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ORIGINAL ARTICLE

Antioxidative Potential of Pomegranate Peel Extract: *In Vitro* and *In Vivo* Studies

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Abstract

Background/Aim: Due to the numerous beneficial effects of pomegranate that can be explained through its antioxidative effects, the aim of this study was to determine the antioxidant potential of pomegranate peel extract (PoPEx) prepared from pomegranate that was harvested in the south-east region of Herzegovina (Bosnia and Herzegovina), through *in vitro* and *in vivo* studies.

Methods: In PoPEx total phenols, flavonoids, flavonols, flavan-3-ols and anthocyanins content was determined, as well as several antioxidative assays, including 2,2 diphenyl-1-picrylhydrazyl assay (DPPH), 2,2'-azino bis(3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS), iron (III) - 2,4,6-tripyridyl-S-triazine complex assay (FRAP), reduction of copper(II) ions (CUPRAC) assay, Briggs-Rauscher oscillatory reactions, neutralisation of OH radicals and lipid peroxidation assay. *In vivo* studies were performed by administrating 100 mg/kg of body weight of PoPEx to the rats by gavage for 7 days, after which the rats were euthanised and prooxidative parameters (thiobabrituric acid reactive substances - TBARS as an index of lipid peroxidation, nitrites - NO_2, hydrogen peroxide - H_2O_2 and superoxide anion radical O_2^-) were determined in plasma, as well as antioxidative parameters (superoxide dismutase - SOD, reduced glutathione - GSH and catalase - CAT) in erythrocyte lysates.

Results: High content of phenolic compounds was found in PoPEx, which resulted in high antioxidative potential in all *in vitro* tests performed. *In vivo* study showed that PoPEx administration caused a significant decrease in TBARS, NO_2^-, as well as an increase in reduced glutathione ($p < 0.05$) in comparison to the control group, while H_2O_2 and O_2^* showed a lowering trend and SOD and CAT showed an increasing trend in PoPEx group, but without statistical significance.

Conclusion: PoPEx demonstrated high antioxidative capacity measured *in vitro* and *in vivo* and can be potentially used as a supplement treatment in the prevention of various inflammatory conditions.

Key words: Pomegranate peel extract; Antioxidative capacity; Phenolic compounds.

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Introduction

Pomegranate (*Punica granatum* L.) is a fruit-bearing tree that is cultivated and consumed worldwide. It is used in folk medicine and has scientifically proven beneficial effects in diseases such as diabetes mellitus type 2,[1] cardiovascular diseases,[2, 3] inflammatory diseases[4] and even cancer.[5] Knowing that non-communicable diseases are the leading cause of morbidity and mortali-

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ty worldwide,[6] there is a high interest in finding potential agents that could be used in the prevention and/or treatment of these diseases. The main active compounds in the pomegranate are different polyphenolic antioxidants such as anthocyanins, tannins and flavonoids.[7] Although pomegranate is mostly consumed in the form of juice produced from the aerial parts of the plant, more than 40 % of the fruit is its peel, which is usually considered as waist.[8] However, studies have shown that the pomegranate peel has 48 different polyphenolic compounds,[9] that have been proven to have high antioxidative capacity.[2, 7, 10] Gallic and ellagic acid, ellagitannin, punicalin, punicagalin, anthocyanins, flavonoids and other phenols, are found in abundance in pomegranate peel extracts (PoPEx) and have been shown to exhibit various biologically beneficial properties such as hypoglycaemic, lipid-lowering, anti-inflammatory, antihypertensive, antimicrobial and antioxidant effects.[11-14]

Therefore, the aim of this study was to investigate the antioxidative potential of a PoPEx produced from pomegranate plants collected in the south-east Herzegovina region using various *in vitro* techniques and an *in vivo* study.

Methods

Materials

Fruits of the pomegranate (*P. granatum*) were harvested in the south-east Herzegovina region (the Republic of Srpska, Bosnia and Herzegovina).

Preparation of PoPEx dry extract

The fruits were washed by hand after which the peel was removed from the fruits and dried for 4-6 days and grounded into fine powder. The pomegranate peel dry extract was prepared using 70 % (v/v) ethanol as a solvent and the method of triple percolation was performed, followed by evaporation of the solvent using a vacuum oven.

