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Effects of Four-Week Intake of Blackthorn Flower Extract on Mice Tissue Antioxidant Status and Phenolic Content

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The study examined the antioxidative physiological effects of phenolics from an ethanol-water extract of blackthorn flowers orally administrated to C57/BL6 mice for 28 days in daily doses of 25 mg of total phenolics/kg body weight. Contents of phenolics in the intestine, liver, and kidneys collected after 1, 7, 14, 21, and 28 days of extract administration were analyzed by UPLC-MS/MS method. In the same tissues, the antioxidative properties were determined as ferric reducing antioxidant power (FRAP), $ABTS^{\bullet+}$ scavenging activity, content of reduced glutathione (GSH), and activity of superoxide dismutase (SOD) and catalase (CAT). The lipid peroxidation in tissues was also evaluated by thiobarbituric acid reactive substances (TBARS) assay. The exposed mice (compared to the control ones) had a lower content of TBARS in all tissues mostly on the third/fourth week of daily consumption. SOD activity and GSH content increased on the 28th day in tissues. CAT activity was higher only in the liver after one week of consumption but remained unchanged in other organs throughout the experiment. Phenolic profiles were different in individual tissues. The most prominent increases compared to the control were determined for contents of 3-*O*-feruloylquinic acid, 4-*O*-*p*-coumaroylqiunic acid, kaempferol pentoside, and quercetin rhamnoside in the intestine; for ferulic acid and quercetin 3-*O*-rutinoside in the liver; and for quercetin 3-*O*-rutinoside, ferulic acid, and 4-*O*-*p*-coumaroylquinic acid in the kidneys. The screened phenolics with different distribution in tissues could be responsible for slight differences in the recorded antioxidative effects.

INTRODUCTION

Blackthorn (*Prunus spinosa* L.) is a perennial shrub belonging to the rose family (Rosaceae), growing throughout Europe, western Asia, and northwest Africa [Elez-Garofulić *et al.*, 2018]. Recently, blackthorn has become interesting both industrially as a food plant and pharmacologically as a nutraceutical or a medicinal plant because it is a rich source of phenolic compounds [Meschini *et al*., 2017; Mikulic-Petkovsek *et al*., 2016; Pinacho *et al*., 2015; Yuksel, 2015]. The fruits of blackthorn, known also as the "sloe", are small round with black skins covered in a blue waxy bloom and extremely acid-tasting, and must be thermally processed prior to consumption. They are used for jams, liqueurs, wines, juices, compote, and tea production. Blackthorn flowers, bark, and root have been traditionally used in folk medicine for diuretic and laxative properties, due to their abilities to remove excess sodium ions and harmful products of metabolism, to reduce blood vessels permeability, and against inflammation of the urinary tract [Elez-Garofulić *et al.*, 2018]. In western and northern Europe and Mediterranean countries, fruits were traditionally consumed but consumption declined in the 20th century [Alarcón *et al.*, 2015; Menendez-Baceta *et al*., 2012]. There is archaeological evidence that fruits were consumed in distant past as well. In the book on palaeoethnobotany of the prehistoric food plants of the Near East and Europe, the blackthorn seeds are mentioned as leftover of fruits that have been found and recovered in a number of European prehistoric sites from the neolithic and iron age, sometimes in large quantities (in barrels) [Renfrew, 1973].

Mikulic-Petkovsek *et al.* [2016] compared the phenolic and other secondary metabolite contents of various *Prunus* spp. wild fruits and concluded that the blackthorn (*P. spinosa*) showed richness of various plant phenolics making it a good candidate species among the genus *Prunus* spp. for the studies of phenolic biological activity upon consumption. Although berries are traditionally consumed, other plant parts such as flowers were studied for the content of phenolics as well. For example, a study of various morphological parts of blueberries (*Vaccinium angustifolium* L.) and lingonberries (*Vaccinium vitis-idaea* L.) has found that the leaves have a much higher phenolic content and antioxidant capacity than fruits [Kelly *et al*., 2017]. Olszewska and her co-authors were among the first who recorded the abundance of phenolics in *P. spinosa* leaf and also in the flower extract [Olszewska *et al.,* 2001; Olszewska & Wolbiś, 2001, 2002]. Marchelak *et al.* [2017] reported that blackthorn flower extract contained

