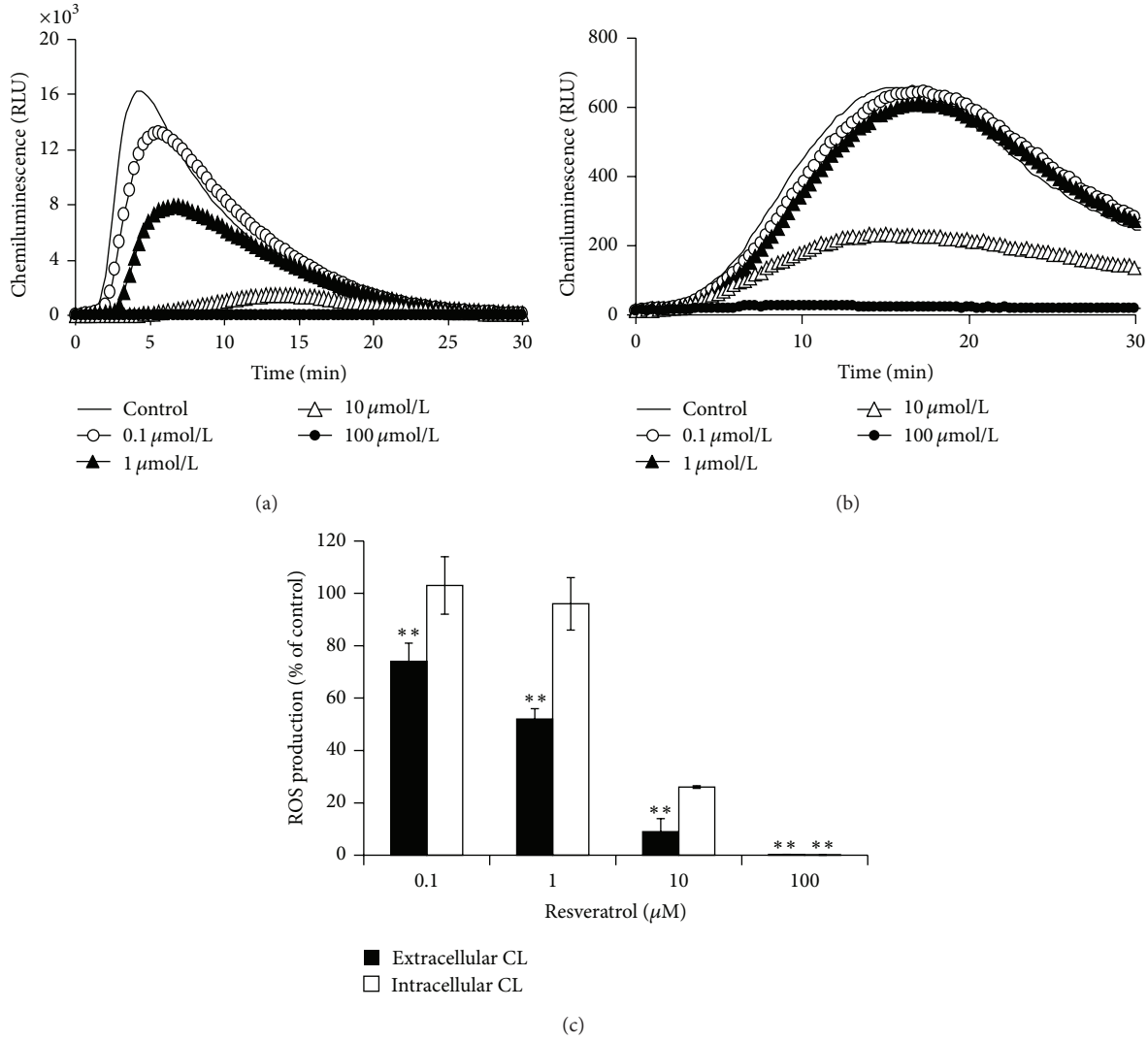

Figure 2: Chemiluminescence of isolated neutrophils. (a) Dose-dependent extracellular representative chemiluminescence (luminol + peroxidase) curves of human isolated neutrophils pretreated with resveratrol and stimulated with PMA (0.05 μM). (b) Intracellular dose-dependent representative chemiluminescence (isoluminol + catalase + superoxide dismutase) curves of isolated human neutrophils pretreated with resveratrol and stimulated with PMA (0.05 μM). (c) Dose-dependent effect of resveratrol on PMA (0.05 μM) stimulated extracellular and intracellular chemiluminescence. n = 6–8; mean ± SEM, $^{**}P \leq 0.01$.

cells. The number of dead cells did not increase. These results show that RES in the optimal concentrations used (1 and 10 μM) did not change apoptosis of isolated human neutrophils.

Figure 3 shows the result of RES on radical scavenging activity in cell free system. RES in concentrations 10 and 100 μM increased hydroxyl scavenging activity (HORAC) to 88 and 114 μM of gallic acid equivalents, respectively, and peroxyl scavenging activity (ORAC) to 34 and 29 of Trolox equivalents, respectively. This effect demonstrates effective scavenging activity of RES on hydroxyl and peroxyl radicals *in vitro*.

Figure 4 shows the inhibitory effects of different concentrations (1, 10, and 100 μM) of RES on production of ROS in cell free system generated by means of luminol + hydrogen peroxide + HRP. RES in 1 μmol/L concentration significantly ($P < 0.01$) inhibited and in 10 μM concentration totally blocked the chemiluminescence of 100 μM hydrogen peroxide in the samples.

Since lipids are very susceptible to lipid peroxidation, we tested also the ability of RES to prevent the peroxidation of polyunsaturated fatty acids induced by hydroxyl radical. It is evident from Figure 5 that RES in all tested concentrations significantly and dose-dependently inhibited lipid peroxidation.

The activation of protein kinase C in isolated neutrophils stimulated with PMA (0.15 μM) in the presence of 10 and 100 μM RES is demonstrated in Figure 6. In both concentrations applied, RES reversed PMA stimulated PKC activation

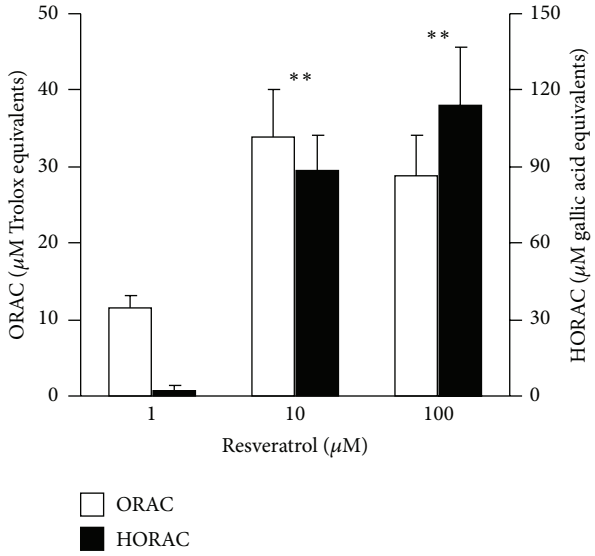

Figure 3: Effect of resveratrol on hydroxyl (HORAC) and peroxyl (ORAC) radical scavenging activity in cell free system expressed as gallic acid and Trolox equivalents, $n = 4$, mean ± SEM, $^{**}P \geq 0.01$.

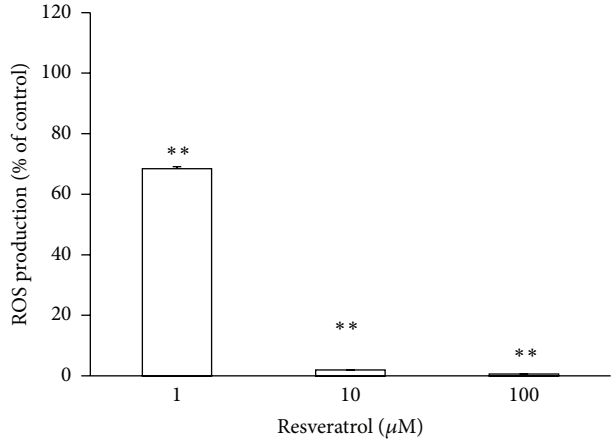

Figure 4: Dose-dependent effect of resveratrol on reactive oxygen species generated in cell free system by means of luminol + hydrogen peroxide + horseradish peroxidase. $n = 3$, mean ± SEM, $^{**}P \geq 0.01$.

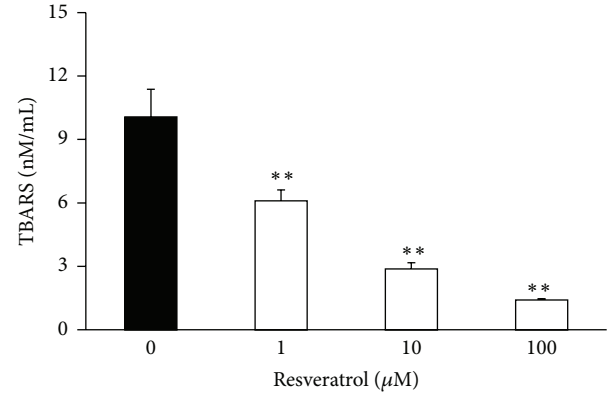

Figure 5: Effect of resveratrol on lipid peroxidation of α-linolenic acid expressed as thiobarbituric acid-reactive substances (TBARS), induced by hydroxyl radicals. $n = 6$, mean ± SEM, $^{**}P \geq 0.01$.

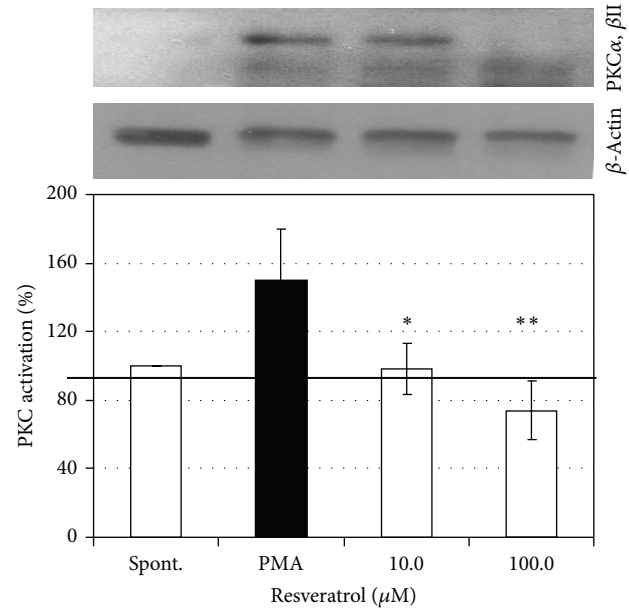

Figure 6: Western blotting analysis of protein kinase C activation in isolated human neutrophils pretreated with resveratrol (10 and 100 μM) and stimulated with PMA (0.15 μM). $n = 3$-4, mean ± SEM; $^{*}P \leq 0.05$.

to spontaneous (control) values indicating a suppressive effect of RES on the activity of protein kinase C, one of the essential regulatory enzymes in reactive oxygen generation.

The effect of RES on nitrite production and iNOS expression in RAW 264.7 cell culture after LPS activation is demonstrated in Figure 7. Figure 7(a) demonstrates that RES in concentrations of 1, 10, and 100 M decreased nitrite concentration in cell supernatants to 82, 65, and 6 percent of the control value, respectively.

Figure 7(b) shows the effect of RES on iNOS expression determined by Western blot analysis. In comparison with the iNOS protein level in the control sample, iNOS protein expression was significantly inhibited only by the highest concentration (100 μM) of RES to 60% of the control value.

4. Discussion

Resveratrol dose-dependently inhibited oxidative burst in human whole blood stimulated with two membrane-bypassing (PMA, A23187) and two membrane-operating stimuli (OpZ, fMLP). There was no significant difference between the stimuli applied and chemiluminescence decrease of whole blood indicating that RES may not act only as an extracellular scavenger but suppresses oxidative burst also intracellularly. This suggestion was confirmed on isolated neutrophils (Figure 2) stimulated with PMA demonstrating that RES in a concentration dependent way inhibited not only extracellularly determined chemiluminescence but effectively suppressed formation of intracellularly generated

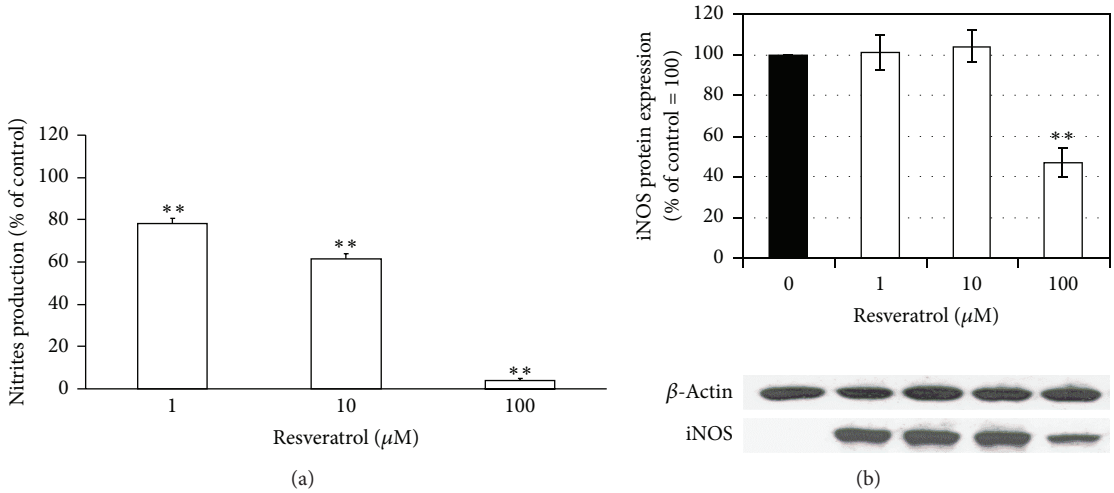

Figure 7: Nitrite production and iNOS expression in RAW 264.7 culture cells. Nitrite production (a) and densitometric analysis together with representative Western blot of iNOS protein expression (b) in LPS stimulated RAW 264.7 cells treated with resveratrol. $n = 3$, mean ± SEM, $^{**}P < 0.01$.

ROS (Figure 2(b)). The difference was evident; RES started to inhibit extracellular chemiluminescence at 0.1 μM, intracellularly at 10 μM concentration (Figure 2(c)).

The stimulated generation of ROS in whole blood and isolated neutrophils was decreased by many polyphenolic compounds like curcumin, pterostilbene, pinosylvin, and N-feruloyl serotonin [11, 14, 31]. Inhibition of fMLP-activated human neutrophil chemiluminescence was accompanied by inhibition of elastase and β-glucuronidase secretion and production of 5-lipoxygenase metabolites leukotriene B4, 6-trans-LTB4, and 12-trans-epi-LTB4 after stimulation with calcium ionophore, indicating that transresveratrol interferes with the release of inflammatory mediators in activated polymorphonuclear leukocytes [32].

Since RES did not liberate ATP from isolated neutrophils, it is evident that even in higher concentrations used (up to 100 μM) there was no disintegration of cells. Moreover, RES dose-dependently decreased spontaneous ATP liberation.

RES did not change significantly the number of dead cells even in the highest concentration used, decreasing the number of live cells by 10.7% (Table 1). In contrast, RES decreased the activity of recombinant caspase-3 activity in cell free system, significantly at 10 μM concentration. This result has to be verified in the cellular model since apoptosis, a programmed cell death, appears to be the most frequent fate of cells treated with RES [33]. Moreover, RES induced autophagy in human U251 glioma cells [34], decreased the intracellular reactive oxygen species level, which correlated with the induction of caspase-8 and caspase-3 cleavage in human colon cancer cells [35] and induced apoptosis in patients with chronic myeloid leukemia cells [36].

The antioxidant properties of RES were analysed via five different methods: ORAC(peroxyl), HORAC(hydroxyl), hydrogen peroxide-peroxidase dependent chemiluminescence, NO scavenging, and lipid peroxidation inhibition. The chosen methods embrace different aspects of the antioxidant action and give a comprehensive view on the antioxidant potential of the sample investigated.

In the following experiments, we tested also the scavenging properties of RES against NO, using electrochemical analysis which is considered to be a reliable method for verifying NO scavenging. However, no scavenging properties of resveratrol against NO were found in any concentration used.

These observations confirmed previous findings that RES is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidant enzymes [37]. The result of decreasing dose-dependently LDL oxidation in cell free system is supportive of its effect on lipid peroxidation and atherosclerotic lesion formation in animal hypercholesterolemic models [38].

RES decreased protein kinase C activation in PMA-stimulated neutrophils, indicating its interference with oxidative burst in neutrophils. Similar results were demonstrated in human gastric adenocarcinoma and CaSki cells [39, 40] and for the polyphenolic compound N-feruloyl serotonin in human neutrophils [14]. By inhibiting the activation of PKC [41], RES may interfere with modulation of intracellular signalling pathways involved in downregulation of COX-2 and iNOS expression and NF-κB activation [10, 42]. Nitric oxide, a member of reactive nitrogen species, is an important molecule involved in the regulation of many physiological and microbicidal processes. RES markedly inhibited NO production by LPS stimulated macrophages. This finding corresponds with the latest results of other authors [43, 44] who also reported suppression of inducible nitric oxide synthase expression and NO production in macrophages after RES administration. Our results showed that resveratrol reduced nitrite accumulation more effectively than it reduced iNOS protein expression in stimulated macrophages through a mechanism which is at least partially independent of the regulation of iNOS protein expression. Nevertheless, in

electrochemical measurements we showed that RES was not able to scavenge NO, suggesting that the direct scavenging activity against NO resulting from the inhibitory action of RES can be excluded.

In conclusion, RES possesses antioxidant activities *in vitro* inhibiting generation of ROS in cell free systems, oxidative burst of stimulated blood and isolated neutrophils both at extracellular and intracellular level, as measured by chemiluminescence.

Oxidative burst inhibition of human blood and isolated neutrophils, suppression of free radical generation and NO formation in cell free system confirmed the antioxidative properties and supported the effort to enlarge clinical studies with RES.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the paper.

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Black chokeberry (*Aronia melanocarpa*) polyphenols reveal different antioxidant, antimicrobial and neutrophil-modulating activities

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ABSTRACT

The current study reports data on antioxidant, antimicrobial and neutrophil-modulating activities of different polyphenolic preparations from black chokeberry fruits: crude extract, purified extract standardized to 20% and 40% anthocyanins, and proanthocyanidins; as well as pure compounds (chlorogenic acid, cyanidin-3-*O*-galactoside, epicatechin, rutin and quercetin) present in black chokeberries. Minor phenolic components - quercetin and epicatechin showed the highest ORAC and TRAP antioxidant activity. Given the amount of individual phenolics in the fruits, proanthocyanidins are the major contributor to antioxidant activity of fresh black chokeberries. Studied polyphenols and preparations had no effect on the spontaneous chemiluminescence (CL) of human neutrophils and only mild effect on PMA-activated CL. Greater effects were observed on OZP-activated CL, being statistically significant ($p < 0.05$) for quercetin and rutin. The antimicrobial activity test against 10 pathogens showed that black chokeberry proanthocyanidins are the most potent antimicrobial agents in the fruit.

1. Introduction

In recent years, considerable attention has been devoted to medicinal plants rich in polyphenols, with antioxidant and antimicrobial properties. With over 8000 known representatives in the plant kingdom, polyphenols are the most abundant dietary antioxidants (Ross & Kasum, 2002). Natural polyphenols differ significantly in their structure and vary from single molecules, such as phenolic acids to polymers, such as tannins (Harborne & Simmonds, 1964). Phenolic compounds are known to possess various pharmacological activities and there is growing interest in polyphenol substances that are given to humans through food, functional foods, nutraceuticals or even medicines.

Black chokeberry (*Aronia melanocarpa*) fruits are particularly rich in polyphenol compounds and are among the fruits with the highest antioxidant activities. Their antioxidant action embraces radical scavenging, suppression of the formation of reactive oxygen and nitrogen species, restoration of antioxidant and inhibition of prooxidant enzymes (Denev, Kratchanov, Číž, Lojek, & Kratchanova, 2012). In the last years, there is an increasing research interest in black chokeberries, their chemical composition (Denev et al., 2012; Kulling & Rawel, 2008), health benefits (Denev et al., 2012; Valcheva-Kuzmanova & Belcheva, 2006) and clinical effectiveness (Chrubasik, Li, & Chrubasik, 2010). Black chokeberry fruits (BCF) are very rich source of anthocyanins, proanthocyanidins and hydroxycinnamic acids. Besides, quercetin, quercetin glycosides and epicatechin are present in the fruits as minor components (Denev et al., 2012; Kulling & Rawel, 2008). There is strong scientific evidence for black chokeberry health benefits. Antidiabetic, antimutagenic, cardioprotective, hepatoprotective and anticarcinogenic effects of the fruits and their extracts have been reported (Denev et al., 2012). Many of the studies were performed with complex extracts, either crude or partly purified and it is not clear which particular polyphenol components are responsible for the observed biological effects. In our previous study, we observed that crude black chokeberry extract possesses high antioxidant activity, measured via different assays and inhibited reactive oxygen species (ROS) production of opsonized zymosan-activated phagocytes (Denev et al., 2014). The extract showed strong antimicrobial properties against broad spectrum of microorganisms and had high mitogenic activity on hamster spleen lymphocytes. Based on these results, we hypothesized that black

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chokeberry polyphenols (BCP) differ in their biological activity and decided to perform detailed study on their antioxidant, antimicrobial and neutrophil-modulating properties. Therefore, the aim of our study was to determine the antioxidant properties of black chokeberry polyphenolic preparations and pure phenolic compounds, and their effect on spontaneous, opsonized zymosan particles (OZP)- and phorbol-myristate-acetate (PMA)-activated human neutrophils. The antimicrobial activity against 10 pathogens was investigated as well. The results of the study shed light on the activity of different classes of polyphenols in black chokeberry and could be used in activity-guided development of functional foods and nutraceuticals with antioxidant, antimicrobial and immuno-modulating properties.

2. Material and methods

2.1. Chemicals

Trolox, fluorescein disodium salt, AAPH, gallic acid, chlorogenic acid, 3,4-dihydroxy benzoic acid, p-coumaric acid, caffeic acid, ferulic acid, ellagic acid, naringin, myrecetin, quercetin, quercetin-3-glucoside, rutin, naringenin, kaempherol, catechin, epicatechin, Amberlite XAD7 and Sephadeh LH20 were obtained from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's reagent was delivered from Merck (Darmstadt, Germany). Cyanidin-3-*O*-galactoside (Cy-3-gal) chloride was purchased from Extrasynthese S.A. (Genay Cedex, France). All other solvents used were of analytical grade and purchased from local distributors.

2.2. Plant materials

Black chokeberry fruits were supplied from local growers in the stage of full maturity, in august 2016. After collection, fresh fruits were put in polyethylene bags, frozen immediately and stored at −18 °C until their analysis. For analytical purposes 200 g berries were freeze dried in Alpha 1–4 LDplus laboratory freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) and extracted according to (Denev et al., 2014). Extract was kept at −18 °C prior to analysis and used for determination of total polyphenol, anthocyanin and proanthocyanidin contents, as well as individual phenolic compounds via HPLC.

2.3. Extraction, purification and isolation of black chokeberry preparations

The scheme for extraction and purification of black chokeberry preparations is depicted on Fig. 1, and different steps are explained bellow.

2.3.1. Extraction

100 g frozen fruits were defrosted at room temperature, mixed with 300 ml ultra-pure water and homogenized in a laboratory blender. Homogenized sample was transferred into extraction tube and subjected to extraction in thermostatic shaker water bath (NUVE, Turkey) for one hour at 60 °C. After that, mixture was centrifuged (20 min, 6200 × *g*) and the obtained supernatant was used for purification and isolation of black chokeberry polyphenol preparations. 50 ml of the extract were freeze dried in Alpha 1–4 LDplus laboratory freeze drier and the dry extract was denoted as Crude Extract (CE). Extraction procedure was repeated two times.

2.3.2. Column chromatography with Amberlite XAD7

Column chromatography was performed on SUPELCO VISIPER system, connected to a vacuum pump (KNF lab) with 50 cm^3 glass cartridges. Amberlite XAD7 was soaked in ultra-pure water and then loaded into the column. After that, the column was rinsed with ultra-pure water and crude extract (50 ml portion) was applied. The impurities were eluted with ultra-pure water and 10 ml fractions were collected until no soluble dry solids were detected. After that, black chokeberry polyphenols were eluted with 200 ml 96% ethanol. Column was washed with 100 ml water and another 50 ml portion of the extract was applied and processed in the same way. The procedure was repeated 7 times in order to purify the whole amount of crude extract. All polyphenol-containing ethanol fractions were combined, concentrated via rotary evaporation at 50 °C and volume was adjusted to 100 ml with ultra-pure water. After that, 20 ml of the concentrated extract containing partially purified polyphenols were freeze dried to obtain powdered dry product denoted as Purified Extract containing 20% anthocyanins (PE20). PE20 was kept at −18 °C until its analysis. The SPE procedure was repeated two times with both extracts obtained in Section 2.3.1.

2.3.3. Column chromatography with Sephadex LH 20

Proanthocyanidins were further purified from the concentrated extract containing partially purified polyphenols (described in Section 2.3.2) by the modified procedure of Howell et al. (2005). Briefly, 20 ml of the concentrated extract were applied to 50 cm^3 cartridge filled with 5 g of Sephadex LH-20 that was equilibrated overnight in ethanol:distilled water (50:50) (v/v). After that, column was washed with 80 ml ethanol:distilled water (50:50) (v/v) in order to remove low-molecular polyphenols. Proanthocyanidin fraction was eluted with 80 ml 80% acetone in distilled water (80:20) (v/v). The same procedure was repeated four times in order to process the whole volume (80 ml) of the partially purified polyphenol extract (see Section 2.3.2.). All ethanol:distilled water (50:50) fractions were collected and combined and ethanol was evaporated via rotary evaporation at 50 °C. Obtained water extract was freeze dried in Alpha 1–4 LDplus laboratory freeze drier and dry extract was denoted as Purified Extract containing 40% anthocyanins (PE40). Aqueous acetone fractions were combined and acetone evaporated via rotary evaporation at 40 °C. Obtained water extract was freeze dried in Alpha 1–4 LDplus laboratory freeze drier and dry extract was denoted as Proanthocyanidins (PACNs) fraction.

2.4. HPLC analysis of phenolic compounds

High Performance Liquid Chromatography (HPLC) analysis of phenolic components was performed according to Denev et al. (2014), on Agilent 1220 (Agilent Technology, USA), HPLC system with binary pump and UV–Vis detector (Agilent Technology, USA). Wavelength of 280 nm was used. Phenolics separation was performed using Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25 °C. The results were expressed in mg per 1 g preparation or fresh weight.

2.5. Total anthocyanins determination

Total anthocyanins content was determined by the pH-differential method according to Lee, Durst, and Wrolstad (2005). The detailed procedure is described in Denev et al. (2010). The results were expressed in mg of cyanidin-3-*O*-glucoside equivalents per 1 g preparation or fresh weight.

2.6. Total proanthocyanidin content analysis

Total proanthocyanidins were determined according to Sarneckis et al. (2006). Proanthocyanidin content was calculated from a calibration curve with catechin solutions and expressed in mg of catechin

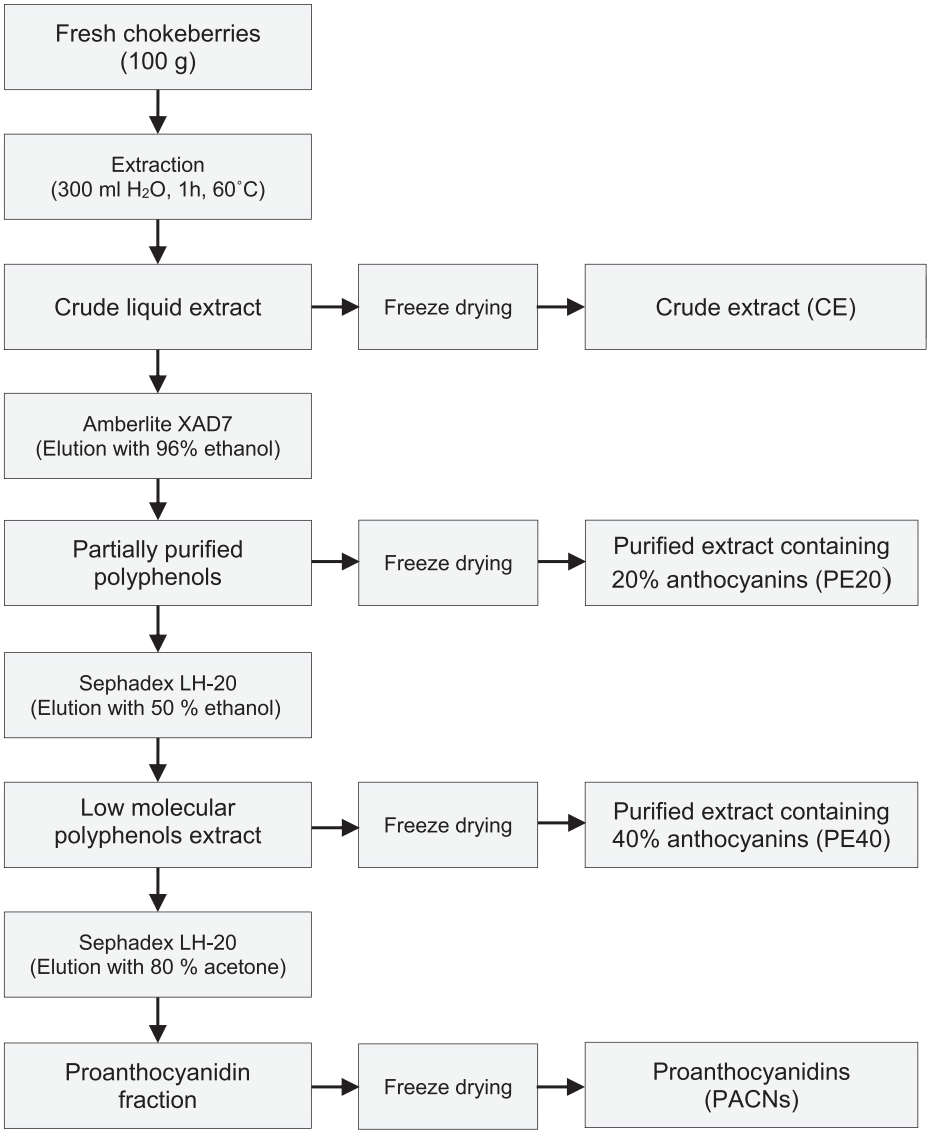

Fig. 1. Scheme for extraction and purification of aronia preparations.

equivalents per 1 g preparation or fresh weight.

2.7. Oxygen Radical Absorbance Capacity (ORAC) assay

Oxygen Radical Absorbance Capacity was measured according to the method of Ou, Hampsch-Woodill, and Prior (2001) with some modifications described by Denev et al. (2010). ORAC analysis was carried out using a plate reader FLUOstar OPTIMA (BMG Labtech, Germany), excitation wavelength of 485 nm and emission wavelength of 520 nm were used. ORAC values were expressed in micromole trolox equivalents (μmol TE) per 1 g preparation or pure compound.

2.8. Total peroxyl radical trapping parameter (TRAP) assay

TRAP method was performed according to (Čížová, Lojek, Kubala, & Číž, 2004). TRAP values are determined from the duration of the time period (T_{sample}) during which the sample quenched the CL signal due to the present antioxidants. TRAP analysis was performed on Luminometer Orion II (Berthold Dection System GmbH, Germany) and activity of the sample was expressed in micromole trolox equivalents (μmol TE) per 1 g preparation or pure compound.

2.9. Oxidative burst

The oxidative burst of phagocytes in whole blood was measured chemiluminometrically on LM-01 microplate luminometer (Immunotech, Czech Republic). The principle of the method was described previously (Číž et al., 2007) and the detailed procedure was performed according to Denev et al. (2014).

2.10. Agar diffusion method

Determination was performed according to Denev et al. (2014). Briefly, saline solution was used to prepare a suspension of each test

microorganism (concentration ~1.10^7 cfu/ml). 1 ml of each microbial suspension was used to inoculate melted PCA (Scharlau) nutrient medium and 15 cm^3 were poured in Petri dishes. 7 mm in diameter wells were made in the solidified agar medium (after cooling) and 50 µl of the samples were pipetted in the wells. Petri dishes were incubated (37 °C, 24–48 h) and inhibition zones were measured. Zones with diameter more than 7 mm were considered as zones of inhibition.

2.11. Minimal inhibiting concentration (MIC) determination

Minimal inhibiting concentrations of the extracts were analyzed as a criterion for their antimicrobial effects, using 96 well microtiter plate method (broth dilution), according to the modified procedure of Gutierrez, Barry-Ryan, and Bourke (2008) and details are described by Denev et al. (2014). MIC was the lowest concentration of the extract or compound that reduced the growth of the tested microorganism to 50% of the positive control.

2.12. Statistical analysis

The processing was repeated two times and the analysis performed at least in triplicate. Results were expressed as mean values ± standard deviations. In the case of chemical analysis and antioxidant activity, one-way analysis of variance (ANOVA) and Student's *t*-test were used to evaluate the differences of the mean between groups. In the case of effects of black chokeberry polyphenols and preparations on the production of reactive oxygen species, the data were analyzed by F-test to test the equality of two variances, followed by Student's *t*-test to evaluate the differences of the mean between individual samples and control. P values less than 0.05 were considered to be significant. Microsoft Excel, 2013 (Microsoft Corporation, Redmond, USA) was used in the analyses.

3. Results and discussion

3.1. Purification and chemical composition of black chokeberry preparations

For the extraction of anthocyanins and other phenolic compounds, different extraction systems have been used (Nicoue, Savard, & Belkacemi, 2007). Organic solvents (ethanol, methanol and acetone) or their water solutions are usually used for extracting polyphenols. Acetone and methanol are effective extragents, but their toxicity limits their application in food processing. Ethanol is suitable for food application but is more difficult to eliminate in the purification process. Since the goal of our project is development of black chokeberry functional foods we choose water as extragent for BCP. Common extraction procedures including the one we used are non-selective and yield solutions with impurities, such as sugars, sugar alcohols, organic acids, amino acids, proteins, etc. It is known that besides polyphenols, black chokeberries and crude extracts are rich source of other substances such as sugars (sorbitol, fructose and glucose) and organic acids (Denev et al., 2018; Kulling & Rawel, 2008). In the current study, we applied several purification steps in order to obtain different black chokeberry preparations: CE, PE20, PE40 and PACNs. Results for the content of total polyphenols, anthocyanins and individual phenolic compounds of these preparations are shown in Table 1.

As it is seen from Table 1, all preparations including the crude extract contain several classes of phenolic compounds: hydroxycinnamic acids (neochlorogenic acid and chlorogenic acid); flavan-3-ols (epicatechin); flavonols (quercetin, rutin and quercetin-3-glucoside) and anthocyanins, which is in agreement with the literature information (Denev et al., 2012; Kulling & Rawel, 2008). The amount of polyphenolic compounds in the crude extract is only 5.35% of which 1.48% are anthocyanins. Crude extract was quite hygroscopic because of the high content of monosaccharides and especially sorbitol, and required further purification. Solid phase extraction using the absorbent Amberlite XAD7 is a fast, simple and reliable method for purification of anthocyanins and other phenolics from complex extracts, containing impurities such as sugars, acids, proteins, pectin etc. (Kraemer-Schafhalter, Fuchs, & Pfannhauser, 1998). In our previous study, we enriched different crude berry extracts with anthocyanins and other phenolics, which led to a significant increase in their antioxidant properties (Denev et al., 2010). As it is evident from Table 1, application of Amberlite XAD7 increased the total polyphenol content of the crude extract more than 12-fold and the amount of anthocyanins reached more than 200 mg/g extract. Sephadex LH 20 resin is broadly used for purification and isolation of proanthocyanidins and other biologically active compounds (steroids, terpenoids, lipids, low molecular weight peptides, etc.) form plant matter. However, in our study we used Sephadex LH 20 for simultaneous isolation of proanthocyanidins and for further purification and enrichment of black chokeberry low-molecular polyphenols. As a result, after elution of the resin with 50% ethanol and further freeze-drying of the extract, we obtained dry black chokeberry extract containing more than 40% anthocyanins, while the amount of total polyphenols reached 84%. In the same time, this allowed us to isolate black chokeberry proanthocyanidins. The purification scheme adopted by us could be easily produced in higher scale, using simple two-step column purification, first with Amberlite XAD 7 followed by Sephadex LH 20.

