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Effects of raspberry fruit extracts and ellagic acid on respiratory burst in murine macrophages

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The mechanism of action of polyphenolic compounds is attributed to their antioxidant, anti-inflammatory, and anti-proliferative properties and their effects on subcellular signal transduction, cell cycle impairment and apoptosis. A raspberry (*Rubus idaeus* L.) fruit extract contains various antioxidant active compounds, particularly ellagic acid (EA); however the exact intracellular mechanism of their action is not fully understood. The aim of the study was to evaluate the antioxidant effect of raspberry extracts, and that of ellagic acid by assessment of the production of the reactive oxygen species (ROS) by murine macrophage J774 cells. Raspberry extracts and their active compound EA did not affect or had very minor effects on cell viability. No significant difference in the ROS generation in arachidonic acid stimulated macrophages was determined for raspberry extracts and EA whereas in the phorbol-12 myristate-13 acetate model ROS generation was significantly ($p < 0.05$) reduced. Our observation that raspberry pomace extracts *in vitro* reduce ROS production in a J774 macrophage culture suggests that raspberry extract and ellagic acid mediated antioxidant effects may be due to the regulation of NADPH oxidase activity.

Introduction

Epidemiological studies confirmed that consumption of polyphenolic compounds reduces the risk of cardiovascular, cancer, and other degenerative diseases.[1] The mechanism of action of polyphenolic compounds is attributed to their antioxidant, anti-inflammatory, and anti-proliferative properties and their effects on subcellular signal transduction, cell cycle impairment and apoptosis.[2,3] The antioxidant effect of polyphenolic compounds may be expressed *via* various mechanisms, mainly by directly scavenging free radicals and inhibiting enzymes, such as NO synthase, xanthine oxidase, cyclooxygenase, lipoxygenase, and NADPH oxidase.[4]

NADPH oxidase is a multicomponent enzyme system, and a prevailing cellular source of reactive oxygen species (ROS), particularly in inflammation.[5] Activated NADPH oxidase produces a superoxide that is toxic not only to pathogens, but damages surrounding normal tissues and cells to cause various abnormalities, such as infection, arteriosclerosis, neurodegenerative diseases and inflammation.[6,7]

Though a lot of antioxidant active compounds from the plant origin have anti-inflammatory properties, the mechanisms of their action are not fully understood. Raspberry (*Rubus idaeus* L.) is a perennial medicinal and edible plant that belongs to the family *Rosaceae* Juss. The species is native to Europe and northern Asia and is commonly cultivated all over the world.[8] Ellagic acid (EA), which is present in the vacuoles of plant cells as hydrolysable tannins called ellagitannins, is the predominant phenolic compound of raspberries.[9] Ellagitannins significantly contribute to the antioxidant activity of red raspberries; they are responsible for up to 60% of the detectable antioxidant capacity of the raspberry fruit.[10] Therefore, EA/ellagitannins are of particular interest from a nutritional and pharmacological point of view. As EA is far more abundant in the seeds of raspberries as compared to pulp and juice,[11] raspberry pomace (residue after juice processing) could be further used as a natural source of ellagitannins and other antioxidants.

Thus, the aim of the study was to evaluate and compare the antioxidant effect of pomace extracts of raspberry cultivars, and that of ellagic acid by assessment of the production of the hydrogen peroxide by murine macrophage J774 cells. Additionally, the effects of raspberry extracts on the viability of macrophages were investigated in order to find the non-toxic concentrations to be used in further experiments.

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Experimental

Chemicals

Trypan blue, Folin–Ciocalteu's phenol reagent, and methanol were purchased from Fluka, Switzerland. Arachidonic acid (AA), phorbol-12 myristate-13 acetate (PMA), phosphate buffered

saline (PBS), horseradish peroxidase, Amplex red, dimethyl sulfoxide, Dulbecco's modified Eagle's medium, acetonitrile (HPLC grade), methanol (HPLC grade), gallic acid, anhydrous sodium carbonate, and ellagic acid were purchased from Sigma-Aldrich, Steinheim, Germany. Concentrated hydrochloric acid and formic acid (98–100%) were purchased from Merck, Darmstadt, Germany. The purified cyanidin-3-glucoside used in this study was obtained from the Danish Inst. of Agricultural Sciences (Dept. of Fruit, Vegetable and Food Science).

