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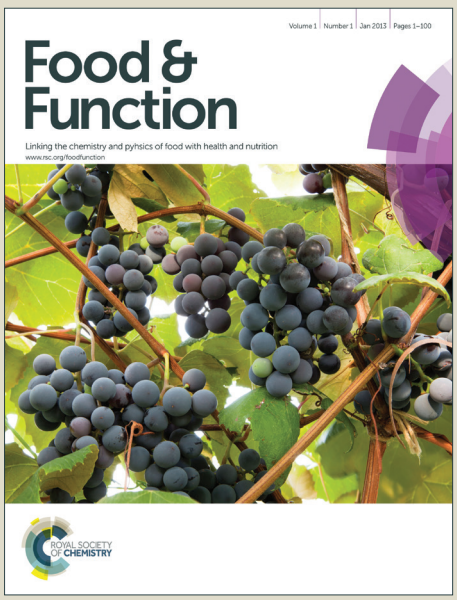

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Effect of raspberry fruit extracts and ellagic acid on respiratory burst in murine macrophages

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Abstract

The mechanism of action of polyphenolic compounds is attributed to their antioxidant, anti-inflammatory, anti-proliferative properties and their effect to subcellular signal transduction, cell cycle impairment and apoptosis. 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 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 either had very minor effects on cell viability. No significant difference in the ROS generation in arachidonic acid stimulated macrophages was determined for raspberry extract and EA whereas in phorbol-12 myristate-13 acetate model ROS generation was significantly ($p<0.05$) reduced. Our observation that raspberry pomace extract *in vitro* reduce ROS production in J774 macrophage culture suggest that raspberry extract and ellagic acid mediated antioxidant effects may be due to the regulation of NADPH oxidase activity.

Keywords: *Rubus idaeus* L., raspberry, ellagic acid, macrophages, antioxidant.

1. 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, anti-proliferative properties and their effect to subcellular signal transduction, cell cycle impairment and apoptosis[2,3]. Antioxidant effect of polyphenolic compounds may be

expressed *via* various mechanisms, mainly by directly scavenging free radicals, inhibiting enzymes, such as NO synthase, xanthine oxidase, cyclooxigenase and lipoxigenase, 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 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 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 family *Rosaceae* Juss. Species is native to Europe and northern Asia and commonly cultivated all over the world[8]. Ellagic acid (EA), which is present in the vacuoles of the plant cell 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 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], therefore 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 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.

Experimental

Chemicals

Trypan blue, Folin-Ciocalteu's phenol reagent, methanol (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, ellagic acid (Sigma-Aldrich, Steinheim, German). Concentrated hydrochloric acid and formic acid (98-100%) (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 pomaces were frozen and stored in deep freezer at – 30 °C. Prior the analysis, the pomaces were thawed and pureed using a blender followed by few minutes homogenization with Polytron PT 1200E homogenizer (Kinematica AG, Luzern, Switzerland) at room temperature. Twenty g 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 used.

Analysis of Ellagic Acid and Ellagitannins

Ellagitannins were determined as EA equivalents after acidic hydrolysis using method described by Koponen, *et al.*[12]. Free EA was analyzed prior to acid hydrolysis. *HPLC analysis of ellagic acid and ellagitannins*. Samples were filtered using 0.22 μm, 13 mm PTFE syringe-tip filters prior 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 (5μm; 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 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. Detection of ellagic acid and its derivatives was performed at 254 nm and quantified following calibration with EA (concentration range 5-100 ml/L, 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 3V, cone voltage 25 V, desolvation gas flow 300 L/h and cone gas flow at 80 L/h.

Analysis of total phenolic compounds

The total phenolic content (TPC) of the samples was determined using the Folin-Ciocalteu 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 calibration curve and expressed in mg of gallic acid equivalents in one gram of 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 dry extract.

2.6 Antioxidant Activity Assay

For antioxidant activity assay 200mg 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] DPPH methanol solution (2 mL, 6 × 10-5 M) was mixed with 20 μL of 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:

TE = c×V/m, μmol/g of dry weight.

c – Trolox concentration 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) / streptomycin (100 μg/mL) medium at a 37ºC in a humidified atmosphere containing 5% of CO_2.