3.2. Antioxidant activity of black chokeberry polyphenols and polyphenolic preparations

After the characterization of black chokeberry preparations by means of their polyphenol content and composition (Section 3.1), we

Table 1
Content of total polyphenols, anthocyanins and individual phenolic compounds in black chokeberry preparations.

	CE	PE20	PE40	PACNs
Neochlorogenic acid	10.2^{c} ± 2.1	130.4^{b} ± 11.2	189.5 ^{a} ± 28.2	12.2^{c} ± 3.1
Chlorogenic acid	8.3^{c} ± 0.9	121.1^{b} ± 8.1	177.1 ^{a} ± 15.4	11.9^{c} ± 1.2
Epicatechin	2.3 ^{d} ± 0.3	29.3^{b} ± 4.2	59.3 ^{a} ± 7.5	15.1^{c} ± 0.9
Rutin	7.8^{c} ± 0.5	45.5 ^{a} ± 5.6	35.3 ^{ab} ± 14.2	24.2^{b} ± 0.6
Quercetin-3-glucoside	1.9^{b} ± 0.1	23.4 ^{a} ± 2.1	20.1 ^{a} ± 3.5	18.2 ^{a} ± 0.4
Quercetin	2.1^{b} ± 0.2	2.0 ^{bc} ± 0.9	1.4^{c} ± 0.1	5.7 ^{a} ± 0.3
Anthocyanins	14.8^{c} ± 0.9	213.5^{b} ± 20.2	400.2 ^{a} ± 37.0	20.1^{c} ± 5.4
Total polyphenols	53.5^{c} ± 4.2	687.2^{b} ± 35.6	840.5 ^{a} ± 62.1	910.2 ^{a} ± 46.3

Results are expressed as mean ± SD in mg/g preparation. There are no significant differences among values marked with the same superscript letters in individual rows ($p < 0.05$).

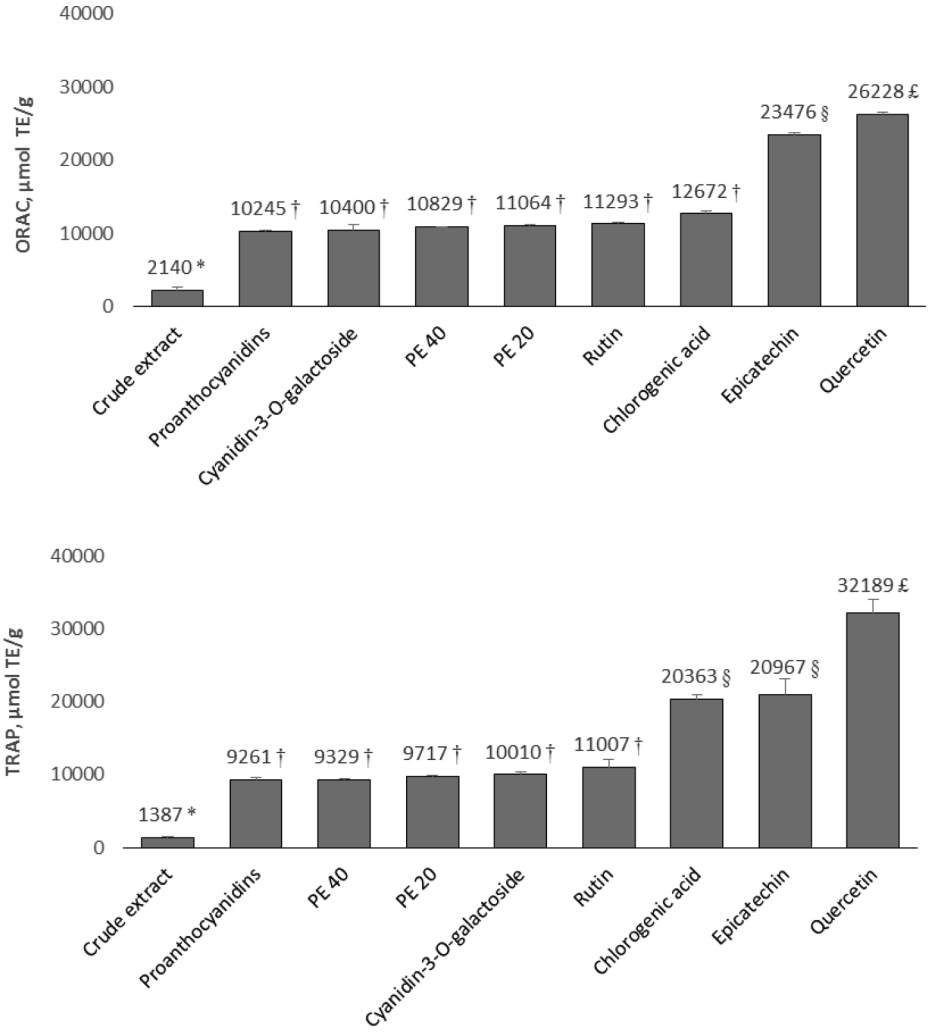

Fig. 2. Antioxidant activity of black chokeberry polyphenols and preparations measured by ORAC and TRAP assays. There are no significant differences among values marked with the same symbols ($p < 0.05$).

continued with determination of their antioxidant properties. In order to better assess the antioxidant activity of black chokeberry polyphenols, we additionally analyzed pure compounds found naturally in these fruits. They belong to different classes of polyphenols and include: cyanidin-3-*O*-galactoside (anthocyanin); chlorogenic acid (hydroxycinnamic acid), epicatechin (flavan-3-ol), quercetin (flavonol aglycone) and rutin (flavonol glycoside). To obtain more detailed information about the antioxidant activity of black chokeberry polyphenols and preparations, we used two complementary assays – ORAC and TRAP. Both assays rely on hydrogen atom transfer and express the ability of the sample to scavenge peroxyl radicals, which are physiologically the most relevant. Moreover, these methods are performed at physiological temperature and pH. Results are presented on Fig. 2.

ORAC and TRAP results of studied compounds and preparations were similar, giving the following rank order via ORAC: quercetin > epicatechin > chlorogenic acid > rutin > PE20 > PE40 > cyanidin-3-*O*-galactoside > proanthocyanidins > crude extract and via TRAP: quercetin > epicatechin > chlorogenic acid > rutin > cyanidin-3-*O*-galactoside > PE20 > PE40 > proanthocyanidins > crude extract. Our literature review revealed that there is only one study on the antioxidant activity of black chokeberry polyphenols (Zheng & Wang, 2003). Authors of the study reported that among them, quercetin glycosides possess the highest ORAC values (13400–15800 µmol TE/g) without providing information about the activity of quercetin and rutin. Our result for rutin – 11293 µmol TE/g is similar to those of other quercetin glycosides, but quercetin itself revealed the highest antioxidant activity – 26228 µmol TE/g. Our ORAC value for cyanidin-3-*O*-galactoside, which is the predominant anthocyanin in black chokeberries is 10400 µmol TE/g which is slightly lower than the result of (Zheng & Wang, 2003) – 11500 µmol TE/g. The biggest discrepancy was obtained for the ORAC of chlorogenic acid – 12672 µmol TE/g in our study, compared to 7400 µmol TE/g in the abovementioned work. TRAP values for pure polyphenols and black chokeberry phenolic preparations further enrich the available data about the antioxidant activity of BCP. As it could be expected, crude polyphenol extract had the lowest activity via both methods. From the purified black chokeberry fractions, proanthocyanidins revealed the lowest TRAP – 9261 µmol TE/g and ORAC activity – 10245 µmol TE/g, despite the fact that they were purified to more than 90% polyphenols. Interestingly, degree of purification of anthocyanins did not affect their antioxidant activity and there was no

Table 2
Content of different phenolics in fresh black chokeberries and their contribution to antioxidant activities of the fruit.

	Content of phenolic components in fresh berries, mg/g	Contribution to ORAC of fresh berries, μmol TE/g	Contribution to TRAP of fresh berries, μmol TE/g	% from total ORAC	% from total TRAP
Anthocyanins *	3.22 ± 0.17	33.5 ± 2.3	32.3 ± 1.2	24.1	22.1
Proanthocyanidins	5.22 ± 0.65	53.5 ± 1.0	48.3 ± 2.4	38.5	33.0
Neochlorogenic acid **	0.91 ± 0.05	11.5 ± 0.3	18.5 ± 0.6	8.4	12.8
Chlorogenic acid	1.02 ± 0.01	12.9 ± 0.3	20.8 ± 0.6	9.3	14.2
Epicatechin	0.65 ± 0.05	15.3 ± 0.1	13.6 ± 1.4	11.0	9.3
Rutin	0.5 ± 0.02	5.6 ± 0.1	5.5 ± 0.5	4.1	3.8
Quercetin-3-glucoside ***	0.27 ± 0.02	3.0 ± 0.1	3.0 ± 0.3	2.2	2.0
Quercetin	0.13 ± 0.03	3.4 ± 0.1	4.2 ± 0.2	2.4	2.8
Total		138.9	146.3	100.0	100.0

* Calculated as cyanidin-3-*O*-galactoside.
** Calculated as chlorogenic acid.
*** Calculated as rutin.

significant difference ($p < 0.05$) between antioxidant activity of PE20, PE40 and pure cyanidin-3-*O*-galactoside. Antioxidant properties of phenolic compounds are influenced by their structural characteristics, such as number and arrangement of OH groups. In the case of flavonoids, the presence of OH group at the 3th position, ortho-dihydroxy substitution in the B ring and a 2,3 double bond conjugated with the 4-oxo function of the C ring are another important structural features for strong antioxidant activity (Bors, Heller, Michel, & Saran, 1990; Rice-Evans, Miller, & Paganga, 1996; Salah et al., 1995). Among black chokeberry polyphenols, quercetin possesses all structural characteristics, necessary for potent antioxidant activity and its high antioxidant activity is proved by our results. Zheng and Wang (2003) estimated that anthocyanins contribute more than 52% to ORAC antioxidant activity of black chokeberries, whereas flavonols and caffeic acid (including its derivative), add additional 8.7% and 38.2%, respectively. In order to calculate the contribution of individual phenolic compounds to ORAC and TRAP activity of black chokeberry fruits, we analyzed the content of anthocyanins and individual polyphenol compounds in fresh berries. Results are presented in Table 2.

As it is evident from the table, proanthocyanidins are the major antioxidant substances in BCF contributing to more than 38% of ORAC value and 33% of TRAP value. This is an experimental evidence for our previous assumption that about 40% of the *in vitro* antioxidant activity of black chokeberry fruits is due to the potency of proanthocyanidins (Denev et al., 2012). The second biggest contributor to antioxidant activity of the berries are anthocyanins, followed by hydroxycinnamic acids and epicatechin. Flavonols are characterized with very high antioxidant activity, but due to their low quantity in fresh BCF, they contribute to their antioxidant activity less than 10%. Plant extracts are complex mixtures of many antioxidants acting simultaneously. Thus, antioxidants could have additive effect in their action, but synergism and antagonism are possible, as well. However, both TRAP and ORAC assays could not account these effects, since they measure the common antioxidant action of all antioxidants in the sample. Furthermore, plant metabolites with low (i.e. ascorbic acid) or no (i.e. sugars, polysaccharides, etc.) antioxidant activity could also interfere with the results. Therefore, further studies with pure antioxidants model systems could be very helpful in order to investigate the possible synergistic or antagonistic effects in their action.

It should be noted that results presented in Table 2 are valid for the particular batch of fruits used in the current study. Fresh black chokeberries differ significantly in their content of individual polyphenols. For example, their proanthocyanidin content vary in a broad range (522–1002 mg/100 g fresh weight), but in all cases they are the most abundant phenolic component in fresh black chokeberries (Denev et al., 2018). Despite their high contribution to antioxidant activity of black chokeberries, proanthocyanidins are barely absorbed in the gastrointestinal tract (GIT), and the majority pass through and reach the colon (Denev et al., 2012). Nevertheless, many of proanthocyanidins metabolites in the colon are of phenolic nature, possess free –OH groups and reveal antioxidant activity, as well. This could be very important, since large numbers of phagocytic cells normally reside in the GIT. Thus, BCP and in particular proanthocyanidins, and their metabolites could directly modulate their function *in vivo*.

3.3. Effect of black chokeberry polyphenols and polyphenolic preparations on the production of ROS from phagocytes

Knowledge of the immunomodulating effects of BCP is scarce. There are just few studies on the effect of black chokeberry crude extracts or isolated fractions mainly on murine macrophages. For example, Ohgami et al. (2005) observed that crude polyphenolic extract from black chokeberries reveal anti-inflammatory effect and suppress LPS-induced iNOS and COX-2 protein expressions in RAW 264.7 cells *in vitro*. In a more recent study, Ho et al. (2014) revealed that cyanidin, procyanidin B2, B5 and C1 and proanthocyanidin-rich fractions from black chokeberry were highly active in the complement-fixing assay. In addition, the oligomeric procyanidins displayed inhibitory effects on LPS-induced NO production in murine RAW 264.7 macrophages. As far as we know, no studies exist on the immunomodulating effects of black chokeberry polyphenolic extracts on human neutrophils, except our earlier report for crude extract (Denev et al., 2014). We determined the neutrophil-modulating activity of black chokeberry polyphenolic preparations and pure phenolic compounds present in the fruits, as their effect on spontaneous, OZP- and PMA-activated production of ROS by human neutrophils. The oxidative burst of neutrophils in whole blood was measured chemiluminometrically. The results are expressed as % of control of the integrals of CL curves (the total production of ROS during 60 min), (Fig. 3). Any of the studied compounds and preparations had no effects on the spontaneous chemiluminescence of human neutrophils while only mild effects on PMA-activated neutrophils were observed. In that case, the highest inhibitory effects were observed for quercetin, cyanidin-3-*O*-galactoside and PE40. Greater effects of BCP were observed in the case of OZP-activated neutrophils. The production of ROS by OZP-activated neutrophils was inhibited most strongly by quercetin and rutin, the effect being statistically significant ($p < 0.05$). This indicate that probably black chokeberry flavonoids interfere with the signaling cascade of phagocyte activation upstream to the protein kinase C activation. Cyanidin-3-*O*-galactoside also inhibited greatly the ROS production, but the effect was statistically insignificant ($p < 0.05$).

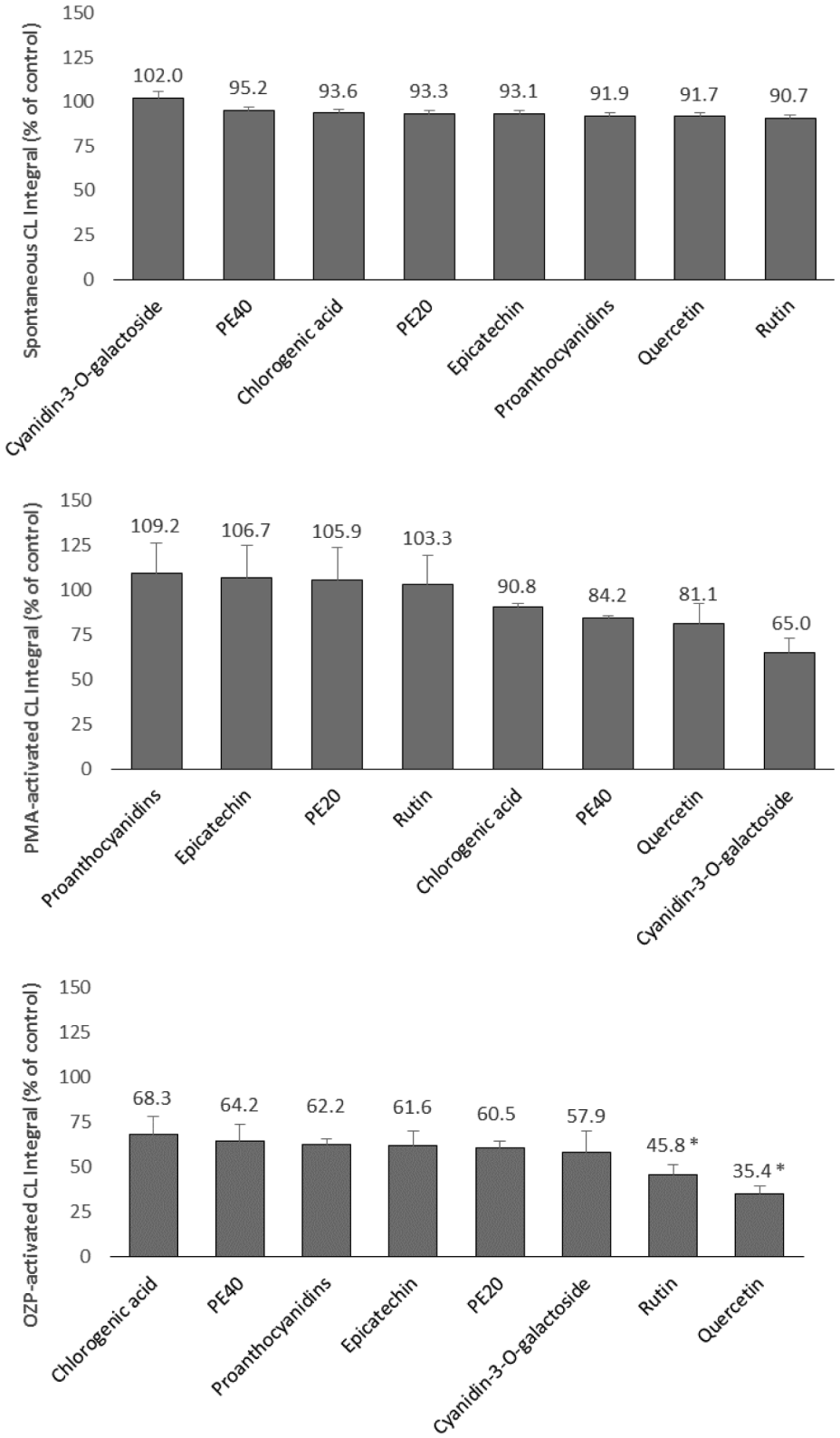

Fig. 3. Effect of black chokeberry polyphenols and preparations on the production of reactive oxygen species from untreated (spontaneous); PMA-activated; and OZP-activated human neutrophils. Values marked with asterisk are significantly different from the control ($p < 0.05$).

There are several studies on the effects of flavonoids on the respiratory burst of neutrophils. Kenny, Shu, Moritoki, Keen, and Gershwin (2009) demonstrated that cocoa flavonoids moderate the LPS-stimulated signaling pathways of neutrophils. They assumed that flavonoids decrease the effect of LPS on the ability of fMLP-activated neutrophills to generate ROS and hypothesized that the possible mechanism is activation of the MAPK pathway. Pincemail et al. (1987) revealed that flavonoid-containing *Ginkgo biloba* extract slowed down the release of ROS from fMLP-stimulated human neutrophils. fMLP is a soluble neutrophil activator that binds to specific receptors on the

Table 3
Antimicrobial activity of black chokeberry polyphenols and preparations.

Microorganism	Viable cell count of microorganism in nutrient medium, .10^7 cfu/cm^3	Crude extract	PE20	PE40	PACNs	Chlorogenic acid	Epicatechin	Quercetin	Cy-3-gal	Ampicillin
Inhibition zones against human pathogens, mm										
Escherichia coli ATCC 8739	4.8	–	–	–	–	–	10*	9*	–	28
Salmonella enterica ssp. *enterica* ATCC BAA-2162	1.2	–	–	–	–	–	12*	9*	–	27
Staphylococcus aureus ATCC 6538P	1.9	–	10	10*	11	–	12*	10*	12*	30
Staphylococcus aureus ATCC 25923	2.7	–	9	10*	9	–	13*	11*	12*	30
Listeria monocytogenes	4.1	–	–	–	–	–	–	–	–	22
Listeria monocytogenes I	2.5	–	–	–	–	–	–	–	–	23
Proteus vulgaris G	2.6	–	8	9*	11	–	–	–	10*	31
Pseudomonas aeruginosa ATCC 9027	4.9	–	–	–	–	–	–	–	–	20
Candida albicans ATCC 10231	1.3	–	–	–	–	–	12	9	–	–
Minimal Inhibiting Concentration (MIC), mg/ml										
Escherichia coli ATCC 8739	–	–	–	–	–	–	–	–	0.0125	
Salmonella enterica ssp. *enterica* ATCC BAA-2162	–	–	–	–	–	–	–	–	0.0063	
Staphylococcus aureus ATCC 6538P	–	2.50	–	1.25	–	–	–	–	0.0016	
Staphylococcus aureus ATCC 25093	–	5.00	–	0.32	–	–	–	–	0.0031	
Listeria monocytogenes	–	–	–	–	–	–	–	–	0.0016	
Listeria monocytogenes I	–	–	–	–	–	–	–	–	0.0008	
Proteus vulgaris G	–	0.63	–	0.16	–	–	–	–	0.0063	
Pseudomonas aeruginosa ATCC 9027	–	–	–	–	–	–	–	–	0.1	
Candida albicans ATCC 10231	–	–	–	–	–	1.25	2.50	–	–	

"-" – no antimicrobial effect.
* Single colonies in the inhibition zone.

membrane. Similarly, OZP bind to the surface opsonin receptors on phagocytes and triggers a signaling cascade leading to the activation of protein kinase C and subsequent NADPH oxidase activation. Wang, Chang, Hsu, Chen, and Kuo (2002) investigated the inhibitory effect of a cirsimaritin against respiratory burst in rat neutrophils and its cellular localization and observed that it inhibited the generation of superoxide radical and neutrophils oxygen consumption. Selloum, Reichl, Muller, Sebihi, and Arnhold (2001) compared the effects of kaempferol, quercetin, rutin and myricetin on the oxidative burst of neutrophils and revealed that they inhibit the pholasin luminescence of fMLP-activate neutrophils. Lee et al. (2010) showed that luteolin effectively blocked MAPK/ERK kinase 1/2 and Akt phosphorylation in fMLP-stimulated neutrophils, which is evidence that it is more likely for flavonoids to inhibit the signaling enzymes, rather than to scavenge superoxide radicals in fMLP-stimulated neutrophils. Furthermore, Qi, Feng, Li, Qi, and Zhang (2017) observed that myricitrin exhibited anti-inflammatory activity in LPS-activated RAW264.7 macrophages by blocking the activation of JAKs and the downstream transcription factor STAT1, which may result from the downregulation of NADH oxidase-dependent ROS production mediated by myricitrin. However, myricitrin had no impact on the MAPK signaling pathway. Myricitrin attenuated the generation of intracellular ROS by inhibiting the assembly of components of the gp91(phox) and p47(phox). Tian, Ding, Peng, and Lu (2017) showed that (−)-epigallocatechin gallate, the main flavonoid present in green tea, dose-dependently inhibited MPO-mediated HOCl formation *in vitro*. They reported that (−)-epigallocatechin gallate bound to the active site of MPO and resulted in the accumulation of compound II, which was unable to produce HOCl. This flavonoid also effectively inhibited HOCl generation in activated neutrophils without influencing MPO and NADP oxidase release and superoxide formation, suggesting that (−)-epigallocatechin gallate specifically inhibited MPO but not NADPH oxidase activity in activated neutrophils.

3.4. Antimicrobial activity

The antimicrobial activity of BCP (as phenolic preparations and pure polyphenols) was tested against 10 human pathogens, via two different methods – primary screening with the agar diffusion method and determination of minimal inhibitory concentration. The broad-spectrum antibiotic ampicillin was used as a positive control. It should be noted that the concentration of the extracts used in the current study was significantly lower than that in our previous study (Denev et al., 2014). In that concentration, crude black chokeberry extract showed no antimicrobial activity against all pathogenic microorganisms included in the study (Table 3).

As already stated, purification of crude extract with Amberlite XAD7 lead to a 12-fold increase in polyphenol content of the preparation. As a result, the PE20 exhibited antimicrobial activity against *Staphylococcus aureus* strains and *Proteus vulgaris* G. From the pure phenolic compounds, only epicatechin and quercetin showed antimicrobial activities against *Candida albicans,* but there was no activity against *Staphylococcus aureus* strains and *Proteus vulgaris* G. In all other instances, where inhibition zones were present, there were single colonies in inhibition zones, indicating that a part of the microbial population was more sensitive to the action of the tested pure substance and hence no antimicrobial activity was attributed to those compounds. Interestingly, the subsequent purification via Sephadex LH20 did not result in further increase of the activity. Black chokeberry proanthocyanidins that are partly present in PE20, exhibited inhibitory effect against the same pathogens as PE20. The inhibition zones diameter reached 11 mm for *Proteus vulgaris* and *Staphylococcus aureus* ATCC 6538P. The positive control - ampicillin exhibited strong inhibitory effect against all bacterial pathogens.

In the second step, we determined minimal inhibitory concentrations (MICs) of phenolic preparations and pure compounds, which displayed antimicrobial activity in the agar-diffusion assay. Although

PE40 contains more anthocyanins and other low-molecular phenolic compounds, it does not contain proanthocyanidins, which could explain the lack of antimicrobial activity. Interestingly, black chokeberry proanthocyanidins revealed respectively 2- and 16-fold higher antimicrobial activity towards *Staphylococcus aureus* strains in comparison to PE20. Similar effect was observed for the antimicrobial activity against *Proteus vulgaris* G. The lowest MIC (0.156 mg/ml) was found for proanthocyanidins and it was four-fold lower than that of PE20. Epicatechin and quercetin MICs of *Candida albicans* ATCC 10231 were 0.078 mg/ml and 2.5 mg/ml, respectively. Based on the results, it could be concluded that antimicrobial effect of black chokeberries is mainly due to the action of proanthocyanidins. Toxicity of tannins towards microorganisms is well documented, but for the first time we demonstrate that black chokeberry condensed tannins are the major antimicrobial agents of the berry. The different mechanisms proposed so far to explain tannin antimicrobial activity include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation (Scalbert, 1991). The study of Taguri, Tanaka, and Kouno (2004) demonstrated that various types of polyphenols including catechins and their oxidation products - proanthocyanidins and hydrolyzable tannins, showed antibacterial activities against four groups of food-borne bacteria. Authors concluded that the sensitivity of bacteria to polyphenols depends on bacterial species and polyphenol structure and showed the importance of 3,4,5-trihydroxyphenyl groups for the observed antimicrobial activity. As it could be expected, antibiotic positive control exhibited lower MICs than the tested polyphenols and preparations against all sensitive pathogens. The lowest MIC displayed by the proanthocyanidins against *Proteus vulgaris* G. was 25 times higher than the activity of the positive control ampicillin. However, it should be noted that there is no evidence that BCP and in particular proanthocyanidins are toxic or have adverse effects, characteristic for antibiotics. Therefore, condensed tannins from black chokeberry fruits could exert their antimicrobial properties against *Staphylococcus aureus* strains and *Proteus vulgaris* G in gastrointestinal tract, more over their concentration in the GIT could be significant (Scalbert & Williamson, 2000).

4. Conclusion

The current study confirmed our hypothesis that black chokeberry polyphenols differ in their antioxidant, antimicrobial and neutrophil-modulating activities. BCP have mild effect on PMA-activated and greater effect on OZP-activated CL of human neutrophils. Quercetin and epicatechin are the strongest antioxidants among BCP, but the amount of proanthocyanidins makes them the major contributor to antioxidant activity of fresh black chokeberries. Besides, we revealed that black chokeberry proanthocyanidins are the most potent antimicrobial agents in the fruit. Despite their high contribution to antioxidant activity of *Aronia melanocarpa* berries, proanthocyanidins are barely absorbed in the gastrointestinal tract and reach the colon. However, their action may be very important, since numbers of phagocytic cells normally reside in the gastrointestinal tract. Therefore, black chokeberry polyphenols and in particular proanthocyanidins could directly modulate their function and express their antioxidant and antimicrobial effects *in vivo*.

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Conflict of interests

Authors declare no conflict of interests.

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Effects of Pectic Polysaccharides Isolated from Leek on the Production of Reactive Oxygen and Nitrogen Species by Phagocytes

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ABSTRACT The current survey investigates the effect of four polysaccharides isolated from fresh leek or alcohol insoluble substances (AIS) of leek on the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from phagocytes. The ability of the polysaccharides to activate serum complement was also investigated. Despite the lack of antioxidant activity, the pectic polysaccharides significantly decreased the production of ROS by human neutrophils. Polysaccharides isolated from AIS markedly activated RAW 264.7 macrophages for RNS production in a concentration-dependent manner. The Western blot analysis revealed that this effect was due to the stimulation of the inducible nitric oxide synthase protein expression of macrophages. The polysaccharides extracted from AIS with water showed the ability to fix serum complement, especially through the alternative pathway. It was found that the polysaccharide that has the highest complement-fixing effect is characterized by the highest content of uronic acids and the highest molecular weight.

KEY WORDS: • ***complement fixing*** • ***immunomodulatory activity*** • ***leek*** • ***macrophages*** • ***neutrophils*** • ***pectic polysaccharides*** • ***reactive nitrogen species*** • ***reactive oxygen species***

INTRODUCTION

PECTIC SUBSTANCES ARE water-soluble heteropolysaccharides with various applications in the food industry.[1] They are industrially produced mainly from apples and citrus fruits, but are ubiquitous in the plant kingdom and are of great importance for the structure of plant cells. There is an increasing interest in pectic polysaccharides from various plant sources, since they have revealed various biological activities.[2,3] Recently, several articles reviewed the obtaining and purifying of plant heteropolysaccharides rich in uronic acids and investigated the relationship between their primary structure and biological activity.[4,5] Among the pharmacological activities of these heteropolysaccharides, immunomodulating activity seems to be the most important, and there is accumulating evidence that they are involved in the modulation of several components of the immune system.[5,6]

Innate immunity serves as an essential first-line of defense against microbial pathogens and foreign substances. Phagocytic cells such as macrophages and neutrophils play a key role in innate immunity because of their ability to recognize, ingest, and destroy pathogens by oxidative and nonoxidative mechanisms. Thus, approaches designed to enhance innate immune mechanisms nonspecifically could increase the defense against microbial infections.[7,8] In response to a variety of stimuli, NADPH oxidase present in neutrophils is activated in a phenomenon described as ''the respiratory burst,'' characterized by the production of superoxide anion, which gives rise to other forms of reactive oxygen species (ROS). In addition to ROS, nitric oxide (NO) and other reactive nitrogen species (RNS) produced mainly by macrophages are one of the important microbicidal tools in the process of inflammation during the fight against pathogenic microorganisms, bacteria, and tumor cells.[9,10] However, excessive or inappropriate ROS and RNS production by phagocytes is associated with oxidative damage to membrane lipids, DNA, proteins, and lipoproteins, resulting in various autoimmune and inflammatory diseases. Thus, the modulation of inflammation and oxidative stress by natural substances can be beneficial.

The complement cascade is another important part of the innate immune defense. Therefore, an activation of the complement system contributes to inflammatory responses and immunological defense reactions. As reported before, interaction with the complement system of polysaccharides due to fixation could be a good therapeutic strategy for treating inflammatory diseases.[11,12]

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Leek (along with onion and garlic) is part of the traditional Balkan diet, and its medicinal action has been known since ancient times. So far, there is scarce data about the chemical composition of leek polysaccharides and their biological activity,[13–15] but it was established that some polysaccharides are promising stimulators of some components of the immune system. That provoked our interest to conduct a more extensive study on the effects of leek pectic polysaccharides obtained through different extraction methods on the production of ROS and RNS by phagocytes.

Taken together, the complement system and phagocytes are both recruited by activation processes. Therefore, the main aim of the present study was to investigate the effects of pectic polysaccharides isolated from leek on ROS and NO production by phagocytes as well as on complement activity.

MATERIALS AND METHODS

Preparation of leek polysaccharides

Fresh leek was purchased from the local market in Plovdiv, Bulgaria. Polysaccharides 1 and 2 (PS1 and PS2, respectively) were obtained from fresh leek by the following procedure: 500 g of leek was homogenized and mixed with 250 mL 0.5% HCl and 250 mL 0.5% citric acid (for PS1) or 500 mL 0.5% HCl (for PS2). The mixtures were incubated with stirring for 1 h at 80°C and centrifuged (4400 *g*, 30 min). The supernatants were removed, coagulated with 96% ethanol (1/1.5; v/v), and left at room temperature for 1 h. The coagulated polysaccharides were then separated by centrifugation (4400 *g*, 15 min). Separated polysaccharides were washed consecutively with 70% ethanol and 96% ethanol and dried at 60°C in a laboratory drier.

Polysaccharides 3 and 4 (PS3 and PS4, respectively) were obtained by the following procedure: fresh leek (1 kg) was cut into small pieces of 8–10 mm and treated with 2500 mL 96% ethanol preheated to 65°C. The obtained mixture was kept for 1 h at 65°C followed by 24 h at room temperature, and then filtered through cheese-cloth, after which, the insoluble material was washed with 96% ethanol. The obtained alcohol insoluble substances (AIS) were dried at 60°C. Fifty grams of dried AIS was treated with 1250 mL water at 80°C for 1 h and then filtered. The filtrate was processed to obtain water extractable pectic PS3. The residue was further extracted with 1000 mL 0.5% HCl for 1 h under the same conditions. The mixture was filtered through cheese-cloth, and the filtrate represents the crude acid extractable pectic PS4. The two obtained aqueous filtrates were coagulated using an equal volume of 96% ethanol and left for an hour. The coagulated crude pectins were separated by filtration, washed with 100 mL 70% acidified ethanol (0.5% HCl), then with 70% ethanol to a neutral pH, and finally with 100 mL 96% ethanol. They were dried at 60°C in a laboratory drier.

Determination of polyuronic content and degree of esterification of pectic polysaccharides

The polyuronic content and degree of esterification were determined by the method of Owens.[16]

Determination of molecular weight

Molecular weights of the polysaccharides were assayed through high-performance size exclusion chromatography (HPSEC) on a Waters (Millipore) system as described by Kratchanova *et al.*[15]

Cell culture

The murine peritoneal macrophage cell line RAW 264.7 (American Type Culture Collection) was cultivated in Dulbecco's modified Eagle's medium (PAN) and supplemented with 10% of fetal bovine serum (PAN). Cells were maintained at 37°C, 5% CO_2.

Whole blood phagocyte-derived ROS production

Heparinized (50 IU/mL) blood samples were obtained from the cubital vein of healthy human volunteers after overnight fasting. The kinetics of ROS production by blood phagocytes was analyzed by luminol-enhanced chemiluminescence (CL) for a period of 60 min, using a microtiter plate Luminometer LM-01T (Immunotech). For activation of ROS production, opsonized zymosan particles (OZP) or phorbol myristate acetate (PMA; Sigma-Aldrich) were used. The detailed procedure is described elsewhere.[17] Spontaneous CL measurements (without any activator) were included in each assay. The recorded values included the intensity of CL emitted during the time interval studied (integral of the CL).

Measurement of nitrite concentration by Griess reaction

The accumulation of nitrites in a medium was measured spectrophotometrically according to Pekarova *et al.*[10] Cells were incubated in 12-well plates at 1×10^6 cells/well for 24 h without/with 0.1 μg/mL lipopolysaccharide (LPS, *Escherichia coli* serotype 0111.B4; Sigma-Aldrich) and pectic polysaccharides at 37°C, 5% CO_2. At the end of the incubation period, culture media were collected from wells and centrifuged at 5000 *g*, 4°C for 5 min. Then, 150 μL of supernatant was mixed with an equal volume of Griess reagent (Sigma) in a 96-well plate, and the mixture was incubated at room temperature and in the dark for 30 min. The absorbance was measured at 546 nm. Sodium nitrite was used as the standard.

Western blot analysis of inducible nitric oxide synthase expression

After removing the supernatant for the nitrite measurement, the remaining cells were washed with cold phosphate-buffered saline and lysed in the lysis buffer (1% sodium dodecyl sulfate, 10^{-1} M Tris pH 7.4, 10% glycerol, 10^{-3} M sodium ortho-vanadate, 10^{-3} M phenylmethanesulfonyl fluoride). Protein concentrations were determined using BCA™ protein assay (Pierce), with bovine serum albumin as a standard. The equal amounts of protein were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, using 7.5% running gel. The expression of inducible nitric oxide synthase (iNOS) protein was quantified by Western blot analysis, as described previously.[18]

Anti–iNOS/NOS Type II mouse monoclonal antibody (BIO-RAD; 1:5000) and ECL™ anti–mouse IgG horseradish peroxidase linked whole antibody (from sheep; Biosciences; 1:2000) were used. The immunoreactive bands were detected using an ECL™ detection reagent kit (Pierce) and exposed to radiographic film (AGFA). Equal loading of proteins was confirmed by determination of β-actin. Relative protein levels were quantified by scanning densitometry using the ImageJ™ program, and the individual band density value was expressed in arbitrary units.