Plant materials and preparation of dry extracts

Raspberries were grown in the Lithuanian Research Centre for Agriculture and Forestry, Institute of Horticulture. Two *Rubus idaeus* L. cultivars were selected for extract preparation: red fruiting 'Novokitaevskoje', and yellow fruiting 'Beglianka'. After juicing raspberries, the obtained pomace was frozen and stored in a deep freezer at −30 °C. Prior to analysis, the pomace was thawed and pureed using a blender followed by few minutes' homogenization with a Polytron PT 1200E homogenizer (Kinematica AG, Luzern, Switzerland) at room temperature. Twenty grams of homogenates were extracted with 150 mL of 90% methanol at room temperature under constant shaking (Sklo Union LT, Teplice, Czech Republic). Coupled extracts were filtered and dried in a rotary vacuum evaporator (to remove methanol) and freeze-dryer (to remove water). Dry extract powders were kept in the hermetically sealed containers in a freezer until being used.

Analysis of ellagic acid and ellagitannins

Ellagitannins were determined as EA equivalents after acidic hydrolysis using the method described by Koponen *et al.*[12] Free EA was analyzed prior to acid hydrolysis. HPLC analysis of ellagic acid and ellagitannins was performed. Samples were filtered using 0.22 μm, 13 mm PTFE syringe-tip filters prior to HPLC injection. The HPLC system consisted of a Shimadzu HPLC equipped with a DAD detector (Shimadzu, Kyoto, Japan). The separation was performed on a LiChroCART LiChrospher 100 RP-18 column (5 μm; 125 × 4 mm; Merck, Darmstadt, Germany). The temperature of the column oven was set at 30 °C. The mobile phase consisted of aqueous 1% formic acid (eluent A) and acetonitrile–methanol (85 : 15, v/v) (eluent B). Gradient elution was as follows: 0–20 min, from 5% to 30% of B; 20–30 min, from 30% to 90% of B; 30–35 min, 90% of B; 35–40 min, from 90% to 5% of B. The flow rate was 1.0 mL min^{-1}. Detection of ellagic acid and its derivatives was performed at 254 nm and quantified following calibration with EA (concentration range: 5–100 mL L^{-1}, $R^2 = 0.995$). Peak identification was performed by comparison of retention times and UV-Vis spectral characteristics with the standard and the literature data.[12,13] In addition, peak identity of hydrolyzed samples was confirmed by using the HPLC–ESI-MS system. It consisted of a Waters 1525 binary pump, a Waters 996 photodiode array detector and a Waters Micromass ZQ mass spectrometer. The mass spectra of the compounds were obtained after electrospray ionization (ESI) in a negative mode. ESI conditions for ionization were as follows: source temperature: 120 °C, capillary voltage: 3 kV, extraction voltage: 3 V, cone voltage: 25 V, desolvation gas flow: 300 L h^{-1} and cone gas flow: 80 L h^{-1}.

Analysis of total phenolic compounds

The total phenolic content (TPC) of the samples was determined using the Folin–Ciocalteu's reagent according to the method of Slinkard and Singleton.[14] The absorbance of all samples was measured at 765 nm using a Genesys-10 UV/Vis (Thermo Spectronic, Rochester, USA) spectrophotometer after incubation at ambient temperature for 1 h. Total concentration of phenolic compounds was determined from the calibration curve and expressed in mg of gallic acid equivalents in one gram of the dry extract.

Analysis of total anthocyanins

Total anthocyanins were determined using the pH differential method of Giusti and Wrolstad.[15] Dried extracts were dissolved in buffer solutions (pH 1.0 and pH 4.5) and absorbance of the solutions was measured using a Cintra 202 UV/Vis spectrophotometer (GBC Scientific Equipment, Australia) at 510 and 700 nm. The concentration of anthocyanins was determined from a calibration curve and expressed in mg of cyanidin-3-glucoside in 1 gram of a dry extract.

Antioxidant activity assay

For antioxidant activity assay 200 mg of dried extracts were dissolved in 0.5 L of water following the addition of 31.5 mL of methanol. The radical scavenging capacity of the extracts against stable 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was determined by a slightly modified spectrophotometric method of Brand-Williams *et al.*[16] A $DPPH^{\bullet}$ methanol solution (2 mL, 6×10^{-5} M) was mixed with 20 μL of the prepared extract. The reaction was carried out at ambient temperature. The decreasing absorbance at 515 nm due to the scavenging of $DPPH^{\bullet}$ was measured with a spectrometer Genesys-10 UV/Vis for a period of 30 min to attain reaction equilibrium. Simultaneously, the absorption of a blank sample containing the same amount of methanol–water and DPPH solution was measured. Radical scavenging capacity of the samples was expressed as Trolox equivalents determined from the calibration curve of Trolox and calculated by the following formula:

$$\mathrm{TE} = c \times V/m, \text{ μmol per g of dry weight.}$$

c – Trolox concentration in μM from the calibration curve; V – volume of the extract, L; m – precise weighted amount of the dry extract, g.