Measurement of hydrogen peroxide production in macrophage culture

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

In incubation model murine macrophage J774 cell suspension (3×10^5 cell/mL) was dispensed into in 6-well plates with 1 mL medium (Dulbecco's Modified Eagle's Medium + 10% fetal calf serum + penicillin (100 U/mL) / streptomycin (100 μg/mL) in a thermostat (at a 37ºC 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 water and dimethyl sulfoxide mixture (1:1). Different concentrations of raspberry extracts (1 – 120 μg/mL medium) and EA solutions (0.0175 – 0.28 μg/mL) were added into wells with cell cultures for the incubation. After 24 h of incubation, medium with extracts was removed, cells were collected, carefully washed from remaining extract with PBS buffer and centrifuged at 1000 × g for 10 min. Hydrogen peroxide production in macrophage culture was measured fluorimetrically using Ascent Fluoroscan plate reader (Thermo Fisher Scientific, Waltham, MA) at an excitation 544 nm and an emission 590 nm). Oxidative burst

fluorimetrically was measured in PBS buffer resuspended macrophages (3×10^5 cell/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 outer cell membrane generates superoxide radical, which is converted to hydrogen peroxide by superoxide dismutase[17]. Horseradish peroxidase uses Amplex red as electron donor for the reduction of hydrogen peroxide to water; reaction product resorufin is colorful and fluorescent component. Measurements were performed in the presence of 1 μM Amplex red and 10 U/mL horseradish peroxidase. Fluorescence signal was evaluated according to the calibration curve of hydrogen peroxide.

Evaluation of macrophage cell viability

For the evaluation of cell viability definite amount of macrophages (3×10^4 cell/mL) was incubated in 24-well plate with 0.5 mL medium (Dulbecco's Modified Eagle's Medium + 10% fetal calf serum + penicillin (100 U/mL) / streptomycin (100 μg/mL) in a thermostat for 2 hours (for the adherence of cells). Raspberry extracts (1 – 120 μg/mL of experimental medium) and ellagic acid solutions (0.11 – 14 μg/mL of experimental medium) were added into wells. Cell cultures were maintained in a thermostat at 37ºC for 24 hours. Cell number was counted after 24 hours using 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. Total amount of live and dead cells was calculated and expressed as cell viability (%).

Statistical analysis

Data was 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 hypothesis on non-linear regression. Level of significance α=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. Total amount of ellagitannins was evaluated as a sum of compounds detected in the sample after acid hydrolysis. Ellagic acid (RT = 15.9 min, λ_{max} 254 and 365 nm, molecular ion m/z 301 $[M-H]^-$), methyl sanguisorboate (RT = 17.8 min, λ_{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, λ_{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 'Novokitaevskoje' pomace extract contains greater amounts of free ellagic acid (2.74 ± 0.07 mg/g), ellagitannins (50.12 ± 1.62 mg/g) and total phenolics (149.34 ± 4.01 mg/g) than 'Beglianka' (Table 1.). Note that only traces of anthocyanins were detected in 'Beglianka' pomace extracts. 'Novokitaevskoje' pomace extract possessed significantly greater radical scavenging activity (TE = 590.2 ± 41.06 µmol/g) compared to 'Beglianka' (Table 1.).

Effect of raspberry pomace extracts on cell viability

In this study we were interested 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 of EA on cell viability. As can be seen from Figure 1, 'Beglianka' and 'Novokitaevskoje' pomace extracts at a concentration range of 1 – 10 µg/mL had no effect

and at 20 – 60 μg/mL inhibited only slightly (on the average 7.3 %) the cell viability. Significant differences between the pomace extracts of cultivars were determined only for the concentration of 120 μg/mL ($p<0.05$) (inhibition by 47.2 % and by 33.2 %, 'Beglianka' and 'Novokitaevskoje' pomace extracts, respectively). Thus, only at high concentrations 'Novokitaevskoje' pomace extract inhibited cell viability more potent than 'Beglianka' pomace extract (At 480 μg/mL and greater concentrations, pomace extracts of both cultivars totally inhibited cell viability (data not shown) and were not used for experiments.

Active compound of raspberry extract EA did not affect or either had very minor effect (up to 5.2 %) on cell viability at concentration range up to 7 μg/mL (Figure 2). At a concentration of 14 μg/mL cell viability by EA was inhibited at 32 % ($p<0.0001$). From the concentration of 28 μg/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) has been used.

Effect 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, 'Beglianka' and 'Novokitaevskoje' pomace extracts significantly ($p<0.01$) inhibited AA stimulated macrophage respiratory burst at 30 and 31 %, respectively (Figure 3). PMA triggered respiratory burst was also significantly ($p<0.01$) inhibited by 53 and 51 % using 10 μg /mL concentration of 'Beglianka' and 'Novokitaevskoje' pomace extracts, respectively (Figure 3). There were no significant differences between the inhibitory effects of 'Beglianka' and 'Novokitaevskoje' pomace extracts in both triggering models. We evaluated the effect 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 (Figure 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 incubation model .