Antioxidant activity determination

The antioxidant activity of the investigated polysaccharides was investigated by total peroxyl radical-trapping antioxidant parameter (TRAP) and oxygen radical absorbance capacity (ORAC) assays.[19,20]

Complement fixation assay

The test is based on the colorimetric measurement of hemoglobin released from target erythrocytes after incubation with normal human serum.[21] The detailed procedure is described elsewhere.[15]

Statistical analysis

The results are presented as mean from at least three independent experiments ± standard error of mean (SEM). Experiments were realized in duplicates or triplicates. Comparisons with the control were performed by analysis of variance (ANOVA), followed by the Newman–Keuls *Post hoc* test. p values of less than .05 and .01 were considered significant.

RESULTS

Characteristics of leek pectic polysaccharides, isolated by different extraction methods

The yield and characteristics of the isolated pectic polysaccharides from leek are presented in Table 1. It is evident that extraction conditions lead to different characteristics of the extracted pectins. Generally, pectic polysaccharides extracted from dry AIS are distinctive, with a lower degree of esterification when compared to the polysaccharides obtained from fresh leek. Water extracted PS3 has the highest polyuronic content -72.8%, and the highest molecular weight -8.8×10^5 Da.

The effect of pectic polysaccharides on ROS production

Phagocytes play a key role in host defense against invading pathogens, and play a crucial role in inflammatory processes. In our study, the effect of pectic polysaccharides on ROS production by whole blood neutrophils was analyzed using luminol-enhanced CL. Typical kinetic curves of spontaneous neutrophil CL and neutrophil CL activated with receptor-bypassing (PMA) and receptor-operating (OZP) stimuli are shown in Figure 1. Spontaneous CL represents the basal production of ROS in nonactivated neutrophils. On the other hand, the activation of neutrophils with both PMA and OZP induced a significant increase in ROS production. The pectic polysaccharides in our study decreased both spontaneous and PMA- or OZP-activated ROS production in a 0.1 mg/mL concentration (Table 2). This effect was expressed even more significantly when a concentration of 0.5 mg/mL was used.

The effect of pectic polysaccharides on nitrite production and iNOS expression

In our previous experiments,[18] the highest concentration of nitrites in a medium was observed in control RAW 264.7 cells after their 1-day stimulation with LPS, and this schedule was chosen for the experiments. Figure 2 demonstrates that while PS1 and PS2, in both concentrations, do not activate resting macrophages (not treated with LPS) to produce nitrites, PS3 at the concentration of 0.1 mg/mL markedly activated macrophages for nitrite production. This effect became significant ($p<.01$) when 0.5 mg/mL PS3 was used. PS4 was even more effective activating macrophages very significantly ($p<.01$) in a 0.1 mg/mL concentration, with a further activation increase in a 0.5 mg/mL concentration. Interestingly, none of the tested pectic polysaccharides influenced the production of nitrites in macrophages treated with LPS (data not shown). The possibility

Table 1. Yield and Characteristics of Pectic Polysaccharides from Leek Obtained by Different Extraction Methods

	Material	*Extragent*	*Polyuronic content (%)*	*Degree of esterification (%)*	*HPSEC fractionation of polysaccharides*		
					%	*Molecular weight (Da)*	*Average molecular weight (Da)*
PS1	Fresh leek	0.5% HCl + 0.5% citric acid	48.8	67.4	80.4 19.6	8.5×10^5 3.3×10^4	6.8×10^5
PS2	Fresh leek	0.5% HCl	49.0	73.1	71.4 28.6	8.4×10^5 2.2×10^4	6.0×10^5
PS3	AIS	Water	72.8	56.3	100 28.7	8.8×10^5 8.8×10^5	8.8×10^5
PS4	AIS	0.5% HCl	49.3	52.2	52.0 19.3	1.2×10^5 3.8×10^4	3.2×10^5

HPSEC, high-performance size exclusion chromatography; PS1–PS4, polysaccharides 1–4.

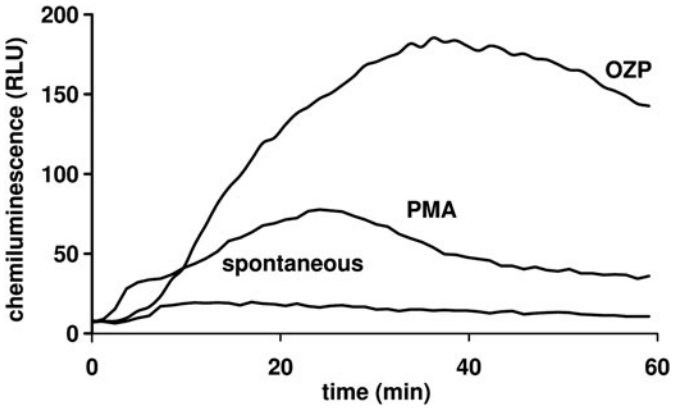

FIG. 1. Kinetics of luminol-enhanced spontaneous and activated chemiluminescence (CL) of human whole blood neutrophils. The results are expressed in relative light units (RLU).

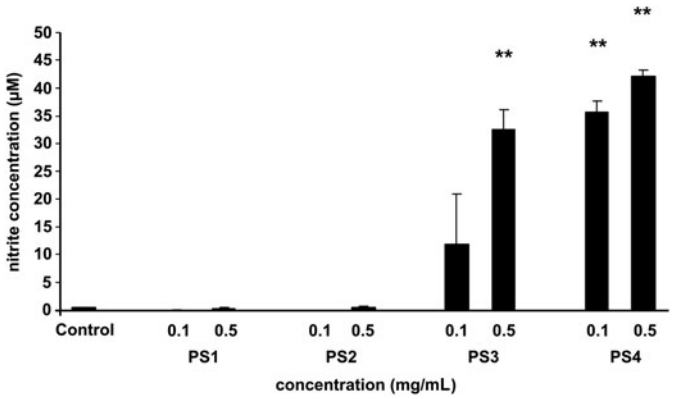

FIG. 2. The effect of pectic polysaccharides on NO production by nonstimulated murine RAW 264.7 macrophages. Data are expressed as mean ± SEM from three independent experiments. **Indicates significance at level $P < .01$ when compared with a relevant control. PS1–4, polysaccharides 1–4; NO, nitric oxide; SEM, standard error of the mean.

that the changes in nitrite concentration induced with PS3 and PS4 were associated with changes in iNOS protein expression was determined using Western blot analysis (Fig. 3). In comparison with the iNOS protein level in the control macrophages without LPS treatment, iNOS protein expression was stimulated with PS3 and PS4 in a manner very similar to the increase in nitrite concentrations in the cultured media shown in Figure 2. The expression of iNOS protein was not changed in LPS-treated macrophages, which also corresponds with our data on NO production.

Scavenging properties against the peroxyl radical or NO

The results of some authors (*e.g.*, Kaur *et al.*[22]) suggest that polysaccharides isolated from plants may play important roles in free radical scavenging. Therefore, we analyzed whether the antioxidant properties of polysaccharides against ROS and NO can interfere with changes in phagocyte-derived ROS and NO production. All assays used (TRAP, ORAC, and direct amperometrical analysis) confirmed that none of the tested pectic polysaccharides exerted significant scavenging properties against the peroxyl radical or NO (data not shown).

Table 2. Effect of Tested Polysaccharides on Spontaneous and Activated Chemiluminescence of Human Neutrophils

	Concentration (mg/mL)	*Spontaneous CL*	*PMA-activated CL*	*OZP-activated CL*
PS1	0.1	76.0 ± 11.0	75.3 ± 8.8*	78.5 ± 3.8**
	0.5	60.7 ± 11.2*	65.1 ± 12.5*	75.2 ± 3.1**
PS2	0.1	77.1 ± 8.1*	75.9 ± 3.6**	85.5 ± 4.2*
	0.5	59.3 ± 10.4*	65.3 ± 13.5*	76.5 ± 3.4**
PS3	0.1	79.3 ± 7.6	80.8 ± 3.8**	78.7 ± 4.0**
	0.5	69.6 ± 10.1*	79.9 ± 7.0*	72.1 ± 2.4**
PS4	0.1	78.6 ± 12.4	80.8 ± 6.2*	85.5 ± 4.4*
	0.5	69.0 ± 10.9*	71.6 ± 9.4*	85.5 ± 4.4**

The data are expressed as % of respective controls and represent mean ± SEM, $n = 3$.

Asterisks indicate significant differences at $^{**}P \leq .01$ or $^{*}P \leq .05$ levels when compared to respective control values (cells incubated without lipopolysaccharide [LPS]).

PMA, phorbol myristate acetate; OZP, opsonized zymosan particles; CL, chemiluminescence; SEM, standard error of the mean.

The effect of pectic polysaccharides on the complement system

The polysaccharides were tested for their ability to fix complement. As shown in Table 3, PS1, PS2, and PS4 showed a weak complement-fixing effect, whereas the addition of pectic PS3 resulted in dose-dependent erythrocyte hemolysis, indicating that this fraction exhibited potent complement-fixing activity. The effect was more profound via the alternative pathway of complement activation. Therefore, the fixation of complement by this polysaccharide can be proposed as a potential therapeutic strategy for treating inflammatory diseases.

DISCUSSION

One of the most promising recent alternatives to classical medical treatment is the use of immunomodulators for enhancing host defense responses. In recent decades, polysaccharides, including pectic substances isolated from plant sources, have attracted a great deal of attention because of their broad spectrum of therapeutic properties and relatively low toxicity.[4,5] While our understanding of the mechanism of action of these substances is still developing, it appears that one of the primary mechanisms involves modulation of the phagocyte and the complement activity.[23] In our study, we investigated the effects of pectic polysaccharides isolated from leek on ROS and NO production by neutrophils and macrophages, as well as on the complement activity.

Only limited information exists on the macrophage immunomodulatory activity of pectic polysaccharides; and, to our knowledge, there are no reports regarding the effects of

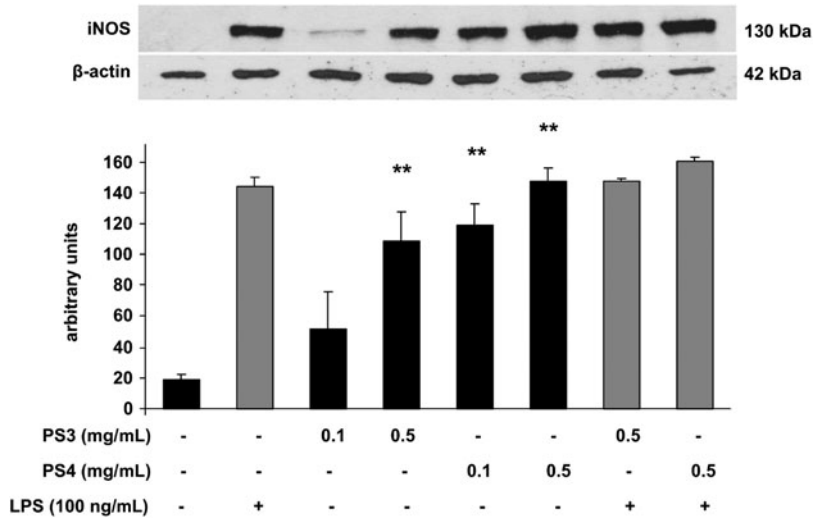

FIG. 3. Densitometric analysis and representative Western blot of iNOS protein expression in LPS-stimulated RAW 264.7 cells treated with pectic polysaccharides. The data represent mean ± SEM from three independent experiments. Asterisks indicate a significant difference (**$P < .01$) when compared to the relevant control value (cells incubated without LPS). iNOS, inducible NO synthase; LPS, lipopolysaccharide.

leek polysaccharides on neutrophils. However, in contrast with studies by other authors, where the stimulation effects of some polysaccharides on neutrophils is reported,[8,24–26] the pectic polysaccharides in our study decreased both spontaneous and PMA- or OZP-activated ROS production in a concentration-dependent manner. The results of some authors[22] suggest that polysaccharides isolated from plants may play an important role in ROS scavenging. Schepetkin *et al.*[27] found that *Opuntia polyacantha* polysaccharide fractions exhibited ROS scavenging activity, with the high molecular weight fractions being the most active. In our study, both TRAP and ORAC assays confirmed that none of the tested pectic polysaccharides exerted antioxidant properties against peroxyl radicals, proving that the decrease in neutrophil-derived CL is not caused by the antioxidant properties of the tested polysaccharides.

It is well established that a wide range of plant polysaccharides exhibit beneficial pharmacological effects via their ability to modulate macrophage function, including the production of NO by iNOS.[23] The macrophage-modulating activity of polysaccharides toward NO production has been reported for polysaccharides isolated from different sources,[8,25,26,28] but very little is known about the structural characteristics of the polysaccharides that determine this activity. Schepetkin *et al.*[27] proposed that the macrophage-immunomodulatory activity of *Opuntia* polysaccharides is positively correlated with the average molecular weight of the polysaccharides. The authors suggested that polysaccharides may activate macrophages via receptor(s) or other surface structures, although the nature of these surface targets is currently unknown. Contrary to these observations, in our study, polysaccharide PS4 exhibited the most potent macrophage-modulatory properties, by activating macrophages to produce NO, even when a concentration of 0.1 mg/mL was used (Fig. 2). HPSEC analysis showed that this acid-extractable polysaccharide is heterogeneous, with an average molecular weight of 3.2×10^5 Da, which is the lowest, compared to other polysaccharides isolated from leek. The homogeneous PS3 with the highest molecular weight among leek pectic polysaccharides (8.8×10^5 Da) also activated macrophages for NO production, although its activity in a concentration of 0.1 mg/mL was lower compared with PS4. At a concentration of 0.5 mg/mL, there was no significant difference in the macrophage-modulatory activity of the two polysaccharides tested. Our results indicate that a high molecular weight is not the only prerequisite for macrophage-immunomodulatory activity. There are probably some structural characteristics that also determine this activity, but further studies are necessary to understand the relationship between the structure and biological activity.

While interpreting results from an *in vitro* study, one has to keep in mind that the absorption of plant polysaccharides into the bloodstream after oral administration is not well understood and is a disputable issue. Nevertheless, the metabolic activity of neutrophils can be modulated by polysaccharides directly in the intestinal lumen, especially during inflammatory conditions. One of the challenges of the immune system is to ensure, on one hand, a balance between the protection of barrier surfaces from pathogens

Table 3. Complement-Fixing Activity of Polysaccharides

	Inhibition of hemolysis [%]				
	Classical pathway		*Alternative pathway*		
	Concentration of polysaccharides [μg/mL]				
	6000	*3000*	*3000*	*1500*	*750*
PS1	7.5 ± 0.23^a	0^a	21.1 ± 0.86^a	5.4 ± 0.46^a	0^a
PS2	6.8 ± 0.69^a	0^a	8.5 ± 0.34^b	0^b	0^a
PS3	38.2 ± 5.08^b	0^a	65.4 ± 10.57^c	54.9 ± 8.32^c	17.3 ± 0.46^b
PS4	21.4 ± 2.6^c	0^a	14.8 ± 4.97^d	0^b	0^a

The data represent mean ± SEM from three independent experiments.

[abcd]Values marked with different superscript letters within individual columns are significantly different ($P \leq .01$).

and, on the other, the establishment of a beneficial relationship with commensal bacteria.[29] In mucosal tissues, the inflammatory response is central to effective host defense against invading pathogens. The mucosal immune system must be tightly regulated to prevent abnormal responses to innocuous environmental antigens and commensal organisms, which could result in an allergy or chronic inflammatory diseases. Many intestinal inflammatory illnesses are marked by neutrophil transepithelial migration and arrival in the lumen, forming crypt abscesses. Here neutrophils not only directly defend the surface, but also release biologically active mediators and stimulate a secretory flush.[30,31] Neutrophil migration across the intestinal epithelium and their presence in the lumen enable pectins to interact directly with the receptors on the surface of neutrophils.

The absorption of plant polysaccharides into the bloodstream after oral administration is another possibility for how pectins can influence the components of the immune system, including neutrophils, monocytes/macrophages, and the complement system. The majority of the intestine is covered with a single layer of columnar epithelial cells, which make up the absorptive surface for water and nutrient absorption.[30] While the intestinal barrier is permeable to digested nutrients as well as fluids, it is, in general, impermeable to macromolecules, particular antigens, and most microorganisms. However, the immune system needs direct contact with antigens or pathogens to generate specific immune responses. For this purpose, the intestine provides specialized epithelial cells, called M cells, which are responsible for the uptake of antigens and microorganisms. The most noticeable feature of M cells is their active transport (by a phagocytic mode of transport that has yet to be fully characterized) of a wide variety of inert material from the gut lumen toward the follicles, from where particles can migrate to the blood via the mesentery nodes and the thoracic lymph duct.[32] Because polysaccharides also behave as hydrated nanoparticles in an aqueous solution, it is postulated that plant polysaccharides become incorporated into the lymphoid follicles of Peyer's patches and isolated lymphoid follicles by a mechanism similar to other nanoparticles, resulting in their dissemination to systemic circulation through the mesenteric lymph nodes and the thoracic duct. Some experimental results suggest that oral administration of certain immunostimulating polysaccharides affects the gastric mucosal immune system through Peyer's patch cells. Otsuka *et al*.[33] reported that peptide–mannan complexes with a molecular weight of 60,000–95,000, when orally administered to rats, reached the lymph and the blood stream. Mitsuhashi *et al*.[34] found that α-glucan isolated from the liquid-cultured mycelium of *Tricholoma matsutakwith,* with a molecular weight over 2.0×10^6 daltons administered as a ^{14}C-labeled derivative, was absorbed in the digestive system and remained in the blood stream even 168 h after administration. The peptide–mannan complex, as well as α-glucan, was observed to accumulate in the mesenteric lymph nodes and Peyer's patches as well as in the liver, spleen, and kidneys. Yunoki *et al*.[35] reported the accumulation of the polysaccharides derived from the basidiomycetes *Coriolus versicolor* in the liver of mice after oral administration, whereas Sakurai *et al*.[36] observed the same for the pectic polysaccharide bupleuran 2IIc, from *Bupleurum falcatum*, which was also accumulated in Peyer's patches. Otsuka *et al*.[33] indicated that peptide–mannan appeared in aortic blood more than 2 h after it was detected in portal blood, suggesting that the peptide–mannan, which was absorbed in the digestive system was initially incorporated by the phagocytic system of the liver, while the excess of peptide–mannan overflowed into the systemic blood stream. It is assumed that the formation of the immune complexes of the pectic polysaccharide-reacting natural IgA antibody with the active pectic polysaccharide in the intestinal fluid may not only participate in effective incorporation of the active pectic polysaccharides into Peyer's patches, but may also activate complement components that result in certain modulations of the intestinal immune system.[37]

The complement can also meet pectins directly in the epithelium or even in the intestinal lumen. In general, the complement system plays an important role as a primary defense against bacterial and viral infections, and appears to be intrinsically associated with several immune reactions, such as the chemotactic attraction of leukocytes, immune adherence, the modulation of antibody production, and increased local vascular permeability.[37,38] According to the study of Kiyohara *et al*.,[37] the pectic polysaccharide-reacting natural and secretory IgA antibody exists in the mucosal sites of the human intestine. Dimeric IgA has been assumed to contribute to activating the alternative complement pathway, producing some biologically active complement fragments, and complement components such as C3 and C4, which are known to be produced by the epithelial cells of the intestinal tract.[39,40] It is also speculated that the formation of immune complexes of the pectic polysaccharide-reacting natural IgA antibody with the active pectic polysaccharides in the intestinal fluid may activate complement components in the fluid, resulting in certain modulations of the intestinal immune system.

As reported by Gasque,[41] complement component proteins can be activated through three cascade pathways: the classical pathway, the alternative pathway, and the antibody-independent lectin pathway. In general, the alternative and lectin pathways contribute to the early natural defense mechanism of the host before production of the antibody. It is known that some plant polysaccharides are good modulators of the immune system, and their activity depends on several factors, such as raw material, extraction conditions, chemical composition, and the structure of the polysaccharide. However, it is disputable which part of the pectic macromolecule is responsible for their immunomodulating properties. Most polysaccharides with immunomodulating properties have been isolated from hot-water extracts of medicinal herbs,[5] and some authors suggest that a high molecular weight and a higher content of galacturonic acids are prerequisites for a higher complement-fixing ability.[3] Our results confirm these observations, and show that PS3 obtained after water extraction of AIS reveals the highest

complement-fixing effect and is characterized by a high content of uronic acids and a high molecular weight.

CONCLUSION

It is evident from the performed study that leek polysaccharides modulate the immune system via different mechanisms. Polysaccharides PS3 and PS4 isolated from AIS exhibited potent macrophage-activating properties resulting in increased generation of NO via the induction of inducible NO synthase. Pectin PS3 obtained after water extraction of AIS revealed the highest complement-fixing effect. These findings make leek a valuable source of biologically active polysaccharides, which could also be considered for further medicinal applications. It was shown that molecular weight is not the only thing that determines the macrophage immunomodulatory activity of leek polysaccharides, and further studies are required to investigate the structural features in relation to the biological activity.

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AUTHOR DISCLOSURE STATEMENT

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Acidic polysaccharide complexes from purslane, silver linden and lavender stimulate Peyer's patch immune cells through innate and adaptive mechanisms

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ABSTRACT

Three polysaccharide complexes (PSCs) were isolated from the aerial parts of common purslane (*Portulaca oleracea* L.), and the flowers of common lavender (*Lavandula angustifolia* Mill.) and silver linden (*Tilia tomentosa* Moench) by boiling water extraction and ethanol precipitation. The chemical composition and immunomodulating effects of isolated PSCs were characterized. The chemical characterization revealed that the three samples contain mainly pectic polysaccharides. They exhibited *ex vivo* intestinal immunomodulating activity through the murine Peyer's patch-mediated bone marrow cell proliferation test at 100 μg/ml concentration. At the same time, they stimulated *ex vivo* human blood T-cell populations ($CD4^+/CD25^+$ and $CD8^+/CD25^+$), phagocytic leukocytes ($CD14^+$ and $CD64^+$ cells) and induced IL-6 production from human white blood cells and Peyer's patch cells. The herbal PSCs stimulated *ex vivo* ROS production from whole blood phagocytes and showed unspecific *in vitro* anti-proliferative activity against normal and A549, HeLa and LS180 tumor cells. This is the first report on immunomodulating studies of linden flower pectins and chemical and biological activity characterization of lavender polysaccharides. Our study demonstrates that similarly to purslane, lavender and silver linden herbal materials contain immunomodulating polysaccharides that could be useful for support of compromised immune system.

1. Introduction

Herbal medicine is a symbol of human knowledge, because it has been accompanying every nation through its development and people will always prepare herbal infusions, decoctions and extracts [1]. The thorough research in the last 30 years in the Chinese and especially the Japanese Kampo medicine, traditional European, African and Indian Ayurvedic medicines has shown that herbal pectic polysaccharides contribute to the biological activity of widely used herbs [2–5]. Already in 1988, polysaccharide hydrocolloids were included in the German Rote Liste® as antitussive, antipyretic, wound healing, nutraceutical, laxative, immune stimulating and gastric agents [6]. Furthermore, examples of commercial products containing immunomodulating polysaccharides are modified citrus pectin, Bazoton®, *Viscum album* extract, *Astragalus* polysac-

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charide extract and Kampo medicine formulations Juzen-taiho-to and Hochuekkito [7–10].

Generally, pectic polysaccharides contain three main building blocks – unbranched homogalacturonan (HG), ramified rhamnogalacturonan (RG) I and II [11]. The unbranched HG is composed of an α-(1 → 4)-linked poly-D-Gal*p*A chain, which COOH groups are methyl-esterified to different extent and some OH groups are acetylated at C-2 and/or C-3. The ramified RGI has a backbone structure of the linear repeating fragment [→2)-α-L-*R*hap-(1 → 4)-α-D-Gal*p*A-(1 →]$_n$. The main fragment carries side chains of α-(1 → 5)-L-arabinans, β-(1 → 4)-D-galactans, arabinogalactan (AG) type I and II at the OH group of C-4 of the L-Rha residues. The AGI is composed of a linear β-D-(1 → 4)-linked galactan, which is branched at O-3 mainly with α-L-(1 → 5)-linked Ara*f* residues. The AG II is composed of a β-D-(1 → 3)-linked galactan backbone with side chains at O-6 of β-(1 → 6)-linked galactans. 1,3- and 1,6-galactans are substituted mainly with α-L-(1 → 3)-linked Ara*f* residues. The glycosidic coupling of L-Ara in the AG structures is highly diverse (1,2; 1,3; 1,5) [2,4,11].

The most important immune active pectic side chains are AGs [2,4]. The immunomodulating effects of herbal pectins in the gastrointestinal tract expand a lot the classical concept for dietary fibers and human health. The pectic polysaccharides from herbal infusions, traditional foods and drinks pass easily through the gastrointestinal tract, where they can interact with mucosal immune system. Stimulation of the mucosal immune system through immunocompetent cells in Peyer's patches by polysaccharides has shown perspective application for supplementary treatment of infectious diseases [10,12–14]. The Peyer's patches contain B-, T-cells and phagocytic leukocytes, such as macrophages, dendritic cells and neutrophils, which can be affected by the pectic polysaccharides [2,3,15]. The pectins activate oxidative burst and phagocytosis of neutrophils and macrophages, thus influencing the innate immune response [16]. Furthermore, the activated macrophages and dendritic cells could stimulate adaptive immune cells [17,18]. Direct or indirect immunomodulating effect of polysaccharides on T-cell subpopulations can be used for prevention and treatment of tumor and autoimmune diseases [19,20].

Silver linden (*Tilia tomentosa*) and common lavender (*Lavandula angustifolia*), together with the edible weed common purslane (*Portulaca oleracea*) are traditionally used in the herbal medicine [21]. Bulgaria is a major exporter of wild and cultivated aromatic medicinal plants and their derived products, which are raw materials for the pharmaceutical, cosmetic and nutraceutical industries. It is the biggest lavender oil producer in the world since 2011 and leading country in the export of Tiliae flos. Interestingly, *T. tomentosa* forms coenoses only in the Balkans and although it is widely used in the gemmotherapy, it is still poorly studied [22]. On the other hand, the medicinal use of lavender is focused on its essential oil. The third herb, purslane is a very popular leaf vegetable in the Indian and Mediterranean cuisine, which is named the "global panacea" by WHO [23]. The immunomodulating activity of the three herbs is related mainly to the lavender essential oil, linden tiliroside, scopoletin and lignans, and only for purslane to polysaccharides [24,25,23]. The purslane contains acidic pectic polysaccharides and neutral AGs, arabinoglucomannans and starch [26–28]. It is shown that purslane polysaccharides have potential to be used in the treatment of tumor diseases because they exhibit immune-enhancing activities, but studies on their intestinal immunomodulating activity are missing [19,29,30]. Kram & Franz [31] reported that small-leaved linden *Tilia cordata* flower mucilage is rich in pectin, as later Schmidgall et al. [32] showed that its raw polysaccharide complex expresses moderate bioadhesion to epithelial tissue *ex vivo*. To our knowledge investigations on the chemical composition and biological activity of silver linden and lavender pectic polysaccharides are absent.

The aim of the current study was to isolate and characterize the chemical composition and immunomodulating effects of polysaccharide complexes from *L. angustifolia* and *T. tomentosa*, and to compare their effects with the known bioactive polysaccharide complex from *P. oleracea*.

2. Materials & methods

2.1. Plant material

Common purslane aerial parts were collected from natural habitats in September 2013 in Plovdiv region, washed, cut into small pieces, freeze-dried and stored in a desiccator until use. The plant material was identified as *Portulaca oleracea* L. (Portulacaceae) by Assoc. Prof. Dr. Ivanka Dimitrova-Dyulgerova (Plovdiv University Paisii Hilendarski). Lavender flowers *Lavandula angustifolia* Miller (syn. *L. vera*) sort "Sevtopolis" (Institute of Roses, Essential and Medical Cultures, Kazanlak, Bulgaria) were provided dried by ET "Ve Pe Pi – Vesko Pipev" (Velingrad, Bulgaria). Linden flowers were collected in June 2013 in Plovdiv, air-dried for two weeks and stored in a desiccator until use. The plant material was identified as silver linden, *Tilia tomentosa* Moench (Tiliaceae), by Dr. Evgeny Tsavkov (University of Forestry, Sofia). A voucher specimen with given number SOA061426 was deposited in the Herbarium of the Agricultural University of Plovdiv.

2.2. Isolation of the herbal PSCs

Preparation of the herbal PSCs was performed partly as the Japanese Kampo medicine decoctions are prepared [33]. Thirty grams of each milled herbal material were mixed with 600 ml water and boiled until the mixture volume was reduced to half. The extracts were filtered through a nylon cloth and the plant residue was rinsed with 150 ml water and re-boiled with 600 ml water again, following the same procedure. The collected extracts and washes were centrifuged (4000 rpm, 20 min), filtered through a Büchner funnel and concentrated four times in volume under vacuum at 50 °C. The plant extracts were centrifuged again and coagulated with 4 vol. 95% ethanol at 4 °C over night. The obtained precipitates were collected by a Büchner funnel separation for linden and purslane PSCs (linden PSC and purslane PSC) or centrifugation (4000 rpm, 30 min, 4 °C) for lavender PSC. Then, the three crude polysaccharides were re-dissolved in water, dialyzed for 72 h against water at 4 °C (VISKING®, SERVA Electrophoresis, MWCO 12–14 kDa), centrifuged, freeze-dried and stored in a desiccator in dark until use.

2.3. General methods

The polyuronic acid content (PUC) in the plant materials was determined titrimetrically, according to Owens et al. [34]. The anhydrouronic acid content (AUAC) and simultaneous determination of GalA and GlcA (in the absence of 4-*O*-methyl-GlcA) in the herbal PSCs were measured colorimetrically by the *m*-hydroxydiphenyl (GalA as a standard) and 3,5-dimethylphenol (GalA and D-glucuronolactone as standards) methods [35,36]. Degree of methoxylation (DM) was analyzed with alcohol oxidase (*P. pastoris*, Sigma-Aldrich) and Purpald® (Sigma-Aldrich) [37]. Degree of acetylation (DA) was assayed by the method of McComb & McCready [38], using β-D-glucose pentaacetate as a standard. Molecular weight was determined by HPSEC-RID (Waters) with Shodex standard P-82 kit (pullulan standards, Showa DENKO, Japan) [39]. Protein content (BSA standard), total phenolics (gallic acid standard) and presence of total saponins were analyzed according to Bradford [40], Singleton & Rossi [41] and Pasaribu et al.

[42], respectively. Only distilled water was used for all experiments. All analyses were performed in duplicates (n = 6).

2.4. Monosaccharide composition

Polysaccharide samples (10 mg) with added *myo*-inositol as an internal standard (1 mg) were pre-hydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 °C. Then, the samples were diluted with water to 1 M H_2SO_4 and further hydrolyzed for 3 h at 100 °C. The released monosaccharides were converted into their alditol acetates, according to Selvendran et al. [43]. The formed alditol acetates were analyzed by GC/MS on a HP 6890 Series Plus gas chromatograph coupled with a HP 5973 mass selective detector (Hewlett–Packard, Palo Alto, USA), on a Supelco SP-2380 column (30 m × 0.25 mm ID × 0.25 μm). The monosaccharide composition (including GalA and GlcA) of the three samples (1 mg) containing 100 μg *myo*-inositol was also determined by HPAEC-PAD after methanolysis with anhydrous 2 M HCl in methanol for 16 h at 80 °C, followed by hydrolysis with 2 M TFA for 1 h at 121 °C [44]. All samples were analyzed in duplicates.

The presence of arabinogalactan type II (AGII) was detected by precipitation with the *ß*-glucosyl Yariv reagent [45].

2.5. Fourier transform infrared spectroscopy (FTIR)

The lavender and linden PSCs were dissolved in water (10 mg/ml) and coagulated several times with 95% ethanol with final alcohol concentration of 70%. After each run the polysaccharides were separated by centrifugation at 4000 rpm for 30 min at 20 °C, dissolved in water and freeze-dried. FTIR spectra of lavender and linden PSCs (2 mg) after an exhaustive removing of alcohol-soluble molecules were obtained on a Nicolet™ Avatar (Thermo Fisher Scientific, Waltham, USA) spectrometer in a KBr tablet. The spectra were recorded in the range 4000 and 400 cm^{-1} and analyzed by the SpectraGryph software (Friedrich Menges).

2.6. Immunomodulatory effect

2.6.1. Ethics

Blood sampling procedure was in accordance with the Helsinki Declaration of 1975, as revised in 1983. All participants signed a written informed consent prior to initiation of the study. The animal experiments were approved by the Animal Research Committee of the Kitasato University, and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at the Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan.

2.6.2. Animal procedure and care

Female C3H/HeJ mice (6–8 weeks old) were purchased from Japan SLC (Shizuoka, Japan), and male ICR mice (4 weeks old) were from Charles River (Tokyo, Japan). The mice were housed in plastic cages in an air-conditioned room at 23 ± 2 °C with a relative humidity of 55 ± 10% under a 12 h-light-dark cycle, and fed a standard laboratory diet with water given *ad libitum*.

2.6.3. Flow cytometric analyses

Peripheral venous blood was collected from healthy volunteers (n = 3; gender = 2 men and 1 women, age range 25–39 years) using Venoject® tubes (Terumo Corporation, Japan). The blood samples were centrifuged at 1500*g* for 15 min. Then, the serum was removed and the red blood cells were lysed using 0.84% NH_4Cl buffer. The samples were washed twice with phosphate-buffered saline (PBS) buffer. The isolated white blood cells were pooled, centrifuged at 1000 rpm for 10 min and the cell pellet was re-suspended in a complete DMEM culture medium (Gibco, Invitrogen, USA) supplemented with 10% fetal calf serum and antibiotics. The cell suspension was seeded in four 25 cm^2 culture flasks (TPP, Switzerland), as one flask served as a control (cells cultured in a standard growth medium), the cells in the other three culture vessels were treated with 100 μg/ml purslane, lavender and linden PSCs, respectively for 48 h in a humidified incubator at 37 °C and 5% CO_2 content. At the end of the incubation period, the control and the PSC-treated cells were harvested and centrifuged at 1000 rpm for 10 min. The cell pellets were re-suspended in FACS buffer (PBS containing 5% fetal calf serum and 0.05% NaN_3). Then, the cells were stained with fluorochrome labeled antibodies specific for CD3, CD4, CD8, CD14, CD20, CD64 markers. The cell samples were incubated with antibody mixes for 10 min at room temperature in dark. After that, the cells were washed twice with the FACS buffer and acquired on a FC500 flow cytometer (Beckman Coulter, USA). The levels of different leukocytes were compared between the control and PSC-treated cells.

2.6.4. Reactive oxygen species (ROS) production from human whole blood phagocytes (WBP)

Heparinized (50 IU/ml) blood samples were obtained from the cubital vein of healthy human volunteers after overnight fasting. The kinetics of ROS production by WBP was analyzed by luminol-enhanced chemiluminescence (CL) for a period of 60 min at 37 °C, using 96-well white flat bottom culture plates on luminometer (Orion II Berthold Detection Systems GmbH, Germany). The principle of the method was previously described [46]. Briefly: 25 μl of ten times diluted blood with Hank's buffered salt solution (HBSS) were mixed with 25 μl 1 mM luminol (10 mM stock solution in 0.2 M borate buffer) HBSS solution and incubated with HBSS solutions of the three PSCs (10 and 100 μg/ml) for spontaneous (without activators) CL. Furthermore, 12.5 μl or 5 μl of ten times diluted blood mixed with 25 μl luminol were used for incubation of the samples with 25 μl (0.5 μg/ml) phorbol myristate acetate (PMA-activated, Sigma-Aldrich) or 25 μl (62.5 μg/ml) opsonized zymosan particles (OZP-activated). The final volume for spontaneous, PMA- and OZP-activated CL tests was 250 μl. The recorded values included the intensity of CL emitted during the studied time interval (integral of CL). The results are expressed as mean percentage of the control ± SEM from six independent experiments.

2.6.5. Ex vivo *immunomodulating activity against murine Peyer's patch (PP) cells*

Immunomodulating activity was measured by the enhanced production of bone marrow cell-proliferative cytokines from the PP cells of pathogen-free C3H/HeJ female mice ($Tlr4^{Lps-d}$), according to Hong et al. [47]. Suspensions (1.2×10^6 cells/ml, 180 μl) of PP cells (5–9 patches obtained per mouse) from the small intestine of the C3H/HeJ mice were cultured with H_2O (negative control), AMOL-1 (pectic polysaccharide from *Astragalus mongholics* as a positive control, Kiyohara et al. [48]) or 100 μg/ml of the three PSCs (20 μl) in a 96-well flat bottom culture plate (FALCON 3072) for 6 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The resulting culture supernatants (50 μl) were further cultured with a bone marrow cell suspension (5.0×10^5 cells/ml) from male ICR mice for 6 days. The numbers of proliferated bone marrow cells were measured by Alamar Blue™, as described previously [47]. IL-6 production from the murine PP cells stimulated by the tested samples was measured in the cell culture supernatants by ELISA, according to the company manual (BD Pharmingen) and as described by Kiyohara et al. [48]. The results are expressed as mean ± SEM.