Liquid chromatography – mass spectroscopy (LC-MS) chemical analysis

The LC-MS analysis of PoPEx was performed with an Agilent Technologies HPLC 1260 Infinity system coupled with a single quadrupole mass detector (Singlequad MS detector 6130). Compounds were separated on Zorbax SB Aq-C18 column (3.0 150 mm; 3.5 m) at a temperature adjusted to 25 ºC. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B) and a gradient elution was applied at a flow rate of 0.3 mL/min. The following gradient program was utilised: 0–30 minutes from 10 % to 25 % B, 30–35 minutes from 25 % to 70 % B and 35–40 minutes from 70 % B to 10 % B. The detection wavelengths were 280 and 350 nm and the range of m/z was 50–2000. The electrospray ionisation method was used to ionise the sample at a pressure of 40 psi, a temperature of 350 ºC, and a flow rate of 10 L/min of nitrogen. Signals from deprotonated molecules and fragmented ions were acquired in full-scan mode at voltages of 100 V and 250 V.

Total phenols, flavonoids, flavonols, flavan-3-ols and anthocyanins determination

For the determination of total phenols, flavonoids, flavonols and *in vitro* antioxidative tests, different concentrations of ethanolic (80 % v/v) solution of PoPEx were used. Total phenols were determined using the spectrophotometric method of Folin-Ciocalteu,[15] by measuring the absorbance at 765 nm and the results were expressed as gallic acid equivalent per dry weight (mgGAE/g_{DW}). Flavonoids were determined spectrophotometrically at 420 nm[16] and expressed as quercetin equivalents per dry weight (mgQc/g_{DW}). Flavonols were determined according to Kumaran and Karunakaran[17] spectrophotometrically at 510 nm and expressed as quercetin equivalents (mgQc/g_{DW}). The method of Revilla et al[18] was used for the determination of total flavan-3-ols. For the determination of total and monomeric anthocyanins, "single" pH method and pH differential methods were used.[19] The content of total and monomeric anthocyanins is expressed as mg cyanidin-3-glucoside (C3G)/g_{DW}.

In vitro determination of antioxidant potential

(1) 2,2 diphenyl-1-picrylhydrazyl (DPPH) method

The antioxidant capacity of the examined samples of PoPEx was determined by measuring its antioxidant capacity to reduce DPPH radicals.[20, 21] $DPPH^{\cdot}$ is a stable free radical that has the ability to delocalise a free electron over the entire molecule. This is the reason why dimerization of $DPPH^{\cdot}$ does not occur, as it happens with most free radicals and because of which the purple coloration of the solution oc-

curs with the absorption maximum at around 520 nm. The reaction of $DPPH^{\cdot}$ with a hydrogen donor creates a yellow-coloured reduced form of the radical (diphenyl picrylhydrazine) and the consequence is the loss of purple coloration. Absorption decrease is linearly dependent on antioxidant concentration.

(2) 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method

The antioxidant capacity of the tested samples was determined by measuring their efficiency in reduction of $ABTS^{\cdot+}$ radicals. ABTS is a compound that upon oxidation with potassium peroxodisulphate ($K_2S_2O_8$) gives a dark-blue cation radical $ABTS^{\cdot+}$, with an absorption maximum at 734 nm. The resulting $ABTS^{\cdot+}$ cation radical reacts with antioxidants, decolourising them and the decrease in colour intensity is proportional to the amount of antioxidants present.[22]

(3) Ferric reducing antioxidant power (FRAP) method

The FRAP method is based on the ability of antioxidants to reduce the iron (III) - 2,4,6-tripyridyl-S-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ in the acidic medium.[23] FRAP values were calculated by measuring the increase in absorbance at 593 nm and comparing them with a standard solution of coloured ions, or a standard solution of antioxidants (Trolox).

(4) Cupric reducing antioxidant capacity (CUPRAC) method

The CUPRAC method[24] is based on monitoring the reduction of copper(II) ions, which with neocuproine (2,9-dimethyl-1,10-phenanthroline), in a neutral environment, builds colourless bis (neocuproine) copper(II)) chelate complex, Cu(II)-Nc. In the presence of a reducing agent, Cu(I)-Nc is formed, an orange-red complex compound that shows an absorbance maximum at 450 nm.