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total phenolics up to 584.07 mg/g of dry weight (dw). Likewise, similar studies in *P. spinosa* flowers were done by Elez-Garofulić *et al.* [2018] and Lovrić *et al.* [2017], confirming the richness of phenolics and the antioxidative properties of the flower extract *in vitro*. However, although this plant phenolic extracts are recently scientifically examined, those experiments describing bioactivity (antioxidative properties and similar physiological modulations) were done mostly under *in vitro* conditions. *In vitro* studies showed that the phenolics from blackthorn extract were potent antioxidants that scavenged DPPH$^{\bullet}$, reduced ferric ions, and inhibited linoleic acid peroxidation and pro-inflammatory enzymes (lipoxygenase and hyaluronidase) activity, as well as effectively protected the isolated human plasma (outside the body, *in vitro*) against peroxynitrite-induced damage by reducing levels of oxidative stress biomarkers: 3-nitrotyrosine, lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) [Marchelak *et al.*, 2017]. Authors proposed the hypothesis concluded from *in vitro* results that the blackthorn extract might enhance the total antioxidant bioactivity if administrated within the organism. There are also *in vitro* antitumor assays as well as a study conducted by our group where the flower extract showed promising antitumor results [Murati *et al.,* 2019].

Based on the described *in vitro* evidence and lack of *in vivo* data, we have previously also conducted the preliminary *in vivo* study by applying only a single acute dose of the blackthorn flower extract in mice to establish whether the phenolics can be absorbed *via* the gastrointestinal system. A significant concentration of phenolics was found in plasma of animals after 24 h, indicating their good gut absorption [Đikić *et al.,* 2018]. Based on those results of blood pharmacokinetic study [Đikić *et al.,* 2018], indicating that the number of phenolics are being absorbed, we set foundations for this 28-day consumption study and evaluation of the antioxidative activity in the organs (intestine, liver, kidney). We wanted to establish, whether mice tissue antioxidative defense markers change due to the subchronic (28 day) intake of blackthorn flower extract.

MATERIALS AND METHODS

Chemicals and standards

HPLC grade formic acid and acetonitrile were purchased from BDH Prolabo, VWR (Lutterworth, England). The following commercial phenolic compound standards: quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, caffeic acid, gallic acid, ferulic acid, chlorogenic acid, and *p*-coumaric acid were purchased from Sigma–Aldrich (Steinheim, Germany). The (+)-catechin, (-)-epicatechin, (-)-epicatechin 3-gallate, (-)-epigallocatechin 3-gallate, apigenin, and luteolin were purchased from Extrasynthese (Genay, France) and quercetin 3-*O*-rutinoside from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Horse heart cytochrome C (type VI), human blood superoxide dismutase (SOD; type I, lyophilized powder, 2400 U/mg protein), xanthine and xanthine oxidase (200 U/mL), Ellman reagent (5,5'-dithiobis-(2-nitrobenzoic acid); DTNB), NADPH, glutathione reductase and hydrogen peroxide (30%) 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,4,6-tripyridil-s-triazine (TPTZ) were all purchased from Sigma-Aldrich. Deionized water of Milli-Q quality (Millipore Corp., Bedford, USA) was used throughout the experiment.

