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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

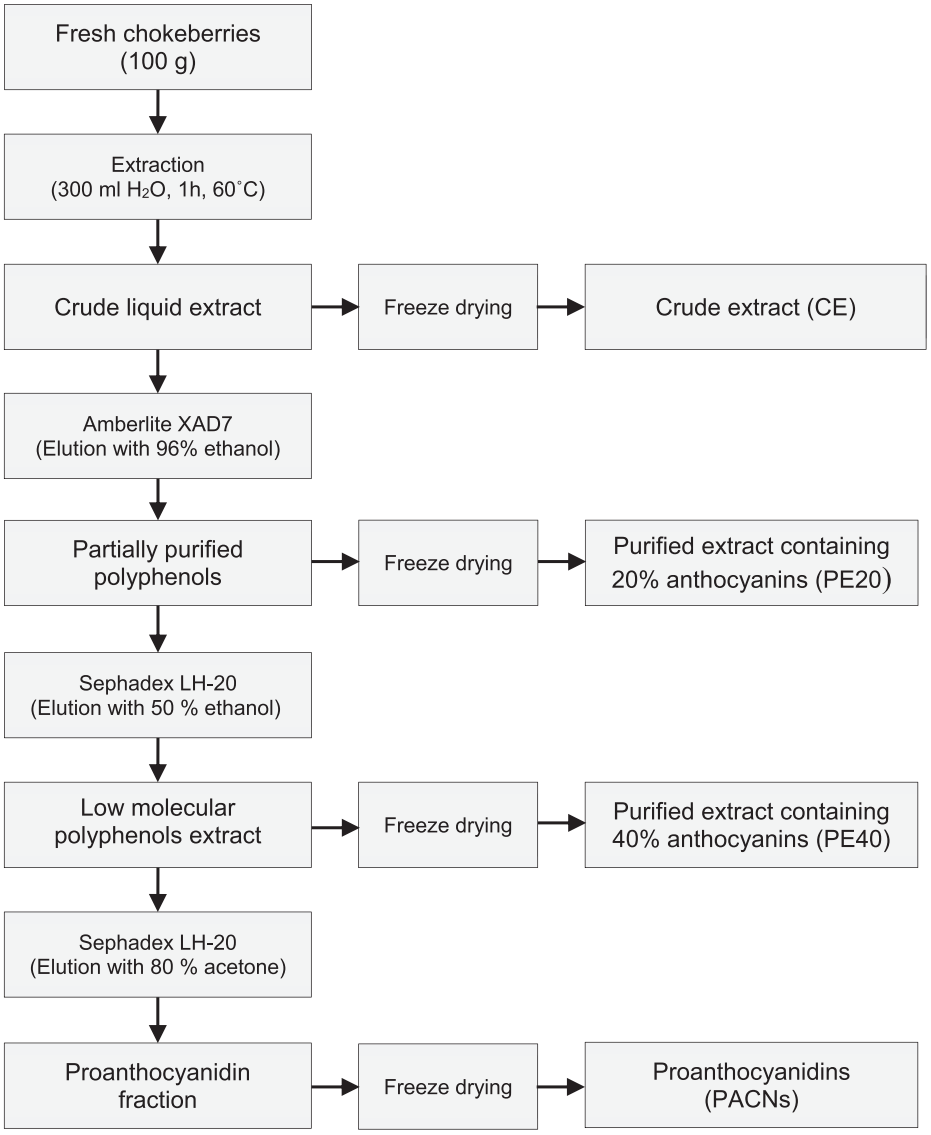


Fig. 1. Scheme for extraction and purification of aronia preparations.

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 ($p < 0.05$).