In this study we were interested 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 have chosen the 'Novokitaevskoje' pomace extract which had a higher phenolic content compared to 'Beglianka' as significant differences during viability test and during direct oxidative burst measurements between the pomace extracts ('Beglianka' and 'Novokitaevskoje') were not determined for the non-toxic concentration range of 1 – 60 μg/mL.

As can be seen from Figure 4, in AA model, 'Novokitaevskoje' pomace extract at a concentration range of 1 and 60 μg/mL had no effect on macrophage H_2O_2 generation when compared with control macrophages. In contrast, in the model of PMA mediated respiratory burst (Figure 4) the same concentrations (1 and 10 μg/mL) had no effect whereas 60 μg/mL 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 of Novokitaevskoje pomace extract) inhibited H_2O_2 generation of incubated macrophages by 14 % ($p<0.05$) in AA and by 38 % in PMA model. We assume that at high (120 μg/mL) concentration the obtained significant inhibitory effect in both AA and PMA models may be derived from the toxicity on cell viability which was comprised of 33 – 47 %.

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

Discussion

In this study we used murine macrophage cell line J774 to test whether 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].

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 only at high raspberry extract concentrations that were inhibited cell viability the ROS production was reduced. Protective effect was obtained using concentrations of raspberry extracts up to 60 µg/mL. On the basis of results, we assume, that the inhibitory action of raspberry extract on ROS production of activated macrophages, involve possibly

potential effect to NADPH oxidase activation processes. Therefore, scientific studies confirming that NADPH oxidase activity can be regulated by biologically active compounds and thus attenuating oxidative stress and inflammation are of special importance in research of effective anti-oxidant/anti-inflammatory compounds[3,22]. When comparing the effect 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 is obvious (Figures 4, 5). Since AA acts as direct activator of NADPH oxidase[23] and PMA acts through the induced protein kinase C (PKC) phosphorylation[24], results suggest that 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 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 (Figures 4, 5). As PKC inhibitors inhibit PMA-stimulated NADPH oxidase activity and p47 translocation[25], we assume that ellagic acid and active ingredients of 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 active complex with p22 and p67 generating superoxides[25]. Lee *et al*. determined a reduction of gp91 and p22phox 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 hyperproduction, via regulation of iNOS and inhibition of NO production[17].

Effect 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*

306 determined that isoflavan glabridin with hydroxylated B ring inhibits the translocation of the
307 NADPH oxidase p47 cytosolic component to the plasma membrane[25]. Hydroxyl groups of
308 flavonoid B ring are necessary for the inhibitory effect. 'Novokitaevskoje' extract contains
309 149 mg/g of phenolic compounds. This includes flavonol glycosides of quercetin and
310 kaempferol[26]. Both flavonols contain OH group in C-4' position in flavonol B ring, which is
311 associated with PKC inhibitory activity. Our results in a direct experimental model (when
312 ROS generation by macrophages was measured without 24 h incubation with extracts, but
313 directly) demonstrated significantly greater inhibition (52 and 34 % of 'Novokitaevskoje' and
314 ellagic acid, respectively, Figure 3) of ROS production in PMA stimulation compared to AA.
315 We assume that this effect may occur due to inhibition of the activity of NADPH oxidase and
316 due to the capabilities of direct scavenging of ROS by phenolic compounds that are present in
317 the extracts. As in incubation model 'Novokitaevskoje' pomace extract had no effect on AA
318 triggered macrophage ROS generation, it can be implied that direct effect in AA model
319 occurred only due to direct scavenging of ROS. We propose that raspberry extract and its
320 active ingredients might act on the assembly of NADPH oxidase and also directly scavenge
321 generated ROS. This is in agreement with Derochette *et al*. experiments with curcumin,
322 which added to the medium before NADPH oxidase assembly, inhibited ROS production [27].
323 We have also revealed that raspberry extracts, particularly at high concentrations, reduced
324 the viability of macrophages. However, the concentration range possessing significant effects
325 to PMA mediated respiratory burst had no or minor impact on cell viability, suggesting that
326 the effect on hydrogen ROS production is not associated with toxicity. In this study we
327 compared the effects of two different raspberry cultivars on cell viability and revealed no
328 evidence of great differences between the effects of the 'Beglianka' pomace extract (where
329 anthocyanins detected only in traces) and 'Novokitaevskoje' pomace extract rich in
330 anthocyanins (6.1 mg/g) (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 minor effect on cell proliferation[28].