Cell culture

Murine macrophage cells of J774 were maintained in Dulbecco's modified Eagle's medium + 10% fetal calf serum + penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1}) medium at 37 °C in a humidified atmosphere containing 5% of CO_2.

Measurement of hydrogen peroxide production in a macrophage culture

Direct measurement of hydrogen peroxide was performed using a macrophage culture (3×10^5 cells per mL) stimulated by arachidonic acid (AA – 30 μM) and phorbol-12-myristate-13 acetate (PMA – 10 μM), 1 μM Amplex red, and 10 U mL^{-1} horseradish peroxidase with or without added raspberry fruit extracts and ellagic acid.

In the incubation model a murine macrophage J774 cell suspension (3×10^5 cells per mL) was dispensed into 6-well plates with 1 mL medium (Dulbecco's modified Eagle's medium + 10% fetal calf serum + penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1})) in a thermostat (at 37 °C in a humidified atmosphere containing 5% of CO_2) for 2 hours (for the adherence of cells). For the analysis raspberry pomace extracts were dissolved in water. EA was dissolved in a water and dimethyl sulfoxide mixture (1 : 1). Different concentrations of raspberry extracts (1–120 μg mL^{-1} medium) and EA solutions (0.0175–0.28 μg mL^{-1}) were added into wells with cell cultures for the incubation. After 24 h of incubation, the medium with extracts was removed, and cells were collected, carefully washed from the remaining extract with a PBS buffer and centrifuged at 1000 × *g* for 10 min. Hydrogen peroxide production in the macrophage culture was measured fluorimetrically using an Ascent Fluoroscan plate reader (Thermo Fisher Scientific, Waltham, MA) at an excitation of 544 nm and an emission of 590 nm. Oxidative burst was measured fluorimetrically in PBS buffer resuspended macrophages (3×10^5 cells per mL) as generation of hydrogen peroxide radicals after stimulation of NADPH oxidase by AA (30 μM) or PMA (10 μM). Macrophage NADPH oxidase located on the outer cell membrane generates superoxide radicals, which are converted to hydrogen peroxide by superoxide dismutase.[17] Horseradish peroxidase uses Amplex red as the electron donor for the reduction of hydrogen peroxide to water; reaction product resorufin is a colorful and fluorescent component. Measurements were performed in the presence of 1 μM Amplex red and 10 U mL^{-1} horseradish peroxidase. The fluorescence signal was evaluated according to the calibration curve of hydrogen peroxide.

Evaluation of macrophage cell viability

For the evaluation of cell viability a definite amount of macrophages (3×10^4 cells per mL) was incubated in a 24-well plate with 0.5 mL medium (Dulbecco's modified Eagle's medium + 10% fetal calf serum + penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1})) in a thermostat for 2 hours (for the adherence of cells). Raspberry extracts (1–120 μg per mL of the experimental medium) and ellagic acid solutions (0.11–14 μg per mL of the experimental medium) were added into wells. Cell cultures were maintained in a thermostat at 37 °C for 24 hours. The cell number was counted after 24 hours using a light microscope. Macrophage viability was evaluated by adding 30 μL of Trypan blue to each well.[18] Live and dead cells were counted in five separate areas. The total amount of live and dead cells was calculated and expressed as cell viability (%).

Statistical analysis

Data were analysed using statistical data analysis packages SPSS 17.0 and Microsoft Excel. All experiments were carried out in triplicate; data were obtained from three independent experiments, and expressed as mean ± standard error. Significant differences were determined using one-way analysis of variance (ANOVA) and paired Student's *t*-test. For the suitability of regression model determination coefficient R^2 and *p*-value were obtained by checking the hypothesis on non-linear regression. Level of significance $\alpha = 0.05$.