2.6.6. In vitro *tumor cell growth inhibition*

The growth inhibitory effect of the three PSCs was studied against FL cell line (ATCC CCL 62, NBIMCC 94) derived from human

amniotic cells, normal human fibroblasts (isolated from testicular tissue; cultures obtained following the 5th passage) and different human tumor cell lines: A549 (ATCC CCL 185), LS180 (ATCC CL-187) and HeLa (ATCC CCL-2). All cell lines were expanded in 75 cm^2 culture flasks (TPP, Switzerland) in a DMEM growth medium, supplemented with 10% fetal calf serum and antibiotics. The cells were trypsinized and adjusted to a concentration of 2×10^5 cells/ml and then seeded on 96-well plates (TPP, Switzerland) with a working volume of 200 μl/well and cultured for 24 h at 37 °C, 5% CO_2 and high humidity. After that, the growth medium was replaced with a fresh medium containing 2, 10, 50, 100 or 200 μg/ml polysaccharide samples and the cells were cultured for 24, 48 and 72 h. At the end of each test-period, MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide) (Sigma–Aldrich) solution in a final concentration of 0.5 mg/ml was added to all wells. The cells were incubated for 4 h in a humidified incubator at 37 °C and 5% CO_2 content. Eventually, the MTT-containing medium was removed; 100 μl DMSO were pipetted to each test well and incubated for 15 min at room temperature on a shaker, in order to dissolve the formazan crystals accumulated in the cells. The absorbance was measured at 570 nm using a Synergy-2 spectrophotometer (BioTek, USA). The percent inhibition of cell growth was calculated using the mean absorbance units from each test sample and the mean data from the control cells incubated in the absence of polysaccharides. All samples were assayed in triplicates and the results are expressed as $IC_{50} \pm$ SEM (48 h).

2.7. Statistical analysis

Statistical significance was analyzed by the Student's *t*-test, using the Statview software (SAS Institute, Inc.). The mean results ± SEM were compared to those from the control. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Isolation and chemical characterization

In Table 1 are presented results from the chemical characterization of the three crude PSCs. The polyuronide content in the initial herbal materials ranges between 14.1–22.2%, which indicates the presence of pectic type polysaccharides in all of them. The differences between the polyuronides in the initial materials and the residues after extraction reveal that about 77%, 57% and 71% of the detected polyuronides in the used parts of purslane, lavender and linden are recovered by the double extraction with boiling water. The corresponding PSCs were obtained with yields of 8.6%, 3.2% and 6.1%, respectively. The three PSCs contain >55% total sugars. The AUAC in the samples is in the range 37.0–45.9%. The most of the AUAC in the purslane and lavender PSCs is represented by GalA according to the 3,5-dimethylphenol method (Table 1). Interestingly for the linden PSC we found 33.6% GalA and 14.3% GlcA by the 3,5-dimethylphenol, which in total is in agreement with the AUAC result (45.9%). The three PSCs contain methoxyl and acetyl esters in different extents, which are normally located in the GalA units in the pectins and rarely found in the neutral sugars [49]. The linden PSC has the lowest methoxyl (2.5%) and the highest acetyl content (3.3%) in comparison with the other two samples. The lavender and purslane PSCs have also relatively high acetyl contents. The AUAC and methoxyl-ester content of the linden PSC are in a good agreement with the data obtained by Yakovlev [50], however he did not mention the presence of acetyl groups. According to the HPSEC-RID analysis, linden PSC is characterized by two main populations with molecular weights 1.23×10^6 and 4.72×10^4 g/mol. The other two samples are characterized by predominant populations with molecular weights in the range 1.5×10^4–5.3×10^4 g/mol.

Table 1
Chemical characterization of the three herbal PSCs. The results are presented as mean (w/w% DS).

	Purslane PSC	Lavender PSC	Linden PSC
Polyuronides[a], initial herb [%]	14.1	15.5	22.2
Polyuronides[a], residue after extraction [%]	3.3	6.6	6.5
Yield [%]	8.6	3.2	6.1
Total sugars[b] [%]	82.5	64.9	56.0
AUAC [%]	43.2	37.0	45.9
GalA[c]	40.1	35.8	33.6
DM[d] [mol%]	68.2	70.8	40.5
(Methoxyl content [%])	(5.0)	(4.6)	(2.5)
DA[d] [mol%]	16.1	25.7	40.4
(Acetyl content [%])	(1.6)	(2.3)	(3.3)
Molecular weight distribution [g/mol]	5.3×10^4 (49.0)	1.6×10^6 (2.5)	1.23×10^6 (67.5)
	1.7×10^4 (51.0)	1.5×10^4 (97.5)	4.72×10^4 (32.5)
Monosaccharide composition [%]			
Ara	9.5[e] (11.0)[f]	5.7 (5.4)	4.4 (4.8)
Rha	3.8 (1.1)	2.5 (0.8)	11.5 (1.2)
Fuc	trace	0.2 (0.1)	trace
Xyl	1.4 (1.0)	1.6 (1.0)	1.2 (0.8)
Man	0.7 (0.5)	2.3 (1.0)	(0.1)
Gal	11.3 (10.8)	6.3 (5.2)	3.3 (2.8)
Glc	13.2 (10.1)	5.1 (3.1)	1.0 (0.5)
GalA	39.1	35.8	24.0
GlcA	3.5	5.4	10.6
Protein content [%]	3.3	2.2[g]	2.2
Total phenolics [%]	0.8	11.0	2.7
Presence of saponins	+	+	+

[a] Determined titrimetrically according to Owens et al. [34].
[b] Sum of the monosacharides determined by the HPAEC-PAD analysis.
[c] Determined on the basis of the differentiation between GalA and GlcA with the 3,5-dimethylphenol method.
[d] Calculated on the GalA basis, moles of methoxyl or acetyl groups per 100 mol of GalA.
[e] Monosaccharide composition determined by HPAEC-PAD analysis.
[f] Neutral monosaccharide composition determined as alditol-acetates.
[g] Determined after an exhaustive treatment with 95% ethanol.

Because of the proposed complex polysaccharide composition of the samples, two different techniques for determination of the monosaccharides were used. When we compared the results from both monosaccharide composition analyses, we found that the neutral monosaccharides and especially Rha are detected in lower amounts after H_2SO_4 hydrolysis, as was already described [31,44] (Table 1). For the comments presented below and for estimation of the total amount of sugars are used the data from the HPAEC-PAD analysis. The monosaccharide composition confirmed that the three samples contain pectic polysaccharides with predominant GalA and the neutral monosaccharides Ara, Gal and Rha. They also have Glc, Xyl, Man and Fuc, as the latter is related to RGII structures [11]. The purslane PSC is characterized by the highest GalA content within the three PSCs, which indicates that the HG fragments in the purslane pectins are in considerable amounts. It has also the highest Glc content and relatively comparable amounts of Gal (11.3%) and Ara (9.5%). The sum GalA + GlcA in this sample is 42.6%, which is in agreement with the determined AUAC (43.2%). The ratio Gal/Ara in the complex is 1.2 and it is comparable to the ratio of 1.1 found by Amin & El-Deeb [26] in mucilage B from the leaves of *P. oleracea*. It was found that *P. oleracea* contains an AG polysaccharide with a low GlcA content that was elucidated to be AGII [27,28]. The high Glc content in the PSC could be attributed to the presence of different glucans, as previously found [27,28].

Similarly, the lavender PSC is characterized by high Gal (6.3%), Ara (5.7%) and Glc (5.1%) contents. To best of our knowledge, there are not studies on the polysaccharides from lavender described in the literature so far. The considerable amount of GalA and the low Rha content indicate that the HG segments are predominantly presented in the lavender polysaccharides. The ratio Gal/Ara in the lavender PSC is 1.1, suggesting the presence of AG structures similarly to the purslane PSC. Furthermore, the detected Fuc is indicative for presence of RGII structures. Interestingly, it has relatively high Man (2.3%) and Glc (5.1%) contents. Both purslane and lavender PSCs contain low amounts of GlcA, which is in agreement with other studies on herbal polysaccharides [13,48]. The results for GalA of both PSCs are in a good agreement with the colorimetric data (Table 1).

The linden PSC is characterized by high Rha and GlcA contents, as the latter finding is confirmed by the colorimetric quantification of GalA and GlcA. The high Rha content, in addition to the high GalA amount suggest the existence of a RGI fragment in a representable proportion in the linden PSC. The GlcA is present in a surprisingly high content, comparable to the amount of Rha. We calculated that GlcA is 30%-31% from uronides in the linden PSC on the basis of the colorimetric and chromatographic determination of both uronic acids. Similarly, Kram & Franz [31] and Yakovlev [50] found high Rha content in the crude linden polysaccharide, and the first group mentioned the simultaneous presence of GalA and GlcA, but they did not provide any quantitative data for both uronic acids. The linden PSC had the lowest Gal content, but we found by the Yariv's test that the sample contains the immune active AGII structures (data not shown).

The purslane and linden PSCs are also characterized by low protein and polyphenol contents. The lavender PSC has a high polyphenol content (11%), which interferes with the quantification of total proteins, as was observed by Kiyohara et al. [9]. After an exhaustive removing of the alcohol-soluble molecules from the lavender PSC, it was determined that it contains 2.2% proteins by the Bradford method. The presence of saponins was evaluated only qualitatively by the vanillin-sulfuric acid method because of a possible Rha interference. The qualitative and quantitative analyses of the non-carbohydrate constituents were necessary for a better explanation of the observed immunomodulatory effects.

3.2. FTIR spectroscopy

After an exhaustive removing of alcohol-soluble molecules from the lavender and linden PSCs, the recovered PSCs were analyzed by FTIR spectroscopy (Fig. 1). Both spectra contain the typical bands for polysaccharides and pectins: 3446, 2920 (2929), 1751, 1734, 1635 and 1420, 1146 (1151) cm^{-1}, arising from O-H of hydroxyl groups, C-H of CH_2, C=O of methoxyl ester group ($COOCH_3$), C=O of acetyl ester group ($OCOCH_3$), C=O of ionized (free) carboxyl group and C-O-C of the glycosidic bond (or within the ring), respectively [51]. For the linden PSC the signal at 1751 cm^{-1} is negligible and the peak at 1734 cm^{-1} is with equal intensity to the signal at 1716 cm^{-1}. The latter is proposed to arise only from ν(C=O) of the acetyl group [51]. The absorption band at 1255 or 1244 cm^{-1} is from ν(C-O-C) of the acetyl group [51]. The absence of a characteristic peak at 1751 cm^{-1}, the higher intensities of the two signals at 1255 and 1716 cm^{-1} in the spectrum of linden PSC than in the lavender PSC spectrum confirm its lower DM and higher DA. Furthermore, the signal at 1373 (1375) cm^{-1} originates from $\delta_s(CH_3)$ of ester groups. It is clearly seen that the intensity of the band at 1635 cm^{-1} for the linden PSC is higher than this in the spectrum of lavender PSC, which is related to the higher AUAC and free COOH derived from the GalA and GlcA in the first sample. The region of 1200–1000 cm^{-1} is the so called "fingerprinting region" for each pectin and it contains mainly the following skeletal ring vibrations: C-OH, C-C and C-O-C glycosidic bond vibrations [52]. We found five signals in this region for the lavender PSC – 1146, 1101 (νC-O ring), 1076, 1051 and 1016 (νC-OH, C-C) cm^{-1} and three signals for the linden PSC – 1151, 1070 and 1041 cm^{-1}. The bands at 1101 and 1019 cm^{-1} are the strongest for the lavender PSC, indicating the predominance of the HG structure in it. Conversely, for the linden PSC the signals at 1070 and 1041 cm^{-1} show that it contains a considerable amount of the RGI structure [53]. In both spectra are found bands in the anomeric region (C1-H, ring) at 822 or 833 cm^{-1} and at 908 (893) cm^{-1} for $\gamma(C\text{-}OH)_{ring}$ characteristic for α- and β-linked aldopyranoses [51]. In the region 1600–1500 cm^{-1} are expected signals derived from different functional groups, such as COOH, NH_2 and aromatic ring vibrations.

3.3. Immunomodulating effects

3.3.1. Effect on human white blood cells

The effect of the three PSCs on various human white blood cells was studied at a concentration of 100 μg/ml by a flow cytometric immunophenotyping analysis (Fig. 2). The purslane PSC stimulated $CD4^+$ cells, whereas the lavender PSC stimulated $CD8^+$ cells. The three samples did not express stimulating activity on $CD20^+$ cells, representing B-cell population. They activated to different extent $CD25^+$ cells, which in addition to the B-cells are also related to T-cells. The linden and lavender PSCs even showed inhibitory activity on $CD20^+$ B-cells, which could be partly associated with the non-carbohydrate constituents. The purslane and lavender PSCs exhibited activity on $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells, and the stimulatory effect of the linden PSC was weak. In addition to the effect on the T-cell subpopulations the herbal polysaccharides can activate phagocytes and thus again exhibit immunostimulating activity. The three PSCs showed activity on $CD14^+$ cells or monocytes. The linden and lavender PSCs had activity on $CD64^+$ cells, representing mainly macrophages. Conversely, the effect of purslane PSC on the $CD14^+$ cells was not observed on the $CD64^+$ cells, which could be difficultly explained. We also found that all samples increased IL-6 production from white blood cells, which was in agreement with the stimulation of monocytes and T-cells (data not included).

3.3.2. Effect on ROS formation by human WBP

The three herbal PSCs stimulated to different extent the oxidative burst in WBP at 10 and 100 μg/ml, which was expressed by the increase in spontaneous CL response, as a measure of ROS generation (Fig. 3). The highest activity at 100 μg/ml was observed for the purslane PSC and then for the linden PSC. Both samples have some important differences in the monosaccharide composition in respect to Gal, Ara, Glc, GalA, Rha and GlcA contents and in their molecular weights. The purslane PSC is characterized with higher Gal, Ara and Glc, which are indicators for presence of immune active AGs and glucans. On the other hand, the lavender PSC expressed a higher stimulating activity at 10 μg/ml than at 100 μg/ml, which was expected because of the mimic antioxidant effect from polyphenols in it. As can be seen from Fig. 3 the control values for PMA- and OZP-activated CL were much higher than those for the spontaneous CL response of the polysaccharide-treated cells. Interestingly, the three PSCs did not increase CL response of the PMA-activated phagocytes. The lavender PSC decreased the PMA-activated CL at 100 μg/ml, expressing a suppressive or protective-like effect on the activated cells. This mode of action was related to polyphenols, as reviewed by Lojek et al. [16]. Similarly, the lavender PSC induced a decrease of the OZP-stimulated CL at 100 μg/ml. Contrary, purslane and linden PSCs increased the CL response in the OZP-treated cells at both studied concentra-

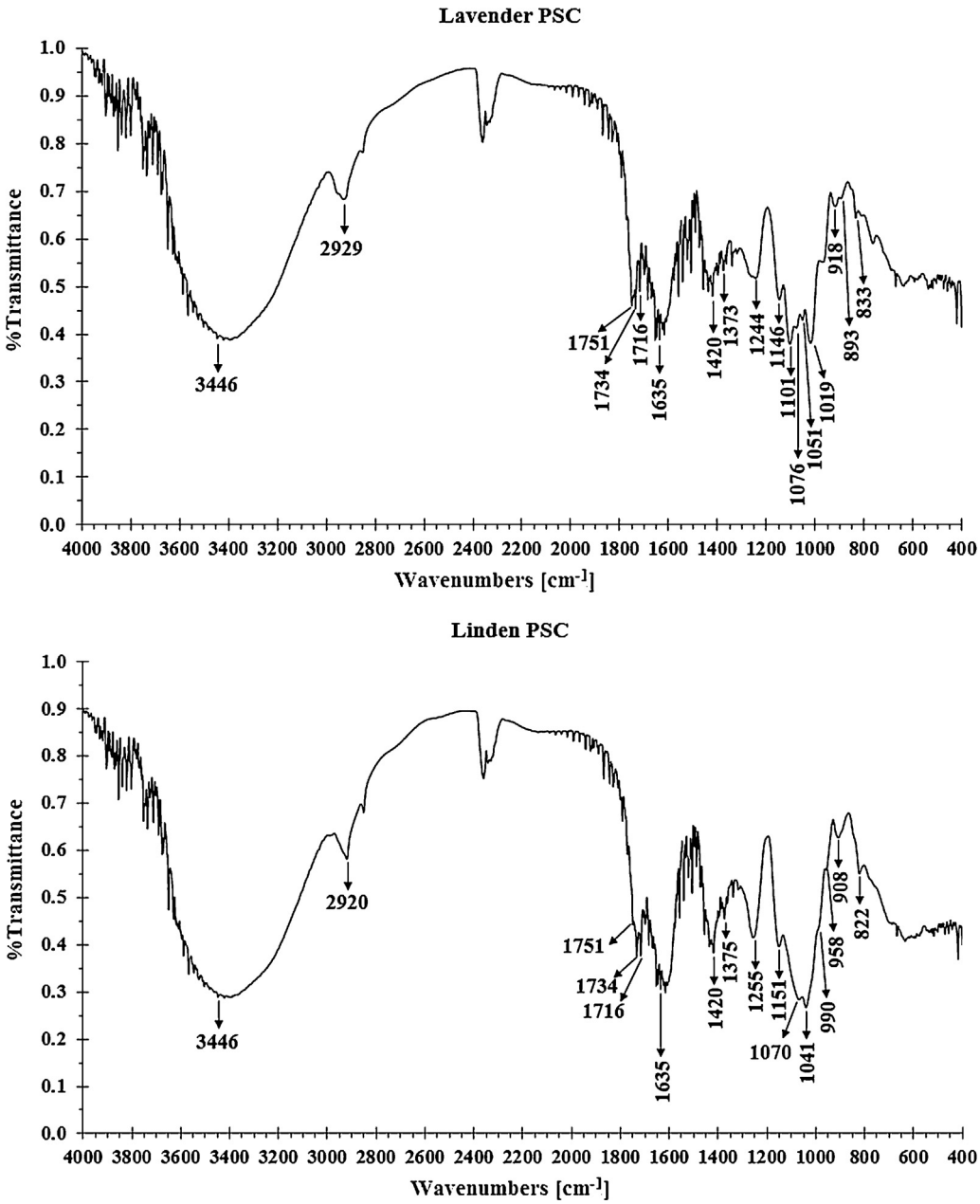

Fig. 1. FTIR spectra of selected herbal PSCs.

tions, which was probably in agreement with the spontaneous ROS generation.

3.3.3. *Intestinal immunomodulating activity*

In the current study the three PSCs exhibited *ex vivo* intestinal immunomodulating activity through the PP-mediated bone marrow cell proliferation test and increased IL-6 production from the PP cells in comparison with the positive control AMOL-1 (Fig. 4). The PP cells were isolated from the LPS low responder ($Tlr4^{Lps-d}$) C3H/HeJ mice, thus it was considered that the observed activity was not a result of possible LPS contamination. The bone-marrow cell proliferation was measured to estimate the relative production of hemopoietic growth factors from the polysaccharide-stimulated PP cells. The highest stimulating activity was expressed by the purslane PSC, followed by the linden and lavender PSCs, as in the same order was IL-6 production. The purslane PSC even induced a higher IL-6 production than the positive control. Therefore, the IL-6 production from the white blood cells induced by the three herbal PSCs was also confirmed by the animal immune cells. The control of IL-6 production in the PP cells is important because this cytokine can induce IgA antibody production through the B-cell proliferation in the patches.

3.3.4. *Anti-tumor activity* in vitro

In Table 2 are presented IC_{50} values of the three PSC samples against the growth of different human tumor cell lines after 48 h of incubation. Normal human fibroblasts (NF) and amniotic cells (FL) were used as controls. The linden PSC was the most active sample, expressing inhibitory activities against A549, HeLa and LS180 cells with IC_{50} values after 48 h of incubation as follows: 92.6 ± 2.7 μg/ml, 96.7 ± 1.6 μg/ml and 185.9 ± 6.3 μg/ml, respectively. The IC_{50} values for A549 and HeLa cell lines after 48 h of culturing with the lavender PSC were 162 ± 2.6 μg/ml and 170 ± 2.5 μg/ml, and for the purslane PSC were 169.9 ± 9.2 μg/ml

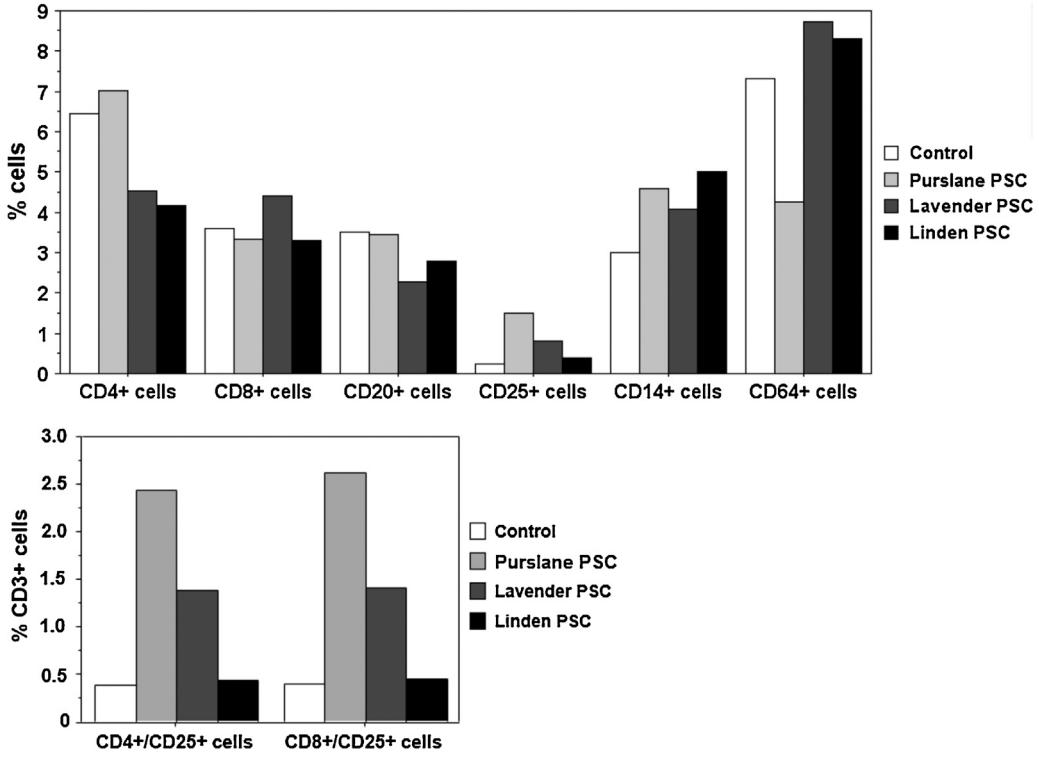

Fig. 2. Flow cytometric analysis for immunophenotyping of human white blood cells treated with 100 μg/ml purslane, lavender and linden PSCs.

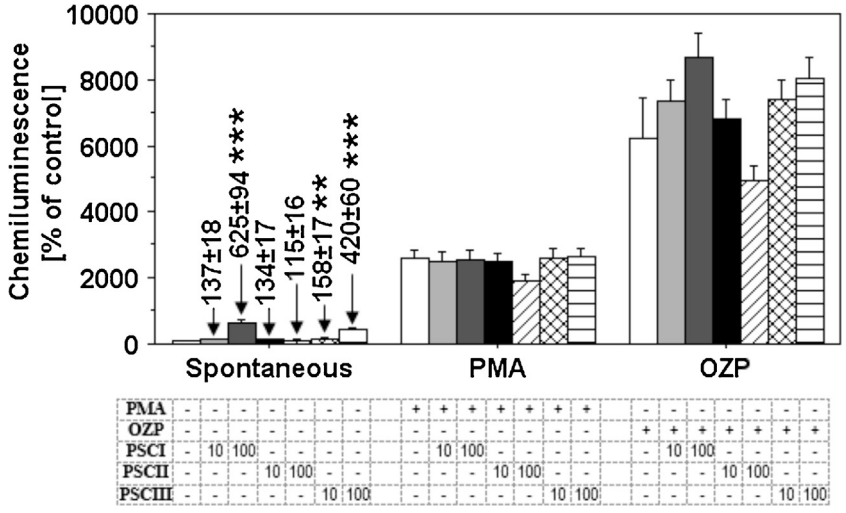

Fig. 3. Effect of purslane (PSCI), lavender (PSCII) and linden PSCs (PSCIII) on ROS production from non-stimulated (spontaneous), PMA- and OZP-activated whole blood phagocytes. 10 (10 μg/ml) and 100 (100 μg/ml). The asterisks indicate statistical difference (**p < 0.01, ***p < 0.001) vs. control.

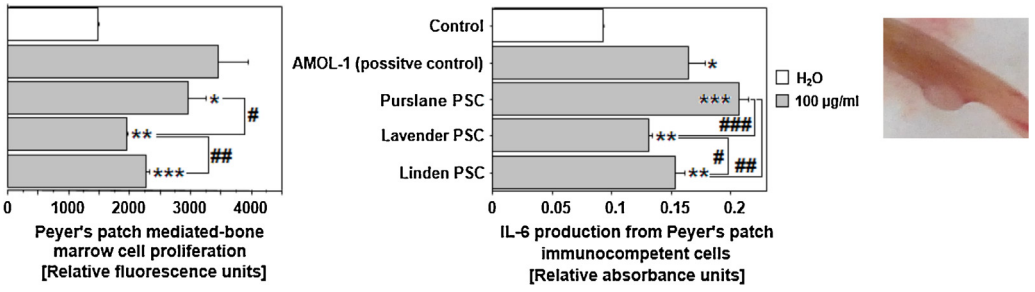

Fig. 4. Enhancement of production of bone marrow cell-proliferating cytokines from Peyer's patch cells cultured in presence or absence of purslane, lavender and linden PSCs. (A). Enhancement of production of IL-6 from Peyer's patch immunocompetent cells cultured in presence or absence of polysaccharides (100 μg/ml) (B). The symbols indicate statistical difference (*p < 0.05, **p < 0.01, ***p < 0.001) vs. water control or among samples (#p < 0.05, ##p < 0.01, ###p < 0.001). AMOL-1 is an active pectic polysaccharide isolated from *A. mongholics*, containing AGII.

Table 2
IC_{50} values (± SEM) of the three herbal PSCs on the growth of different cell lines after 48 h of treatment.

Polysaccharide complex	Cell line	IC_{50} [μg/ml]
Purslane PSC	NF[a]	>200
	FL[b]	173.9 ± 1.6
	A549[c]	169.9 ± 9.2
	HeLa[d]	195.4 ± 0.9
	LS180[e]	>200
Lavender PSC	NF	>200
	FL	183.7 ± 5.4
	A549	162.0 ± 2.6
	HeLa	170 ± 2.5
	LS180	>200
Linden PSC	NF	>200
	FL	167.5 ± 17.2
	A549	92.6 ± 2.7
	HeLa	96.7 ± 1.6
	LS180	185.9 ± 6.3

[a] **NF** (normal fibroblasts).
[b] **FL** (normal amniotic cells).
[c] **A549** (lung adenocarcinoma).
[d] **HeLa** (cervical adenocarcinoma).
[e] **LS180** (colon adenocarciona cells).

and 195.4 ± 0.9 μg/ml. The three PSCs were the most effective against the lung adenocarcinoma A549 cells. It was found that only the linden PSC expressed anti-proliferative activity against the growth of colon adenocarcinoma LS180 cells with IC_{50} value under 200 μg/ml. However, the results from the control treatment of NF and FL cells with the three PSCs showed that control cells were inhibited in a similar manner like abnormal cells. This means that the three samples exhibited non-specific inhibitory activity on the basis of the chosen control cell lines.

4. Discussion

Only on the basis of the monosaccharide composition data and the ester content (Table 1) could be suggested that the purslane and lavender PSCs contain methoxylated and acetylated pectic polysaccharides with predominant HG fragments, followed by AG structures most probably attached in the RGI fragments. The chemical features of the purslane PSC, such as the highest Gal, Ara and Glc contents among the three PSCs must be important for the potent activity of this sample in most of the observed effects. Similarly, it is proposed that the linden PSC contains high molecular weight pectins with considerable DA and low DM and with high RGI and GlcA contents, which could also influence the observed activities. From the three studied PSCs only polysaccharides of the purslane are investigated previously for expression of *in vitro* and *in vivo* immunomodulating activity [19,29]. Our findings on the stimulation of human white blood cells (Fig. 2) showed that the purslane PSC elevated the $CD4^+$ and $CD25^+$ cells, $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells, and $CD14^+$ monocytes. The lavender PSC stimulated the $CD8^+$ and $CD25^+$ cells, T-cells and phagocytes. The linden PSC expressed activity mainly against the $CD14^+$ and $CD64^+$ cells. CD14 and CD64 proteins related to innate immune response are expressed on phagocytes, whereas CD4 and CD8 are related mainly to T-cells and adaptive immunity. CD64 or FcγRI receptor is expressed on monocytes, macrophages, neutrophils and other granulocytes and it interacts with antibody-coated antigens (IgG-Ag), thus triggering cell activation, such as phagocytosis and antibody-dependent cell-mediated cytotoxicity [54]. Its increased CD64 expression in treated with the studied herbal PSCs blood cells could mean Ig interaction with polysaccharides or other molecules in them [55]. When herbal pectins and probably their AG side chains interact with neutrophils through different receptors, they serve as antigens and similarly to macrophages activate phagocytosis with production of ROS [2]. The neutrophils comprise between 50 and 70% of all leukocytes in the blood and they are considered as main contributor to the total luminol CL [16]. Furthermore, the neutrophils exhibit more rapid rates of phagocytosis and a more intense oxidative response than the macrophages. Only the lavender PSC showed inhibitory effects on the PMA- and OZP-activated ROS generation (Fig. 3). Unlike the lavender, purslane and linden PSCs have low phenolic content and did not show any prominent antioxidant activity by the ORAC method (data not included). Similar effects to these of the lavender PSC on the spontaneous, PMA- and OZP-activated WBP were observed by Nikolova et al. [56] for leek pectic polysaccharides, which also showed antioxidant activity through the ORAC method. PMA is a potent inflammatory activator of protein kinase C, which could induce NADPH-oxidase activity and further the neutrophil degranulation and death [57]. OZP are opsonized zymosan particles that are coated with immunoglobulins and some complement factors which could be recognized through Fc and CR3 on neutrophils or other phagocytic leukocytes [58]. The stimulation of WBP by the three studied PSCs was in agreement with the elevated levels of $CD14^+$ and $CD64^+$ cells (Fig. 2). Therefore, the studied herbal PSCs could activate phagocytic leukocytes, which are involved in the first innate mechanism for immune defense against different pathogens and in inflammation processes. Interestingly, polysaccharides can affect positively the immune potentiation and negatively the inflammation [3]. This dual mode of action makes them perspective candidates for development of polysaccharide-derived healthcare products.

It was proposed that the studied herbal PSCs stimulated Tregs cells, represented most probably by the $CD4^+/CD25^+$ T-cells. The $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cell subpopulations are responsible for regulation of the immune response in autoimmune, tumor diseases and immune homeostasis. The stimulatory effect of herbal polysaccharides on T-cells could be useful in the supplementary therapy of infectious and tumor diseases [59]. For example, Shen et al. [19] demonstrated that polysaccharide POP from *P. oleracea* exhibited a potent anti-tumor activity *in vivo* and increased the animal's immune responses, including increase in the number of white blood cells and $CD4^+$ T-cells, as well as the ratio of $CD4^+/CD8^+$ cells. Similarly, Shu et al. [60] showed that polysaccharide PSSC from *Salvia chinensis* expressed an anti-tumor immunostimulatory activity *in vivo* and elevated $CD4^+$ T-cells in both spleen and lymph nodes and the cytotoxic activity of NK cells and $CD8^+$ T-cells.

Herbal polysaccharides are mainly administrated in the human organism by herbal infusions, foods or other formulations. They can express intestinal immunomodulating activity through interaction with immunocompetent cells in the PPs localized in the small intestine [2]. Although, polysaccharides are little absorbed by intestinal epithelial cells, their communication with the epithelium could also lead to immunomodulation of the underlying immunocompetent cells [10]. The AGII, together with the AGI structures in pectins are important for expression of the intestinal immunomodulating activity through the PPs [2]. It was found by the Yariv's test that the linden PSC contains AGII. Similarly, literature data indicate that the purslane pectins also contain AGII [28]. Therefore, the purslane PSC logically showed the most potent activity because it had the highest amount of Gal, which was assumed to be mostly linked in the AGII and AGI structures. It also contained a considerable amount of Glc, derived probably from starch that activates PP cells as well [61]. Our results were in a good agreement with those obtained for a lignin-carbohydrate complex from the Kampo extract preparation TJ48 (Juzen-Taiho-To), BP-II pectic fraction from *Biophytum petersianum* and RP-1 pectic fraction from *Rosa damascena* [9,13,62].

Murine PPs contain about 60% B-cells ($B220^+$), 25% T-cells ($CD3^+$), 10% dendritic cells ($CD11c^+$), and less than 5% macrophages ($F4/80^+$) or polymorphonuclear neutrophils ($Ly\text{-}6G^+$). Among the

T-cells 45% are $CD4^+$, 35% are $CD8^+$ and 20% are $CD4^-/CD8^-$ T-cells. Among the $CD4^+$ T-cells, 85% are memory T-cells ($CD25^-CD45RB^{lo}$), 10% are Naïve ($CD25^-CD45RB^{hi}$) and 5% are regulatory T-cells ($CD25^+CD45RB^{lo}$). Furthermore, bone marrow contains T-cells, B-cells, neutrophils, dendritic cells and monocytes, as it has 8%–20% lymphocytes with T/B-cell ratio of 5:1. The mouse bone marrow contains 1–5% $CD3^+$, 1.5% $CD4^+$, 2.0%–2.5% $CD8^+$ T-cells. Importantly, $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells are found to be about 10% and 5% in the human PPs and 5–30% of the $CD4^+$ T-cells in the mouse bone marrow are $CD4^+/CD25^+$ Tregs cells [63,64]. Therefore, on the basis of our results with human white blood cells (Fig. 2) and WBP (Fig. 3), it could be proposed that the studied polysaccharides expressed activity against the PP mediated-bone marrow cell proliferation through the phagocytic leukocytes and T-lymphocytes. Additionally, the three samples induced IL-6 production from PP cells and human white blood cells, which is produced by T-cells and macrophages [2]. Administration of herbal polysaccharides active against the intestinal immune system could support the compromised immune health. For example, Hou et al. [59] found that treatment (i.p.) of mice suffering from polymicrobial sepsis with 100 and 200 mg/kg *Astragalus* polysaccharide APS increased the percentage of activated Th cells in the PPs.

When it was found that the herbal PSCs expressed immunostimulating activity against innate and adaptive human immune cells and intestinal murine PP cells, it was also important to be tested their effect on abnormal cells. This was necessary in respect to the perspective application of the herbal PSCs and related products in the prophylaxis and prevention of infectious, autoimmune and tumor diseases. Only the anti-tumor activity of purslane polysaccharides was investigated, so far [29,30]. Zhao et al. [30] obtained IC_{50} = 489 μg/ml after 48 h treatment of HeLa cells with polysaccharide fraction POL-P3b isolated from *P. oleracea*, which was 2.5 fold higher than those we obtained for the purslane PSC (Table 2). The purslane PSC expressed a higher *in vitro* activity, but this could be related primarily to the presence of a mixture of purslane neutral and pectic polysaccharides, as well as to the supplementary effect of smaller molecules, which were expected to be <18% (Table 1). Our results with the A549 cells for the purslane and lavender PSCs were in agreement with those obtained for crude polysaccharides extracted from the fruits of *Cyphomandra betacea*, which showed IC_{50} = 170 μg/ml after 48 h with A549 cells [65]. Even though, the combinatorial effects of different compounds in herbal extracts are rather complex, it cannot be excluded that the polysaccharides and some of the non-carbohydrate constituents act in a synergistic manner. For example, it was found that supplementation of breast cancer patients undergoing postoperative radiation therapy with aronia juice enriched with apple pectin led to an immune system stimulation expressed by a significant increase of the $CD4^+$ and $CD8^+$ T-cells levels [66]. Although, studies of the polysaccharide effect on LS180 cells are still scarce, the anti-tumor activity of the herbal polysaccharides against gastrointestinal tumors expands their immunomodulating potential and deserves more studies for their protective and suppressive effects. However, our results showed that the studied herbal PSCs expressed non-specific *in vitro* anti-proliferative activity against normal and tumor cells. This effect needs to be further studied with the purified polysaccharide fractions, otherwise it seems rather unspecific and not promising.