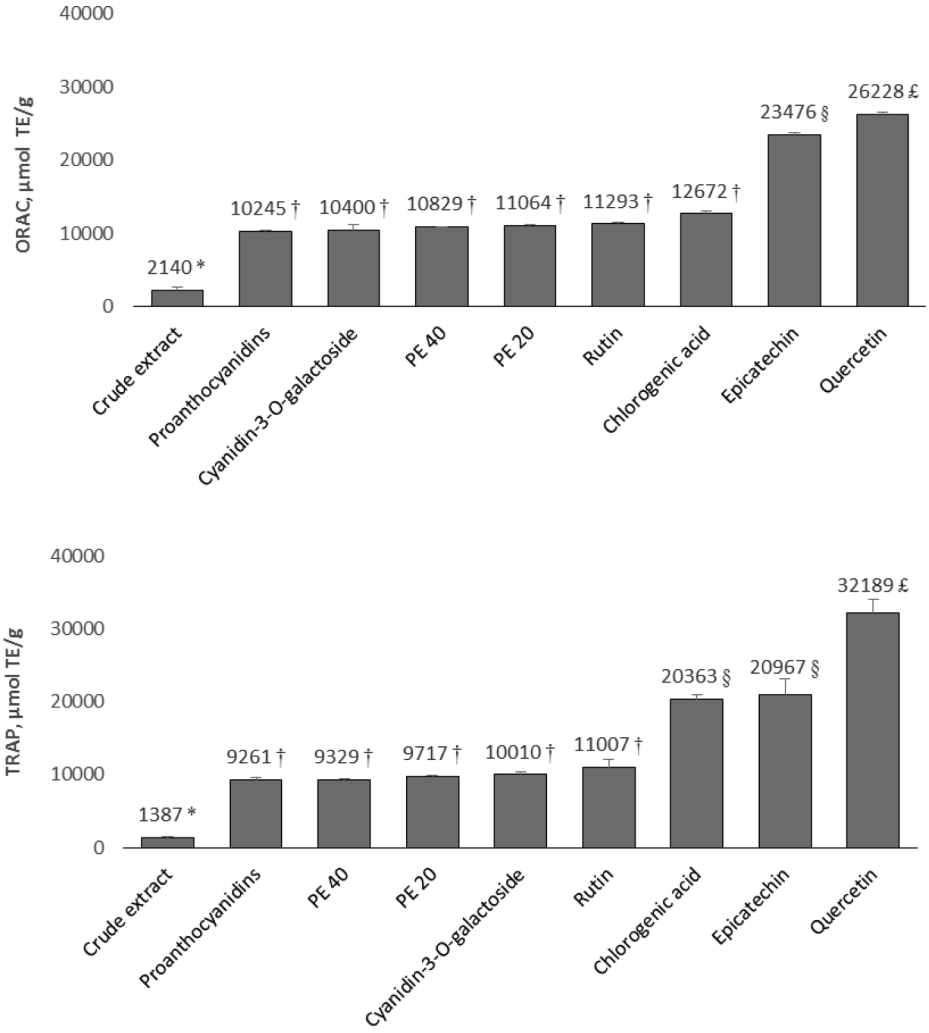


Fig. 2. Antioxidant activity of black chokeberry polyphenols and preparations measured by ORAC and TRAP assays. There are no significant differences among values marked with the same symbols ($p < 0.05$).

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 ($p < 0.05$).