(5) Briggs-Rauscher reactions

The mechanism of Briggs-Rauscher oscillatory reactions is quite complex and takes place through two processes, one of which is radical and the other non-radical.[25] Briefly, the Briggs-Rauscher reaction represents the oxidation of malonic acid in the presence of hydrogen peroxide and iodate in an acidic medium, which is catalysed by manganese ions. In doing so, various reaction intermediates are created. The main intermediates in the reactions are the iodide ion, the oxoiodine species HOI, HOIO and IO• and the hydrogen peroxide radical HOO•. The addition of various antioxidants to the reaction mixture leads to the "neutralisation" of free radicals, synthesised in the reactions, which disrupts the kinetics of the reactions. Oscillations in Briggs-Rauscher reactions were monitored potentiometrically by measuring the potential of the tested mixture using a platinum electrode and a reference electrode $Ag/AgCl/Cl_{sat}$. (+197 mV compared to saturated hydrogen electrode SHE). The results were expressed as mmol Trolox/g extract.

(6) Neutralisation of OH radicals

The method is based on measuring the degree of degradation of 2-deoxy-D-ribose under the influence of the hydroxyl radical generated in the Fenton reaction. The resulting reactive hydroxyl radical in the presence of 2-deoxy D-ribose and oxygen builds malondialdehyde, which is determined in the thiobarbituric acid (TBA) test. In doing so, a pink coloured complex with maximum absorption at a wavelength of 530 nm is formed.[26] The original method was modified because it was found that organic solvents, especially ethanol, significantly increase the degree of inhibition of OH radicals.[27, 28]

(7) Lipid peroxidation

As the primary products of lipid peroxidation, unstable hydroperoxides are formed, which break down to give secondary compounds, one of which is malondialdehyde. TBA was used for its determination. Two molecules of TBA and one molecule of MDA participated in this reaction, resulting in a pink complex with an absorption maximum at around 530 nm.[29]

In vivo studies of antioxidant potential

(1) Experimental animals

For the in vivo studies of antioxidant potential, male Wistar rats (n = 11) with body weight (bw) of 250 ± 25 g were used. The study was conducted at the Centre for Biomedical Research of the Faculty of Medicine, University of Banja Luka. The laboratory animals were kept in standard laboratory conditions with ambient temperature of 21 ± 2 ^{0}C, humidity of 55 ± 5 % and 12/12 h light/dark cycle with water and food access ad libitum. The study

was approved by the Ethics Committee for the Protection and Welfare of Experimental Animals in Biomedical Research, Faculty of Medicine, University of Banja Luka (Decision No 18/1.51/21-7; 9th March 2021).

(2) Experimental design

The animals were divided into two groups:
1. Control (C) group (n = 5) which received 0.5 % Na-carboxymethyl cellulose water solution (1 mL/kg) for 7 days by gastric gavage and
2. P100 group (n = 6) which received 100 mg/kg PoPEx for 7 days by gastric gavage.

At the end of experiment, on eighth day the animals were anesthetised with a combination of ketamine (30 mg/kg bw) and xylazine (5 mg/kg bw) intraperitoneally (ip) and euthanised. Blood was collected into 3.2 % Na-citrate tubes, after which plasma was separated by centrifugation at 3,000 rpm for 10 minutes and the erythrocytes were lysed using deionised water. Plasma was used for the determination of O_2^-, H_2O_2, NO_2^-, TBARS and erythrocyte lysates were used the determination of SOD and catalase CAT activity as well as for the amount of total reduced glutathione (GSH).

(3) Determination of superoxide anion radical (O_2^-)

The determination of the amount of superoxide anion radical (O_2^-) in plasma was based on the reaction of O_2^- with nitro tetrazolium blue (Nitro Blue Tetrazolium - NBT) to nitroformazan blue.[30] Spectrophotometrically measurement was performed at the wavelength of maximum absorption λmax = 550 nm.

(4) Determination of hydrogen peroxide (H_2O_2)

The determination of the amount of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red using hydrogen peroxide in peroxidase from horse radish enzyme-catalysed reaction (Horse Radish Peroxidase - HRPO). This reaction resulted in the formation of a compound whose maximum absorption was λmax = 610 nm.[31]

(5) Determination of lipid peroxidation index (TBARS)

The lipid peroxidation index, as one of the parameters of oxidative stress, was determined indirectly through the products of the lipid peroxidation reaction with thiobarbituric acid. The level of TBARS in plasma was determined spectrophotometrically at 530 nm.[32]

(6) Determination of nitrites (NO_2^-)

Biochemically, this method was based on the use of Griess-reagent, which with nitrites builds a diazo-complex that gives a purple colour. After colour stabilisation at room temperature for 5-10 minutes the concentration of released nitrites were measured spectrophotometrically at wavelength of λ = 550 nm.[33]