Further immunomodulating characterization of the purslane PSC is necessary because of the increasing popularity of purslane as a food and its use in the functional and dietary nutrition [23]. The immunomodulating studies on the lavender and linden PSCs are important primarily due to the traditional use of these herbs and their water extracts in the folk medicine and cosmetics. The increasing lavender oil production implies better utilization of the bioactive compounds, including the pectins in the distilled residue into secondary value-added products, such as water extracts for nutraceuticals and cosmetics. We found that the polyuronides, representing pectins in industrially distilled lavender flowers are 18.9% with a very close value of 19.1% for the stalks, thus the obtained by-product could be a source of bioactive polysaccharides in addition to smaller molecules.

For further explanation of the biological results it was necessary to differentiate between the herbal polysaccharides and the other constituents in the samples. This was done for linden and lavender PSCs in subsequent studies, confirming the polysaccharide role in the observed immunomodulating activities and will be published.

5. Conclusion

We demonstrated that similarly to the edible herb purslane, the other two widely used medicinal plants – common lavender and silver linden contain also immune active acidic PSCs rich in pectic polysaccharides. Interestingly, GlcA is about 31% of the uronides (HPAEC-PAD analysis basis) in the linden PSC, which is also characterized by high Rha content in addition to high GalA amount, suggesting the existence of a considerable amount of a RGI fragment in this sample. The three PSCs expressed *ex vivo* stimulating activity against different blood T-cell populations and phagocytic leukocytes. These herbal PSCs also stimulated *ex vivo* ROS production from WBP and inhibited non-specifically the growth of different tumor cell lines *in vitro*. Furthermore, they showed *ex vivo* intestinal immunomodulating activity against PP cells at 100 μg/ml. It was proposed that the studied herbal PSCs can modulate the PP immunocompetent cells through the $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells and phagocytes, because they are also found in the patches and in the bone marrow. The purslane PSC expressed the highest stimulating activity against normal immune cells and the linden PSC showed the highest inhibitory activity against tumor cells with an IC_{50} value of 93 μg/ml for A549 cells. Our results suggest that similarly to the purslane polysaccharides, these isolated from lavender and silver linden have also perspectives to be studied their supplementary role in treatment of health conditions characterized with compromised immune system.

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The common lavender (*Lavandula angustifolia* Mill.) pectic polysaccharides modulate phagocytic leukocytes and intestinal Peyer's patch cells

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ABSTRACT

Two pectic (chPS-L1, chPS-L2) and one polyphenolic (chPP-L) fractions were obtained from lavender flowers after boiling water extraction, exhaustive removing of alcohol-soluble molecules and SEC. chPS-L1 (52.4 kDa) contains mainly low-acetylated and high-methoxylated homogalacturonans (HG), and smaller rhamnogalacturonan (RG) I backbone fragments rich in 1,3,5-branched arabinan and arabinogalactan (AG) II side chains. chPS-L2 (21.8 kDa) contains predominantly similarly esterified HG, followed by RGI with AGII structures and RGII. The prevalence of catechin and epicatechin in chPP-L indicates that they form weak interactions with pectins. chPS-L1 and chPS-L2 enhanced β_2-integrin expression on neutrophils, inducing ROS generation and macrophage NO production. Both the effects on β_2-integrin and high complement fixation activity of chPS-L1 were proposed for its inhibitory action against PMA- and OZP-activated ROS formation. This, together with suppression of NO generation after co-stimulation with chPS-L1 and LPS, suggested anti-inflammatory activity of studied pectins. Lavender polysaccharides expressed intestinal Peyer's patch immunomodulating activity.

Abbreviations: AGII, arabinogalactan type II; AUAC, anhydrouronic acid content; BSA, bovine serum albumin; CD18, marker for $\beta_2$integrin; CD62L, marker for L-selectin; chPP-L, polyphenolic fraction obtained after SEC from lavender PSC2; chPS-L1, first fraction of SEC purified lavender pectic polysaccharides; chPS-L2, second fraction of SEC purified lavender pectic polysaccharides; CL, chemiluminescence; CR3, omplement receptor 3; DA, degree of acetylation; DM, degree of methoxylation; DSS, sodium 4,4-dimethyl-4-silapentane-sulfonate; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; FTIR, Fourier transform infrared spectroscopy; HBSS, Hank's buffered salt solution; HG, homogalacturonan; IC_{50}, a concentration giving 50% hemolysis of erythrocytes; iNOS, inducible nitric oxide synthase; KDO, 3-deoxy-D-manno-2-octulosic acid; Lavender PSC, crude pectic polysaccharide complex from *Lavandula angustifolia*; Lavender PSC2, obtained from lavender PSC after exhaustive ethanol treatment; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; NMR, Nuclear magnetic resonance spectroscopy; NO, nitric oxide; OZP, opsonized zymosan particles; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PP, Peyer's patches; RGI, rhamnogalacturonan type I; ROS, reactive oxygen species; SEC, size-exclusion chromatography; WBP, whole blood phagocytes.

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1. Introduction

In Bulgaria common lavender, *Lavandula angustifolia* Miller (Lamiaceae) is cultivated predominantly in the Rose valley and since 2011 the country is the biggest lavender oil producer worldwide. Already Dioscorides (c. 40–90 AD) documented that lavender has laxative and invigorating properties and advised its tea for chest complaints (Castle & Lis-Balchin, 2002). The German Commission E has approved *L. angustifolia* tea for treatment of restlessness, insomnia and nerve disorders of the intestines. The lavender flowers express also anti-microbial, repellent, anti-inflammatory, antioxidant and diuretic activities, which are mainly attributed to the essential oils (Laekeman, 2012). However, the lavender contains also non-volatile biologically active constituents, such as triterpenoids, flavonoids and lignans (Harborne & Williams, 2002).

In a previous study, it was shown that a crude pectic polysaccharide complex (lavender PSC) from *L. angustifolia* expresses *ex vivo* immunomodulating activity against phagocytic leukocytes, T-lymphocytes and intestinal Peyer's patch cells (Georgiev, Ognyanov, Kiyohara et al., 2017). Pectic polysaccharides express different bioactivities outside the plants when they are properly extracted. Pectins contain three main building blocks – HG, RG type I and II and substituted galacturonans (Voragen, Coenen, Verhoef, & Schols, 2009). Generally, the immunomodulating activity of pectic polysaccharides is explained by the presence of immune active AG type I and II structures O-4 attached to Rha of the RGI domain (Yamada & Kiyohara, 2007; Wangensteen, Diallo, & Paulsen, 2015). Additionally, in a recent study we found that highly glucuronidated RGI isolated from *Tilia tomentosa* also exhibits immunomodulating activity (Georgiev, Paulsen et al., 2017). Immune cells express signaling receptors, such as complement receptor 3 (CR3), Fc receptor, toll-like receptors and scavenger receptors that can interact with polysaccharides to initiate up- or down-regulation of numerous immunological responses (Leung, Liu, Koon, & Fung, 2006). The immunomodulating properties of pectins are shown by exhibition of moderate complement fixation activity, activation and regulation of phagocytic leukocytes, B- and T-lymphocytes, thrombocytes, suppression of tumor cells, etc (Yamada & Kiyohara, 2007; Wangensteen et al., 2015). Because of these effects, pectins are included in *in vivo* studies and some clinical trials for their supplementary role for treatment of socially significant diseases, as reviewed by Georgiev, Ognyanov, Denev et al. (2017). Furthermore, together with smaller molecules, pectic polysaccharides are partly extracted during preparation of lavender teas or decoctions. In this way, polysaccharides are coupled with naturally occurring compounds like polyphenols, forming bioactive mixtures with combinatorial effects (Jakobek, 2015). At the same time, the increasing rate of lavender cultivation for perfume and healthcare industries expands development of different lavender-derived products and needs a proper utilization of the waste after distillation.

The aim of the current study is to characterize the chemical structure and immunomodulating properties of *L. angustifolia* pectic polysaccharides purified from the immune active crude lavender PSC obtained with boiling water (Georgiev, Ognyanov, Kiyohara et al., 2017).

2. Materials & methods

2.1. Plant material

Lavender flowers from *Lavandula angustifolia* Miller (syn. *L. vera* DC) sort "Sevtopolis" (Institute of Roses, Essential and Medical Cultures, Kazanlak, Bulgaria) were provided dried by ET "Ve Pe Pi – Vesko Pipev" (Velingrad, Bulgaria).

2.2. Isolation and purification of lavender flower polysaccharides

The crude lavender polysaccharide complex (lavender PSC) was extracted with boiling water, as previously described (Georgiev, Ognyanov, Kiyohara et al., 2017).

Lavender PSC was dissolved in water (10 mg/mL) and precipitated five times (each for 24 h) with three volumes of 95% ethanol. This step was for partly removing of ethanol-soluble molecules prior to size exclusion chromatography (SEC). After each run, the polysaccharide-containing precipitates were obtained by centrifugation at 4000 rpm for 30 min at 20 °C. Then, the recovered polysaccharide-containing fraction (lavender PSC2) was dissolved in water, centrifuged and the supernatant was applied to a Sephacryl® S-200 Superfine (GE Healthcare Life Sciences) column (100 × 1.5 cm) coupled with a FPLC BioLogic® LP system (BioRad). The sample was eluted with 0.2 M NaCl at 0.2 mL/min flow rate and 5 mL fractions were collected. Two pectic fractions chPS-L1 and chPS-L2, and one polyphenolic fraction chPP-L were obtained. The carbohydrate peaks were visualized by the *m*-hydroxydiphenyl and phenol-sulfuric acid methods, and polyphenols by the Folin-Ciocalteu reagent, and UV–Vis fingerprinting (200–800 nm). The chPP-L fraction was concentrated under vacuum to 50 mL and loaded on an Amberlite® XAD7HP (Sigma-Aldrich) column (12 × 3 cm), followed by column elution with 200 mL water and the polyphenols were eluted with 150 mL 95% ethanol. The polyphenolic fraction was evaporated to dryness under vacuum, dissolved in water and freeze-dried. chPS-L1 and chPS-L2 were concentrated under vacuum, dialyzed against water (MWCO 12–14 kDa, 72 h), filtered through 0.2 μm cellulose nitrate filters (Sartorius) and freeze-dried. The three samples were stored in a desiccator in dark.

2.3. General methods

Anhydrouronic acid content (AUAC) was determined by the *m*-hydroxydiphenyl method in Skalar San++ autoanalyzer (Analytical BV, Breda, The Netherlands), using galacturonic acid as a standard (Blumenkrantz & Asboe-Hansen, 1973). Total amount of sugars was quantified by the phenol-sulfuric acid method, using 1:1 mixture of glucose and galacturonic acid as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Degree of methoxylation (DM) was determined by alcohol oxidase (*P. pastoris*, Sigma-Aldrich) and Purpald® (Sigma-Aldrich) (Anthon & Barrett, 2008). Degree of acetylation (DA) was measured according to McComb and McCready (1957), using β-D-glucose pentaacetate as a standard. The relative molecular weight was determined by HPSEC-RID (Waters) with Shodex standard P-82 kit (pullulan standards, Showa DENKO, Tokyo, Japan) (Georgiev, Ognyanov, Yanakieva, Kussovski, & Kratchanova, 2012). Total phenolics (gallic acid standard) and protein content (BSA standard) were analyzed according to Singleton and Rossi (1965) and Bradford (1976), respectively.

2.4. Monosaccharide composition and glycosidic linkage determination

Polysaccharide samples (1 mg) with added 100 μg mannitol as an internal standard were subjected to methanolysis with anhydrous 3 M HCl in methanol for 24 h at 80 °C. The formed methyl glycosides of different monosaccharides were converted into their corresponding TMS derivatives and analyzed by GC-FID on a Focus GC system (Thermo Scientific, Milan, Italy), according to Chambers & Clamp (1971) and Barsett, Paulsen, & Habte (1992). The presence of KDO (3-deoxy-D-manno-2-octulosic acid) was detected by the thiobarbituric acid assay (York, Darvill, McNeil, & Albersheim, 1985).

The glycosidic linkage composition of chPS-L1 (2 mg) was examined after methylation. Prior to methylation uronic acids in the sample were reduced with $NaBD_4$ to 6,6′-dideuterio-sugars that could be differentiated from the neutral sugars (Kim & Carpita, 1992). The procedure was completed by methylation, acid hydrolysis and preparation of alditol acetates, partly as described by Ciucanu & Kerek (1984). The hydrolysis was performed with 2.5 M TFA for 2 h at 100 °C. The partly methylated alditol acetates were analyzed by a GC-EI/MS-QP2010 system (Shimadzu, Kyoto, Japan), coupled with a Restek Rxi-5MS column (30 m, 0.25 mm i.d., 0.25 μm film) in a Scan mode (40–450 ions), as described by Ho, Zou, Aslaksen, Wangensteen, and Barsett (2016). The detection and quantification of the relative amounts of each glycosidic linkage type was based on the chromatographic behavior (RT), ionization patterns and results from the monosaccharide composition.

The presence of AGII was detected by precipitation with the Yariv *ß*-glucosyl reagent (Van Holst & Clarke, 1985).

2.5. Contamination with lipopolysaccharides (LPS)

Possible LPS contamination was checked by the detection of 3-hydroxy fatty acids as their specific chemical markers in the form of acetylated fatty acid methyl esters on the GC-EI/MS-QP2010 system (Shimadzu, Kyoto, Japan), coupled with a Restek Rxi-5MS column (30 m, 0.25 mm i.d., 0.25 μm film) in a SIM mode (257 ion intensity for calculation), as described by de Santana-Filho et al. (2012) and Ho et al. (2016).

2.6. Fourier transform infrared spectroscopy (FTIR)

Infrared (IR) spectra of chPS-L1 (2 mg) were obtained on a Nicolet™ Avatar (Thermo Fisher Scientific, Waltham, USA) spectrometer in a KBr tablet. The spectra were recorded in the range 4000 and 400 cm^{-1} and analyzed by the SpectraGryph software (Friedrich Menges).

2.7. Nuclear magnetic resonance (NMR) spectroscopy

chPS-L1 (12 mg) was dissolved in D_2O, freeze-dried and redissolved in 0.7 mL D_2O. 0.5 mL of chPS-L1 was investigated by the following NMR experiments. 1D ^1H, ^{13}C, DEPT 135 and 2D $^1H/^{13}C$ HSQC, $^1H/^1H$ COSY, $^1H/^1H$ TOCSY, $^1H/^1H$ ROESY, $^1H/^1H$ NOESY, $^{13}C/^1H$ HMBC experiments were performed on a Bruker Avance III HD Ascend 800 MHz spectrometer operating at 800.03 MHz (Bruker, Fällanden, Switzerland) with the Bruker TopSpin™ 3.5 software at a temperature of 60 °C. The chemical shifts were referenced to the internal standard DSS (sodium 4,4-dimethyl-4-silapentane-sulfonate).

2.8. Immunomodulatory effect

2.8.1. Ethics

Blood sampling procedure was in accordance with the Helsinki Declaration of 1975, as revised in 1983. Animal experiments were approved by the Animal Research Committee of the Kitasato University, and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at the Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan.

2.8.2. Animal procedure and care

Female C3H/HeJ mice (6–8 weeks old) were purchased from Japan SLC (Shizuoka, Japan), and male ICR mice (4 weeks old) were from Charles River (Tokyo, Japan). Mice were housed in plastic cages in an air-conditioned room at 23 ± 2 °C with a relative humidity of 55 ± 10% under a 12 h-light-dark cycle, and fed a standard laboratory diet with water given *ad libitum*.

2.8.3. Complement fixation assay

The inhibition of complement hemolysis (complement fixation) of target antibody sensitized sheep red blood cells (classical pathway) or rabbit erythrocytes (alternative pathway) by normal porcine serum pre-treated with chPS-L1, chPS-L2 or chPP-L was studied. The analyses were performed according to Klerx, Beukelman, Dijk, and Willers (1983) and Klerx et al. (1985), as described by Georgiev et al. (2012). The complement fixation activity induced by the test samples was calculated on the basis of a colorimetric measurement of hemoglobin released from lysed cells after incubation with PS-treated normal porcine serum by the formula: $[A_{control} - A_{test}]/A_{control} \times 100\%$. From these data a dose-response curve was constructed and the concentration of test sample giving 50% inhibition of hemolysis (IC_{50}) was calculated (Fig. S2). The samples were analyzed in triplicates.

2.8.4. Reactive oxygen species (ROS) production from human whole blood phagocytes (WBP) and isolated neutrophils

Heparinized (50 IU/mL) blood samples were obtained from the cubital vein of healthy human volunteers after overnight fasting. The isolation of human neutrophils from venous blood was accomplished according to the procedure of Nauseef (2014). The kinetics of ROS production by WBP and isolated neutrophils was analyzed by luminol-enhanced chemiluminescence (CL) for a period of 60 min at 37 °C, using 96-well white flat bottom culture plates on luminometer (Orion II Berthold Detection Systems GmbH, Germany). The principle of the method was previously described (Vasicek, Lojek, Jancinova, Nosal, & Ciz, 2014). Briefly: 25 μL of ten times diluted blood with Hank's buffered salt solution (HBSS) or 0.2×10^6 cells/well of isolated neutrophils in phosphate-buffered saline (PBS) were mixed with 25 μL 1 mM luminol (10 mM stock solution in 0.2 M borate buffer) HBSS solution and incubated with HBSS solutions of chPS-L1, chPS-L2 or chPP-L (50 and 100 μg/mL) for spontaneous (non-stimulated with known activators) CL. Furthermore, 12.5 μL or 5 μL of ten times diluted blood (or 0.2×10^6 cells/well) mixed with 25 μL luminol were used for incubation of each fraction with 25 μL (0.5 μg/mL) phorbol myristate acetate (PMA-activated, Sigma-Aldrich) or 25 μL (62.5 μg/mL) opsonized zymosan particles (OZP-activated). The final volume for spontaneous, PMA- and OZP-activated CL tests was 250 μL. The recorded values included the intensity of CL emitted during the studied time interval (integral of CL). The results are expressed as percentage of the control (mean ± SEM) from five independent experiments.

2.8.5. Determination of the cell surface expression of adhesion molecules

The measurements were performed according to the method described by Gallova, Kubala, Ciz, and Lojek (2004). Briefly, isolated human neutrophils (1×10^5 cells) were incubated in plastic tubes (Falcon, USA) with chPS-L1 or chPS-L2 (100 μg/mL) for 1 h and with the activators fMLP (N-formyl-met-leu-phe, 0.1 μM) and PMA (0.01 μg/mL) for 10 min. After incubation anti-CD18 (anti-β_2-integrin) and anti-CD62L (anti-L-selectin) monoclonal antibodies (5 μL of each) were added. The samples were incubated at 4 °C for 15 min, subsequently centrifuged, re-suspended in PBS and analyzed by a flow cytometer FACSVerse™ (Becton Dickinson, USA). The median fluorescence intensity was determined and corrected for unspecific staining by subtracting the fluorescence of cells stained with the control antibody (isotype control). The data are expressed as $CD62L^-/CD18^+$ population (mean% of total leukocytes ± SEM) from five independent experiments.

2.8.6. Nitric oxide (NO) production from murine macrophages

Murine macrophage cell line RAW 264.7 (ATCC, USA) was incubated in 12-well flat bottom culture plates at 1×10^5 cells/well with chPS-L1 or chPS-L2 (50 and 100 μg/mL) alone in DMEM-high Glc supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (500 μL final volume) (HyClone™, GE Healthcare Life Sciences) for 24 and 48 h at 37 °C and 5% CO_2. The cells were also cultured by combination of polysaccharides with 10 ng/mL LPS (*E. coli*/026:B6, Sigma-Aldrich). The generation of NO was determined indirectly as the accumulation of nitrites in the cell culture supernatants. At the end of the incubation period the culture media were collected and centrifuged at 16 000 × *g*, 4 °C for 10 min. Then, 150 μL of supernatants were mixed with an equal volume of Griess reagent (Sigma-Aldrich) in a 96-well plate and the mixture was incubated at room temperature for 30 min in dark. The absorbance was measured at 546 nm and $NaNO_2$ (0–52 μM) was used as a standard (Lojek et al., 2011). The results are expressed as mean ± SEM from three independent experiments.

2.8.7. Western blot analysis of inducible nitric oxide synthase (iNOS)

RAW 264.7 cells, which were obtained as described in **2.8.6.**, were lysed in the lysis buffer (1% SDS, 0.1 M Tris pH 7.4, 10% glycerol, 0.001 M sodium *o*-vanadate, 0.001 M PMSF). The protein concentrations were determined by using the BCA™ protein assay (Pierce, USA), with BSA as a standard. Equal amounts of protein (18 μg) were then subjected to SDS-PAGE, using 10% separating gel. The expression of iNOS protein was quantified by a Western blot analysis (Pekarova et al., 2011). Anti-iNOS/NOS Type II mouse monoclonal antibody (Cell signaling Technology, 1:5000) and ECL™ anti-rabbit IgG horseradish peroxidase linked whole antibody (from goat; Cell Signaling Technology, 1:2000) were used. The immunoreactive bands were detected using an ECL™ detection kit (Pierce) and exposed to a radiographic film (AGFA). The relative protein levels were quantified by scanning densitometry, using the ImageJ™ programme, and the individual band density value was expressed in arbitrary units. The results are shown as mean ± SEM from three independent experiments.

2.8.8. Cytotoxicity assay

Cytotoxicity was estimated by lactate dehydrogenase (LDH) activity in the cell culture supernatants of RAW 264.7 cells, as obtained in **2.8.6.**, using the LDH Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Switzerland). The results are expressed as mean ± SEM from three independent experiments (Fig. S4).

2.8.9. Immunomodulating activity against murine Peyer's patch (PP) cells

Immunomodulating activity was measured by the enhanced production of bone marrow cell-proliferative cytokines from PP cells of pathogen-free C3H/HeJ mice ($Tlr4^{Lps-d}$), according to Hong, Matsumoto, Kiyohara, and Yamada (1998). Suspensions (1.2×10^6 cells/mL, 180 μL) of PP cells (5–9 patches obtained per mouse) from the small intestine of C3H/HeJ mice were cultured with H_2O (negative control), AMOL-1 (pectic polysaccharide from *Astragalus mongholics* as a positive control, Kiyohara et al., 2010) or 100 μg/mL of the pectic fractions (20 μL) in a 96-well flat bottom culture plate (FALCON 3072) for 6 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The resulting culture supernatants (50 μL) were further cultured with a bone marrow cell suspension (5.0×10^5 cells/mL) from male ICR mice for 6 days. The numbers of proliferated bone marrow cells were measured by Alamar Blue™, as described previously (Hong et al., 1998). The results are shown as mean ± SEM.

2.9. Statistical analysis

The statistical significance was analyzed by the Student's *t*-test, using the Statview software (SAS Institute, Inc.). The mean results ± SEM were compared to those from the control. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Purification and structural characterization of lavender pectic polysaccharides

3.1.1. Purification and chemical composition of lavender fractions

After the ethanol pretreatment of lavender PSC, the obtained lavender PSC2 having 41% AUAC and low protein content (2.9%) was subjected to SEC (Fig. 1(A)). The presence of non-carbohydrate compounds with diene and aromatic structure (e.g. polyphenols, fatty acids, proteins) in all fractionation tubes was estimated by reading of the absorbance at 254/280 nm. This in addition to the UV–Vis fingerprinting of selected tubes helped for determination of two polysaccharide and one polyphenolic fractions. As can be seen from Fig. 1(B) the three lavender fractions are distinguished by the intensity of two domains at 201–202 nm and around 280 nm. The first domain originates from different π-bonds, such as C=O of carboxyl group in pectin (202 nm) and other compounds and the second has λ_{max}=276 nm typical for catechin and tannins.

The total yield of the two polysaccharide fractions in dried lavender flowers is 1.6% (w/w). The monosaccharide composition of these fractions shows that they contain mainly pectic polymers with the highest content of GalA, followed by Gal, Ara and Rha (Table 1). This result is in agreement with the relatively high AUAC between 41 and 45%. chPS-L1 and chPS-L2 give a cross-reaction with the *ß*-glucosyl Yariv reagent, which indicates that both fractions contain 3,6-linked galactans or AGII. The reaction ring for chPS-L1 is more intensive, thus it has higher AGII content than chPS-L2 (gels not shown). Furthermore, the presence of small amounts of GlcA, Xyl and Fuc, which are specific for some pectic side chains are detected. The presence of the rare monosaccharides 2-OMeXyl and 2-OMeFuc in chPS-L2 indicated that it contains RGII, which was further confirmed by the presence of KDO. Additionally, negligible contamination with other polysaccharides containing Man and Glc is also found. Both polysaccharides are classified as high methoxylated pectins with DA <20%. As was expected chPS-L2 (2.18×10^4 Da) has lower molecular weight (relative to pullulan) than chPS-L1 (5.24×10^4 Da) and higher total polyphenol content (see Fig. 1(A) & (B)).

Direct SEC fractionation of lavender PSC2 was chosen because of the interest for identification of some polyphenols retained associated to pectins after an exhaustive extraction of ethanol-soluble molecules from lavender PSC. The yield of chPP-L is 9.2% from lavender PSC2. Interestingly, 10 phenolic acids and 8 flavonoids were identified in minor amounts in chPP-L (Table S1). Ferulic acid, protocatechuic acid, quercetin, catechin and epicatechin are presented in the highest concentrations. For comparison, in the alcohol-soluble fraction obtained from lavender PSC, only the catechin (385.4 μg/100 mg) and epicatechin (497.7 μg/100 mg) contents are much lower than in chPP-L based on the identical polyphenols detected in the two samples. Both flavonoids are ethanol-soluble, thus weak interactions with pectic polysaccharides might occur and they could be broken during the saline SEC fractionation.

3.1.2. Glycosidic linkage determination

The quantitative glycosidic linkage analysis of chPS-L1 indicated that it contains mainly 1,4-linked Gal*p*A (47.3 mol%) with considerably lower amounts of 1,2- and 1,2,4-linked Rha*p*. It is

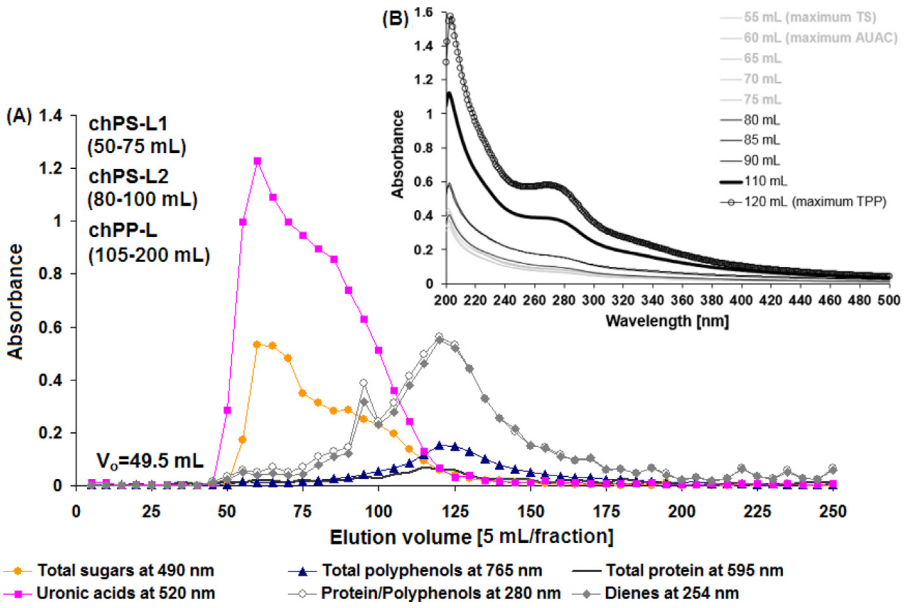

Fig. 1. Purification and fractionation of lavender pectic polysaccharides. (A) Elution profile of lavender PSC2 on Sephacryl S-200. (B) UV–Vis fingerprinting analysis of obtained pectic (chPS-L1 and L2) and polyphenolic (chPP-L) fractions. TS (total sugars), TPP (total polyphenols).

rich in AGII structures (26.7 mol%), shown by 1,3,6-linked Gal*p* (6.9 mol%), T-Ara*f* (5.6 mol%) and 1,5-linked arabinan (3.3 mol%) (Table 2). chPS-L1 is a pectic type polymer composed mainly of HG with a smaller RGI backbone fragment, containing 1,3,5-branched arabinan and highly branched AGII side chains. The molar ratio 1,2:1,2,4-linked Rha*p* in chPS-L1 is 41:59, indicating a high degree of branching on the Rha*p* present in RGI. The 3,6-linked Gal and 1,5-arabinans in the Rha side chains are 25.8 and 12.4 mol% calculated from the total galactan and arabinan structures. The predominance of 3,6-linked Gal is in a good agreement with the Yariv's test results. chPS-L1 is also characterized with a minor amount of 1,3,4-linked Gal*p*A, which probably indicates the presence of some substituted galacturonan, such as RGII or others. The other uronic acid in the fraction – GlcA is T-, 1,2- and 1,2,4-linked most probably in AG side chains (Yamada & Kiyohara, 2007). Additionally, the methylation analysis showed that Glc in chPS-L1 is T- and 1,4-linked probably in an independent glucan polymer, such as starch. Concluding, HG (GalA – Rha) and RGI (2Rha + Ara + Gal) content in chPS-L1 are roughly estimated to be 47.6 mol% and 39.1 mol%, respectively (Table 1) (Denman & Morris, 2015).

3.1.3. FTIR analysis

The IR spectrum of chPS-L1 (Fig. S1) contains the typical bands for polysaccharides and pectins, as follows: 3408, 2935, 1743, 1616, 1331, 1146 and 1101 cm^{-1}, arising from: O—H of hydroxyl groups, C—H of CH_2, C=O of methoxyl ester group ($COOCH_3$), C=O of ionized (free) carboxyl group, C—H in the ring, C—O—C of the glycosidic bond (or within the ring) and C—C or C—O in the ring, respectively (Synytsya, Čopíková, Matějka, & Machovič, 2003). According to the formula: $[A_{1743}/(A_{1743} + A_{1616})] \times 100$, where A is the area of the corresponding signal, the DM of chPS-L1 is 61.4%. This result is in agreement with the already determined DM of 63.4% (Table 1). Additionally, the signal at 1373 cm^{-1} is attributed to $\delta_s(CH_3)$ of methoxyl ester and two overlapped bands at 1242–1259 cm^{-1} originate from ν(C—O—C) of acetyl esters (Synytsya, Čopíková, Matějka et al., 2003). The signals at 1146, 1101 and 1018 cm^{-1}, as well as the peaks at 833 (C1-OH, α-anomer), 764, 638 and 536 cm^{-1} confirm the predominance of the HG structure in the studied polysaccharide (Kačuráková, Capek, Sasinková, Wellner, Ebringerová, 2000). The presence of arabinogalactorhamnoglycan structure or the hairy region in chPS-L1 is demonstrated by the characteristic signals at 1078, 1050 and 914 cm^{-1} (Kačuráková et al., 2000).

3.1.4. NMR structural studies

In Table 3 are presented ^{13}C and ^{1}H assignments for the identified structural fragments of chPS-L1 found from specific correlations in the HSQC spectrum, as well as from the COSY, TOCSY, ROESY and HMBC spectra. The NMR result interpretation was done with the help of literature data and in accordance with the monosaccharide and glycosidic linkage compositions (Shakhmatov, Atukmaev, & Makarova, 2016). The absolute configurations were assumed to be the same as already found for pectins (McNeil, Darvill, Albersheim, 1980). The detailed NMR study confirmed that chPS-L1 is a pectic polysaccharide with a predominant HG fragment with existence of methoxyl and acetyl esters, followed by a RGI branched with arabinans and AGII.

The presence of a HG fragment, revealing the 1,4-linked α-D-polygalacturonic acid in chPS-L1 is shown by the correlations H1/H4 of α-D-Gal*p*A-6-OMe units at 4.95/4.45 ppm (**A**) and H1/H4 of α-D-Gal*p*A units at 5.10 (5.05)/4.45 ppm (**B**) in the ROESY spectrum (Fig. 2). This was also proved by through bond correlations in the COSY spectrum. The C/H correlations at 55.5/3.81 ppm for methyl carbon and proton of CH_3O group, at 22.8/2.08 ppm and 22.5/2.17 ppm for methyl carbon and proton of CH_3CO group in the HSQC spectrum show the presence of methoxyl and acetyl esters in chPS-L1. The intensive through space correlations between the CO signal at 173.4 ppm and the $\underline{CH_3}O$ group at 3.81 ppm, as well as the cross-peaks at 173.4/5.05 ppm and 173.4/5.10 ppm in the HMBC spectrum indicate that the methoxyl groups are located at C6 of α-D-Gal*p*A units in the HG. The calculated DM by NMR is 62%, using the introduced by Synytsya, Čopíková, and Brus (2003) formula:

$$DM = A_{\underline{COOCH_3}}/A_{C\text{-}6\,tot.} \times 100\,(\%) \quad (1)$$

where A is the peak area and $A_{C\text{-}6\ tot.}$ includes the combined area of the C6 peak regions of GalA. The ratio between the integral intensities of the proton signals of CH_3CO- at 2.08 and 2.17 ppm is 69:31. According to the literature data the peak at 2.08 ppm is attributed to O-3 acetylation and this at 2.17 ppm to O-2 acetylation of GalA in the HG (Perrone et al., 2002). The CO signal of the acetyl group attached at O-3 of GalA was determined with the help of a

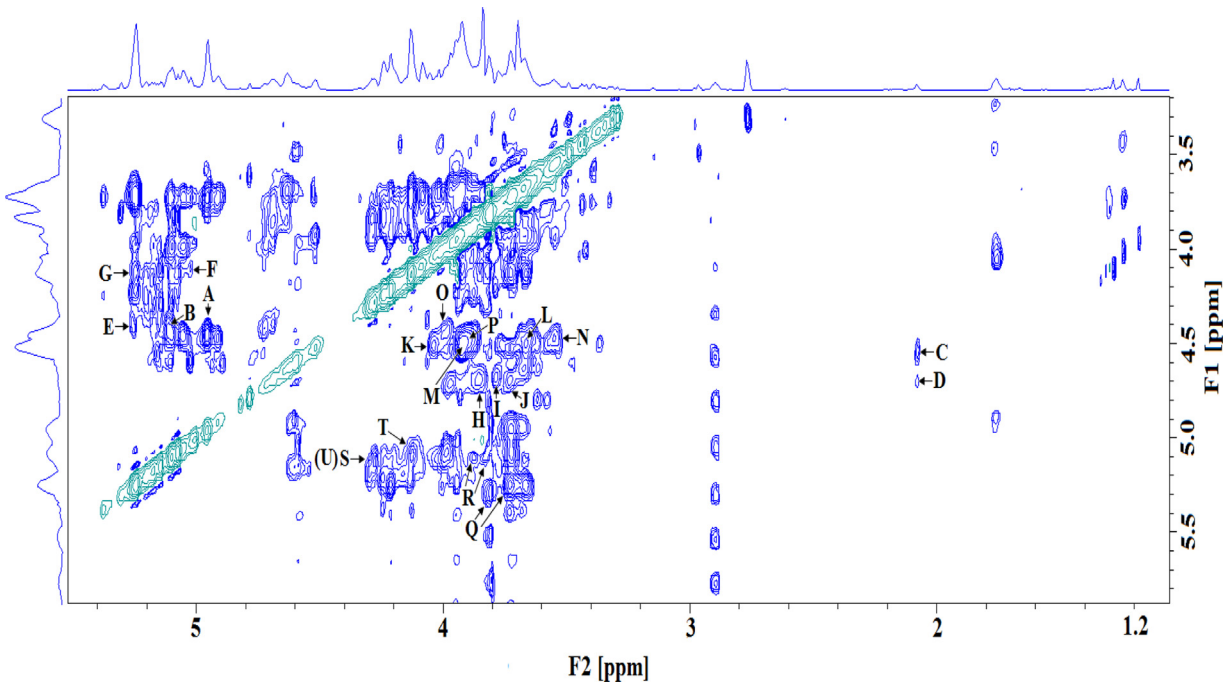

Fig. 2. $^{1}H/^{1}H$ ROESY spectrum of chPS-L1 pectic fraction. Each letter (see Section 3.1.4.) corresponds to important correlations in the HG fragment (**A**–**D**); RGI backbone (**E**–**G**); 1,3-Gal chain (**H**–**J**); 1,6-Gal (**N**–**P**); 1,3,6-Gal (**K**–**M**); 1,5-Ara (**Q**–**R**); 1,3,5-Ara (**S**–**U**).