Preparation and analysis of blackthorn flower extract

The samples of dry blackthorn flowers were bought from Suban Ltd. company (Samobor, Croatia), a certified collector and producer of medicinal plants, and were part of the batch number 63451. The preparation of the blackthorn flower extract (ethanol/water extraction with microwave assistance) was described in detail by Elez-Garofulić *et al.* [2018] and Lovrić *et al.* [2017]. We have utilized the described methods because they yielded the best extraction of phenolics and proved 50% (*v*/*v*) ethanol/water used as a solvent to be safe for mammalian (mice) consumption in comparison to the other organic solvents which might remain as residues. For the *in vivo* experiment, the total phenolic (TP) content was analyzed in the original ethanol/water extract. The TP content of blackthorn flower extracts was determined using the method with the Folin-Ciocalteu reagent previously reported by Lovrić *et al.* [2017]. A volume of 100 μL of the extract (5-fold diluted) was mixed with 200 μL of the Folin–Ciocalteu reagent and 2 mL of distilled water, and after 3 min, 1 mL of 20% Na_2CO_3 was added. This mixture was incubated at 50°C for 25 min. The absorbance was measured at 765 nm using a spectrophotometer (model UV-1600PC; VWR International, Leuven, Belgium). The blank contained 100 μL of the solvent used for extraction instead of the extract. The TP content was calculated according to the gallic acid standard calibration curve, and expressed in mg of gallic acid equivalents (GAE) per mL of extract. The TP content of original extract was 0.5 mg GAE/mL. The extract was further evaporated under reduced pressure at 45°C to remove ethanol and concentrate the solution of polyphenolics. Before application to the mice, the concentrated solution was re-dissolved and further diluted with water to achieve the final applied solution with removed alcohol suitable for use in a dose of 25 mg TP GAE/kg body weight of mice (this water-based working solution for mouse doses, was applied in the volume of 0.2 mL per mouse weighing on average 30 grams). The phenolic composition of blackthorn flower extract and details of the UPLC-MS/MS method used for its determination were shown in our previous study [Đikić *et al.,* 2018; Elez-Garofulić *et al.,* 2018]. It was reported that the highest concentrations were detected for 3-*O*-caffeoylquinic, 3-*O*-*p*-coumaroylquinic, and 3-*O*-feruloylquinic acid among phenolic acids; then for (+)-catechin and (-)-epicatechin among flavan-3-ols; and finally for kaempferol glycosides (kaempferol pentoside and kaempferol rhamnoside) and quercetin glycosides (quercetin 3-*O*-rutinoside, quercetin pentoside, and quercetin rhamnoside) among flavonols.

Experimental animals and husbandry, study design *in vivo*

For this experiment, a total of 50 male inbred C57BL/6 mice, weighing 30±1.5 g were obtained from the Department of Animal Physiology, Faculty of Science University of Zagreb, Croatia. Animals were fed a standard laboratory

diet, tap water *ad libitum*, and received 12 h of light per day. The standardized diet was 4 RF 21, Mucedola (Settimo Milanese, Italy). The composition of standardized pellet mouse feed included wheat, wheat straw, hazelnut skins, maize, soy bean hulled, corn gluten feed, fishmeal, dicalcium phosphate, sodium chloride, whey powder, soybean oil, yeast; and contained 12% moisture, 18.5% protein, 3% fats, 6% crude fibers, 7% crude ash, E672 (vitamin A), E671 (vitamin E), E1 (Fe), E2 (I), E3 (Co), E4 (Cu), E5 (Mn), and E6 (Zn). Phenolic content of Mucedola feed pellets was analyzed in our previous study and results are given in Đikić *et al.* [2018]. The analysis revealed that all phenolic compounds that were detected in Mucedola standard mouse feed were in substantially lower contents than in the blackthorn flower extract [Đikić *et al.,* 2018].

Bioethical standards in animal husbandry and experimental protocol

Maintenance and care of all experimental animals was performed according to the guidelines applicable in the Republic of Croatia [NN 55/2013]. The experimental procedures were approved by the Bioethics Committee of the Faculty of Science, University of Zagreb [Bioethic approval, 2014] and were conducted according to the Guidelines on *in vivo* experiments and accepted and international standards on the Guide for the care and use of laboratory animals [2011].

Treatment groups and doses

Animals were randomly divided according to treatment into two separate groups, namely the control group and the blackthorn flower extract group. Within those two groups, the subgroups were formed based on the time of sacrifice post treatment. Animals from both control or blackthorn flower extract groups were sacrificed on the day 1, 7, 14, 21, and 28 post treatment (the subgroups). Each subgroup contained 5 animals. Saline for the control and blackthorn flower extract for the exposed group were administered daily as a single oral dose for the period of 28 days by gavage in a volume of 0.2 mL per animal. Treatment of all animals took place between 8–10 a.m. in order to equalize circadian differences between treatments and avoid differences in metabolism. The blackthorn flower extract-treated groups were dosed 25 mg of TP of blackthorn extract per kg of body weight of mice (mg TP/kg bw). The dose was derived from pilot experiments.

Tissue preparation

At designated experimental days for organ collection, animals were anesthetized by halothane and perfused through with 10 mL of phosphor buffer saline (PBS) and sacrificed by cervical dislocation, 24 h after the last administered dose on the particular day of experiment. Intestine, liver, and kidneys were extracted. Such tissue samples were used for antioxidative activity assays and for the determination of individually bioaccumulated phenolics by the UPLC MS/MS method. Prior to the measurement of antioxidative parameters and UPLC-MS/MS analysis, the tissue samples were placed in 50 mM phosphate buffer (pH=7.4) and homogenized (10% of homogenate, by tissue mass per volume of PBS) with an ultrasonic homogenizer (SONOPLUS Bandelin HD2070, Bandelin Electronic GmbH & Co KG, Germany) using an MS73 probe (Bandelin, Electronic GmbH & Co KG Germany). Thereafter, homogenates were sonicated on ice for 30 s in three 10-s intervals, centrifuged at 20,000×g for 15 min at 4°C, and immediately frozen at -80°C until analysis. Further details of supernatant treatment for antioxidative activity determination or UPLC-MS/MS analysis are described in each section separately.