Fruits rich in health promoting bioactive food components as phenolic compounds can be used for the prevention of inflammation, cardiovascular, 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 significant rise in urinary total antioxidant capacity after intake of cherries product in human individuals[30,31]. Gonzalez-Flores *et al*., showed that consumption of plums increased significantly the participants' 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 effect[33]. As compared with raspberries, cherries, plums and red grapes also contain high amounts of anthocyanins and other phenolics. Anthocyanins from cherries possess *in vitro* antioxidant and anti-inflammatory activities. 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, 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 raspberries 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 ant its active ingredient ellagic acid may be a 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 antioxidative, anti-inflammatory, anti-proliferative, anticarcinogenic and chemopreventive effects, inhibits lipid peroxidation[17]. EA and urolithins determine the biological effects to 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 – 0.035 µg/mL, 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 [39] which would correspond to 53.6 - 105.6 nM. Thus, in our experiment certain tested concentrations of EA that possessed the effect on PMA mediated oxidative burst corresponds (Figure 5) to concentrations in human plasma.

There is accumulating evidence that bioactive compounds taken up by cells could affect cellular oxidative state[25]. Another important point should be discussed is the possibility of accumulation of biological active compounds from raspberry extracts in macrophages during incubation phase. Derochette *et al*. showed that phenolic compound curcumin inhibited NADPH oxidase radical production when it was removed from the medium before measurements, therefore it could have entered or in 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 raspberry extract constituents or their metabolites penetrate into the cells resulting antioxidant activity.

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, they provide comprehensive and comparative information on the total antioxidant capacity of the sample. As Folin-Ciocalteau regent reacts with any reducing component, it reflects not only 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 polyphenol-rich berry crop that contains many phenolic compounds with potential health benefits. The amounts and content of phenolic compounds varies between different cultivars. Our novel findings were the observation that raspberry pomace extract *in vitro* reduce reactive oxygen species production in J774 macrophage culture in PMA model rather than in AA model. Inhibition in PMA model occurred due to possible effect on NADPH oxidase assembly. The antioxidant effect of ellagic acid is at similar extent as the effect of raspberry pomace extract, it can be assessed as one of bioactive anti-inflammatory compounds of the extract. Polyphenolic-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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485 **Table 1.** Phenolic composition (mg/g of dry weigh) and radical scavenging capacity (μmol
486 TE/g) of raspberry pomace extracts.

Extract	Anthocyanins	Phenolics	Ellagitannins	Free ellagic acid	μmol TE/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

487 *TE – Trolox equivalents.

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491 FIGURES

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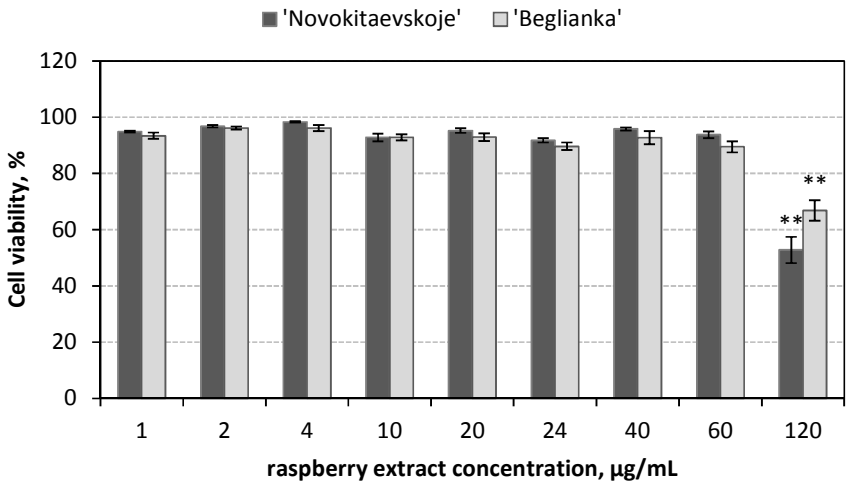

493

494 **Figure 1.** The effect of raspberry pomace ('Novokitaevskoje' and 'Beglianka') extracts
495 on J774 macrophage cell culture viability. Statistical significance is based on the
496 difference when compared with the cells without treating extracts (control) (*$p < 0.05$,
497 **$p < 0.01$, ***$p < 0.001$).