Table 1
Yields and chemical characterization of lavender pectic fractions obtained after SEC of lavender PSC2.

	chPS-L1	chPS-L2
Yield [% of lavender PSC]	26.9	19.6
Yield [% of lavender PSC2]	35.2	25.6
Yield [% of dried flowers]	0.9	0.7
Total sugars [%]	73.7	58.5
AUAC [%]	40.8	44.9
DM[a] [mol%] (Methoxyl content [%])	63.4 (4.7)	61.0 (5.0)
DA[a] [mol%] (Acetyl content [%])	16.8 (1.7)	15.3 (1.7)
Protein content [%]	1.1	4.2
Total polyphenols [%]	0.7	2.1
LPS [%]	n.f.	n.f.
Monosaccharide composition [w/w%]		
Ara	10.1[b] (12.2)[c]	4.6 (5.7)
Rha	5.6 (6.2)	3.7 (4.2)
Fuc	0.7 (0.8)	0.9 (1.0)
Xyl	2.8 (3.4)	2.1 (2.6)
Man	1.7 (1.7)	0.5 (0.5)
Gal	14.3 (14.5)	6.5 (6.7)
Glc	4.7 (4.7)	3.7 (3.8)
GlcA	2.9 (2.8)	1.5 (1.4)
GalA	57.3 (53.8)	76.6 (73.9)
2-OMeXyl	n.f.	+
2-OMeFuc	n.f.	+
Kdo	n.d.	++
AGII	++	+
Relative molecular weight [Da]	5.24×10^4	2.18×10^4

n.d. – not determined. n.f. – not found.
[a] Calculated on AUAC basis, moles of methoxyl or acetyl groups per 100 mol of GalA.
[b] ww%, calculated on total carbohydrate content basis.
[c] mol% of total carbohydrate content.

Table 2
Glycosidic linkage composition of chPS-L1 fraction.

Glycosidic linkages	chPS-L1
T-Ara*f*	4.6[a] (5.6)[b]
1,2-Ara*f*	trace
1,3-Ara*f*	1.4 (1.7)
1,5-Ara*f*	2.8 (3.3)
1,3,5-Ara*f*	1.3 (1.5)
T-Rha*p*	1.4 (1.6)
1,2-Rha*p*	1.7 (1.8)
1,4-Rha*p*	0.1 (0.2)
1,2,4-Rha*p*	2.4 (2.6)
T-Gal*p*	3.3 (3.4)
1,3- Gal*p*	2.0 (2.1)
1,6-Gal*p*	2.1 (2.2)
1,3,6-Gal*p*	6.8 (6.9)
T-Glc*p*	1.9 (1.9)
1,4-Glc*p*	2.8 (2.8)
T-Glc*p*A	0.7 (0.6)
1,4-Glc*p*A	1.7 (1.7)
1,2,4-Glc*p*A	0.5 (0.5)
T-Gal*p*A	4.7 (4.5)
1,4-Gal*p*A	50.4 (47.3)
1,3,4-Gal*p*A	2.2 (2.0)

[a] w/w%, calculated on total carbohydrate content basis.
[b] mol% of total carbohydrate content.

correlation at 176.2/2.08 ppm in the HMBC spectrum. The proton sequence within α-D-Gal*p*A-3-OAc unit was determined according to Perrone et al. (2002) by the following correlations: 2.08/4.52 ppm (**C**) and 2.08/4.72 ppm (**D**) in the ROESY spectrum, revealing interactions between the $\underline{CH_3}CO$- with H4 and H5 of GalA, respectively; 176.2/4.09 ppm and 176.2/4.52 ppm in the HMBC spectrum, showing through space interaction between the $CH_3\underline{C}O$- with H2 and H4 of GalA; finding of the adjacent protons in the COSY spectrum and checking the correlations in the TOCSY spectrum. The gross calculation of DA of chPS-L1 by NMR is 15.4%, using the formula (Synytsya, Čopíková, Matějka et al., 2003):

$$DA = A_{O\underline{C}OCH_3} / A_{C\text{-}6\,tot.} \times 100\,(\%) \quad (2)$$

Table 3
^{13}C and ^{1}H NMR chemical shift assignment (δ in ppm) of chPS-L1 pectic fraction, referenced to DSS.

Residue	C-1	C-2	C-3	C-4	C-5	C-6	$\underline{CH_3}CO$[b]
	H-1	H-2	H-3	H-4	H-5:5′	H-6:6′	
→4)-α-Gal*p*A-6-OMe-(1→	102.7	70.6	71.4	81.5	73.2	173.4[a]	
	4.95	3.72	4.00	4.45	5.10; 5.05		
→4)-α-Gal*p*A-3-OAc-(1→	n.d.	71.6	73.5	79.3	74.0	177.2	22.8
	5.31	4.09	5.14	4.52	4.72		2.08
→4)-α-Gal*p*A-(1→	102.0	70.6	71.4	81.5	74.0	177.2	
	5.10	3.75	4.00	4.45	4.72		
→4)-α-Gal*p*A-(1→2	100.3	71.4	n.d.	81.5	74.4	177.4	
	5.02	3.93	4.13	4.45	4.69		
→2)-α-Rha*p*-(1→	101.7	79.3	71.9	74.7	71.9	19.2	
	5.25	4.13	4.00	3.44	3.72	1.25	
→2,4)-α-Rha*p*-(1→	101.7	79.3	72.6	82.8	72.1	19.4	
	5.25	4.13	4.09	3.67	3.82	1.30	
α-Ara*f*-(1→	111.8	84.0	79.4	86.7	64.1	–	
	5.25	4.21	3.98	4.09	3.72; 3.81		
→3,5)-α-Ara*f*-(1→	110.2	n.d.	85.1	83.6	69.6	–	
	5.10	4.28	4.13	4.29	3.81; 3.93		
→5)-α-Ara*f*-(1→	110.2	84.0	79.4	85.1	68.4	–	
	5.11	4.13	4.03	4.21	3.81; 3.87		
→3)-β-Gal*p*-(1→	106.1	72.9	84.8	71.1	n.d.	63.9	
	4.69	3.78	3.87	4.24	3.73	3.81	
→6)-β-Gal*p*-(1→	106.1	74.7	75.5	71.3	n.d.	63.9	
	4.45	3.54	3.67	3.98		3.93	
→3,6)-β-Gal*p*-(1→	106.1	73.2	83.6	71.4	76.4	72.6	
	4.51	3.67	4.03	4.13	3.93	3.93; 4.03	

[a] -O$\underline{CH_3}$ δ at 55.5/3.81 ppm.
[b] $CH_3\underline{CO}$- δ at 176.2 ppm.

where A_{OCOCH_3} presents the area of the peak at 176.2 ppm, as found in the HMBC spectrum. This result is in agreement with the colorimetric determination of DA (16.8%).

The following important through space cross-peaks are also found in the ROESY spectrum (Fig. 2): H1 of 1,2(,4)-α-L-Rha*p* and H4 of 1,4-α-D-Gal*p*A residues at 5.25/4.45 ppm (**E**); H2 of α-L-Rha*p* and H1 of α-D-GalpA residues at 4.13/5.02 ppm (**F**); H1 of α-L-Rha*p* with H3 of α-D-Gal*p*A units at 5.25/4.13 ppm (**G**). These correlations confirm the presence of the RGI backbone structure [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]$_n$ in chPS-L1. The ratio between the integral intensities of H6 of 1,2:1,2,4-α-L-Rha*p* gives 45:55, showing the prevalence of O-4 substituted Rha in agreement with the glycosidic linkage results.

The anomeric proton at 4.69 ppm shows through bond correlation peaks between H1/H3 (4.69/3.87 ppm) (**H**), H1/H2 (4.69/3.78 ppm) (**I**) and H1/H5 (4.69/3.73 ppm) (**J**) within β-D-Gal*p* residues in the ROESY spectrum confirming the presence of [→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→]$_n$ fragment (Makarova, Patova, Shakhmatov, Kuznetsov, & Ovodov, 2013). Additionally, the through space interaction between C1 and H6 of β-D-Gal*p* units at 106.1/3.93 ppm in the HMBC spectrum demonstrates 1,6-glycosylation in the galactan chains. The presence of 3,6-galactan structure was elucidated by the COSY correlations between the adjacent protons and the following interactions in the ROESY spectrum: H1/H3 (4.51/4.03 ppm) (**K**), H1/H2 (4.51/3.67 ppm) (**L**) and H1/H6 (4.51/3.93 ppm) (**M**) of the β-D-Gal*p* units. Similarly, the found adjacent proton correlations for 1,6-β-D-Gal*p* units in the COSY spectrum and H1/H2 (4.45/3.54 ppm) (**N**), H1/H4 (4.45/3.98 ppm) (**O**) and H1/H6 (4.45/3.93 ppm) (**P**) of the same units in the ROESY spectrum demonstrate the presence of [→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→]$_n$ fragment (Shakhmatov, Toukach, Kuznetsov, & Makarova, 2014). Generally, the 3,6-galactans in pectins are linked to O-4 of α-L-1,2,4-Rha*p* through the 1,3-galactan backbone (Voragen et al., 2009). The ROESY spectrum also shows the presence of [→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→]$_n$ structure by the cross-peaks 5.25/3.81;3.72 ppm (**Q**) for H1/H5 of the terminal Ara*f* residues and 5.10/3.87;3.81 ppm (**R**) for 1,5-Ara*f* residues (Shakhmatov et al., 2014). Furthermore, the correlations H1/H2 (5.10/4.28 ppm) (**S**); H1/H3 (5.10/4.13 ppm) (**T**) and H1/H4 (5.10/4.29 ppm) (**U**) for 1,3,5-substituted Ara*f* units are found in the ROESY spectrum in addition to the respective COSY correlations (Makarova et al., 2013). This confirms the presence of 1,3,5-branching of the Ara*f* units most probably presenting the 1,3,5-arabinans attached to O-4 of α-L-1,2,4-Rha*p* units (Voragen et al., 2009). The presence of terminal α-L-Ara*f* units linked to O-6 and/or O-3 of β-D-Gal*p* residues within the 3,6-galactans is also suggested because of the positive reaction of chPS-L1 with the β-glucosyl Yariv reagent.

3.2. Immunomodulatory effect

chPS-L1 and chPS-L2 were tested for presence of LPS, which are considered to be contaminants that might influence the biological activity of studied compounds. It appears that there is no LPS contamination in the two fractions because the LPS marker 3-hydroxy tetradecanoic acid (3-OH-C14:0) was not detected (de Santana-Filho et al., 2012).

3.2.1. Complement fixation activity

The IC_{50} values of the pectic fractions chPS-L1 and chPS-L2 via the classical pathway were 94.0 and 456.3 μg/mL, revealing the higher activity of the first fraction (Fig. S2). The IC_{50} values of chPS-L1 and chPS-L2 through the alternative pathway were 38.9 and 689 μg/mL, as LPS were not detected in both fractions. It was important to be tested the complement fixation activity of the polyphenolic fraction chPP-L, because it practically accompanies polysaccharides in tea and water extracts. The IC_{50} values of chPP-L were 86.9 and 539.4 μg/mL via the classical and alternative pathways, respectively. In this case polyphenols expressed a similar effect to the most active chPS-L1, which means that they could also contribute to the observed polysaccharide activity in a proper combined formulation.

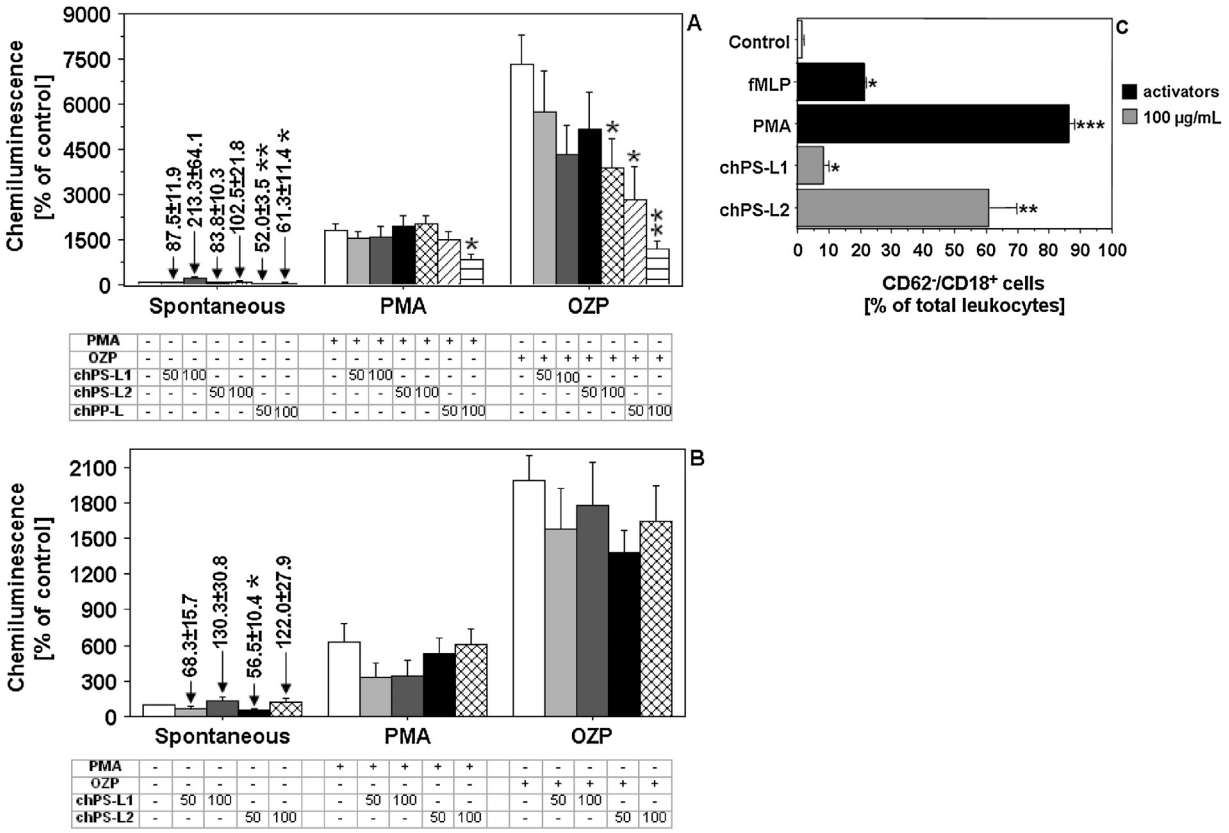

Fig. 3. Effect of lavender pectic (chPS-L1 and chPS-L2) and polyphenolic (chPP-L, only in A) fractions on **A**) ROS production from non-stimulated (spontaneous), PMA- and OZP-activated human whole blood phagocytes. **B**) ROS production from isolated neutrophils. **C**) Proportion of $CD62L^{-}/CD18^{+}$ population of total human neutrophils treated with lavender pectins. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) vs. control. 50 (50 μg/mL) and 100 (100 μg/mL).

3.2.2. *Effects on phagocytic leukocytes*

3.2.2.1. *Human whole blood and isolated neutrophils.*

The effect of lavender fractions on WBP and isolated neutrophils was evaluated *ex vivo* by the change of CL emitted by the cells in response to ROS formation. From both studied concentrations of the three samples, only chPS-L1 enhanced the spontaneous ROS generation from WBP at the higher dose of 100 μg/mL (Fig. 3A). Its effect was negligible in comparison with the independent PMA and OZP treatments alone. Under the co-stimulation with PMA, chPP-L expressed an inhibitory activity against the ROS production in comparison with the PMA control. This inhibition was observed also in the absence of activator and it could be explained by the ROS-scavenging properties of the polyphenols. Interestingly, under the co-stimulation with OZP, the three lavender samples showed an inhibitory activity against the ROS formation, as it was statistically significant for chPS-L2 and chPP-L at 100 μg/mL.

At the higher concentration, chPS-L1 and chPS-L2 slightly increased the CL response of isolated human neutrophils, but again their effect was much lower in comparison with the effect of PMA and OZP activators (Fig. 3B). Because the activity of chPP-L on generation of free radicals was predictable, it was not investigated further. Both pectic fractions did not increase the CL response of PMA-activated cells and chPS-L1 showed an evident inhibitory activity. The observed inhibitory effect of chPS-L2 on the OZP-treated WBP was also seen with isolated neutrophils, but it was not so clearly expressed like in the blood environment. Furthermore, both pectic fractions exhibited a statistically significant induction of the neutrophil activity, which was accompanied with an increased expression of CD18 and a decreased expression of CD62L (Figs. 3 C & S3). Interestingly, the effect of chPS-L2 was much higher than those of fMLP. It was proposed that both polysaccharide fractions could interact with CD18. Our findings suggest that lavender pectins are active ingredients in the crude lavender PSC, affecting ROS production of human WBP and neutrophils.

3.2.2.2. *Murine macrophages.*

The effect of the lavender pectins on macrophages was estimated *in vitro* by measurement the production of NO from treated cells. The pectin behavior in a simulated macrophage infection by a co-stimulation with LPS was also studied. Firstly, both polysaccharide fractions increased in a similar manner the expression of iNOS after 24 h of incubation (Fig. 4A). The enzyme expression in the growth medium control was negligible, which showed that the cells were not artificially activated by the plate surface. As was proposed, under the co-stimulation with LPS the induction was higher than this for pectin-treated cells. Contrary to chPS-L1, chPS-L2 did not increase the iNOS levels of the independent polysaccharide and combined with LPS treatments at the higher concentration of 100 μg/mL. After 48 h of incubation, the effects on the iNOS expression of both fractions alone or in the combinations with LPS were comparable (Fig. 4A). chPS-L2 again did not increase its induction ability at 100 μg/mL, as the iNOS level was the highest at 50 μg/mL with LPS. The enzyme expression with this fraction at the two border points – 50 μg/mL alone and under the co-stimulation with LPS at 100 μg/mL was almost identical.

There was no a significant difference between the activity of the two lavender polysaccharides, concerning stimulation of NO production after 24 or 48 h. However, chPS-L2 showed a negligible prevalence after 24 h (Fig. 4B). Furthermore, the enhancement of NO production induced by chPS-L1 or chPS-L2 at 100 μg/mL after 48 h of culturing were 9 or 6 fold (on mean value basis) higher than the corresponding NO release after 24 h. These results demon-

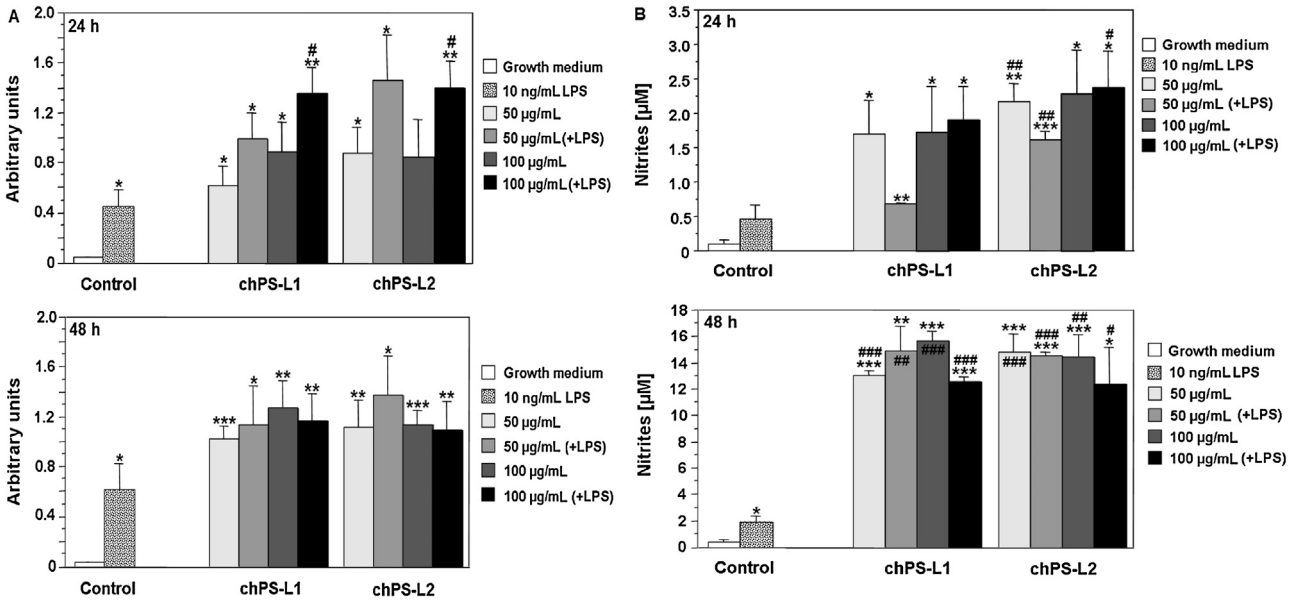

Fig. 4. Effect of lavender pectic fractions on A) iNOS protein expression in murine macrophages Raw 264.7 cells cultured with samples alone or co-stimulated with LPS. B) NO production from murine macrophages cultured with samples alone or co-stimulated with LPS. LPS from *E. coli* were used as a positive control. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) vs. control – untreated cells (growth medium) and (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$) vs. LPS.

strate that lavender pectins similarly to other already investigated polysaccharides could unlock macrophage activation. Additionally, lavender pectins were not toxic for the macrophages (Fig. S4). Under the conditions of co-stimulation with LPS and both fractions, the NO production increased at the higher concentration of pectins used after 24 h. After 48 h, the macrophage stimulation with chPS-L1 did not lead to the same effect, as at 100 μg/mL the NO level was decreased in the presence of LPS and elevated in the absence of LPS. Generally, it was observed that combining 100 μg/mL lavender pectins with 10 ng/mL LPS did not lead to a synergistic elevation of the iNOS expression and NO production after 48 h. Interestingly, the results from the LDH test for co-stimulation of macrophages with 100 μg/mL pectins and 10 ng/mL LPS after 48 h gave absorbance values close to that from the control cells (Fig. S4).

3.2.3. *Intestinal immunomodulating activity*

Normally, polysaccharides are administered orally (e.g. tea, folk remedies and food) and they can interact with the intestinal immune system through different immunocompetent cells localized in the PP, including the phagocytes. We investigated the intestinal immunomodulating activity of the lavender polysaccharides by the PP-mediated bone marrow cell proliferation test (Fig. 5). It was found that both lavender fractions express intestinal immunomodulating activity ($p < 0.01$) comparable to that of the positive control AMOL-1. This result confirmed that lavender pectins are involved in the exhibited PP stimulating activity of the crude lavender PSC (Georgiev, Ognyanov, Kiyohara et al., 2017).

4. Discussion

The lavender pectic polysaccharides are chemically characterized with predominant HG fragments that are acetylated (<20%) and highly methoxylated (>60%), followed by smaller RGI domains, which are extremely branched with arabinans and AGII structures. chPS-L1 has a higher Mw and AGII content than chPS-L2, which could explain its higher complement fixation and intestinal immunomodulating activities (Yamada & Kiyohara, 1999; 2007). chPS-L2 is immune active on PP cells and phagocytes and it has a lower Mw than chPS-L1 and contains monosaccharides characteristic for RGII. It exhibited very potent stimulating activity against CD18 expression (Figs. 3 C & S3).

Interestingly, chPS-L1 expressed a high complement fixation activity through both tested pathways, but it was more potent through the alternative pathway. Therefore, it was suggested that the complement activation by chPS-L1 was not only antibody-dependant, but it could be a result of a direct polysaccharide (AGII) interaction with C3 component (C3b), initiating the alternative pathway (Michaelsen, Gilje, Samuelsen, Høgåsen, & Paulsen, 2000; Kiyohara, Matsumoto, Nagai, Kim, & Yamada, 2006). In fact, Wang et al. (2016) found that pectic fraction Fs-8-ba2 from *Forsythia suspensa* selectively interacts with C1q, C1r, C1s, C2, C3 and C9, but not with C4 and C5 complement proteins.

It was supposed that the high complement fixation activity of chPS-L1 would reflect on the phagocyte immune response, because the fraction could activate antibody-initiated and direct C3-initiated complement system (Leung et al., 2006). chPS-L1 and chPS-L2 induced CD18 expression on isolated neutrophils (Fig. 3C). Thus, the inhibitory effect of lavender pectins on the OZP-activated ROS production from WBP (Fig. 3A) could be due to their direct interaction with CD18 and the complement fixation activity developed in the tested blood environment. The CD18 is a constructive unit of CR3, CR4 and LFA-1 receptor expressed on leukocytes. CR3 is expressed on phagocytes and involved in the phagocytosis via iC3b-bound antigens. Zymosan is a complement activator via the alternative pathway by binding to (i)C3b and it can interact with CR3 on neutrophils inducing phagocytosis and superoxide generation (Fearon & Austen, 1977; Ross & Větvička, 1993). OZP (62.5 μg/mL) and the pectins (50 and 100 μg/mL) could compete for the neutrophil receptor recognition. The inhibitory effect of lavender pectins on the OZP-activated ROS generation from isolated neutrophils was less pronounced probably because of the lack of blood environment containing complement components and immunoglobulins (Fig. 3B). Pectins (100 μg/mL) induced higher CL responses with WBP than with isolated neutrophils. Conversely, chPS-L1 expressed a better inhibitory effect against the PMA-activated ROS generation with isolated neutrophils. PMA induced the CD18 expression, as it can bind to CR3, initiating phagocytosis and oxidative burst (Ross & Větvička, 1993). Thus, chPS-L1 could compete with PMA for a direct CD18 binding.

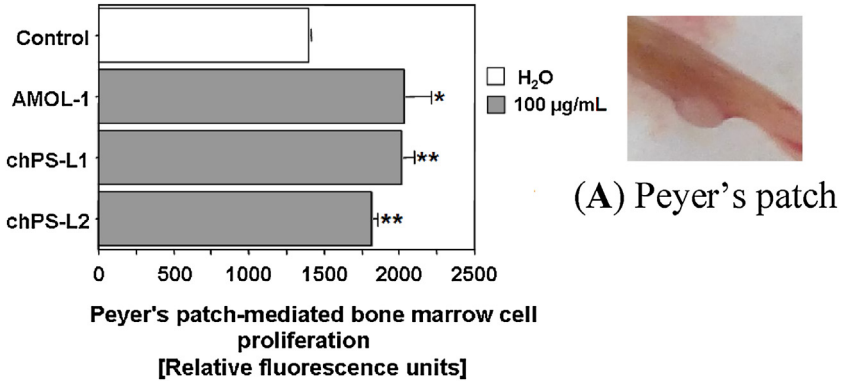

Fig. 5. Enhancement of production of bone marrow cell-proliferating cytokines from Peyer's patch cells cultured in presence or absence of lavender pectic fractions. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$) vs. water control. AMOL-1 is an active pectic polysaccharide from *A. mongholics* used as a positive control. (**A**) is a picture of a Peyer's patch.

The elevation of iNOS levels and NO production induced by the lavender pectins confirmed that they could stimulate the macrophage activation. Therefore, the lavender polysaccharides are at least partly responsible for the observed enhancement of $CD14^+$ monocytes and $CD64^+$ cell population (phagocytes) provoked by the lavender PSC (Georgiev, Ognyanov, Kiyohara et al., 2017). CD64 is a marker for Fc-γ receptor 1 expressed on macrophages, which can be activated by the pectic ramified RGI regions, which are also present in the studied pectins (Matsumoto et al., 1993). In a previous study it was found that *T. tomentosa* pectins exhibit suppression-like effect on LPS-induced iNOS expression and NO production from macrophages (Georgiev, Paulsen et al., 2017). chPS-L1 (100 µg/mL) showed a clear reduction of the NO generation under the co-stimulation with LPS in comparison with its separate action after 48 h. According to Chen et al. (2006), the observed phenomenon could be a result from a pectin-LPS interaction or a competition for the receptor binding (TLR4), but not a pectin inhibition of the LPS-signaling protein activities in the macrophages. Gallet et al. (2009) found that pectic hairy regions inhibiting the LPS-induced NO production in J774.2 macrophages reduced the TNF-α production stimulated by LPS. Therefore, chPS-L1 may exhibit anti-inflammatory effects in the simulated microbial infection, regulating the synthesis of inflammatory molecules. This regulation could result in a reduction of the NO generation, which was initially induced by chPS-L1 alone.

Pectins are little absorbed by the intestinal epithelium, but they may interact with it and most importantly with the mucosal immune system through immunocompetent cells in the PP. Functionally modulated immunocompetent cells by polysaccharides are proposed to result in modulations of several mucosal and systemic immune systems (Yamada & Kiyohara, 2007). Undoubtedly, the complement fixation activity and the stimulatory effect on the neutrophils and macrophages of lavender pectins contributed to the PP-mediated bone-marrow cell proliferation. However, lavender PSC stimulated not only phagocytes, but also $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T cells that constitute about 10% and 5% of PP cells, as 5–30% of $CD4^+$ T-cells in the mouse bone marrow are $CD4^+/CD25^+$ Treg (Georgiev, Ognyanov, Kiyohara et al., 2017; Jung, Hugot, & Barreau, 2010; Zhao et al., 2012). Therefore, in the intestinal immunomodulating activity of lavender pectins might be involved also T-cells. And as well, the CD18 expression, which was found to be induced by the lavender pectins is important for establishment of the intestinal mucosal T-cell compartment (Huleatt & Lefrançois, 1996).

Investigation of the type of attached polyphenols to pectin enables a better understanding of the chemistry of the polyphenol-polysaccharide complexes and the polysaccharide affinity to different bioactive polyphenols. In a previous study, it was found that pectic polysaccharides in rosehip are at least partly involved in the observed synergism in ORAC antioxidant activity after combining rosehip with other herbal extracts (Kratchanova, Denev, & Kratchanov, 2014). The physico-chemically attached polyphenols to pectin have valuable physiological activities in the human gastrointestinal tract (Jakobek, 2015). From a technological point of view, the observed affinity of catechin and epichatechin to the studied polysaccharides reveals the pectin potential for reduction of catechin astringency in drinks. Interestingly, Slavov, Kiyohara, and Yamada, (2013) showed that distilled rose petals from *Rosa damascena* contain pectins with intestinal immunomodulating activity. Méndez-Tovar, Herrero, Pérez-Magariño, Pereira, and Asensio-S.-Manzanera (2015) reported that a distilled lavender waste could serve for recovery of polyphenolic antioxidants. Therefore, the lavender by-product could be subjected to a further extraction of bioactive non-volatile compounds for production of infusion formulations and nutraceuticals with immunomodulating and antioxidant activities.

5. Conclusion

The detailed chemical analysis revealed that chPS-L1 and chPS-L2 are acetylated and highly methoxylated pectic type polymers with a predominant HG fragment, followed by a RGI with AGII structural motifs. Our findings with chPS-L1 and chPS-L2 confirmed that lavender pectins are at least partly responsible for the observed *ex vivo* and *in vitro* effects for lavender PSC. The lavender pectins activated innate and adaptive immune response through the complement system, WBP, neutrophils, macrophages and PP immunocompetent cells. Therefore, they could be considered as immunomodulating compounds in the lavender flowers. The observed inhibitory effects of chPS-L1 against the PMA- and OZP-activated ROS production, and the suppression of NO generation under the co-stimulation between chPS-L1 and LPS suggested anti-inflammatory activity of the studied pectins. chPP-L showed a high complement fixation activity via the classical pathway and was effective against the PMA and OZP inflammatory activation of WBP. The proper formulation or individual use of lavender polysaccharides and polyphenols could be of benefit in the supplementary treatment of a compromised immune system and control of inflammation. Furthermore, different immunomodulating and antioxidant nutraceutical formulations could be developed on the basis of the polysaccharide and polyphenol interaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.07.011.

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Tilia tomentosa pectins exhibit dual mode of action on phagocytes as β-glucuronic acid monomers are abundant in their rhamnogalacturonans I

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ABSTRACT

Silver linden flowers contain different pectins (PSI-PSIII) with immunomodulating properties. PSI is a low-esterified pectic polysaccharide with predominant homogalacturonan region, followed by rhamnogalacturonan I (RGI) with arabinogalactan II and RGII (traces) domains. PSII and PSIII are unusual glucuronidated RGI polymers. PSIII is a unique high molecular weight RGI, having almost completely O-3 glucuronidated GalA units with >30% O-3 acetylation at the Rha units. Linden pectins induced reactive oxygen species (ROS) and NO generation from non-stimulated whole blood phagocytes and macrophages, resp., but suppressed OZP-(opsonized zymosan particles)-activated ROS generation, LPS-induced iNOS expression and NO production. This dual mode of action suggests their anti-inflammatory activity, which is known for silver linden extracts. PSI expressed the highest complement fixation and macrophage-stimulating activities and was active on intestinal Peyer's patch cells. PSIII was active on non-stimulated neutrophils, as it induced β_2-integrin expression, revealing that acetylated and highly glucuronidated RGI exhibits immunomodulating properties via phagocytes.

Abbreviations: AGII, arabinogalactan type II; AUAC, anhydrouronic acid content; BSA, bovine serum albumin; CD18, marker for β_2-integrin; CD62L, marker for L-selectin; CL, chemiluminescence; DA, degree of acetylation; DM, degree of methoxylation; DSS, sodium 4,4-dimethyl-4-silapentane-sulfonate; fMLP, N-Formylmethionyl-leucyl-phenylalanine; FTIR, Fourier transform infrared; GlcA-RGI, glucuronidated RGI; HBSS, Hank's buffered salt solution; HG, homogalacturonan; IC_{50}, a concentration giving 50% hemolysis of erythrocytes; iNOS, inducible nitric oxide synthase; KDO, 3-deoxy-D-manno-2-octulosic acid; LDH, lactate dehydrogenase; Linden PSC, crude polysaccharide complex from *Tilia tomentosa*; Linden PSin70, obtained from linden PSC after an exhaustive ethanol treatment; LPS, lipopolysaccharides; NMR, Nuclear magnetic resonance; NO, nitric oxide; OZP, opsonized zymosan particles; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PP, Peyer's patches; PSI-PSIII, polysaccharide fractions I, II, and III obtained after anion-exchange chromatography from linden PSin70; RGI and II, rhamnogalacturonan type I and II; ROS, reactive oxygen species; SRBC, sheep red blood cells; WBP, whole blood phagocytes.

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1. Introduction

Tilia genus (Tiliaceae) comprises around 45 species of deciduous trees, which are native to the Northern hemisphere, as silver linden (*T. tomentosa*, syn. *T. argentea*) forms coenoses only in the Balkans (Hritcu & Cioanca, 2016; Radoglou, Dobrowolska, Spyroglou & Nicolescu, 2009). Linden (lime tree, basswood or "lipa" in Slavic) is famous in the European mythology, literature, folklore and medicine. Since the middle ages, the linden flower tea has been used as a diaphoretic to promote perspiration. The traditional use of the linden blossom is for common cold, feverish colds, cold-related coughs, catarrhs, mental stress, high blood pressure, headache, indigestion, diarrhea, skin ailments, etc (Chinou, 2012a; WHO, 2010). *Tilia* flos (*T. cordata*, *T. platyphyllos*, *T. vulgaris*) is included in the European Pharmacopeia and approved by the German Commission E for internal use for colds and cold-related coughs. It is listed by the Council of Europe as a natural source of food flavouring and with GRAS status from FDA. *T. tomentosa* buds are popular and used worldwide for preparation of tea and dietary supplements, such as the commercially available "silver linden bud extract" used in gemmotherapy (Allio et al., 2015). The largest suppliers of the vegetal product now are China, Bulgaria, Poland, Russia, Romania and Turkey (Hritcu & Cioanca, 2016).