(7) Determination of superoxide dismutase (SOD) activity

Superoxide dismutase was determined by the adrenaline method according to Beutler. The measurement was carried out spectrophotometrically at the wavelength of λ = 470 nm.[34]

(8) Determination of catalase (CAT) activity

Catalase was determined by measuring the decrease in absorbance for 1 minute at 240 nm of the reaction mixture that was comprised of the sample and 10 mM hydrogen peroxide.[35]

(9) Determination of reduced glutathione (GSH)

The activity of reduced glutathione, was measured in erythrocyte lysate using a spectrophotometric method. This method was based on the reaction of oxidation of glutathione with 5.5-dithio-bis-6.2-nitrobenzoic acid, according to the method of Beutler.[36]

Statistical analysis

In vitro experiments were performed in 3 parallel repetitions and the results were expressed as mean value ± standard deviation. IC50 values were calculated by linear regression analysis using Origin 6.0 software. All *in vivo* results were expressed as mean ± standard deviation (STDEV) and statistical program SPSS 18.0 was used for the analysis. Statistical significance was set up at $p < 0.05$.

Results

Qualitative and quantitative analyses

The LC-MS analysis of the PoPEx identified 14 major compounds, including bioactive compounds such as punicalin, punicalagin and tellimagran-

Table 1: Compounds identified in pomegranate peel ethanol extract by LC-MS method

RT (280 nm)	λmax	MW	[M – H] – (m/z) (100 V)	MS data (m/z) (250V)	Compound name
3.321	258, 376	482	481	275, 301	HHDP-hexoside
3.777	260, 380	782	781	601, 721	Punicalin isomer (α or β) (4,6-gallagyl-glucose)
3.961	260, 380	782	781	601, 721	Punicalin isomer (α or β)
4.982	260, 380	784	783	481, 301	Penduculagin I isomer (bis-HHDP-hexoside)
6.072	258, 378	1416	933	783, 633	bis-(HHDP-galloylglucose)-pentose
6.767	256, 376	952	951	933, 301	galloyl-HHDP-DHHDP-hexoside (granatin B)
8.285	258, 380	1084	1083	781, 601	Punicalagin isomer 1 (HHDP-gallagyl-glicoside)
11.129	258, 380	1084	1083	781, 601	Punicalagin isomer 2 (HHDP-gallagyl-glucoside)
12.537	220, 266	1086	1085	783, 633, 451, 301	Digalloyl-gallagyl-hexoside
13.542	230, 278	1568	1567	935, 783, 633	Sanguiin H-10 isomer (digalloyl triHHDP-dihexoside)
14.222	266, 362	634	633	463, 301	Galloyl - HHDP hexoside
15.226	218, 274	786	785	755, 301	Tellimagrandin I
17.489	252, 304, 362	464	463	301, 463	Quercetin-hexoside
25.513	252, 308, 368	628	627	301	Ellagic acid derivate