Results

Phenolic composition and total radical scavenging activity of raspberry cultivars

The amounts of anthocyanins, phenolics, ellagitannins and free ellagic acid in these extracts are shown in Table 1. The total amount of ellagitannins was evaluated as a sum of compounds detected in the sample after acid hydrolysis. Ellagic acid (RT = 15.9 min, $\lambda_{max} = 254$ and 365 nm, molecular ion $m/z = 301$ $[M - H]^-$), methyl sanguisorboate (RT = 17.8 min, $\lambda_{max} = 369$ and 371 nm, molecular ion $m/z = 483$ $[M - H]^-$ which fragmented to m/z 315 and m/z 301) and methyl gallate (RT = 8.1 min, $\lambda_{max} =$ 218 and 274 nm, molecular ion $m/z = 183$ $[M - H]^-$) were considered as ellagitannin hydrolysis products in the acid-hydrolyzed raspberry sample.

Quantitative analysis revealed that a 'Novokitaevskoje' pomace extract contains greater amounts of free ellagic acid (2.74 ± 0.07 mg g^{-1}), ellagitannins (50.12 ± 1.62 mg g^{-1}) and total phenolics (149.34 ± 4.01 mg g^{-1}) than 'Beglianka' (Table 1). Note that only traces of anthocyanins were detected in 'Beglianka' pomace extracts. 'Novokitaevskoje' pomace extracts possessed significantly greater radical scavenging activity (TE = 590.2 ± 41.06 μmol g^{-1}) compared to 'Beglianka' (Table 1).

Effects of raspberry pomace extracts on cell viability

In this study we were interested in knowing whether antioxidant properties of raspberry extracts might affect the ability of macrophages to produce hydrogen peroxide. In order to select non-toxic concentrations, first we tested the effects of 'Beglianka' and 'Novokitaevskoje' pomace extracts and EA on cell viability. As can be seen from Fig. 1, 'Beglianka' and 'Novokitaevskoje' pomace extracts at a concentration range of 1–10 μg mL^{-1} had no effect and at 20–60 μg mL^{-1} inhibited only slightly (on the average of 7.3%) the cell viability. Significant differences between the pomace extracts of cultivars were determined only for the concentration of 120 μg mL^{-1} ($p < 0.05$) (inhibition by 47.2% and by 33.2%, by 'Beglianka' and 'Novokitaevskoje' pomace extracts, respectively). Thus, only at high concentrations 'Novokitaevskoje' pomace extracts inhibited cell viability more potently than 'Beglianka' pomace extracts (at 480 μg mL^{-1}) and at greater concentrations, pomace extracts of both cultivars totally inhibited cell viability (data not shown) and were not used for experiments.

The active compound of raspberry extracts EA did not affect or had a very minor effect (up to 5.2%) on cell viability at the

Table 1 Phenolic composition (mg per g of dry weight) and radical scavenging capacity (μmol TE per g) of raspberry pomace extracts

Extract	Anthocyanins	Phenolics	Ellagitannins	Free ellagic acid	μmol TE[a] per g
'Beglianka' pomace	Traces	101.24 ± 3.23	32.13 ± 1.01	1.73 ± 0.04	503.0 ± 31.73
'Novokitaevskoje' pomace	5.25 ± 0.14	149.34 ± 4.01	50.12 ± 1.62	2.74 ± 0.07	590.2 ± 41.06

[a] TE – Trolox equivalents.

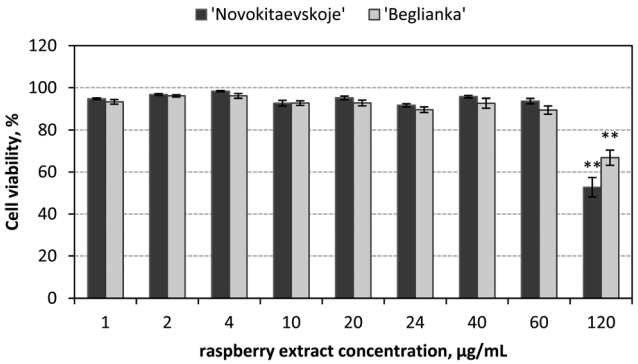

Fig. 1 Effects of raspberry pomace ('Novokitaevskoje' and 'Beglianka') extracts on J774 macrophage cell culture viability. Statistical significance is based on the difference when compared with the cells not treated with extracts (control) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

concentration range of up to 7 μg mL^{-1} (Fig. 2). At a concentration of 14 μg mL^{-1} cell viability was inhibited by EA at 32% ($p < 0.0001$). From the concentration of 28 μg per mL of EA, cell viability was inhibited totally (data not shown). Therefore, for further experiments (measurements of hydrogen peroxide production in macrophage cells) only concentrations without toxic effects (0.0175–0.28 μg mL^{-1}) were used.