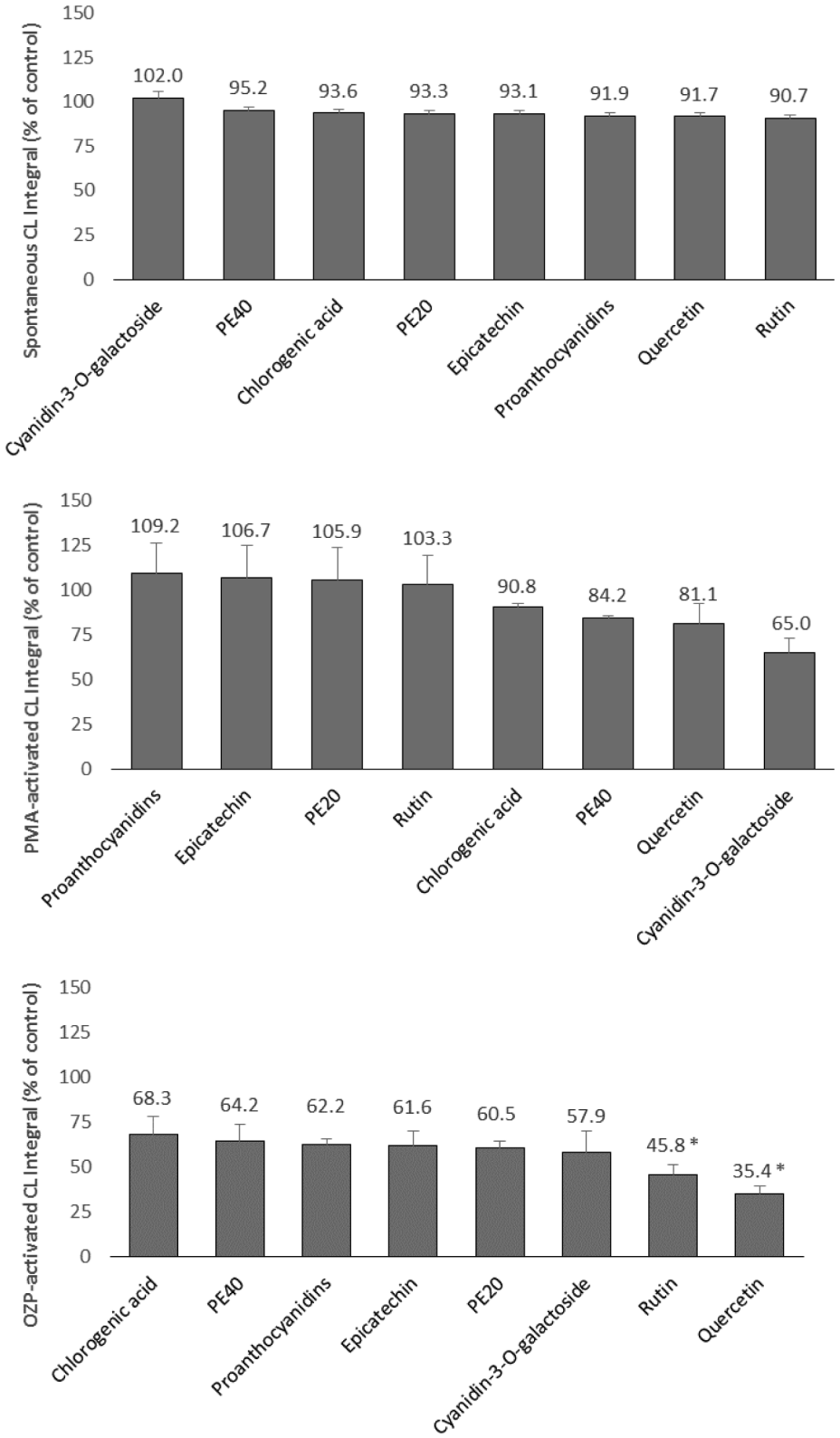


Fig. 3. Effect of black chokeberry polyphenols and preparations on the production of reactive oxygen species from untreated (spontaneous); PMA-activated; and OZP-activated human neutrophils. Values marked with asterisk are significantly different from the control ($p < 0.05$).

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 1	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 1		–	–	–	–	–	–	–	–	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.

References

Bors, W., Heller, W., Michel, C., & Saran, M. (1990). Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods in Enzymology, 186*, 343–355.

Chrubasik, C., Li, G., & Chrubasik, S. (2010). The clinical effectiveness of chokeberry: A systematic review. *Phytotherapy Research, 24*(8), 1107–1114.

Číž, M., Komrsková, D., Prachařová, L., Okénková, K., Čížová, H., Moravcová, A., ... Nosá, R. (2007). Serotonin modulates the oxidative burst of human phagocytes via various mechanisms. *Platelets, 18*(8), 583–590.

Čížová, H., Lojek, A., Kubala, L., & Číž, M. (2004). The effect of intestinal ischemia duration on changes in plasma antioxidant defense status in rats. *Physiological Research, 53*(5), 523–531.

Denev, P., Číž, M., Ambrozova, G., Lojek, A., Yanakieva, I., & Kratchanova, M. (2010). Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chemistry, 123*(4), 1055–1061.

Denev, P., Kratchanov, C., Číž, M., Lojek, A., & Kratchanova, M. (2012). Bioavailability and antioxidant activity of black chokeberry (*Aronia melanocarpa*) polyphenols: *In vitro* and *in vivo* evidences and possible mechanisms of action: A review. *Comprehensive Reviews in Food Science and Food Safety, 11*(5), 471–489.

Denev, P., Kratchanova, M., Číž, M., Lojek, A., Vasicek, O., Nedelcheva, P., ... Vojtek, L. (2014). Biological activities of selected polyphenol-rich fruits related to immunity and gastrointestinal health. *Food Chemistry, 157*, 37–44.

Denev, P., Kratchanova, M., Petrova, I., Klisurova, D., Georgiev, Y., Ognyanov, M., & Yanakieva, I. (2018). Black chokeberry (*Aronia melanocarpa* (Michx.) Elliot) fruits and functional drinks differ significantly in their chemical composition and antioxidant activity. *Journal of Chemistry,* 11.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology, 124*(1), 91–97.

Harborne, J. B., & Simmonds, N. W. (1964). *Biochemistry of phenolic compounds.* London: Academic Press.

Ho, G., Bräunlich, M., Austarheim, I., Wangensteen, H., Malterud, K. E., Slimestad, R., & Barsett, H. (2014). Immunomodulating activity of *Aronia melanocarpa* polyphenols. *International Journal of Molecular Sciences, 15*, 11626–11636.

Howell, A. B., Reed, J. D., Krueger, C. G., Winterbottom, R., Cunningham, D. G., & Leahy, M. (2005). A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. *Phytochemistry, 66*(18), 2281–2291.