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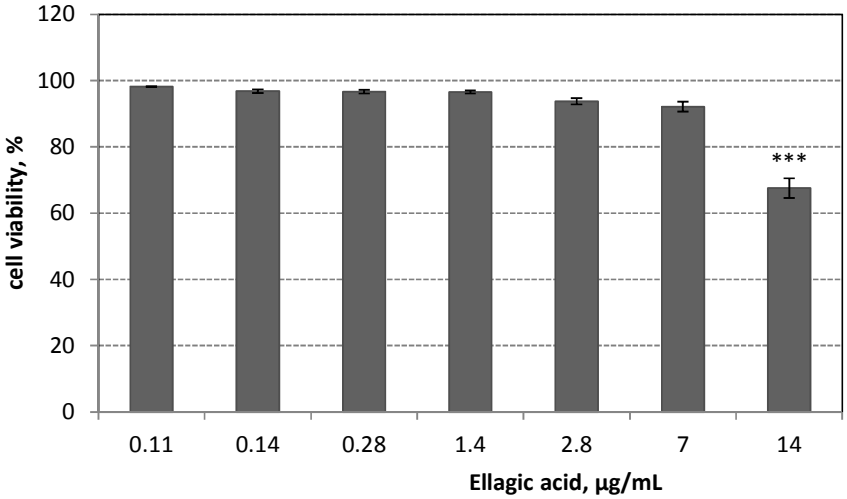

501

502 **Figure 2.** The effect of ellagic acid on J774 macrophage cell culture viability.
503 Statistical significance is based on the difference when compared with the cells without
504 treating extracts (control) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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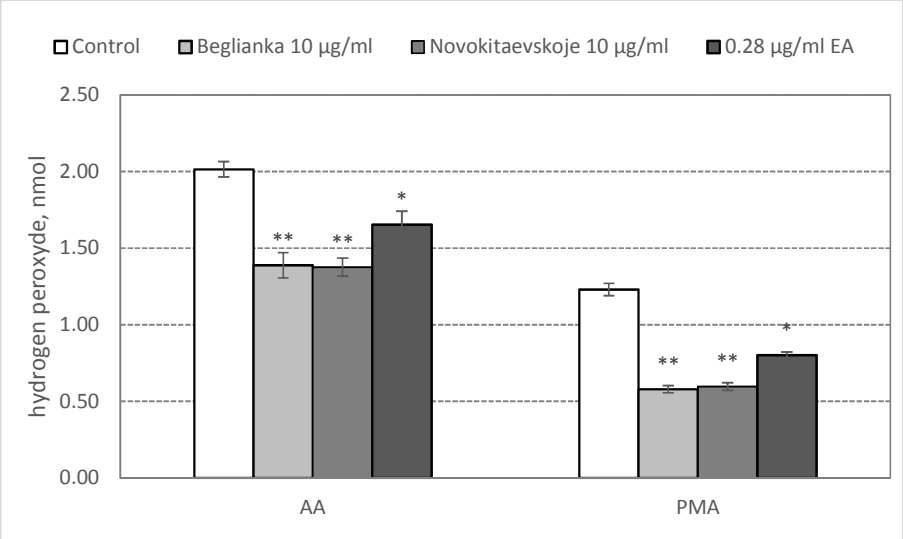

507

508 **Figure 3.** Direct effect of raspberry pomace extracts ('Beglianka' and
509 'Novokitaevskoje') and ellagic acid (EA) on hydrogen peroxide production (nmol) in
510 J774 macrophages stimulated with AA and PMA. Statistical significance is based on
511 the difference when compared with the cells without treating extracts (control) (*$p <$
512 0.05, **$p < 0.01$, ***$p < 0.001$).

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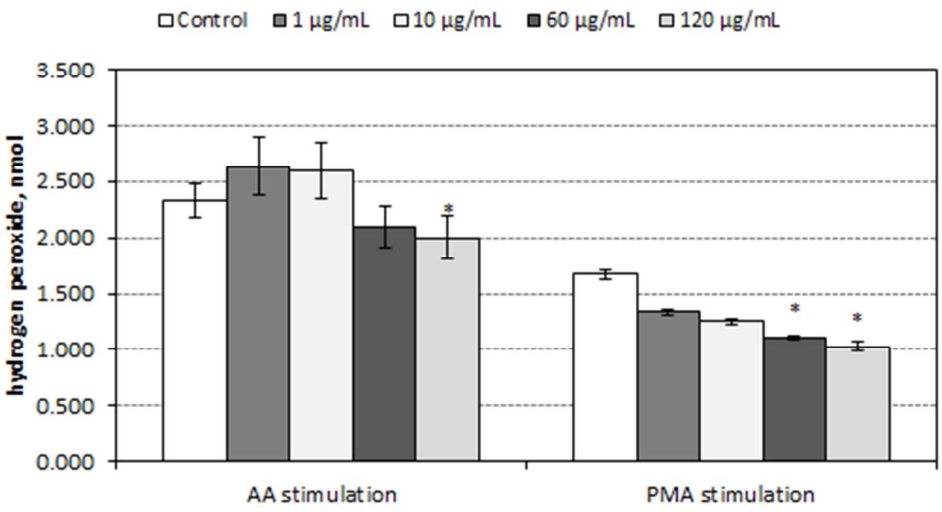