Effects of raspberry pomace extracts on H_2O_2 production by macrophages

Direct measurement of macrophage respiratory burst was performed using AA and PMA triggered ROS production in the presence and in the absence of raspberry extracts without incubation. At the extract concentration of 10 μg mL^{-1}, 'Beglianka' and 'Novokitaevskoje' pomace extracts significantly ($p < 0.01$) inhibited AA stimulated macrophage respiratory burst at 30 and 31%, respectively (Fig. 3). PMA triggered respiratory burst was also significantly ($p < 0.01$) inhibited by 53 and 51% using 10 μg mL^{-1} concentration of 'Beglianka' and 'Novokitaevskoje' pomace extracts, respectively (Fig. 3). There were no significant differences between the inhibitory effects of 'Beglianka' and 'Novokitaevskoje' pomace extracts in both triggering models. We evaluated the effects of ellagic acid, one of the compounds of raspberry extracts, on PMA and AA stimulated macrophages. Significant ($p < 0.05$) inhibition of radical generation was determined in AA and PMA stimulated macrophages treated with ellagic acid at a concentration of 0.28 μg mL^{-1} (Fig. 3). Since our results demonstrate that raspberry fruit extracts and ellagic acid can directly scavenge hydrogen peroxide from 10% up to 80% (data not shown), depending on the concentration, we performed an experiment with the incubation model.

In this study we were interested in knowing whether respiratory burst induced after stimulation of NADPH oxidase by AA or PMA may be reduced after pre-incubation for 24 hours of macrophages with raspberry extracts or EA. For this purpose we chose the 'Novokitaevskoje' pomace extract which had a higher phenolic content compared to 'Beglianka' as significant differences during the viability test and during direct oxidative burst measurements between the pomace extracts ('Beglianka' and

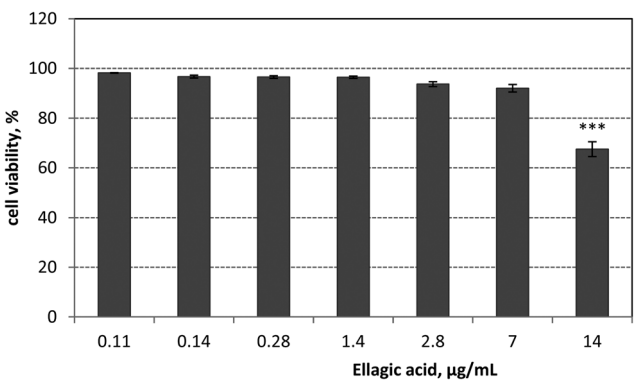

Fig. 2 The effect of ellagic acid on J774 macrophage cell culture viability. Statistical significance is based on the difference when compared with the cells not treated with extracts (control) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

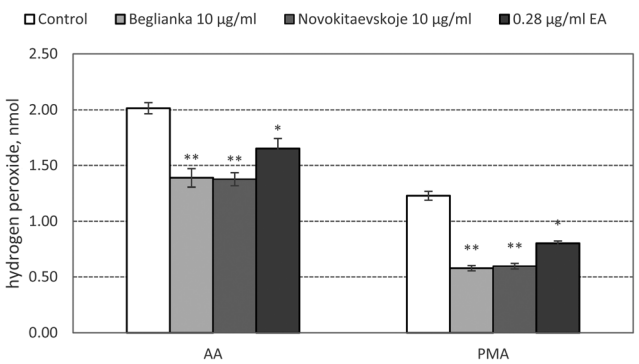

Fig. 3 Direct effects of raspberry pomace extracts ('Beglianka' and 'Novokitaevskoje') and ellagic acid (EA) on hydrogen peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Statistical significance is based on the difference when compared with the cells not treated with extracts (control) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

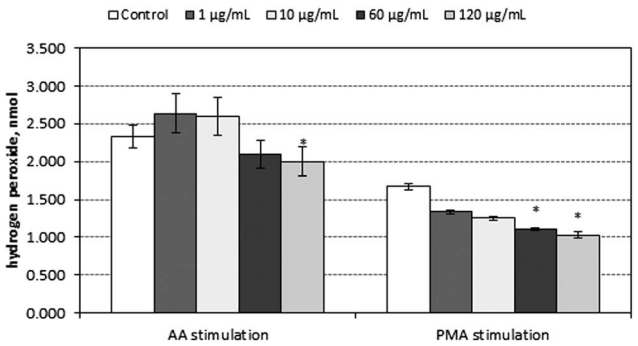

Fig. 4 Effect of a raspberry pomace extract ('Novokitaevskoje') on hydrogen peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Cells were incubated for 24 h with the Novokitaevskoje pomace extract. Statistical significance is based on the difference when compared with the cells not treated with extracts (control) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

'Novokitaevskoje') were not determined for the non-toxic concentration range of 1–60 µg mL^{-1}.