The active constituents in the linden inflorescences are flavonoids, phenolic acids, tannins, essential oils, mucilage, triterpenes and sterols (Hritcu & Cioanca, 2016; Kosakowska et al., 2015). There are not many studies on the biologically active constituents of *T. tomentosa* blossom, except for its flavonoids having anti-inflammatory activity (Chinou, 2012b; Matsuda, Ninomiya, Shimoda, & Yoshikawa, 2002; Viola et al., 1994). It is found that the mucilage is the main component of the linden flower bracts and it is also detected in the subglandular parenchyma tissue of sepals (Hritcu & Cioanca, 2016). The traditional use of the linden mucilage is related to its emollient properties and antitussive activity, but many of its immunomodulating compounds, such as the anti-inflammatory constituents are unknown (Hritcu & Cioanca, 2016). It has been reported that *Tilia* flower mucilage is rich in pectic polysaccharides, however their structural diversity and biological activity are little studied. Kram and Franz (1983, 1985), and Yakovlev (1985) found that pectins are abundant in *Tilia cordata* blossoms. Earlier, Simson and Timell (1978) found that a cambial tissue from basswood, *T. americana*, contains 50–60% pectin. The immunomodulating activity of the linden blossom is attributed to its flavonoids (tiliroside, rutin), coumarins (scopoletin) and monoterpenes, but there are not such studies on the linden pectins (Arcos et al., 2006; Manuele, Ferraro, & Anesini, 2008; Matsuda et al., 2002). Interestingly, aqueous extracts from linden flowers, which should contain pectins, have shown *in vitro* stimulatory effects on lymphocyte proliferation and antitumor activity (Brizi, Marrassini, Zettler, Ferraro, & Anesini, 2012). In search of the mucilaginous effect on irritated buccal membranes, Schmidgall, Schnetz, and Hensel (2000) showed that a raw polysaccharide complex from the flowers of *T. cordata* expresses a moderate bioadhesion to epithelial tissue *ex vivo*.

Pectic polysaccharides contain three main building blocks covalently linked together – unbranched homogalacturonan (HG), ramified RGI and II. The linear HG is methyl-esterified and O-acetylated to different extent. RGI is also acetylated but branched at C-4 of the L-Rha residues with side chains of α-(1 → 5)-L-arabinans, β-(1 → 4)-D-galactans, arabinogalactan (AG) type I and II (Ridley, O'Neill, & Mohnen, 2001; Schols & Voragen, 2002). The AG structural motifs are extremely important for the expression of immunomodulating activity. This could clearly be seen from the works of Yamada & Kiyohara on pectic polysaccharides isolated from the Japanese Kampo medicine formulations and Paulsen and coworkers on pectins from Malian medicinal plants (Paulsen & Barsett, 2005; Wangensteen, Diallo, & Paulsen, 2015; Yamada & Kiyohara, 2007). RGII is composed of a partly methyl-esterified galacturonan backbone, carrying four oligosaccharide side chains rich in rare sugars (2-Me-Fuc, 2-Me-Xyl, Api, Kdo and Dha) (Ridley et al., 2001). Pectins have beneficial effects on the immune, digestive, excretory, cardiovascular, endocrine, nervous and respiratory systems (Groudeva, Kratchanova, Panchev, & Kratchanov, 1997; Fan et al., 2012; Liu, Dong, Yang, & Pan, 2016; Šutovská, Capek, Fraňová, Pawlaczyk, & Gancarz, 2012; Xie, Zhang, & Zhang, 2015; Yamada & Kiyohara, 2007). The pectic polysaccharides exhibit moderate immunomodulating effects against T-cells, B-cells, NK-cells, neutrophils, macrophages, dendritic cells, thrombocytes and abnormal tumor cells (Bijak et al., 2013; Holderness et al., 2011; Inngjerdingen et al., 2007; Inngjerdingen et al., 2008; Ma et al., 2013; Yamada & Kiyohara, 2007). These actions combined with their regulatory effects on immune signaling proteins, complement system and probiotic bacteria could be useful for prevention and supplementary therapy of infectious, tumor, gastrointestinal and inflammatory diseases.

In our preliminarily study a crude polysaccharide complex from *T. tomentosa* (linden PSC) expressed *ex vivo* immunomodulating activity against human phagocytic leukocytes, T-cells, murine Peyer's patch cells and anti-tumor activity *in vitro* (Georgiev et al., 2017). The aim of the current study is to characterize the chemical diversity and immunomodulating properties of purified pectic polysaccharides from *T. tomentosa* Moench blossoms. This will help in elucidation of the structure-activity relationship of these possible pharmacologically active linden constituents.

2. Materials & methods

2.1. Plant material

Linden flowers were collected in June 2013 in Plovdiv (Bulgaria), air-dried for two weeks and stored in a desiccator until use. The plant material was identified as silver linden, *Tilia tomentosa* Moench (Tiliaceae), by Dr. Evgeny Tsavkov from the Department of Dendrology in the University of Forestry, Sofia. A voucher specimen (SOA061426) was deposited in the Herbarium of the Agricultural University of Plovdiv.

2.2. Isolation and purification of silver linden flower polysaccharides

Linden PSC was extracted with boiling water as previously described (Georgiev et al., 2017).

The linden PSC was dissolved in water (10 mg/ml) and precipitated twice with 95% ethanol (24 h each) with a final alcohol concentration of 70%. This step was for partly removing of smaller molecules. After each run polysaccharide precipitates were separated by centrifugation at 4000 rpm for 30 min at 20 °C. Then, the recovered highest in yield (47.1% on linden PSC basis) polysaccharide-containing fraction insoluble in 70% ethanol (linden PSin70) was applied to a DEAE-Sepharose FF column (1.5 × 50 cm) in HCO^-_3 form, coupled with a FPLC BioLogic® LP system (BioRad). The column was eluted successively with 170 ml water, then with 300 ml of: 0.35 M NH_4HCO_3; 0.45 M NH_4HCO_3 and 1.0 M NH_4HCO_3, at 1 ml/min flow rate. Three pectic polysaccharide fractions PSI-PSIII were obtained, as 7 ml fractions were collected and peaks were visualized by the phenol-sulfuric acid (not shown) and *m*-hydroxydiphenyl methods (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Blumenkrantz & Asboe-Hansen, 1973). The samples were concentrated under vacuum, dialyzed against water (MWCO 12–14 kDa, 72 h at 4 °C), filtered through 0.2 μm cellulose nitrate filters (Sartorius), freeze-dried and stored in a desiccator in dark.

2.3. General methods

Total amount of sugars was quantified by the phenol-sulfuric acid method, using 1:1 mixture of glucose and galacturonic acid as a standard (Dubois et al., 1956). Anhydrouronic acid content (AUAC) was determined by the *m*-hydroxydiphenyl method, using galacturonic acid as a standard (Blumenkrantz & Asboe-Hansen, 1973). Degree of methoxylation (DM) was determined after methanol release with 0.5 M NaOH and its further oxidation to formaldehyde by alcohol oxidase (*P. pastoris*, Sigma-Aldrich), and measuring of the absorbance of the obtained colored product with Purpald® (Sigma-Aldrich) at λ = 550 nm (Anthon & Barrett, 2008). Degree of acetylation (DA) was measured according to McComb and McCready (1957), using β-D-glucose pentaacetate as a standard. Molecular weight was determined by HPSEC-RID (Waters) with Shodex standard P-82 kit (pullulan standards, Showa DENKO, Tokyo, Japan) (Georgiev, Ognyanov, Yanakieva, Kussovski, & Kratchanova, 2012). The elution profile of PSIII (1.9 mg/1 ml) was also analyzed by FPLC on a Sephacryl S300 column (100 × 1.5 cm) at a flow rate of 0.5 ml/min 0.2 M NaCl and 5 ml fractions were collected. Protein content (BSA standard) was analyzed according to Bradford (1976). Only distilled water was used for all experiments.

2.4. Monosaccharide composition and glycosidic linkage determination

Polysaccharide samples (1 mg) with added 100 μg mannitol as an internal standard were subjected to methanolysis with anhydrous 3 M HCl in methanol for 24 h at 80 °C. The formed methyl glycosides of different monosaccharides were converted into their corresponding TMS derivatives and analyzed by GC-FID on a Focus GC system (Thermo Scientific, Milan, Italy), according to Chambers and Clamp (1971) and Barsett, Paulsen, and Habte (1992). The presence of KDO (3-deoxy-D-manno-2-octulosic acid) was detected by the thiobarbituric acid assay, as modified by York, Darvill, McNeil, and Albersheim (1985).

Glycosidic linkage composition of PSI and PSIII (2 mg) were examined by the methylation approach. Prior to methylation uronic acids in the polysaccharides were reduced with $NaBD_4$ to 6,6′-dideuterio-sugars that could be differentiated from the neutral sugars (Kim & Carpita, 1992). The procedure was completed by methylation, acid hydrolysis and preparation of alditol acetates, partly as described by Ciucanu and Kerek (1984). First hydrolysis was performed with 2.5 M TFA for 2 h at 100 °C. Secondly, only for PSIII (2 mg) formolysis with 90% HCOOH (1 ml) for 6 h at 120 °C and a further hydrolysis by addition of 5 ml water for 1 h at 100 °C was carried out on the methylated polymer. This was necessary for the complete hydrolysis of the glycosidic bonds in PSIII. The partly methylated alditol acetates were analyzed by a GC-EI/MS-QP2010 system (Shimadzu, Kyoto, Japan), coupled with a Restek Rxi-5MS column (30 m, 0.25 mm i.d., 0.25 μm film) in a Scan mode (40–450 ions), as described by Ho, Zou, Aslaksen, Wangensteen, and Barsett (2016). The detection and quantification of the relative amounts of each glycosidic linkage type was based on the chromatographic behavior (RT), ionization patterns and results from the monosaccharide composition.

The presence of AGII was detected by precipitation with the ß-glucosyl Yariv reagent (Van Holst & Clarke, 1985).

2.5. Contamination with microbial lipopolysaccharides (LPS)

Eventual LPS contamination was checked by a possible detection of 3-hydroxy fatty acids as their specific chemical markers in the form of acetylated fatty acid methyl esters on the GC-EI/MS-QP2010 system (Shimadzu, Kyoto, Japan), coupled with the Restek Rxi-5MS column (30 m, 0.25 mm i.d., 0.25 μm film) in a SIM mode (257 ion intensity for calculation), as described by de Santana-Filho et al. (2012) and Ho et al. (2016).

2.6. Nuclear magnetic resonance (NMR) spectroscopy

PSIII (12 mg) was dissolved in D_2O, freeze-dried, re-dissolved in 700 μl D_2O and 500 μl sample was used. 1D ^1H, ^{13}C and 2D $^{13}C/^1H$ HSQC, $^1H/^1H$ COSY 90, $^1H/^1H$ TOCSY, $^1H/^1H$ ROESY, $^{13}C/^1H$ HMBC, $^{13}C/^1H$ HSQC-NOESY experiments were performed on a Bruker Avance III HD Ascend 800 MHz spectrometer operating at 800.03 MHz (Bruker, Fällanden, Switzerland) with the software Bruker TopSpin™ 3.5 at a temperature of 333.15 K. The chemical shifts were referenced to an internal standard DSS (sodium 4,4-dimethyl-4-silapentane-sulfonate).

2.7. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of PSIII before and after saponification with NaOH were obtained on a Nicolet™ FTIR spectrometer (Thermo Fischer Scientific, USA) controlled by the OMNIC 3.2 software. The sample was pressed onto the attenuated total reflectance (ATR) crystal using a high pressure tower to provide consistent results. No more than 2 mm^3 of the sample was used to cover the ATR diamond cell.

2.8. Small-angle X-ray scattering (SAXS)

SAXS experiments of PSIII aqueous solutions in different concentrations and at different temperatures were carried out using a Bruker Nanostar instrument (RECX-Norway, Cu-Kα radiation λ = 1.54 Å). The instrument setting allows to access a Q-range between 0.009 $Å^{-1}$ to 0.2 $Å^{-1}$, where Q is defined by $Q = 4\pi\sin(\theta/2)/\lambda$, as θ is the scattering angle and λ the wavelength. The samples were measured in a Quartz cell of 1 mm path length. The data were analysed on an absolute scale (water as a primary standard), using a coexistence model of a linear polymer chain:

$$I(Q) = I_{cluster}(Q) + I(Q)_{chain}$$

where the contribution from the dissolved polymer chains can be written as:

$$I(Q)_{chain} = \frac{\varphi M_w}{d_p}(\rho_p - \rho_0)^2 P(Q)_{chain}$$

Here φ is the volume fraction of a polymer, M_w is the weight average molecular weight, $d_p = 1.2$ g/ml is the density for typical hydrogen-bonding polymers (Sommer, Pedersen, & Stein, 2004), ρ is the electron density for the solvent, $\rho_0 = 9.4 \times 10^{10}$ cm^{-2} and the polymer ($\rho_p = 1.1 \times 10^{11}$ cm^{-2}). $P(Q)_{chain}$ is the form factor derived by Beaucage (1996):

$$P(Q)_{chain} = \exp\left(-\frac{Q^2R_g^2}{3}\right) + \frac{d_f}{(QR_g)^{d_f}}\Gamma\left(\frac{d_f}{2}\right)\cdot\left(\frac{\left[erf\left(\frac{Q\cdot Rg}{\sqrt{6}}\right)\right]^3}{Q}\right)^{df}$$

where R_g is the radius of gyration, d_f is the fractal dimension ($d_f = 2.0$, 1.7 for θ and a good solvent, respectively). Γ(x) is the gamma function and erf(x) is the error function. The cluster contribution can be taken into account by the term:

$$I(Q)_{blob} = \frac{C}{(1 + Q^2\xi^2)^2}$$

where ξ is the characteristic size of the cluster (correlation length), and C is a numerical constant.

2.9. Immunomodulatory effect

2.9.1. Ethics

Blood sampling procedure was in accordance with the Helsinki Declaration of 1975, as revised in 1983. Animal experiments were approved by the Animal Research Committee of the Kitasato University, and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at the Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan.

2.9.2. Animal procedure and care

Female C3H/HeJ mice (6–8 weeks old) were purchased from Japan SLC (Shizuoka, Japan), and male ICR mice (4 weeks old) were from Charles River (Tokyo, Japan). The mice were housed in plastic cages in an air-conditioned room at 23 ± 2 °C with a relative humidity of 55 ± 10% under a 12 h-light-dark cycle, and fed a standard laboratory diet with water given *ad libitum*.

2.9.3. Complement fixation assay

The inhibition of complement hemolysis (complement fixation) of target antibody sensitized sheep red blood cells (SRBC) by a normal human serum pre-treated with PSI, II or III was performed according to Michaelsen, Gilje, Samuelsen, Høåsen, and Paulsen (2000) (Method A). A highly active pectic polysaccharide BP-II from the aerial parts of *Biophytum petersianum* was used as a positive control (Grønhaug et al., 2011). The complement fixation activity induced by the test samples was calculated on the basis of a colorimetric measurement of hemoglobin released from lysed SRBC after incubation with a normal human serum pretreated with PSI, II or III by the formula: $[A_{control} - A_{test}]/A_{control} \times 100\%$. From these data a dose-response curve was constructed and the concentration of a test sample giving 50% inhibition of lysis (IC_{50}) was calculated. The results are obtained from two independent experiments.

2.9.4. ROS production from human whole blood phagocytes (WBP) and isolated neutrophils

Heparinized (50 IU/ml) blood samples were obtained from the cubital vein of healthy human volunteers after overnight fasting. The isolation of human neutrophils from venous blood was accomplished according to the procedure of Nauseef (2014). Kinetics of ROS production by WBP and isolated neutrophils was analyzed by luminol-enhanced chemiluminescence (CL) for a period of 60 min at 37 °C, using 96-well white flat bottom culture plates on a luminometer (Orion II Berthold Detection Systems GmbH, Germany). The principle of the method was previously described (Vasicek, Lojek, Jancinova, Nosal, & Ciz, 2014). Briefly: 25 μl of ten times diluted blood with Hank's buffered salt solution (HBSS) or 0.2×10^6 cells/well of isolated neutrophils in phosphate-buffered saline (PBS) were mixed with 25 μl 1 mM luminol (10 mM stock solution in 0.2 M borate buffer) HBSS solution and incubated with HBSS solutions of PSI, II or III (50 and 100 μg/ml) for spontaneous (non-stimulated with known activators) CL. Furthermore, 12.5 μl or 5 μl of ten times diluted blood (or 0.2×10^6 cells/well) mixed with 25 μl luminol were used for incubation of each linden polysaccharide with 25 μl (0.5 μg/ml) phorbol 12-myristate 13-acetate (PMA-activated, Sigma-Aldrich) or 25 μl (62.5 μg/ml) OZP (OZP-activated). The final volume for spontaneous, PMA- and OZP-activated CL tests was 250 μl. PMA and OZP were used as ROS production activators. The recorded values included the intensity of CL emitted during the time interval studied (integral of CL). The results are expressed as percentage of the control (mean ± SEM) from five independent experiments.

2.9.5. Determination of the cell surface expression of adhesion molecules

The measurements were performed according to the method previously described (Gallova, Kubala, Ciz, & Lojek, 2004). Briefly, human neutrophils (1×10^5 cells) were incubated in plastic tubes (Falcon, USA) with PSI, II or III (100 μg/ml) for 1 h and with the activators fMLP (N-formyl-met-leu-phe, 0.1 μM) and PMA (0.01 μg/ml) for 10 min. After the incubation anti-CD18 (anti-β_2-integrin) and anti-CD62L (anti-L-selectin) monoclonal antibodies (5 μl of each) were added. The samples were incubated at 4 °C for 15 min, subsequently centrifuged, re-suspended in PBS and analyzed by a flow cytometer FACSVerse™ (Becton Dickinson, USA). The median fluorescence intensity was determined and corrected for unspecific staining by subtracting the fluorescence of cells stained with the control antibody (isotype control). The data are expressed as $CD62L^-/CD18^+$ population (mean% of total leukocytes ± SEM) from five independent experiments.

2.9.6. Nitric oxide (NO) production from murine macrophages

Murine macrophages cell line RAW 264.7 (ATCC, USA) were incubated in 12-well flat bottom culture plates at 1×10^5 cells/well with PSI, II or III (50 and 100 μg/ml) alone in DMEM-high Glc supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (500 μl final volume) (HyClone™, GE Healthcare Life Sciences) growth medium for 24 and 48 h at 37 °C and 5% CO_2. The cells were also incubated with polysaccharides in combination with 10 ng/ml LPS (*E. coli*/026:B6, Sigma-Aldrich). The generation of NO was determined indirectly as the accumulation of nitrites in the cell culture supernatant. At the end of the incubation period, the culture media were collected and centrifuged at 16 000×*g*, 4 °C for 10 min. Then, 150 μl of supernatants were mixed with equal volumes of the Griess reagent (Sigma-Aldrich) in a 96-well plate and the mixtures were incubated at room temperature for 30 min in dark. The absorbances were measured at 546 nm and $NaNO_2$ (0–52 μM) was used as a standard (Lojek et al., 2011). The results are expressed as mean ± SEM from three independent experiments.

2.9.7. Western blot analysis of inducible nitric oxide synthase (iNOS)

RAW 264.7 cells, which were obtained as described in 2.9.6., were lysed in the lysis buffer (1% SDS, 0.1 M Tris pH 7.4, 10% glycerol, 0.001 M sodium *o*-vanadate, 0.001 M PMSF). The protein concentrations were determined by using the BCA™ protein assay (Pierce, USA), with BSA as a standard. Equal amounts of protein (18 μg) were then subjected to SDS-PAGE using a 10% separating gel. The expression of iNOS protein was quantified by a Western blot analysis (Pekarova et al., 2011). Anti-iNOS/NOS Type II mouse monoclonal antibody (Cell signaling Technology, 1:5000) and ECL™ anti-rabbit IgG horseradish peroxidase linked whole antibody (from goat; Cell Signaling Technology, 1:2000) were used. The immunoreactive bands were detected using the ECL™ detection kit (Pierce) and exposed to a radiographic film (AGFA). The relative protein levels were measured by scanning densitometry using the ImageJ™ programme, and the individual band density value was expressed in arbitrary units. The results are expressed as mean ± SEM from three independent experiments.

2.9.8. Cytotoxicity assay

Cytotoxicity was estimated by lactate dehydrogenase (LDH) activity in the cell culture supernatant of RAW 264.7 cells, as obtained in 2.9.6., using the LDH Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Switzerland). The results are expressed as mean ± SEM from three independent experiments.

2.9.9. Immunomodulating activity against murine Peyer's patch (PP) cells

Immunomodulating activity was measured by the enhanced production of bone marrow cell-proliferative cytokines from PP cells of specific pathogen-free C3H/HeJ mice ($Tlr4^{Lps-d}$) according to Hong, Matsumoto, Kiyohara, & Yamada (1998). Suspensions (1.2×10^6 cells/ml, 180 μl) of the PP cells (5–9 patches obtained per mouse) from the small intestine of C3H/HeJ mice were cultured with H_2O (negative control), AMOL-1 (pectic polysaccharide from *Astragalus mongholics* as a positive control, Kiyohara et al., 2010) or 100 μg/ml of the polysaccharide fractions (PSI, II or III, 20 μl) in a 96-well flat bottom culture plate (FALCON 3072) for 6 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The resulting culture supernatants (50 μl) were further cultured with a bone marrow cell suspension (5.0×10^5 cells/ml) from male ICR mice for 6 days. The number of proliferated bone marrow cells was measured by Alamar Blue™, as described previously (Hong et al., 1998). The results are shown as mean ± SEM.

2.9.10. Tumor cell growth inhibition in vitro

Growth inhibitory effect of some of the linden polysaccharides was studied against FL cell line (ATCC CCL 62, NBIMCC 94) derived from normal human amniotic cells and different human tumor cell lines: A549 (ATCC CCL 185), LS180 (ATCC CL-187), HT29 (ATCC HTB-38), HeLa (ATCC CCL-2), CaOV (NBIMCC 1108), Jurkat (ATCC, TIB-152) and MOLT-4 (ATCC, CRL-1582). The studied cell lines were treated with PSI or III (50 and 25 μg/ml) for 24, 48 and 72 h, and the cell viability was tested with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide) (Sigma-Aldrich) reagent as previously described (Georgiev et al., 2017). The percent inhibition of the cell growth was calculated using the mean absorbance units from each test sample and the mean data from the control cells incubated in the absence of linden polysaccharides. The samples were analyzed in triplicates.

2.10. Statistical analysis

The statistical significance was analyzed by the Student's *t*-test, using the Statview software (SAS Institute, Inc.). The mean ± SEM were compared to those from the control. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Purification and structural characterization of the linden polysaccharides

3.1.1. Purification and chemical composition of the linden polysaccharide fractions

The linden PSin70 fraction (AUAC = 43.4%) was subjected to anion-exchange chromatography on a DEAE-Sepharose FF ($-HCO_3^-$) column and three defined acidic polysaccharide fractions PSI-III were obtained by a stepwise elution with 0–1 M NH_4HCO_3 (Fig. S1). After sample loading the column was eluted with 170 ml water, but this gave no neutral fractions. We did not find any further carbohydrate-containing fractions when the column was successively washed with 2 M NaCl after the stepwise elution. In Table 1 results from the chemical characterization of the obtained three pectic fractions are presented. The combined yield of the studied samples is 42.7% and it is 20.2% and 1.25% from the linden PSC and dried *T. tomentosa* flowers, respectively. The results from the AUAC analysis (39.9-46.8%) of the samples show that the isolated polysaccharides have acidic character. Kram and Franz (1983) reported between 10 and 63% AUAC in the obtained four acidic fractions from *T. cordata* flowers. Methoxyl esters are detected only in PSI, which is found to be a low methoxylated pectin with DM = 13.1%. The total acetyl content in the three fractions varies between 0.6-3.3%, as PSII and PSIII are characterized with higher acetyl content than PSI, which indicates that these fractions are considerably acetylated. The three polysaccharide samples contain all monosaccharides typical for pectins and also small amounts of Glc and Man. Interestingly, the Rha content is high in all fractions, originating from the high Rha content (11.5%) in the linden PSC for which was shown by a FTIR analysis that it is rich in RGI backbone structures (Georgiev et al., 2017). The values for Rha and GalA in PSII and III are in the same range. Theoretically, the ratio GalA:Rha in the RGI backbone tends to be 1:1, as this ratio for PSI, II and III is 6.2, 1.3 and 1.1, respectively. According to Denman and Morris (2015) the difference between GalA and Rha could give the approximate content of HG (%) in pectins. For PSI the HG content is estimated to be 57%, following 8.3% for PSII and 2.0% for PSIII. It was calculated that GlcA is 30–31% from the uronides in the linden PSC on the basis of colorimetric and chromatographic determination of both uronic acids (Georgiev et al., 2017). Interestingly, the GlcA content is high in PSII and III, compared to the other monosaccharides present. Generally, GlcA and/or 4-O-Me-GlcA are presented in minor amounts linked to the AG side chains, thus expected to be lower than Gal + Ara (Ridley et al., 2001). This is the case in PSI but it is not the same for PSII and III. Additionally, GlcA:Rha ratio is 0.7 and 1.0 for II and III, which shows that the uronic acid is not a rare substituent in our RGI. PSI appears to be a typical pectic polymer with high HG content, PSII and III are unusual RGsI, due to the high content of GlcA. The gross calculation for the glucuronic acid-containing polymers indicates that they are presented in more than 0.7% in the dried flowers.

PSI has a considerably lower M_w (28.2 kDa) than PSII and III – 949 and 382 kDa, respectively. Furthermore, PSIII was eluted as a single peak with 0.2 M NaCl on a Sephacryl S300 column and its molecular weight was determined to be 352 kDa, using pullulan calibration standards (0.59×10^4–40.4×10^4 Da). This value is in the range between M_w and the number average molecular weight M_n calculated for PSIII from the HPSEC-RID results (Table 1). The polydispersity index M_w/M_n for PSII and PSIII is 1.39 and 1.22, respectively. The thiobarbituric acid assay shows the presence of KDO in PSI, which is used as an indicator for presence of RGII. The specific immunodiffusion with the *β*-glucosyl Yariv reagent for detection of 3,6-galactans or AGII is positive only for PSI and II. PSI is distinctive with the highest content of Ara and Gal, followed by PSII. The isolated linden pectins are also characterized by a very low protein content <1.0%.

The three purified pectic fractions (PSI-PSIII) were tested for the presence of LPS, which are considered to be contaminants during the purification procedure by detection of some characteristic LPS fatty acid constituents using GC–MS analysis. It appears that there is no LPS contaminants in these polysaccharide fractions because the LPS marker 3-hydroxy tetradecanoic acid (3-OH-$C_{14:0}$) was not detected (de Santana-Filho et al., 2012).

3.1.2. Linkage determination

In Table 2 are shown the results for estimation of the linkage composition in PSI and III. Based on the quantitative linkage results, we found that PSI is composed predominantly of 1,4-linked Gal*p*A (59.4%) and 1,2-linked Rha*p* (8.4%) with a minor amount of 1,2,4-linked Rha*p* (1.4%) most probably substituted with arabinans and highly branched AGs with AGII structural characteristics. This means that PSI contains mainly HG and RGI structures. These findings are in a good agreement with the calculated HG content according to Denman and Morris (2015) and the Yariv's test for AGII. It is estimated that the 1,2,4-linked Rha*p* is 12.7% from the total Rha in PSI (Table 2). The presence of 1,3,4-linked Gal*p*A units in both samples is not unusual and it could be an indicator for RGII structures and other substituted galacturonans, such as xylogalac-

Table 1
Yields and chemical characterization of silver linden pectic fractions obtained after anion-exchange chromatography of linden PSin70.

	PSI	PSII	PSIII
Yield [% of linden PSC]	9.0	2.6	8.6
Yield [% of linden PSin70]	19.1	5.4	18.2
Yield [% of dried flowers]	0.56	0.16	0.53
Total sugars[a] [%]	55.2	67.5	64.8
AUAC [%]	46.8	39.7	39.9
DM[b] [mol%]	13.1	n.f.	n.f.
(Methoxyl content [%])	(1.1)		
Acetyl content [%]	0.6	3.3	2.9
Protein content [%]	0.4	n.d.	0.5
LPS [%]	n.f.	n.f.	n.f.
Monosaccharide composition [w/w%]			
Ara	7.5[c] (9.2)[d]	2.3 (2.7)	1.9 (2.2)
Rha	11.0 (12.3)	29.2 (32.3)	29.8 (33.1)
Fuc	trace	trace	trace
Xyl	1.9 (2.3)	0.9 (1.1)	1.0 (1.2)
Man	0.1 (0.1)	0.4 (0.4)	0.2 (0.2)
Gal	6.7 (6.8)	4.7 (4.8)	3.4 (3.5)
Glc	3.3 (3.4)	3.6 (3.6)	2.2 (2.3)
GlcA	1.6 (1.5)	21.4 (20.0)	29.6 (27.8)
GalA	67.9 (64.3)	37.5 (35.1)	31.8 (29.8)
Kdo	+	n.d.	n.d.
AGII	+	+	n.f.
Molecular weight [Da]	2.82×10^4	9.73×10^5 (68.0%)	3.91×10^5 (78.3%)
		1.31×10^5 (14.7%)	5.05×10^4 (15.4%)
(M_w)[e]		0.14×10^4 (17.3%)	0.14×10^4 (6.3%)
(M_n)[f]		(9.49×10^5)	(3.82×10^5)
		(6.81×10^5)	(3.14×10^5)

n.f. – not found; n.d. – not determined.
[a] Mixture of 1:1 Glc:GalA was used as a standard.
[b] Calculated on the AUAC basis, moles of methoxyl groups per 100 mol of GalA.
[c] w/w%, calculated on the total carbohydrate content basis.
[d] mol% of the total carbohydrate content.
[e] Weight average molecular weight.
[f] Number average molecular weight.

Table 2
Glycosidic linkage composition of two linden pectic fractions obtained after anion-exchange chromatography of linden PSin70.

Glycosidic linkages	PSI[c]	PSIII[d]
T-Ara*f*	3.4[a] (4.2)[b]	–
1,3-Ara*f*	0.3 (0.4)	–
1,5-Ara*f*	3.0 (3.6)	–
1,3,5-Ara*f*	0.8 (1.0)	–
T-Rha*p*	1.2 (1.4)	–
1,2-Rha*p*	8.4 (9.4)	29.8 (33.1)
1,2,4-Rha*p*	1.4 (1.6)	–
1,3-Fuc*p*	trace	–
1,4-Xyl*p*	1.9 (2.3)	–
T-Gal*p*	2.6 (2.6)	–
1,3- Gal*p*	0.9 (0.9)	–
1,6-Gal*p*	1.1 (1.1)	–
1,3,6-Gal*p*	2.1 (2.2)	–
1,4-Glc*p*	3.3 (3.4)	2.2 (2.3)
T-Glc*p*A	1.6 (1.5)	29.6 (27.8)
T-Gal*p*A	5.3 (5.0)	–
1,4-Gal*p*A	59.4 (56.2)	1.8 (1.7)
1,3,4-Gal*p*A	3.3 (3.1)	30.0 (28.1)

[a] w/w%, calculated on the total carbohydrate content.
[b] mol% of the total carbohydrate content.
[c] 2.5 M TFA for 2 h at 100 °C.
[d] 90% HCOOH for 6 h at 120 °C and further hydrolysis by addition of 5 ml d. water for 1 h at 100 °C.

turonans (Schols & Voragen, 2002). Normally, these fragments do not occupy high proportion in the initial not enzymatically treated pectins (Grønhaug et al., 2011). It could be stated that the 1,3,4-linked Gal*p*A in PSI might be related to RGII structures because this sample is KDO positive.

The methylated PSIII was subjected to formolysis in order to get a complete hydrolysis of the strong glycosidic bonds in this polysaccharide. Similar approaches have been applied for studying of other acidic polymers (Ohtani, Okai, Yamashita, Yuasa, & Misaki, 1995). After formolysis of the methylated PSIII, 30% of the 1,3,4-linked Gal*p*A units are obtained, which is in a comparable amount to that of the 1,2-linked Rha*p* (29.8%) and T-linked Glc*p*A (29.6%). The obtained 1:1:1 ratio of 1,2-Rha*p*:1,3,4-Gal*p*A:T-Glc*p*A reveals that the Glc*p*A monomers are O-3-bound to the Gal*p*A units in RGI. The ratio 1,4-linked Gal*p*A:(1,4+1,3,4)-linked Gal*p*A for PSIII from Table 2 gives a value of 5.7%, which shows that a minor amount of the GalA units are not O-3 glucuronidated. These GalA units could originate from HG structures. According to the polysaccharide classification, the glucuronidated RGI could be named a glucurono-rhamnogalacturonan I (GlcA-RGI) or an acetylated glucurono-rhamnogalacturonan I in this case.

Because of the low yield of PSII, it was not possible to be performed a linkage determination of this sample. PSII is characterized by a similar acetyl content to PSIII and a high GlcA content of 21.4%, therefore it is assumed that the Glc*p*A units might be also O-3 linked to the Gal*p*A.

3.1.3. NMR structural studies

On the basis of the monosaccharide, glycosidic linkage composition data and yield, PSIII was chosen for further detailed NMR analyses. One- and two-dimensional homo- and heteronuclear NMR experiments on a high-resolution 800 MHz spectrometer were performed in order to elucidate the structural features on the substitution of the GalA with GlcA and to localize the acetyl esters in PSIII. In Table 3 are presented ^{13}C and ^{1}H assignments found from COSY, TOCSY, ROESY, HSQC, HMBC and HSQC-NOESY spectra of PSIII, identified according to literature data and in accordance with the monosaccharide and glycosidic linkage compositions (Ravenscroft et al., 2015; Renard, Crépeau, & Thibault, 1999; Shakhmatov, Atukmaev, & Makarova, 2016; Sengkhamparn

Table 3
^{13}C and ^{1}H NMR chemical shift assignment (δ in ppm) of PSIII – acetylated glucurono-rhamnogalacturonan I, referenced to DSS.

Residue	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C-6 H-6	CH_3CO
→4)-α-GalpA-3-OGlcpA-(1→2	100.3 5.02	70.1 4.10	81.3 4.20	80.3 4.66	73.5 4.77	176.2	
→2)-α-Rhap-(1→	101.3 5.25	79.3 4.11	72.2 3.87	74.7 3.42	71.8 3.71	19.2 1.24	
→2)-α-Rhap-3-OAc-(1→	101.3 5.37	70.4 4.20	– 5.09	78.2 3.61	72.8 4.11	19.2 1.28	22.9 2.09
β-GlcpA-(1→	106.6 4.71	76.4 3.49	78.4 3.52	74.3 3.61	78.2 3.81	177.1	

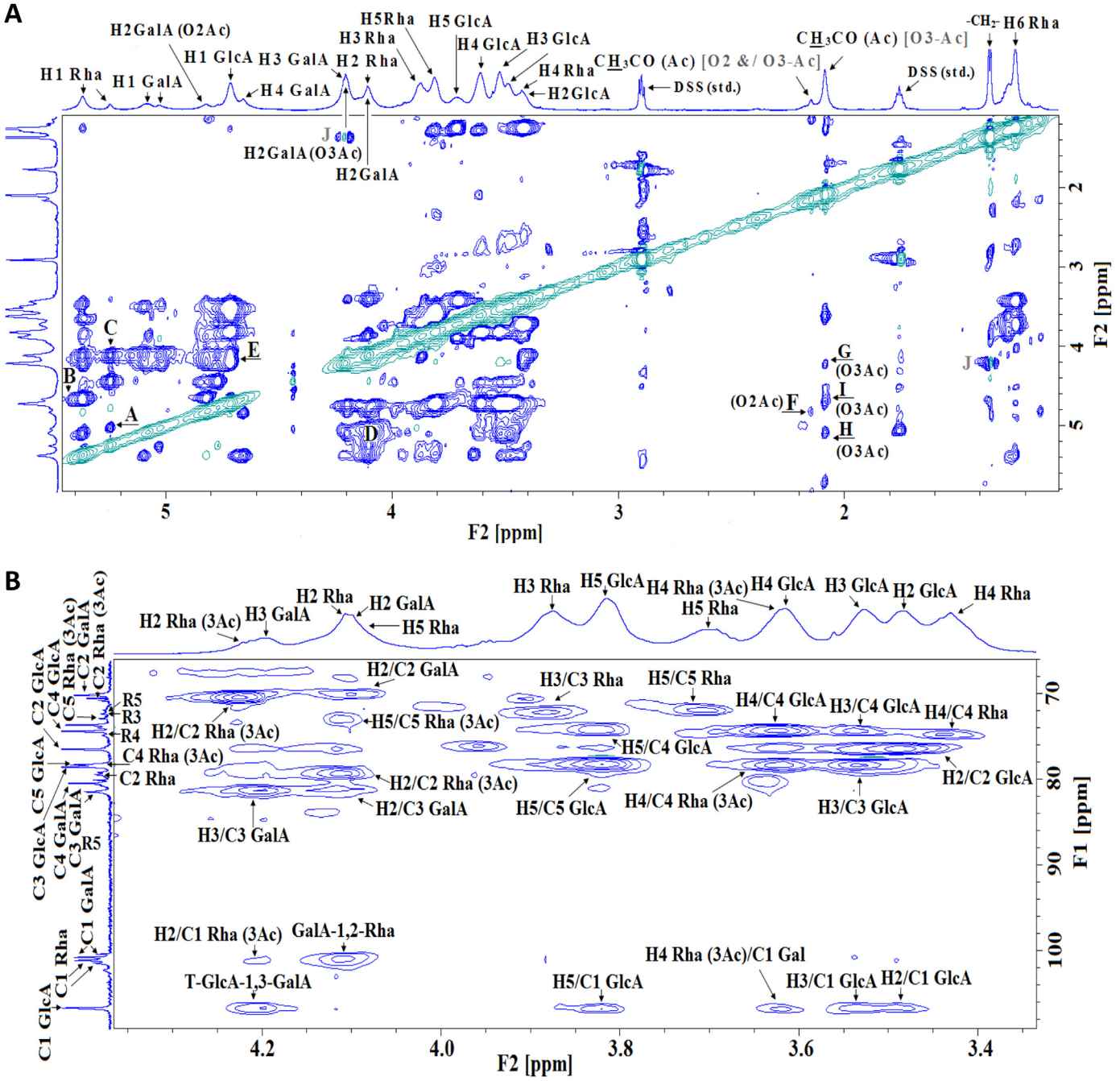

Fig. 1. 2D NMR analysis of linden pectic fraction PSIII. A) H^1/H^1 ROESY spectrum. Each letter (see section 3.1.3.) corresponds to important correlations. B) Partial $^{13}C/H^1$ HSQC-NOESY spectrum. R3-R5 are C3-C5 of the Rha residues.

et al., 2009; Shimizu & Tomoda, 1985). The absolute configurations were assumed to be the same as already found for RGI (McNeil, Darvill, & Albersheim, 1980). The detailed NMR study confirmed that PSIII is a GlcA-RGI, which is predominantly acetylated at O-3 of the α-L-Rha*p* units. The ROESY spectrum(Fig. 1A) reveals the following cross-peaks: H1 of α-L-Rha*p* and H1 of α-D-Gal*p*A residues at 5.25/5.02 ppm (**A**); H1 of internal α-L-Rha*p* with H4 of α-D-Gal*p*A units at 5.37/4.66 ppm (**B**); H1 of α-L-Rha*p* with H3 of α-D-Gal*p*A residues at 5.37/4.20 ppm (**C**); H2 of α-L-Rha*p* with H1 of α-D-Gal*p*A units at 4.11/5.02 (5.09) ppm (**D**). These correlations confirm the presence of the RGI backbone structure [→4)-α-D-Gal*p*A-(1 → 2)-α-L-Rhap-(1 → 4)-α-D-Gal*p*A-(1 →]$_n$ in PSIII. The absence of a correlation between H1 and H4 of α-D-Gal*p*A units confirms that the HG is really a minor part of the polymer.