516

517 **Figure 4.** Effect of raspberry pomace extract ('Novokitaevskoje') on hydrogen
518 peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Cells
519 were incubated for 24 h with Novokitaevskoje pomace extract. Statistical significance
520 is based on the difference when compared with the cells without treating extracts
521 (control) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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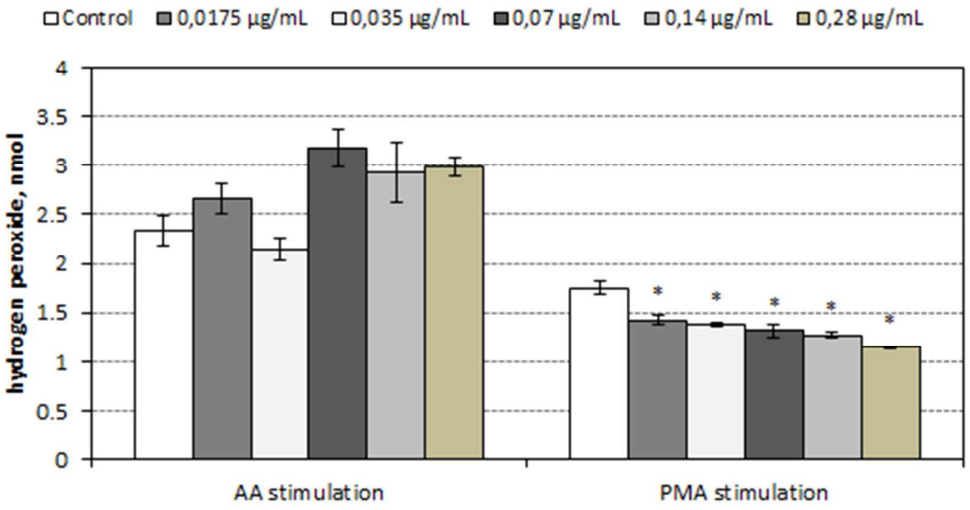

524

525 **Figure 5.** Effect of ellagic acid on hydrogen peroxide production (nmol) in J774
526 macrophages stimulated with AA and PMA. Cells were incubated for 24 h with ellagic
527 acid. Statistical significance is based on the difference when compared with the cells
528 without treating extracts (control) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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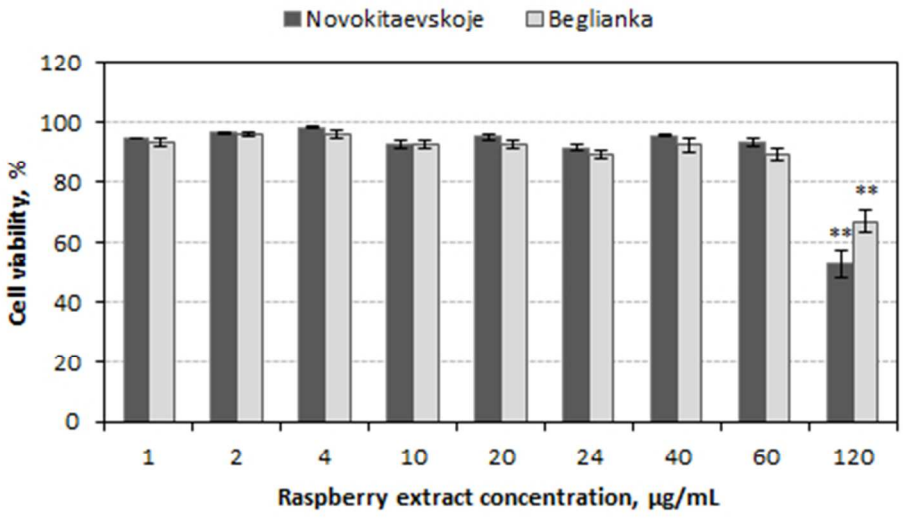