Antioxidant status of tissues

Tissue supernatant samples that were stored until analysis as described in the above section were slowly thawed at +6°C on cooling pads until liquid again. Afterwards, they were centrifuged at 20,000×g for 15 min at 4°C. The supernatants of the centrifuged tissue homogenates were further used for analysis of ferric reducing antioxidant power (FRAP), $ABTS^{\bullet+}$ scavenging activity, TBARS content, CAT and SOD activity, and GSH content following protocols described below.

Determination of the ferric reducing antioxidant power of tissues

The FRAP assay for tissue homogenates was conducted according to Katalinic *et al*. [2005] method adopted for animal organs and modified from the assay by Benzie & Strain [1996]. The FRAP reagent was prepared from 5 mL of a TPTZ solution (10 mM) in HCl (40 mM) and 5 mL of an $FeCl_3$ solution (20 mM) mixed with 50 mL of an acetate buffer (0.3 M, pH=3.6). Such freshly prepared FRAP reagent (1.5 mL) was mixed with 200 μL of water and 50 μL of the tissue sample or as a blank standard sample with 50 μL water, and incubated for 4 min at room temperature. After 4 min of incubation, the absorbance was measured at λ=595 nm with a Libro S22 spectrophotometer (Biochrom Ltd. Cambridge, UK). The results of the ferric reducing ability of the tissue homogenate were calculated according to the standard curve and expressed as nmol Fe^{2+} per mg of protein in a tissue homogenate.

Determination of the antioxidant capacity of tissues by ABTS assay

The ABTS assay for tissue homogenates was conducted according to Katalinic *et al*. [2005] method adopted for animal organs and modified from assay by Re *et al*. [1999]. The volume of 20 μL of the tissue supernatant was mixed with 2 mL of an $ABTS^{\bullet+}$ solution and after 6 min of incubation, the absorbance was measured at a wavelength of 734 nm with a Libro S22 spectrophotometer (Biochrom). The $ABTS^{\bullet+}$ solution was prepared by oxidizing the 7 mM $ABTS^{\bullet+}$ solution with a freshly prepared 140 mM potassium peroxydisulfate solution mixed in equal proportions. On the day of analysis, the solution was diluted with PBS (pH 7.4) and incubated at 30°C so that the absorbance of this solution was 0.700 (±0.020). As a blank sample-free mix without tissue sample, a 1 mL of $ABTS^{\bullet+}$ solution and 20 μL phosphate buffer was used and the mix of $ABTS^{\bullet+}$ solution with tissue sample was compared to it. The results are expressed as nmol Trolox equivalents per mg of protein in the tissue homogenate.

Markers of tissue oxidative stress defense systems

Protein concentration in tissues

Protein concentration in the samples was determined with the method of Lowry *et al*. [1951], with bovine serum albumin (BSA) used as the standard. Protein concentration in tissue samples was used to express the values of the measured oxidative stress parameters (TBARS, FRAP, $ABTS^{\bullet+}$, SOD, and CAT).

Lipid peroxidation in tissues

Lipid peroxidation was determined by measuring the content of TBARS using a modified method of Ohkawa *et al*. [1979] described in Landeka Jurčević *et al*. [2017]. A centrifuged sample of 200 μL of the homogenized tissue was mixed with 200 μL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid (pH=3.5), and 1.5 mL of 0.81% thiobarbituric acid, and incubated at 95°C for 60 min. After cooling on ice, the absorbance was measured at 532 and 600 nm with a Libro S22 spectrophotometer (Biochrom). The total absorbance was determined using the formula:

$$A_{total} = A_{532\ nm} - A_{600\ nm} \quad (1)$$

TBARS levels were determined using the molar absorption coefficient for malondialdehyde-thiobarbiturate (MDA–TBA) complex of $1.56 \times 10^5\ M^{-1}cm^{-1}$ and expressed as nmol/mg protein of tissues homogenate.