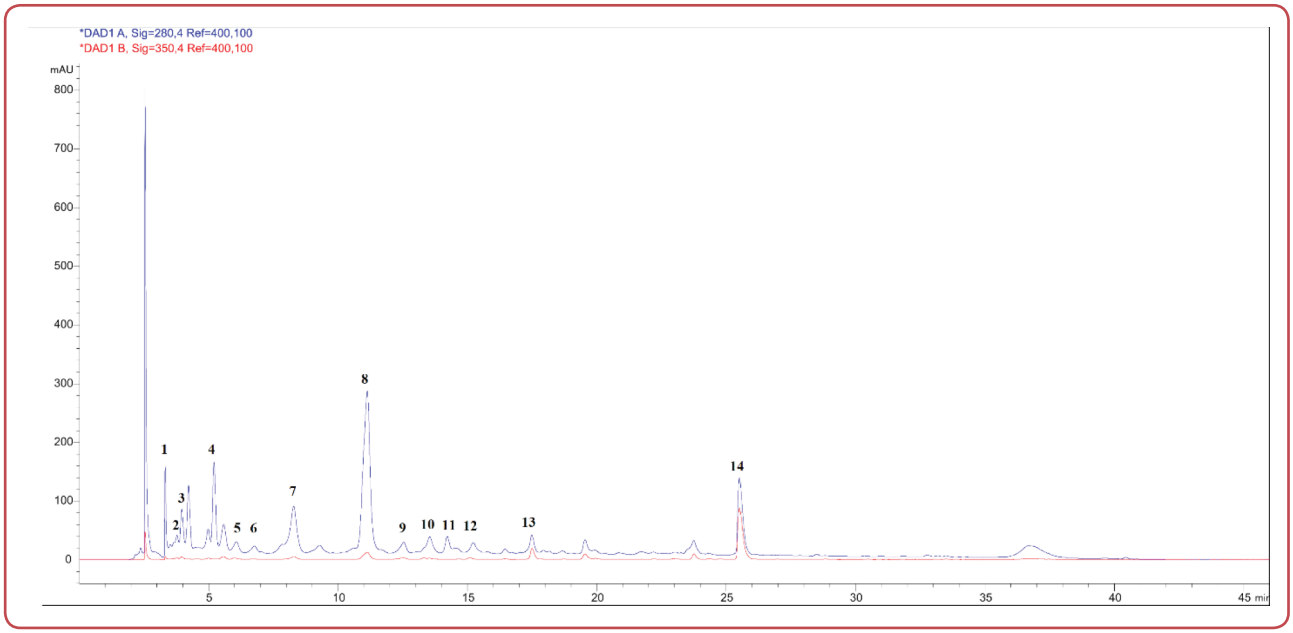

Figure 1: Chromatogram of PoPEx (5 mg/mL) analysed by the LC–MS

PoPEx: pomegranate peel extract; The LC-MS analysis of the PoPEx identified 14 major compounds, including bioactive compounds such as punicalin, punicalagin and tellimagrandin I. Punicalagin was the most prevalent compound.

din I. According to the results, punicalagin was the most prevalent compound (Table 1 and Figure 1).

The content of total phenols in the samples was extremely high and amounted to 621.13 mg GAE/g_{DW} sample. Total flavonoids were relatively high, amounting to 63.3 mg Qc/g_{DW}. A high content of total flavonols was found in the sample and it was 90.84 mg Qc/g_{DW}. In the sample, a relatively low content of flavan-3-ol was found compared to other phenols, only 71.44 μg CAT/g_{DW}. In this sample, the content of total monomeric anthocyanins was 0.55 mg C3G /g_{DW}. On the other hand, the content of total anthocyanins, polymerised (degraded) and monomeric, was 6.25 mg C3G /g_{DW} (Table 2).

Table 2: Content of total phenols, flavonoids, flavonols, total and monomeric anthocyanins

Compounds	Content
Total phenols (mgGAE/g_{DW})	621.13 ± 32.33
Flavonols (mgQc/g_{DW})	90.84 ± 3.56
Flavan-3-ols (μg CAT/g_{DW})	71.44 ± 7.80
Flavonoids (mgQc/g_{DW})	63.30 ± 0.43
Total anthocyanins (mg C3G/g_{DW})	6.25 ± 0.01
Monomeric anthocyanins (mg C3G/g_{DW})	0.55 ± 0.02

In vitro studies of antioxidant potential

In vitro measured antioxidative capacity of PoPEx, with Trolox used as a reference antioxidant, showed significantly better capacity in neutralising OH radical ($p < 0.05$), while the other tests showed slightly lower potency in comparison to Trolox (Table 3).

Table 3: Antioxidative capacity of PoPEx (mean ± standard deviation)

Reaction	PoPeX (mmol Trolox/g_{DW}Ex)	IC_{50} µg/mL	IC_{50} Trolox µg/mL	mmol Fe/g	mmol Fe/g trolox
B-R	0.815 ± 0.074	-	-		
DPPH	2.142 ± 0.037	13.260 ± 0.110	7.110 ± 0.190		
ABTS	2.735 ± 0.137	3.606 ± 0.233	2.469 ± 0.106		
CUPRAC	2.891 ± 0.167	-	-		
FRAP	3.422 ± 0.044	-	-	7.137 ± 0.057	8.532 ± 0.095
Lipid peroxidation	3.945 ± 0.079	19.130 ± 0.240	18.890 ± 4.380		
OH	5.020 ± 0.090	49.920 ± 0.930	62.690 ± 2.040		

PoPEx: pomegranate peel extract; BR: Briggs-Rauscher reactions; DPPH: 2,2 diphenyl-1-picrylhydrazyl assay; ABTS: 2,2'-azino bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; CUPRAC: Cupric reducing antioxidant capacity assay; FRAP: ferric reducing antioxidant potency assay;