The effect of raspberry pomace ('Novokitaevskoje' and 'Beglianka') extracts on J774 macrophage cell culture viability. Statistical significance is based on the difference when compared with the cells without treating extracts (control) (*p < 0.05, **p < 0.01, ***p < 0.001).
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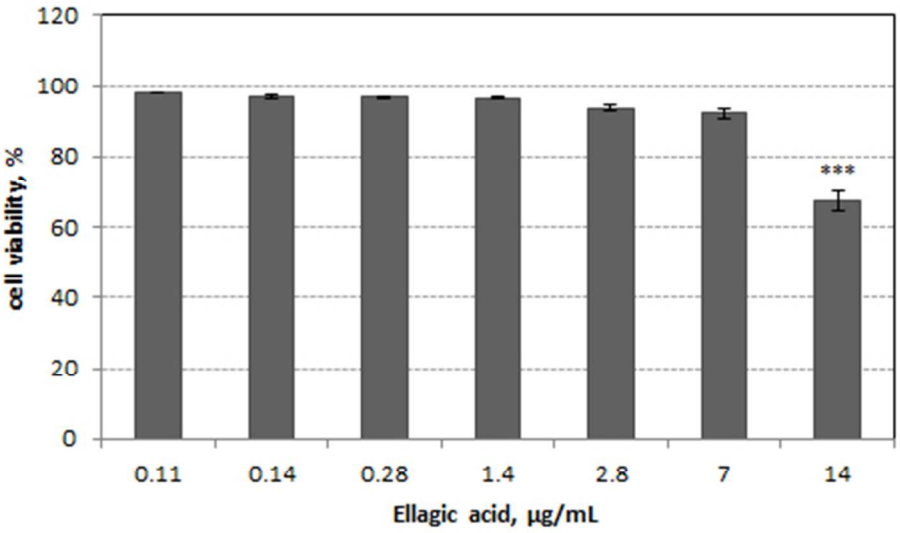

The effect of ellagic acid on J774 macrophage cell culture viability. Statistical significance is based on the difference when compared with the cells without treating extracts (control) (*p < 0.05, **p < 0.01, ***p < 0.001).
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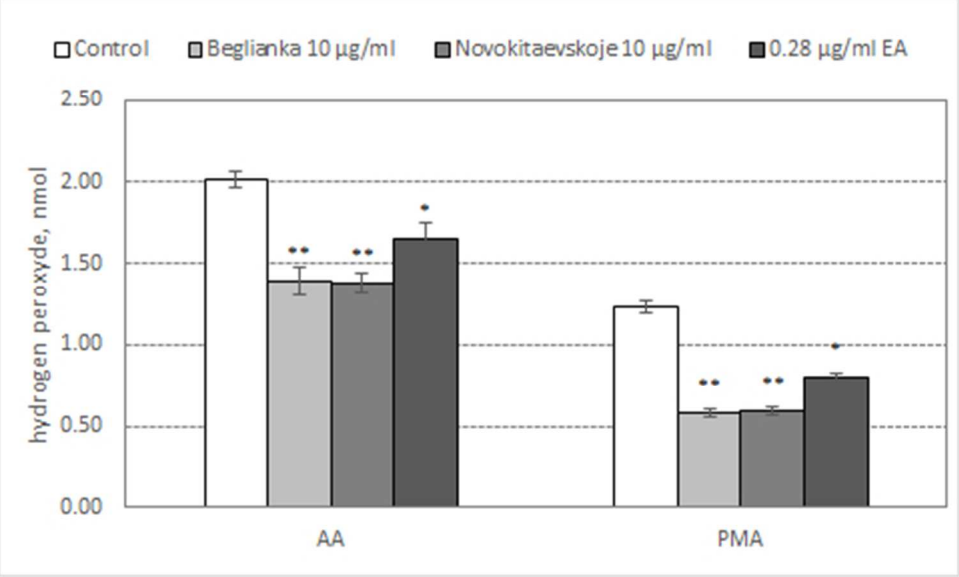
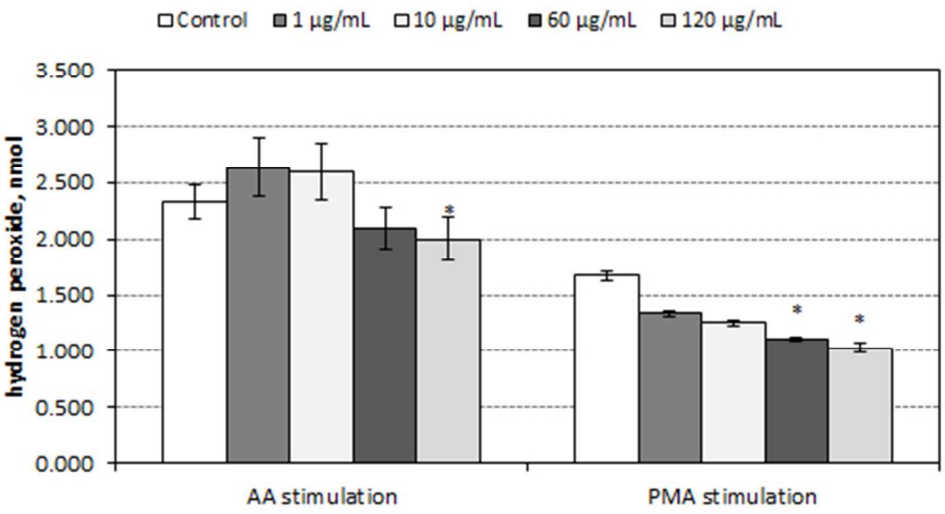