Kenny, T. P., Shu, S. A., Moritoki, Y., Keen, C. L., & Gershwin, M. E. (2009). Cocoa flavanols and procyanidins can modulate the lipopolysaccharide activation of polymorphonuclear cells in vitro. *Journal of Medicinal Food, 12*, 1–7.

Kraemer-Schafhalter, A., Fuchs, H., & Pfannhauser, W. (1998). Solid-phase extraction (SPE) - A comparison of 16 materials for the purification of anthocyanins from *Aronia melanocarpa* var Nero. *Journal of the Science of Food and Agriculture, 78*(3), 435–440.

Kulling, S., & Rawel, H. (2008). Chokeberry *(Aronia melanocarpa)* – A review on the characteristic components and potential health effects. *Planta Medica, 74*(13), 1625–1634.

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *Journal of AOAC International, 88*(5), 1269–1278.

Lee, J. P., Li, Y. C., Chen, H. Y., Lin, R. H., Huang, S. S., Chen, H. L., ... Kuan, Y. H. (2010). Protective effects of luteolin against lipopolysaccharide-induced acute lung injury involves inhibition of MEK/ERK and PI3K/Akt pathways in neutrophils. *Acta Pharmaceutica Sinica B, 31*, 831–838.

Nicoue, E. E., Savard, S., & Belkacemi, K. (2007). Anthocyanins in wild blueberries of Quebec: Extraction and identification. *Journal of Agricultural and Food Chemistry, 55*(14), 5626–5635.

Ohgami, K., Ilieva, I., Shiratori, K., Koyama, Y., Jin, X., Yashida, K., ... Ohno, S. (2005). Anti-inflammatory effects of *Aronia* extract on rat endotoxin-induced uiveitis. *Investigative Ophthalmology & Visual Science, 46*, 275–281.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619–4626.

Pincemail, J., Thirion, A., Dupuis, M., Braquet, P., Drieu, K., & Deby, C. (1987). *Ginkgo biloba* extracts inhibits oxygen species production generated by phorbol myristate acetate stimulated human leukocytes. *Experientia, 43*, 181–184.

Qi, S., Feng, Z., Li, Q., Qi, Z., & Zhang, Y. (2017). Myricitrin modulates NADPH oxidase-dependent ROS production to inhibit endotoxin-mediated inflammation by blocking the JAK/STAT1 and NOX2/p47(phox) pathways. *Oxidative Medicine and Cellular Longevity* Article ID 9738745.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine, 20*(7), 933–956.

Ross, J. A., & Kasum, C. M. (2002). Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annual Review of Nutrition, 22*(1), 19–34.

Salah, N., Miller, N. J., Paganga, G., Tijburg, L., Bolwell, G. P., & Rice-Evans, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics, 322*(2), 339–346.

Sarneckis, C. J., Dambergs, R. G., Jones, P., Mercurio, M., Herderich, M. J., & Smith, P. A. (2006). Quantification of condensed tannins by precipitation with methyl cellulose: Development and validation of an optimised tool for grape and wine analysis. *Australian Journal of Grape and Wine Research, 12*(1), 39–49.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry, 30*(12), 3875–3883.

Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition, 130*, 2073S–2085S.

Selloum, L., Reichl, S., Muller, M., Sebihi, L., & Arnhold, J. (2001). Effects of flavonols on the generation of superoxide anion radicals by xanthine oxidase and stimulated neutrophils. *Archives of Biochemistry and Biophysics, 395*, 49–56.

Taguri, T., Tanaka, T., & Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biological and Pharmceutical Bulletin, 27*(12), 1965–1969.

Tian, R., Ding, Y., Peng, Y. Y., & Lu, N. (2017). Inhibition of myeloperoxidase- and neutrophil-mediated hypochlorous acid formation *in vitro* and endothelial cell injury by (-)-epigallocatechin gallate. *Journal of Agricultural and Food Chemistry, 65*(15), 3198–3203.

Valcheva-Kuzmanova, S. V., & Belcheva, A. (2006). Current knowledge of *Aronia melanocarpa* as a medicinal plant. *Folia Medica, 48*(2), 11–17.

Wang, J. P., Chang, L. C., Hsu, M. F., Chen, S. C., & Kuo, S. C. (2002). Inhibition of ormyl-methionyl-leucyl-phenylalaninestimulated respiratory burst by cirsimaritin involves inhibition of phospholipase D signaling in rat neutrophils. *Naunyn-Schmiedeberg's Archives of Pharmacology, 366*, 307–314.

Zheng, W., & Wang, S. Y. (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *Journal of Agricultural and Food Chemistry, 51*(2), 502–509.