As can be seen from Fig. 4, in the AA model, a 'Novokitaevskoje' pomace extract at a concentration range of 1 to 60 µg mL^{-1} had no effect on macrophage H_2O_2 generation when compared with control macrophages. In contrast, in the model of PMA mediated respiratory burst (Fig. 4) the same concentrations (1 and 10 µg mL^{-1}) had no effect whereas 60 µg mL^{-1} had the significant inhibitory effect (decrease by 25%) on macrophage H_2O_2 generation. It should be mentioned that at this concentration range there was no or minor effect on cell viability. Higher concentrations (120 µg mL^{-1} of the Novokitaevskoje pomace extract) inhibited H_2O_2 generation of incubated macrophages by 14% ($p < 0.05$) in the AA and by 38% in the PMA model. We assume that at high (120 µg mL^{-1}) concentration the significant inhibitory effect attained in both AA and PMA models may be derived from the toxicity on cell viability which amounted to 33–47%.

Similar experiments were performed with EA, one of the active compounds of raspberry extracts. For this, we chose the non-toxic concentrations of EA (between 0.0175 and 0.28 µg mL^{-1}) which corresponded to those in raspberry extracts at concentrations of 6–120 µg mL^{-1} used for experiments. These concentrations of EA had no effect on cell viability. As can be seen from Fig. 5, after 24 hours' incubation with 0.0175–0.28 µg mL^{-1} EA, no significant difference in the H_2O_2 generation in AA stimulated macrophages was determined (Fig. 5) whereas in the PMA model ROS generation was significantly ($p < 0.05$) reduced (by 18–34%). Thus, a significant difference in effectiveness between these two stimulations was obvious.

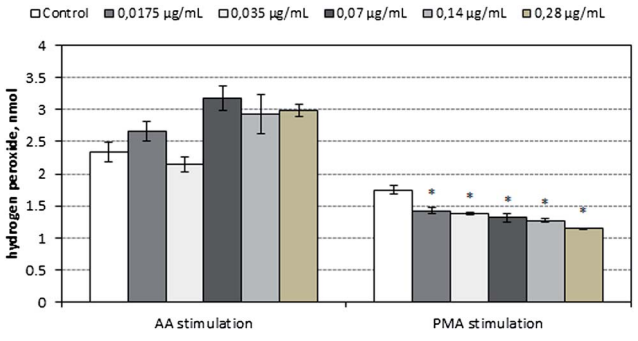

Fig. 5 Effect of ellagic acid on hydrogen peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Cells were incubated for 24 h with ellagic acid. Statistical significance is based on the difference when compared with the cells not treated with extracts (control) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Discussion

In this study we used murine macrophage cell line J774 to test whether a raspberry extract is implicated in anti-oxidant and anti-inflammatory pathways *via* suppression of PMA or AA mediated NADPH oxidase dependent ROS production. It is well known that ROS produced by NADPH oxidases play an important role in inflammation pathological processes occurring in heart, liver, lungs, and other organs.[19] ROS and induced cellular damage are markers of chronic inflammation and are important in cancer pathogenesis.[20] The consumption of polyphenolic compounds reduces the risk of morbidity; although the mechanisms by which the extracts affect the targets in the organism are still not clear.[21]