Direct effect of raspberry pomace extracts ('Beglianka' and 'Novokitaevskoje') and ellagic acid (EA) on hydrogen peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Statistical significance is based on the difference when compared with the cells without treating extracts (control) (*p < 0.05, **p < 0.01, ***p < 0.001).
127x76mm (96 x 96 DPI)



Effect of raspberry pomace extract ('Novokitaevskoje') on hydrogen peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Cells were incubated for 24 h with Novokitaevskoje pomace extract. Statistical significance is based on the difference when compared with the cells without treating extracts (control) (*p < 0.05, **p < 0.01, ***p < 0.001).
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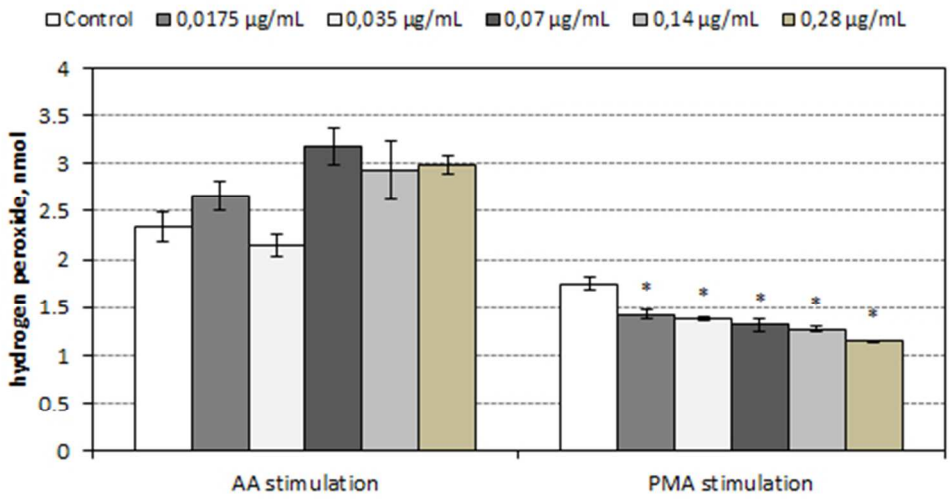
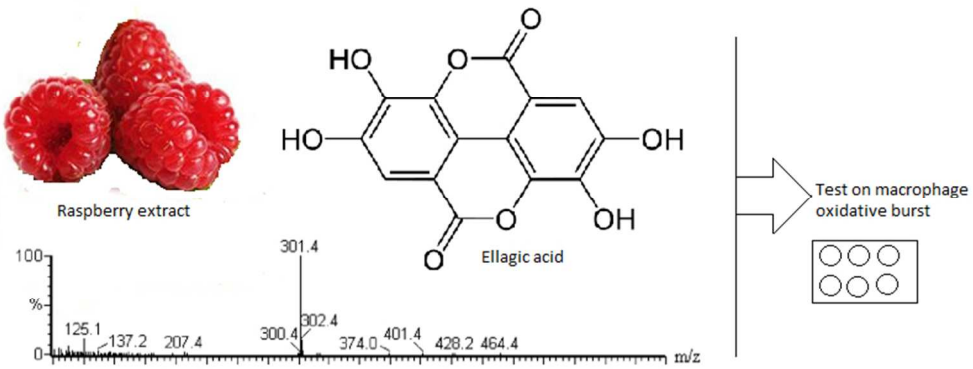


Effect of ellagic acid on hydrogen peroxide production (nmol) in J774 macrophages stimulated with AA and PMA. Cells were incubated for 24 h with ellagic acid. Statistical significance is based on the difference when compared with the cells without treating extracts (control) (*p < 0.05, **p < 0.01, ***p < 0.001).
143x76mm (96 x 96 DPI)



The main finding in our study is that raspberry extract and ellagic acid inhibits reactive oxygen species production in PMA stimulated macrophages
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