More intriguing is a the through space cross-peak between H1 of terminally linked β-D-Glc*p*A units and H3 of 1,4-α-D-Gal*p*A residues at 4.71/4.20 ppm (**E**) in the ROESY spectrum (Fig. 1A). A correlation between C1 of β-D-Glc*p*A and H3 of 1,4-α-D-Gal*p*A at 106.6/4.20 ppm in the HSQC-NOESY spectrum is also found (Fig. 1B). This NMR information reveals that 1,4-α-D-Gal*p*A units in the RGI are O-3 substituted with β-D-Glc*p*A monomers. In fact, the chemical shifts for H2 (4.10 ppm), H3 (4.20 ppm) and H4 (4.66 ppm) of α-D-Gal*p*A, and H1 of 1,2-α-L-*R*ha*p* show markedly movement to the downfield region in comparison with literature data for pectins (Shakhmatov et al., 2016). This was also observed by Renard et al. (1999) and it indicates that α-D-Gal*p*A is substituted out of the RGI backbone and that the substitution influences the nearly located Rha. Beta anomeric configuration of Glc*p*A is suggested directly by the identified chemical shift of the H1 proton at 4.71 ppm (Renard et al., 1999).

The cross-peaks in the HSQC and HSQC-NOESY spectra for carbon and proton signals from methyl group of acetic acid ester at 22.9/2.09 ppm and 22.9/2.15 ppm are found, which confirms the presence of ester-linked acetic acid in PSIII. Correlations between the acetyl methyl protons and acetyl carbonyl carbons are observed in the C6 region of uronic acids in the HMBC spectrum. C6 signals of both uronic acid residues and $CH_3\underline{C}O$ group are overlapped. The presence of two peaks for $-\underline{C}H_3CO$ group at 2.09 ppm and 2.15 ppm suggests O-3 and O-2 acetylation in PSIII. The signal at 2.09 ppm is more intensive (integral ratio 2.09:2.15 ppm 3,8:1) and according to literature data attributed to O-3 acetylation of GalA in the HG, as well as of the Rha in RGI (Renard & Jarvis, 1999; Sengkhamparn et al., 2009). The proton methyl signal at 2.09 ppm is correlated with the peaks at 1.28 ppm (**F**), 3.61 ppm (**G**), 4.71 ppm (**H**) and 5.09 ppm (**I**) in the ROESY spectrum (Fig. 1A). The correlation **G** is the most intensive, supporting the closeness between the proton at 3.61 ppm with the acetyl protons. The correlation **F** indicates that the acetyl groups are within the Rha ring. The detailed analysis of the COSY, TOCSY, ROESY, HMBC and HSQC-NOESY spectra is in agreement with the predominant localization of the acetyl groups at O-3 of 1,2-α-L-*R*ha*p* units (Fig. 2). This is demonstrated by the downfield shift of H2 (4.20 pm), H3 (5.09 ppm), H4 (3.61 ppm) and H5 (4.11 ppm) of the 1,2-α-L-*R*ha*p* residues. By calculating the DA of PSIII on Rha basis instead of GalA basis, it is estimated that 33.3% of the Rha units are acetylated. The ratio between integral intensities of H6 of 1,2,-α-L-*R*ha*p*:1,2-α-L-*R*ha*p*-3OAc is 2.16:1, indicating that approximately 32% of the Rha units are acetylated. Similarly, Sengkhamparn et al. (2009) observed downfield shift of H2 (4.22 ppm), H3 (5.24 ppm) and H5 (3.91 ppm) of the O-3 acetylated 1,2,4-α-L-*R*ha*p* in pectin isolated from okra. Interestingly, *A. esculentus* (okra) belongs to Malvaceae family, where the linden was classified initially. The correlation between the resonances at 2.09 ppm and 4.71 ppm in the ROESY spectrum suggests that the proton at 4.71 ppm is conformationally close to the acetyl group. The monosaccharide composition and preliminarily glycosidic linkage analysis of PSIII indicate that it contains a small amount of Gal that might be linked to O-4 of 1,2-α-L-Rha*p*-3OAc. Therefore, it is proposed that the signal at 4.71 ppm contains the anomeric proton of T-β-D-Gal*p* units (Shakhmatov, Udoratina, Atukmaev, & Makarova, 2015). This is supported by the finding that the proton at 4.71 ppm (106.6 ppm) is adjacent to the proton at 3.52 ppm (74.3 ppm) in the COSY spectrum and by one cross-peak between 106.6 ppm and 3.61 ppm in the HSQC-NOESY spectrum (Fig. 1B).

The assignment of carboxyl carbonyl carbons C6 of both uronic acids was done from the HMBC spectrum, where the following information revealed, the proton H5 of 1,3,4-α-D-Gal*p*A at 4.77 ppm is correlated with the carbons at 176.2 ppm. Similarly, the H5 proton of β-D-Glc*p*A resonating at 3.81 ppm is connected with the carbons at 177.1 ppm. The absence of a signal at 53–55 ppm arising from $-O\underline{C}H_3$ of methyl ester group confirmed that PSIII is with very low DM or not methyl-esterified (Shakhmatov et al., 2016). From additional analysis of the COSY, TOCSY, ROESY and HMBC spectra, it was suggested that the carbon and proton resonances respectively at 183.3 ppm and 1.35-1.36 ppm arise from −CO and $-CH_3$ of fatty acids (RCOOH), which were detected in the initial linden PSC and probably partly left in PSIII after the alcohol pretreatment and anion-exchange chromatography of the linden PSin70 (not shown). A model structure of PSIII with some important H/H and C/H interactions from the NMR analyses are presented on Fig. 2.

3.1.4. FTIR analysis

The presence of acetyl esters is also confirmed by the FTIR study (Fig. S2), where bands with similar intensities at 1248 cm^{-1} ν(C—O—C)$_{Ac}$ and 1721 cm^{-1} ν(C═O)$_{Ac}$ are detected and disappear when the sample is saponified with NaOH (Paulsen, Fagerheim, & Øverbye, 1978). The bands at 3347 cm^{-1}, 2935 cm^{-1}, 1600 cm^{-1} and 1417 cm^{-1} for υ(OH), υ(CH), ν_{as}(COO$^-$) and ν_s(COO$^-$) are also observed (Filippov, Luknár, & Kohn, 1978). The presence of the signals at about 1151, 1037, 991, 958 and 819 cm^{-1} are also found in the linden PSC (Georgiev et al., 2017). Most of these signals, in addition to those at 916 and 844 cm^{-1} registered in the FTIR spectrum of PSIII are related to the RGI backbone structure according to the literature data (Kačuráková, Capek, Sasinková, Wellner, Ebringerová, 2000).

3.1.5. Small-angle X-ray scattering studies

In order to investigate the nanostructure of PSIII in water, SAXS experiments were performed. The absolute macroscopic scattering cross-section $d\Sigma/d\Omega(Q)$ as a function of modulus of the scattering vector Q ($Q = 4\pi \sin\left(\frac{\theta}{2}\right)/\lambda$, where λ = 1.54 Å and θ is the scattering angle) is depicted on Fig. 3. The function represents a typical polymer-like scattering at an intermediate and a high Q, while at a lower Q a significant upturn is observed with a close to Q^{-4} power-law characteristic for large clusters. This indicates the presence of larger aggregates (> 100 nm) in coexistence with chain-like molecules. Interestingly, the structure is essentially independent of concentration and temperature, suggesting rather long-lived stable aggregates. More quantitatively, the data can be described using a simple coexistence model of clusters and single chain (see section 2.8.). The fits provide an average molecular weight of 332 kDa for the dissolved chain, R_g = 4.6 nm and d_f = 1,7. The clusters are larger than what can be resolved by SAXS (> 100 nm) and could not be analyzed in details. The aggregation of pectins in aqueous solutions could be expected, thus different salts were used in the HPSEC and preparative SEC analyses to prevent it. Despite of the aggregation, the calculated M_w is in a good agreement with the determined values from HPSEC (382 kDa) and preparative SEC analyses (352 kDa).

3.2. Immunomodulating studies

We have already found that the linden PSC possesses *ex vivo* immunomodulating potential against human phagocytic leuko-

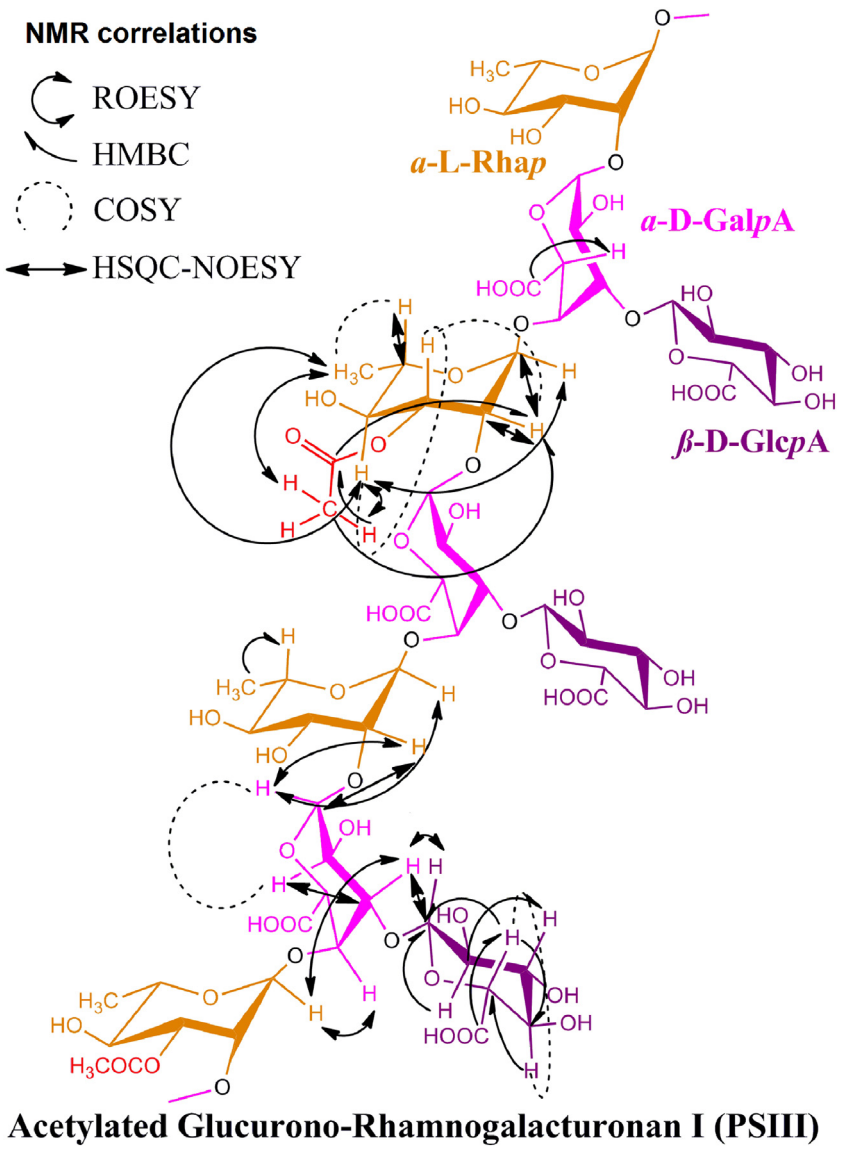

Fig. 2. Chemical structure of linden pectic fraction PSIII, elucidated by GC–MS glycosidic linkage analysis and 2D NMR spectroscopy. The most important H/H and C/H interactions are shown.

cytes, T-cells, murine PP cells and anti-tumor activity *in vitro* (Georgiev et al., 2017). In order to investigate the role of linden pectins in the observed immunomodulating activity of the initial linden PSC and to evaluate immune potency of each purified linden pectic fraction were performed some important biological experiments. In the following sections are presented the results from these experiments with purified linden pectic fractions.

3.2.1. Complement fixation activity

The three linden pectic fractions were tested against inhibition of human complement hemolysis of target SRBC *in vitro* (Fig. S3). PSI expressed the highest complement fixation activity with IC_{50} of 154.5 μg/ml, which was 4.7 fold higher than the IC_{50} value of the positive control, the highly active pectic polysaccharide BP-II from *B. petersianum* (Grønhaug et al., 2011). PSII and PSIII expressed lower activities, as IC_{50} value of PSIII was 491.6 μg/ml. Most probably the prevalence of the complement fixation activity of PSI was related to the presence of AGII structures active on the human complement system (Ho et al., 2016).

3.2.2. Phagocyte immune-response

3.2.2.1. Human WBP and isolated neutrophils

The typical pectic polysaccharide (PSI) and the two GlcA-RGsI (PSII and PSIII) increased ROS production from WBP at 100 μg/ml and only PSIII increased CL response from isolated neutrophils (Fig. 4A and B). Meanwhile, with co-stimulation by PMA, PSI and PSIII did not increase the ROS production from WBP, however PSII slightly enhanced the ROS formation at a concentration of 100 μg/ml (Fig. 4A). Interestingly, under the condition on a co-stimulation with OZP, PSI and PSII clearly decreased the ROS production at the higher concentration of 100 μg/ml in comparison with the control of the OZP stimulation alone (Fig. 4A). However, all polysaccharide fractions did not show a clear enhancing activity on the ROS formation from the isolated neutrophils in the presence of PMA and OZP (Fig. 4B). PMA and OZP significantly increased the CL signal of both WBP and isolated neutrophils. In the case of spontaneous ROS formation the CL signal caused by the linden pectins at the higher concentration of 100 μg/ml was much lower than those

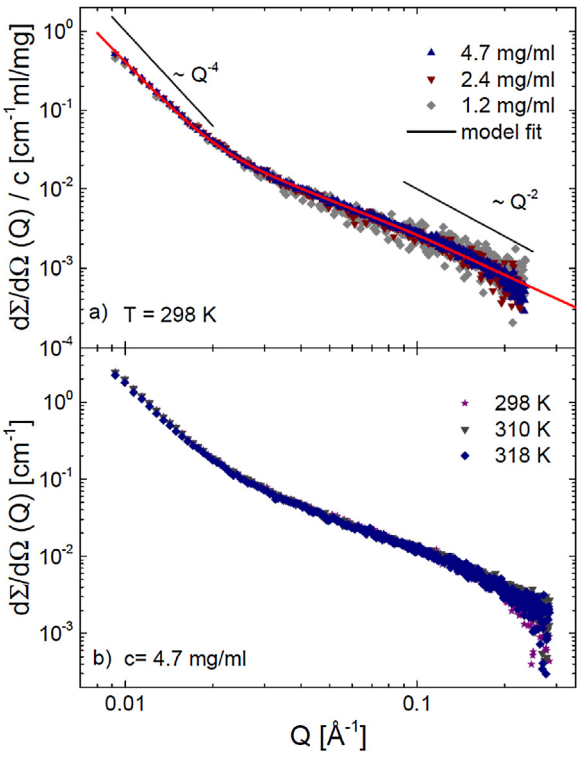

Fig. 3. Small-angle X-ray scattering data showing the absolute macroscopic scattering cross-section $d\Sigma/d\Omega(Q)$ as a function of modulus of the scattering vector, Q for PSIII aqueous solutions. a) at different concentrations and b) at different temperatures.

in the presence of PMA or OZP (Fig. 4A and B). When the effect of the polysaccharide fractions on the expression of CD18 and CD62L on isolated neutrophils was analyzed, only PSIII showed a weak effect on CD18 expression (Fig. S4). This was in agreement with the results from the spontaneous ROS production from both WBP and isolated neutrophils. It was proposed that the unique glucuronidated RGI structure of PSIII might be involved in the observed effects.

3.2.2.2. *Murine macrophages*

Macrophage activation can be evaluated by the release of antimicrobial NO under the catalytic action of iNOS. The purified linden pectins did not have a cytotoxic effect on the RAW 264.7 cells on the basis of the LDH test (Fig. S5). When the iNOS induction was analyzed by western blotting, the stimulation with all linden pectic fractions for 24 h increased the iNOS protein level of the RAW 264.7 cells. The co-stimulation with LPS (10 ng/ml) in the presence of the polysaccharides significantly enhanced the iNOS protein level by comparison with the stimulating condition with the purified pectins alone (Fig. 5A). Otherwise, the simultaneous treatment of the macrophages with LPS and linden polysaccharides led to statistically significant lower iNOS levels at 100 μg/ml than at 50 μg/ml for PSI and PSIII. This effect was similar to the reduction of the ROS formation from WBP co-stimulated with OZP and PSI or PSII (Fig. 4A). When the RAW 264.7 cells were stimulated with the linden pectic fractions for 48 h, the iNOS production reached almost to the same level as that observed in the co-stimulation condition with LPS and linden fractions (Fig. 5A). Similarly to the results from 24 h, the iNOS levels in the cells co-treated with LPS and PSI or PSIII at 100 μg/ml after 48 h were slightly decreased in comparison with the pectic fractions alone. But unlike the results from 24 h, where there was not observed any clear difference in the iNOS expression induced by three samples alone, after 48 h PSI exhibited a higher induction activity at the both studied concentrations.

Meanwhile, the stimulation effect of polysaccharide fractions, alone or in a combination with LPS was analyzed on the NO production from RAW 264.7 cells (Fig. 5B). The NO production was dramatically increased by the stimulation with all fractions for 24 h at concentrations of 50 and 100 μg/ml compared with the growth medium control. The NO release in the control medium was negligible, therefore the adherent cells were not activated by the plate surface. The stimulating activity of PSII seemed to be weaker than those of PSI and PSIII, and there was no a significant difference between the stimulatory activities of PSI and PSIII. However, the combinatorial effect of linden polysaccharides with LPS was tested, and the NO production induced by all linden pectins at 100 μg/ml were lower in comparison with the production in the stimulation with the polysaccharide fractions alone (Fig. 5B). When the stimulation was prolonged for 48 h, PSI induced more than 2 fold higher NO production than PSIII, which could be explained by the different structural features of both samples ($p < 0.001$). This result was in agreement with the iNOS expression after 48 h of incubation (Fig. 5A). After 48 h NO production in the co-stimulation condition was reduced again at 100 μg/ml in comparison with the separate polysaccharide treatments ($p < 0.001$) (Fig. 5B). This effect was in agreement with the iNOS expression levels after 24 h and 48 h of macrophage stimulation. From the cytotoxicity data, combinations with LPS showed relative absorbance units close to the control, which was another confirmation for the suppression-like effect on the LPS-induced inflammatory response in macrophages (Fig. S5). For elucidation of this phenomenon more studies on the effect of linden polysaccharides on the iNOS expression with higher doses are needed.

3.2.3. *Intestinal immunomodulating activity*

It has been found that the linden PSC exhibited *ex vivo* intestinal immunomodulating activity through the PP-mediated bone mar-

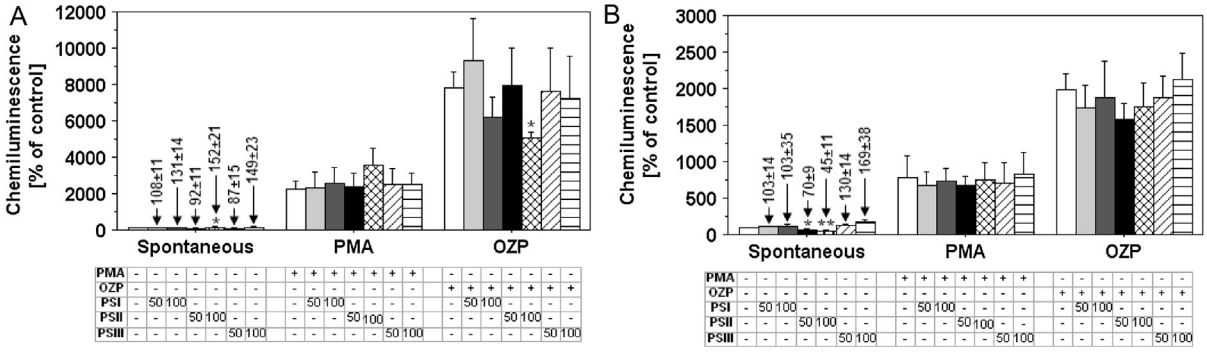

Fig. 4. Effect of linden pectic fractions (PSI-PSIII) on A) ROS production from non-stimulated (spontaneous), PMA- and OZP-activated human whole blood phagocytes. B) ROS production from isolated neutrophils. 50 (50 μg/ml) and 100 (100 μg/ml). The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$) vs. control.

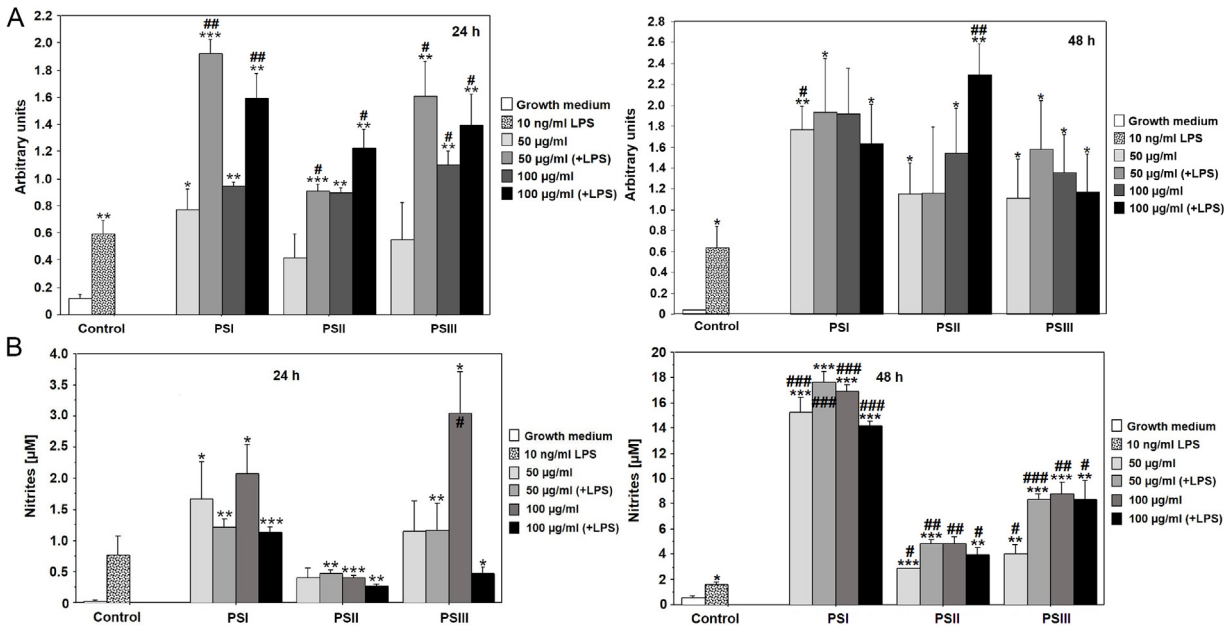

Fig. 5. Effect of linden pectic fractions on A) iNOS expression in RAW 264.7 cells cultured with samples alone or co-stimulated with LPS. B) NO production from RAW 264.7 cells cultured with samples alone or co-stimulated with LPS. LPS from *E. coli* was used as a positive control. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) vs. control – growth medium (untreated cells) and (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$) vs. LPS.

row cell proliferation test and increased IL-6 production from the PP cells (Georgiev et al., 2017). In order to clarify whether partially purified polysaccharides (PSI-PSIII) also show activity, their stimulatory potential was investigated as well. However, only PSI expressed a weak activity ($p < 0.01$) and PSII and PSIII were inactive (Fig. S6).

3.2.4. Anti-tumor activity

When we found that the linden pectins exhibited immunomodulating properties against phagocytes, we decided to test their effect on abnormal cells. In a previous study, the linden PSC expressed a non-specific inhibitory activity against different tumor cell lines and normal amniotic cells and fibroblasts *in vitro* (Georgiev et al., 2017). On the basis of our preliminarily screening results with PSI and PSIII, we found that in addition to the tumor cells, polysaccharides inhibited in a similar degree the growth of the studied FL cells (Fig. S7).

4. Discussion

The need of a further characterization of the silver linden pectins was provoked by elucidation of the role of different pectic fractions as immunomodulating compounds in *Tilia* flowers and also due to the traditional use of the linden tea and mucilage. It was found that PSI is a low-esterified typical pectic polysaccharide with HG and RGI, which is O-4 substituted at the Rha units with 1,3,5-arabinans and highly branched bioactive AGII structures. Furthermore, it was suggested that PSI contains trace amounts of RGII on the basis of the KDO test, the presence of 1,3,4-linked GalA residues and the trace amounts of Fuc. PSII and PSIII are two unusual high molecular weight free RGI polymers that are highly acetylated and highly glucuronidated, almost lacking neutral sugar side chains. Renard et al. (1999) estimated that one GalA unit out of 72 are O-3 substituted with GlcA in sugar beet RGI. Sengkhamparn et al. (2009) found for the first time that Rha in okra pectic RGI can be O-3 acetylated. Later, Leijdekkers, Huang, Bakx, Gruppen, and Schols (2015) reported that a single GalA unit in the RGI from sugar beet pectin could be both acetylated and substituted with GlcA. In the present study it was demonstrated that silver linden flowers contain a unique RGI (PSIII) in which almost every GalA unit is O-3 glucuronidated and at the same time significantly acetylated (>30%) at O-3 of the Rha residues. The O-3 glucuronidated RGI core structure was found in the mucilage of the inner bark of *Hydrangea paniculata* Sieb. in 1976–1977 (Tomoda & Satoh, 1977). The structure is not found often in plants and to our knowledge, it has not been isolated from flowering plant organs (Shimizu & Tomoda, 1985).

Phagocytic leukocytes, such as macrophages and neutrophils, producing ROS and NO play a key role in the innate immunity. They are also involved in the acute inflammatory diseases, thus a proper modulation of their activation is often needed. Our findings suggest that the three purified polysaccharide fractions (PSI-PSIII) are active ingredients in the crude linden PSC for enhancing activity on the ROS production of human WBP and neutrophils (Georgiev et al., 2017). Prevalence in the activity was observed for PSII and PSIII, as only PSIII increased the ROS production from isolated neutrophils. It was suggested that the unique glucuronidated RGI structure of PSIII was important for expression of the activity, which was supported by the weak increase of the surface β_2-integrin levels on neutrophils induced by the sample (Fig. S4). However, the effect of the three polysaccharides was negligible in comparison with the potent activators PMA, OZP and fMLP (Figs. 4 & S4). Furthermore, the effect of PSI-III against the spontaneous ROS generation from WBP at 100 μg/ml was lower than this for the crude linden PSC (420 ± 60%), which could be explained by the combinatorial action of different active compounds in the complex (Georgiev et al., 2017). On the other hand, PSI and PSIII expressed a suppression-like activity on the OZP-induced activation of WBP at the higher concentration of 100 μg/ml, which was not observed with the linden PSC (Georgiev et al., 2017). This effect was also not observed in the experiments with isolated neutrophils. Kiyohara, Matsumoto, Nagai, Kim, & Yamada (2006) showed a significant positive correlation between the reactivity of pectic polysaccharides with IgG and the degree of complement-activating ability. PSI expressed a high complement fixation activity, which supposed that the linden polysaccharides could interact with some blood serum components like antibodies and then act in such a manner to negatively influence the OZP-initiated oxidative burst. Interestingly, Popov, Ovodova, Popova, Nikitina, and Ovodov (2005) reported that a pectic polysaccharide

(100 μg/ml) from *Comarum palustre* does not influence the OZP-activated ROS generation from human neutrophils, but it could reduce the PMA-induced ROS production. The crude linden PSC also enhanced the spontaneous ROS production from WBP and did not increase the CL signal under the co-stimulation with PMA (Georgiev et al., 2017). The three pectic fractions did not contribute to a synergistic increase of the ROS production from the PMA- and OZP-treated neutrophils.

The induction of NO production by the linden pectins did not lead to severe cytotoxic effects under the studied conditions (Fig. S5). PSI expressed a higher macrophage stimulating activity than PSII and PSIII, as probably the RGI AG side chains are important for the macrophage activation, as described in the literature (Grønhaug et al., 2010). Generally, our results from the NO production demonstrated that linden pectins could activate macrophages and this was in agreement with the increased human $CD14^+$ and $CD64^+$ white blood cells and induction of IL-6 production by the linden PSC (Georgiev et al., 2017). The linden pectins exhibited suppression-like effect on the LPS-induced inflammatory response in macrophages, shown by reduction of the iNOS expression and NO production. However, both LPS and linden polysaccharides alone were inductors of the iNOS and NO production. Therefore, there was not a synergism between the microbial antigen and the plant antigen-mimetic, as was expected and even observed by Zhu et al. (2012) for a combination between LPS and a polysaccharide from *Chaenomeles speciosa* fruit. The observed effect suggests that the linden pectins have a potential for stimulation of the innate immunity and suppression of the LPS-induced macrophage inflammatory process similarly to the reduction of the OZP-induced ROS generation from WBP. This could be explained by the proposed polysaccharide anti-inflammatory behavior, which could be useful in supplementation during treatment of autoimmune and infectious diseases. In fact, *T. tomentosa* is famous with its anti-inflammatory action which is attributed mainly to its flavonoids, but probably polysaccharides also contribute to this effect (Allio et al., 2015; Matsuda et al., 2002; Viola et al., 1994). Similar to the dual effect observed in our study was also reported for fucoidan, which stimulated the NO release from RAW 264.7 cells at 100 μg/ml alone, but in a combination with LPS at concentrations higher than 10 μg/ml it inhibited the LPS-stimulated NO release and iNOS expression (Nakamura, Suzuki, Wada, Kodama, & Doi, 2006; Yang, Yoon, Oh, Kim, & Kang, 2006). Authors concluded that the fucoidan may not act on the extracellular receptors, but on the intracellular signaling machinery, such as the process of transcription factor activation. Chen et al. (2006) reported that the LPS-induced (100 ng/ml) iNOS expression level in RAW 264.7 cells was similar to those obtained by a co-stimulation with LPS and DE90 citrus pectin, but increasing of the pectin concentration resulted in a decrement of the iNOS protein expression. They found that DE90 pectin had a potential to inhibit the downstream signals of LPS, and proposed that the inhibition was not a result of the DE90 directly inhibiting the activities of signal proteins, but from the pectin-LPS interactions or different affinity to cell-surface receptors.

The role of GlcA in plant polysaccharides and its impact on the biological activity are intriguing but still not fully elucidated (Grønhaug et al., 2011; Kiyohara et al., 2010). Interestingly, Duan, Chen, Dong, Ding, and Fang (2010) showed that pectin from *D. kaki* structurally similar to PSIII could inhibit the LPS-induced B-cell proliferation and had no effect on the Concanavalin A-induced T-cell proliferation. This, together with our results also suggests that GlcA might be involved in the suppression effect of the linden pectins on the LPS-induced and OZP-induced inflammatory response in macrophages and WBP.

Normally, herbal polysaccharides are contained in infusions, extracts, traditional foods and are administered orally from these formulations. Pectins are little absorbed by the intestinal epithelium, but they may interact with the mucosal immune system through immunocompetent cells in the PP localized in the small intestine. By this mode of action of the active polysaccharides, functionally modulated immunocompetent cells are proposed to result in modulations of several mucosal and systemic immune systems (Yamada & Kiyohara, 2007). Only PSI showed a weak activity ($p < 0.01$) probably related to RGI AG side chains as found previously (Grønhaug et al., 2011; Kiyohara et al., 2010). Maybe acetylated RGI backbone and attached GlcA in PSIII were not enough for expression of activity.

The neutrophils and macrophages are involved in the regulation of both innate and adaptive immunity in various inflammatory processes including tumor diseases. The phagocytes present in the tumor microenvironment can promote or inhibit cancer formation and development (Mantovani, 2014). Brizi et al. (2012) reported that aqueous and ethanol extracts from the flowers of *Tilia x viridis* (*T. tomentosa* and *T. americana* hybrid) exhibited anti-proliferative effects on BW 5147 cells (T-cell lymphoma) and normal Concanavalin A-stimulated lymphocytes *in vitro*, as they related the activity to polyphenols. Our preliminarily screening study with the MTT test demonstrated that linden pectins expressed a non-selective growth inhibitory activity against tumor cells and the studied normal FL cells (Fig. S7). However, PSI-PSIII did not show cytotoxic effects (LDH test) on murine macrophages (Fig. S5) and they did not inhibit the proliferation of murine PP immunocompetent cells (Fig. S6). The difference between the polysaccharide effect on the FL cells and the studied immune cells could be explained primarily by their different functions in the organism. Generally, the immune cells could be activated by complex pectins unlike the tumor cells and probably FL cells.

5. Conclusions

In the current paper, we show that the silver linden blossoms contain two structurally different pectic type polysaccharides – a low-esterified typical pectic polysaccharide PSI (M_w = 28.2 kDa) and unusual considerably acetylated (2.9-3.3%) and glucuronidated RGI polymers PSII (M_w = 949 kDa) and PSIII (M_w = 382 kDa). Furthermore, PSIII is a unique RGI macromolecule, which GalA residues are almost completely O-3 glucuronidated and >30% acetylated at O-3 of the Rha units. The three pectic fractions could stimulate the innate immune response through induction of the ROS and NO generation by neutrophils and macrophages in a non-aggressive manner. At the same time, silver linden pectins suppressed the OZP-activated ROS generation and the LPS-induced iNOS expression and NO production by WBP and murine macrophages, respectively. This dual mode of action on the phagocytes revealed the anti-inflammatory potential of the silver linden polysaccharides. Furthermore, PSI showed the highest complement fixation (IC_{50} = 154.5 μg/ml) and macrophage stimulating (almost 18 μM NO production after 48 h) activities and at the same time, it was the only sample expressing intestinal immunomodulating activity against PP cells. It was shown for the first time that acetylated and highly glucuronidated RGI may exhibit immunomodulating properties via neutrophil and macrophage activation, and suppression of viability of different tumor cell lines. On the basis of our results, we conclude that pectins in *T. tomentosa* inflorescences are immune-active compounds. The silver linden pectins deserve to be included in the supplementary therapy research for prevention and treatment of immune related diseases like inflammatory diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.07.073.

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