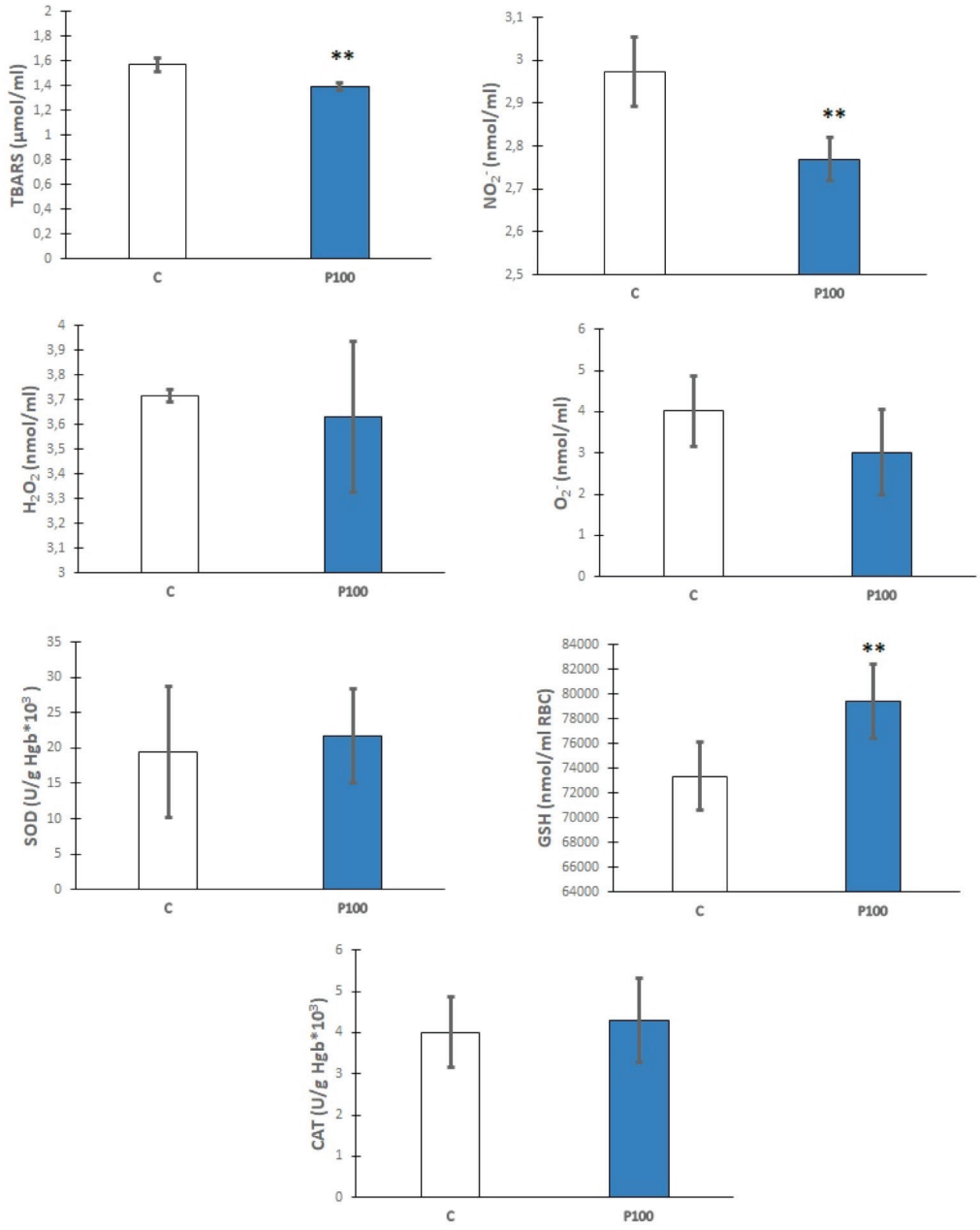

Figure 2: Effects of pomegranate peel extract on prooxidant and antioxidant parameters

*TBARS-thiobabrituric acid reactive substances, NO_2^- - nitrites, H_2O_2 – hydrogen peroxide and O_2^- - superoxide anion radical and antioxidative parameters: SOD – superoxide dismutase, GSH- total reduced glutathione and CAT - catalase. C-control group, P100- pomegranate peel extract 100 mg/kg bw group. Data are presented as means ± standard deviation. **-statistically significant difference (t-test, α = 0.05) vs control group;*

In vivo studies of antioxidant potential

After 7 days of PoPEx administration, oxidative markers (TBARS and NO_2^-) were significantly lowered in the PoPEx group compared to the control, while GSH was significantly increased ($p < 0.05$). Other two oxidative parameters (H_2O_2 and $O_2^{\cdot}$) showed a lowering trend in the values measured after PoPEx administration, as well as two antioxidative parameters (SOD and CAT) which showed an increasing trend in PoPEx group, but without statistical significance (Figure 2).