Superoxide dismutase activity in tissues

The SOD assay is a modification of the method by Flohé & Ötting [1984] described in Landeka Jurčević *et al*. [2017]. An undiluted sample of the tissue homogenate (25 μL) was mixed with 1.45 mL of a reaction solution (cytochrome C, 0.05 mM; xanthin, 1 mM mixed in a 10:1 (*v*/v) ratio with DTNB). A volume of 20 μL of xanthine oxidase (0.4 U/mL) was added to start the reaction. The absorbance of the reaction mixture was measured at 550 nm over 3 min with a Libro S22 spectrophotometer (Biochrom). One unit of SOD activity was defined as the amount of enzyme required to achieve 50% inhibition of superoxide anion formation within the sample which was started by the reaction of xanthine oxidase. The results were expressed as units per mg of protein in tissue homogenate (U/mg protein).

Catalase activity in tissues

The CAT activity was assayed by measuring the initial rate of H_2O_2 degradation according the method modified from Aebi [1984] and described in Landeka Jurčević *et al*. [2017]. The reaction mixture was prepared by mixing 33 mM H_2O_2 in 50 mM phosphate buffer, pH=7.0. This reaction mixture (900 μL) was mixed with the supernatant of the tissue homogenate (100 μL). The absorbance was measured at 240 nm for 3 min using the Libro S22 spectrophotometer (Biochrom). The CAT activity was calculated using the molar absorption coefficient of $43.6\ M^{-1}cm^{-1}$ for H_2O_2. The results were expressed as U/mg protein.

Reduced glutathione in tissues

The reduced glutathione (GSH) assay is a modification of the method first described by Tietze [1969] and then in Landeka Jurčević *et al*. [2017]. In a 96-well plate, 40 μL of 10 mM DTNB (Ellman's Reagent) was mixed with 20 μL of the tissue supernatant (obtained as described above) pretreated with 40 μL of 0.035 M HCl, incubated for 10 min. Then, 100 μL of the reaction solution prepared earlier by mixing 9980 μL of 0.8 mM NADPH and 20 μL of glutathione reductase, 0.2 U/mL, was added and the absorbance was read at 412 nm every minute for 5 min in an ELISA plate reader (Biorad Laboratories, Hercules CA, USA). The GSH levels were determined from the calibration curve of GSH standards. The results are expressed as μM/mg proteins.

Determination of the phenolic content in tissues

The UPLC-MS/MS analysis of phenolics in the tissues of mice post-sacrifice

The qualitative and quantitative analysis of individual phenolic compounds in mice tissues was performed with the Ultra High Performance Liquid-Chromatography Tandem Mass Spectrometry (UPLC-MS/MS). The methods are described in detail (with respective standard curves) by Elez-Garofulić *et al.* [2018]. Briefly, the Agilent 1290 UPLC system (Agilent Technologies, Santa Clara, CA, USA) with the Zorbax Eclipse Plus C18 column (100×2.1 mm, 1.8 μm; Agilent, Santa Clara, CA, USA) was used. Column oven temperature was set at 35°C and flow rate was 0.35 mL/min. The mobile phase consisted of 0.1% (*v/v*) formic acid (A) and acetonitrile with 0.1% (*v/v*) formic acid (B). The QQQ 6430 triple quadrupole mass spectrometer and the Agilent MassHunter Workstation Software (Agilent, Santa Clara, CA, USA) were used for mass spectrometry. The positive and negative electrospray ionization (ESI) mode and dynamic multiple reaction monitoring (dMRM) mode were applied. Capillary voltage was +4000/-3500 V, nitrogen drying temperature was 300°C, flow rate was 11 L/h, and the pressure of the nebulizer was 40 psi. The total analytical time was 12.5 min. The analytes were identified by comparing their retention times and mass spectra with the corresponding standards. For unavailable standards, the structural identification of phenolic compounds was done by comparing the mass fragment ions with the previously reported mass fragmentation patterns; and quantification was performed using the calibration curve of standards from the same phenolic group. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively.