The main finding of our study is that raspberry pomace extracts (and active compound EA) *in vitro* inhibited ROS production in PMA stimulated macrophages, whereas in AA stimulated macrophages ROS production was reduced only at high raspberry extract concentrations that inhibited cell viability. A protective effect was obtained using concentrations of raspberry extracts up to 60 µg mL^{-1}. On the basis of these results, we assume that the inhibitory action of raspberry extracts on ROS production of activated macrophages causes possibly potential effects leading to NADPH oxidase activation processes. Therefore, scientific studies confirming that NADPH oxidase activity can be regulated by biologically active compounds thus attenuating oxidative stress and inflammation are of special importance in research of effective anti-oxidant/anti-inflammatory compounds.[3,22] When comparing the effects of the raspberry extract (and EA) on PMA or AA mediated respiratory burst of macrophages, the differences in the effectiveness in inhibiting ROS production in PMA-stimulated macrophages rather than AA-stimulated macrophages are obvious (Fig. 4 and 5). Since AA acts as a direct activator of NADPH oxidase[23] and PMA acts through the induced protein kinase C (PKC) phosphorylation,[24] results suggest that a raspberry pomace extract and its possible active ingredient EA have no direct inhibitory effect on the active NADPH oxidase complex, but rather an inhibitory effect on cellular processes that lead to NADPH oxidase activation. Our results indicate that the mode of action of raspberry extract and EA depends not only on concentration but also on the mechanism that triggers the activation of NADPH oxidase (Fig. 4 and 5). As PKC inhibitors inhibit PMA-stimulated NADPH oxidase activity and p47 translocation,[25] we assume that ellagic acid and active ingredients of a raspberry extract might affect the translocation of p47 and thus inhibit NADPH oxidase activity. NADPH oxidase

activates by phosphorylation of cytosolic component p47 and translocation of components to the plasma membrane where they form an active complex with p22 and p67 generating superoxides.[25] Lee *et al.* determined a reduction of gp91 and p2 hox protein expression in endothelial cells pretreated with ellagic acid due to suppression of the membrane assembly of the NADPH oxidase complex.[17] It has been reported that protective effects of EA are expressed *via* inhibition of NADPH oxidase induced superoxide hyper-production, *via* regulation of iNOS and inhibition of NO production.[17]

Effects on the translocation of the cytosolic p47 component and NADPH oxidase assembly might also be mediated by other phenolic compounds present in the sample. Rosenblat *et al.* determined that isoflavan glabridin with a hydroxylated B ring inhibits the translocation of the NADPH oxidase p47 cytosolic component to the plasma membrane.[25] Hydroxyl groups of the flavonoid B ring are necessary for the inhibitory effect. A 'Novokitaevskoje' extract contains 149 mg per g of phenolic compounds. This includes flavonol glycosides of quercetin and kaempferol.[26] Both flavonols contain an OH group in the C-4′ position in the flavonol B ring, which is associated with PKC inhibitory activity. Our results from a direct experimental model (when ROS generation by macrophages was measured without 24 h incubation with extracts, but directly) demonstrated significantly greater inhibition (52 and 34% of 'Novokitaevskoje' and ellagic acid, respectively, Fig. 3) of ROS production by PMA stimulation compared to AA stimulation. We assume that this effect may occur due to inhibition of the activity of NADPH oxidase and due to the capabilities of direct scavenging of ROS of phenolic compounds that are present in the extracts. As in the incubation model the 'Novokitaevskoje' pomace extract had no effect on AA triggered macrophage ROS generation, it can be implied that direct effects in the AA model occurred only due to direct scavenging of ROS. We propose that a raspberry extract and its active ingredients might act on the assembly of NADPH oxidase and also directly scavenge generated ROS. This is in agreement with Derochette *et al.*'s experiments with curcumin, which when added to the medium before NADPH oxidase assembly inhibited ROS production.[27]

We have also revealed that raspberry extracts, particularly at high concentrations, reduced the viability of macrophages. However, the concentration range causing significant effects on PMA mediated respiratory burst had no or minor impact on cell viability, suggesting that the effect on hydrogen ROS production is not associated with toxicity. In this study we compared the effects of two different raspberry cultivars on cell viability and no evidence of great differences between the effects of the 'Beglianka' pomace extract (in which anthocyanins were detected only in traces) and the 'Novokitaevskoje' pomace extract rich in anthocyanins (6.1 mg g^{-1}) was revealed (Table 1). Therefore we assume that anthocyanins may determine the minor effects on macrophage viability. This is in agreement with the study of Liu *et al.* with different raspberry cultivars indicating that anthocyanins contributed to antioxidant activity but had a minor effect on cell proliferation.[28]