Discussion

There is a large number of studies that investigated the content of total phenols in extracts of pomegranate peel.[37] The data show that the content of total phenols ranged from 53.65 to 841.5 mg GAE/g_{DW} sample. The results of presented study are well above the average and are close to the results obtained for ethyl acetate extracts of Italian pomegranate peel, which were 3.75 mmol GAE/g_{DW} = 638 mg GAE/g_{DW}.[38] The content of total flavonoids was higher than the content found in ethanol extracts of pomegranate peels in Turkey, which ranged from 12.83-17.27 mg Qc/g_{DW}[39] and lower than the content of samples from Mauritania where a value of 180.1 mg Qc/g_{DW} was found in the methanol extract.[40] Content of flavonols in the samples was significantly higher than the total flavonols in the ethanol extract of blackberry pomace, which showed significant antioxidant and antimicrobial effects and where the values ranged from 2.53–6.39 mgQc/g_{FW}.[41] Low level of flavan-3-ols is not surprising because a high level of tannin (14.15 %) was found in the sample, so probably a large part of flavan-3-ol was used for the synthesis of these compounds. The presence of total monomeric anthocyanins in different pomegranate genotypes, from different geographical latitudes and in different solvents varies in a wide range from 0.68-102.2 mg C3G /g_{DW}.[37] Significant differences in values of monomeric and total anthocyanins in the samples indicate that the anthocyanin content is significantly higher in the polymerised, biologically less valuable form. Such a large range in contents of total phenols, flavonoids, flavonols, total and monomeric anthocyanins, is an obvious consequence of the diversity of samples considering the geographical and climatic area, genotype, degree of maturity, method of extraction and especially the type of solvent used for the extraction of these compounds.

Due to the complexity of oxidative processes, multiple methods are required to determine antioxidant capacity; otherwise, the obtained results cannot be accurately interpreted and confirmed.[42] The results showed that the extract had a very low antioxidant capacity in relation to Briggs Rausher's oscillatory reactions. The value was only 0.815 mmolTr/g of extract and it was significantly lower than the capacity of wild and tame blackberry pomace extracts, which ranged from 17-28 μgTr/μg of extract.[43] Briggs-Rauscher's oscillatory reactions take place at low pH, close to the pH in the stomach, where the basic free radical that initiates the oscillatory reaction system is the hydroperoxyl radical HOO·. From the results, it could be concluded that pomegranate peel extract has a weak antioxidant effect at low pH values and on the hydroperoxyl radical.

The antioxidant capacity of the 70 % ethanol PoPEx against the stable DPPH radical was lower than that of the synthetic antioxidant Trolox. The values were 2.142 mmol Tr/g, or if the value is expressed as IC50, 13.26 μg/mL while Trolox had an IC50 of 7.11 μg/mL. The obtained values indicate that the antioxidant capacity of the extract against the stable DPPH radical is higher than the capacity of ethanol extract of pomegranate by other authors who found the following values: 1.71 - 2.0 mmol Tr/g,[44] 225 μmolTr/g_{dw},[45] IC50 = 69-91 μg/mL.[39] On the other hand, some authors found a higher antioxidant capacity of ethanol extract of pomegranate IC50 = 4.9 μg/mL.[46] Besides, some authors tested pomegranate extracts in different solvents and obtained significantly different results. When pure methanol was used as a solvent, a higher antioxidant capacity was obtained (IC50 = 8.3 μg/mL;[47] IC50 = 8.73 μg/mL[48]), whereas other solvents yielded significantly lower antioxidant capacity, as observed in the study of different varieties of pomegranate in Tunisia (IC50 = 56-65 μg/mL).[39] When 70 % methanol was used, the antioxidant capacity of pomegranate peel was slightly different, IC50 = 14.67.[48]