For the UPLC-MS/MS analysis, the frozen samples that were stored until analysis as described in the above section were slowly thawed at +6°C on cooling pads until liquid again. Afterwards, they were centrifuged at 20,000×g for 15 min at 4°C. The supernatants (200 μL of the supernatant of the tissue homogenate) was then mixed with 10 μL of a mixture of β-glucuronidase (250 units) and sulfatase (20 units), and then incubated at 37°C for 45 min. The reaction mixture was extracted twice with ethyl acetate, to remove tissue debris of the homogenate. The combined ethyl acetate solutions were added to 10 μL of a 20% ascorbic acid solution and evaporated to dryness in a vacuum centrifuge concentrator. Prior to the chromatographic analysis, the samples were reconstituted in 300 μL of a 10% aqueous acetonitrile solu-

tion (*v/v*) and centrifuged at 6500×g for 5 min. The supernatant was transferred to an injection vial and then an aliquot of 2.5 μL of the supernatant was injected onto the UPLC column [Ganguly *et al*., 2016; Gonzales *et al*., 2015].

The results of the UPLC-MS/MS analysis were presented as AUClast, and C_{max} values. These were obtained from the curves of the phenolic content in the tissues *versus* the time (T, days) of blackthorn flower extract administration to mice as the area under the curve and the maximum concentration, respectively. To compute the mean AUClast, $C_{max,}$ and T_{max} values, a non-compartmental pharmacokinetic analysis was done using Phoenix WinNonlin 8.0. software (Certara, Princeton, NJ, USA).

Statistical analysis

Data of TBARS, FRAP, and ABTS assays; tissue content of antioxidative defense molecules; and the AUClast and C_{max} values were presented as means and standard deviation. All data were compared for statistically significant differences ($p \leq 0.05$) by the t-test between the control group and the blackthorn flower extract-treated group within each individual tissue. The Kruskal-Wallis ANOVA was used for testing the statistically significant differences ($p \leq 0.05$) between days of treatment for antioxidative parameters. All statistical analyses were conducted in the SPSS version 17.0 software (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Lipid peroxidation, antioxidative properties, and the content of antioxidative defense molecules in the mice tissues after blackthorn flower extract administration

Compared to the control animals, the administration of blackthorn flower extract to mice significantly ($p \leq 0.05$) lowered the lipid peroxidation (content of TBARS) in tissues (Figure 1 A-C). The inhibited lipid peroxidation was observed in all three assessed organs (intestine, liver, and kidneys) after three weeks of administration. Since no *in vivo* results can be found in literature for blackthorn, the closest study resembling ours was that evidencing the similar lipid peroxidation reduction recorded with *Aronia melanocarpa* extract [Broncel *et al.,* 2010].

The antioxidative capacity of the tissues (Figure 2 and 3) was measured as FRAP and $ABTS^{\bullet+}$ scavenging activity. Each method specifically showed slightly different mechanisms of tissue antioxidative capacity. In the intestine and liver, the FRAP value of blackthorn flower extract-treated group was slightly but significantly ($p \leq 0.05$) higher compared to the control group on the 28th day, while in the kidneys it did not differ statistically during the experiment. In turn, the antioxidant capacity determined as the $ABTS^{\bullet+}$ scavenging activity of the intestine was the highest ($p \leq 0.05$) already on the 7th and 28th day of the experiment. In the liver, FRAP was significantly higher in the exposed ($p \leq 0.05$) animals on the 28th day only. In the kidneys, the antioxidative capacity measured as both FRAP and $ABTS^{\bullet+}$ scavenging activity was not significantly different between the control and the blackthorn flower extract-treated animals. In general, it seems that at least four weeks of consumption is necessary to observe positive antioxidative effects in the intestine and liver using FRAP and/or $ABTS^{\bullet+}$ method. Similar trends were found for other phenolic- and polyphenol-rich plants. For example, Nakhaee *et al.* [2009] and Salahshoor *et al.* [2019] showed an increased antioxidative potential (determined with the FRAP method) of liver tissue of streptozotocin-induced diabetic rats after dosing *Eucalyptus globulus* and *Fucaria vulgaris*, respectively.