Fruits rich in health promoting bioactive food components such as phenolic compounds can be used for the prevention of inflammation, cardiovascular and neurodegenerative diseases and cancer.[29] Experiments have been carried out for the evaluation of the impact of natural antioxidants from various fruits on human antioxidant capacities.[29] Garrido *et al.* determined a significant rise in urinary total antioxidant capacity after intake of cherry products in human individuals.[30,31] Gonzalez-Flores *et al.* showed that consumption of plums increased significantly the participant's urinary total antioxidant capacity levels.[32] Gonzales-Flores *et al.* determined that grape juice consumption increases human urinary and plasma antioxidant levels and urinary 6-sulfatoxymelatonin, thus providing protecting antioxidant effects.[33] As compared with raspberries, cherries, plums and red grapes also contain high amounts of anthocyanins and other phenolics. Anthocyanins from cherries cause *in vitro* antioxidant and anti-inflammatory activities. The aging process is associated with increased oxidative damage and elevated inflammatory processes.[29] Delgado *et al.* determined that consumption of cherry products modulates the balance of pro- and anti-inflammatory cytokines in experimental animals.[34] As bioactive compounds from fruits reduce the levels of pro-inflammatory cytokines, their consumption may retard age related inflammatory processes that lead to neurodegeneration and atherosclerosis.[35] Activated NADPH oxidase mediates oxidation of LDL and contributes to the process of atherosclerosis.[36] Suh *et al.* determined that raspberry juice consumption reduces levels of LDL-cholesterol and triglycerides in hamsters.[37] Elevated levels of LDL, triglycerides and total cholesterol are risk factors of atherosclerosis.[36,37] As NADPH oxidase superoxide production is involved in atherosclerosis formation, raspberries and their active ingredient ellagic acid may be promising agents with antioxidant effects that could participate in anti-inflammatory mechanisms and reduce the risk of age related chronic diseases.

In this study raspberry extract mediated antioxidant properties may be due to potent action of EA. EA possesses anti-oxidative, anti-inflammatory, anti-proliferative, anticarcinogenic and chemopreventive effects, and inhibits lipid peroxidation.[17] EA and urolithins determine the biological effects in the organism, because in the gastrointestinal tract ellagitannins are metabolized into them.[22,38] According to our experimental design, the concentration of EA was between 0.00175 and 0.035 μg mL^{-1}, which would correspond to 6–120 nM. Seeram *et al.* evaluated that concentrations of free EA in plasma after intake of EA and ellagitannins was in the range of 0.0162–0.0319 μg mL^{-1} (ref. 39) which would correspond to 53.6–105.6 nM. Thus, in our experiment certain tested concentrations of EA that led to effects on PMA mediated oxidative burst correspond (Fig. 5) to concentrations in human plasma.

There is accumulating evidence that bioactive compounds taken up by cells could affect the cellular oxidative state.[25] Another important point that should be discussed is the possibility of accumulation of biologically active compounds from raspberry extracts in macrophages during the incubation phase. Derochette *et al.* showed that the phenolic compound curcumin inhibited NADPH oxidase radical production when it was removed from the medium before measurements; therefore

it could have entered or interacted with the cells.[27] In our incubation model macrophages were carefully re-washed from remaining extract traces; it seems likely that at least some of the raspberry extract constituents or their metabolites penetrate into the cells resulting in antioxidant activity.

A raspberry fruit contain notable amounts of phenolics, ellagitannins and ellagic acid with expressed antioxidant properties (Table 1). Several methods should be applied for the quality control and antioxidant capacity evaluation of natural products.[40] Folin–Ciocalteu phenolics assay and DPPH radical scavenging are most commonly used methods, as they provide comprehensive and comparative information on the total antioxidant capacity of the sample. As Folin–Ciocalteau's reagent reacts with any reducing component, it not only reflects the amount of phenolic compounds, but also evaluates the total reducing capacity of the sample.[41] Both investigated raspberry extracts ('Beglianka' and 'Novokitaevskoje') showed strong antioxidant properties in DPPH and TPC assays and also significantly scavenged ROS generated by triggered NADPH oxidase. Constituents with antioxidant and anti-inflammatory properties of raspberry extracts could be further added to food products improving their functional properties.[42]

Conclusions

Raspberry is a polyphenol-rich berry crop that contains many phenolic compounds with potential health benefits. The amounts and contents of phenolic compounds vary between different cultivars. Our novel findings were the observation that raspberry pomace extracts *in vitro* reduce reactive oxygen species production in a J774 macrophage culture in the PMA model rather than in the AA model. Inhibition in the PMA model occurred due to possible effects on NADPH oxidase assembly. The antioxidant effect of ellagic acid occurs to a similar extent to that of a raspberry pomace extract, and it can be assessed as one of the bioactive anti-inflammatory compounds of the extract. Polyphenol-rich fractions from natural products with enhanced nutraceutical potential could be further used as functional food;[23] therefore, more science based evidence about the mechanisms of action and the possible risks of dietary supplements is needed to ensure their efficacy and safety.

Conflict of interest

The authors declare no conflict of interest.

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