The obtained values for the antioxidant capacity against the stable ABTS radical were 2.735 mmol Tr/g, ie IC50 = 3.606 μg/mL, therefore the extract showed a weaker effect than Trolox (IC50 = 2.469 μg/mL) and from some literature values 2.8-4.41 mmol Tr /g.[44] On the other hand, the values were significantly higher than those in the literature for extracts in ethanol IC50 = 5.013 μg/mL,[38] as well as for some extracts in methanol (6.5-8.5 mmolTr/100g).[49] The highest antioxidant capac-

ity was shown by the extract of pomegranate in ethyl acetate; IC50 = 1.725 μg/mL.[38]

The FRAP results indicated a lower antioxidant capacity than Trolox, as the values were 3.42 mmol Tr/g or 7.157 ± 0.057 mmol Fe/g, compared to 8.532 ± 0.095. Also, the results for ethanol extracts were lower than those reported in the literature, which were 12.4 mmol Fe/g dw;[39] 4.05-6.64 mmolTr/g.[44] On the other hand, some other authors found lower values of 21.24-21.5 mmol Tr/100g_{dw} for the methanol extract,[50] 9.07 mmol/100 g,[51] 82.11 mmol/100 g.[52]

Due to the favourable redox potential in a neutral medium, the CUPRAC method is preferable for simulating physiologically significant redox reactions of antioxidant compounds, such as serum antioxidants. The values determined by the CUPRAC method (2.89 mmol Tr/g) indicate that the antioxidant capacity of pomegranate peel extract is weaker than that of Trolox. They were also lower than the literature values, which were 3756 μmolTr/g_{dw}.[45]

The values for the inhibition of lipid peroxidation were 3.945 mmol Tr/g, ie IC50 = 19.13 μg/mL, which was slightly lower than the capacity of the standard antioxidant Trolox, which showed IC50 = 18.89 μg/mL. Also, from the results, it can be seen that the ethanol extract of pomegranate showed a better capacity to inhibit lipid peroxidation than the extract in methanol, which showed only IC50 = 32.4 μg/mL.[47]

PoPEx showed significant antioxidant capacity against the OH radical and was 5.020 mmol Tr/g, ie IC50 = 49.92 μg/mL. These values were lower, ie the extract showed a better antioxidant capacity than the standard antioxidant Trolox, which had an IC50 value of 62.69 μg/mL. The antioxidant capacity against the OH radical of extracts in different solvents was tested and the following values were obtained: IC50 = 322; 126; 13.6; 54.9; 289 μg/mL: hexane, ethyl acetate, methanol, 70 % methanol, water, respectively.[48] By comparison, it can be seen that the extract showed better results, except in the case of the extract in pure methanol. Based on the results, it can be concluded that pomegranate extract can significantly inhibit the formation of OH radicals.

The antioxidative capacity of PoPEx was further investigated in *in vivo* conditions. A seven-day consumption of PoPEx (100 mg/kg) lead to a significant decrease of the index of lipid peroxidation (TBARS) and plasma NO_2^-, as well as a decrease of other two free radicals – superoxide anion radical and hydrogen peroxide, when compared to the control group, but without statistical significance. These results suggest that PoPEx acts as a free radical scavenger. Besides, PoPEx consumption also led to an increase of the levels of the two antioxidative enzymes (CAT, SOD) and GSH. The SOD acts through conversion of superoxide radicals into hydrogen peroxide, that is afterwards converted to molecular oxygen and water via the activity of CAT.[53] GSH, on the other hand, led to the reduction of the hydrogen peroxide radicals. Knowing that the levels of these two enzymes and GSH are decreased in the presence of free radicals, these findings are in concordance with the hypothesis that PoPEx acts as a free radical scavenger. The cornerstone of the antioxidative capacity of the PoPEx is considered to be its high polyphenolic content. This was also shown in previous studies that investigated free radical scavenging capacity of the PoPEx.[54] The main mechanism through which polyphenols scavenge free radicals is considered to be donation of hydrogen atoms.[55]

Conclusion

From the results, it can be seen that PoPEx shows significant antioxidant activity both, *in vitro* and *in vivo*. Besides, different values were obtained for the antioxidant capacity against different radicals, depending on the method used. The values for the antioxidant capacity are arranged in the following order sequence: B-R < DPPH < ABTS < CUPRAC < FRAP < Lipid peroxidation < OH. Such high values of antioxidant capacity measured *in vitro*, as well as the significant decrease of TBARS and NO_2 radical on the one hand and an increase in levels of GSH measured *in vivo* on the other hand are the result of a high content of total phenols, flavonoids, flavonols and monomeric anthocyanins with high radical scavenging potential of PoPEx.

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Conflict of interest

None.

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