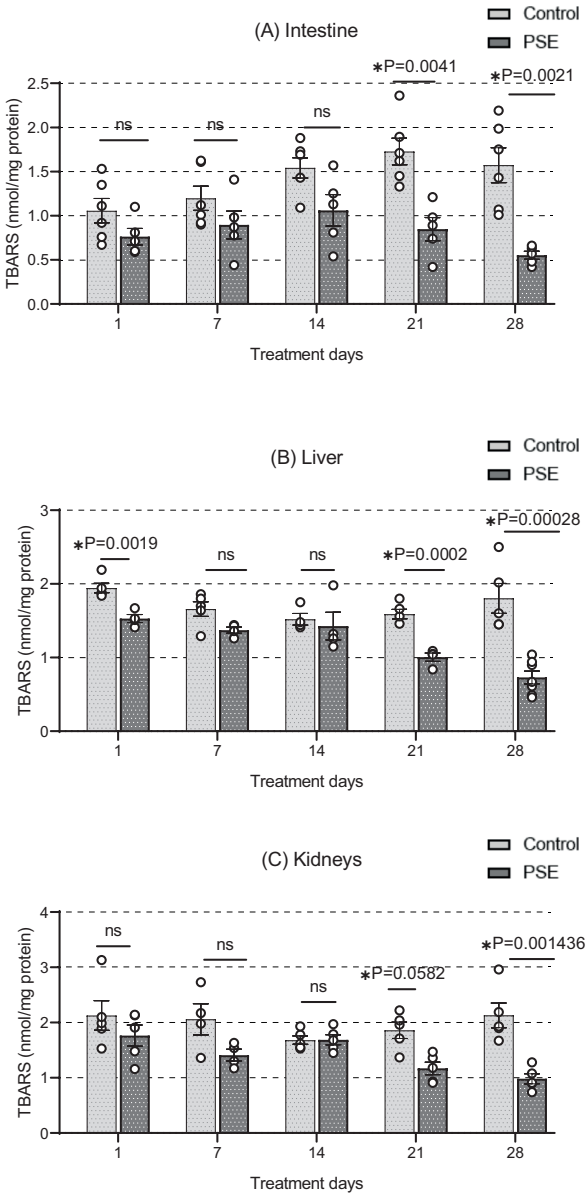

FIGURE 1. The lipid peroxidation expressed as the content of thiobarbituric acid reactive substances (TBARS) in the (A) intestine, (B) liver, and (C) kidneys; PSE – blackthorn flower extract-treated group. The values that are statistically different between control and PSE-treated group are marked with p value of t-test. ns – no statistically significant differences ($p > 0.05$).

In concordance with the pattern of described TBARS, FRAP, and $ABTS^{\bullet+}$ scavenging activity changes, the SOD activity of intestine, liver, and kidneys (Table 1) showed somewhat different increase specific for each organ. The SOD activity of intestine and liver of the treated animals was significantly

TABLE 1. The activity of superoxide dismutase (SOD) and catalase (CAT), and content of reduced glutathione (GSH) in the mice tissues after 1–28 days of daily repeated oral administration of the blackthorn flower extract (PSE) compared to these of untreated control mice.

	Intestine		Liver		Kidneys	
	Control	PSE	Control	PSE	Control	PSE
SOD activity (U/mg proteins)						
1 day	6.03±0.54^{b}	8.17±1.63^{c}	14.26±1.87^{a}	10.43±3.08^{c}	10.83±3.53^{b}	12.38±2.04^{b}
7 day	9.06±1.23^{a}	6.68±0.46^{c}	11.42±4.97^{a}	12.4±0.84^{c}	10.41±1.53^{b}	12.51±1.93^{b}
14 day	7.85±1.33^{b}	7.32±1.76^{c}	10.86±2.11^{a}	13.31±2.04^{c}	16.97±1.54^{a}	21.38±1.17^{a}
21 day	5.55±2.42^{b}	10.39±1.00#^{b}	12.78±2.91^{a}	17.31±1.08#^{b}	11.18±1.94^{b}	14.04±1.92^{b}
28 day	10.17±2.31^{a}	14.53±2.17#^{a}	15.44±1.49^{a}	22.93±3.21#^{a}	12.78±1.81^{b}	21.42±3.93#^{a}
CAT activity (U/mg proteins)						
1 day	5.8±1.96^{b}	5.93±3.63^{b}	115.11±12.17^{a}	100.51±20.19^{a,b}	106.23±28.06^{a}	95.86±8.26^{a}
7 day	7.03±4.03^{a}	6.94±1.07^{d,a}	75.44±11.62^{b}	92.06±5.28#^{b}	117.16±30.49^{a}	91.26±9.46^{a}
14 day	6.12±3.64^{a}	11.1±1.07^{a}	66.95±19.21^{b}	80.61±13.76#^{b}	88.11±29.09^{a}	98.28±39.31^{a}
21 day	7.47±3.81	3.53±1.43^{b}	50.9±15.29^{a}	70.36±21.53#^{b}	78.37±44.21^{a}	86.48±25.37^{a}
28 day	9.49±4.94^{a}	9.78±3.89^{a}	93.91±25.84^{a}	151.89±27.96#^{a}	61.13±19.76^{b}	83.42±24.72^{a}
GSH content (μM/mg proteins)						
1 day	41.44±9.94^{b}	36.26±6.91^{c}	61.77±9.56^{b}	55.59±22.22^{b}	38.61±14.34^{b}	36.87±5.47^{b,c}
7 day	57.86±16.81^{a}	53.76±6.08^{b}	49.77±14.33^{b}	66.95±24.75^{b}	31.24±9.64^{b}	31.89±6.64^{c}
14 day	52.73±12.39^{a}	36.78±4.22^{c}	50.79±12.52^{b}	71.65±22.66^{b}	33.19±7.63^{b}	55.04±12.78#^{b}
21 day	55.43±18.91^{a}	65.99±6.84^{b}	60.01±11.09^{b}	79.13±15.71#^{b}	38.61±16.47^{b}	56.94±14.76^{b}
28 day	76.79±12.69^{a}	114.12±9.94#^{a}	109.81±25.45^{a}	138.15±26.51#^{a}	62.64±9.23^{a}	93.58±28.76#^{a}

The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group on the day of sampling (within rows). The different superscript letters (a-e) show statistically significant difference ($p \leq 0.05$) between the 1st, 7th, 14th, 21st, 28th day of sampling within the control or the PSE group (within columns). The values are expressed as means±standard deviation (SD).

higher compared to that of control groups ($p \leq 0.05$) after three weeks of consumption (from 21st until 28th day). In the kidneys, a significant increase ($p \leq 0.05$) in SOD activity occurred later and was recorded only after four weeks of treatment. Since there is no previous data on *in vivo* antioxidative effects of *P. spinosa* flower extract, the results could be only compared to other plant species rich in polyphenols. For example, Nardi *et al*. [2016] investigated and compared the anti-inflammatory and antioxidative effects of goji berry, blueberry, and cranberry extracts administrated to mice. Mice were treated for 10 days with 50 and 200 mg of extract per kg bw. The antioxidant status of liver was determined by testing GSH concentration and CAT activity. Both were the highest in the group that received goji berries extract and the lowest in the group that obtained the blueberries extract. Similarly, Jin & Yin [2012] investigated the antioxidant effect of polyphenols from leaves of bamboo plants on the aging process of mice. Three groups of mice were treated with the leaf extract at doses of 20, 40, and 80 mg/kg bw. Their antioxidant status was measured by determining SOD, glutathione peroxidase (GSH-Px), and CAT activity, and total antioxidant capacity with the ORAC method. Compared with the control group, the activity of all enzymes in the serum and liver were statistically significantly higher in the groups treated with the leaf extract. In turn, phenolics from grapes and wine modulated SOD, GSH, and CAT levels in mice liver and kidneys [Landeka Jurčević *et al.,* 2017]. Interestingly, the CAT activity (Table 1) increased ($p \leq 0.05$) only in the liver as a result of blackthorn flower extract treatment, compared to the control animals. The onset of activation in the liver was very early in the experiment, *i.e.* from the 7th day onwards. Intestine and kidneys did not show significantly different activity in the treated animals compared to their controls, respectively. The GSH contents (Table 1) in all tissues of blackthorn flower extract administered animals were higher compared to these of controls. They increased ($p \leq 0.05$) by the end of the four-week treatment (on the 28th day). However, only in the liver such a significant increase ($p \leq 0.05$) started earlier – on the 21th day of the experiment (Table 1). In general, liver was the most prominent organ where induced activity of antioxidative defense molecules was recorded, while kidneys were the least responsive organ for the induction of tissue antioxidative markers.

Does the blackthorn flower extract has antioxidant properties in mammalian organism?

This important fundamental question addressed in this work was based on the previous publications that demonstrat-

ed antioxidant and other bioactive properties of the blackthorn flower extract under *in vitro* conditions [Elez-Garofulić *et al.,* 2018; Lovrić *et al.,* 2017; Marchelak *et al*., 2017; Meschini *et al*., 2017; Mikulic-Petkovsek *et al*., 2016; Olszewska & Wolbis, 2001; Pinacho *et al*., 2015]. The results presented in Figure 1 A-C and Table 1 indirectly show the reduction of oxidative stress that occurred *in vivo* in all major entry and excretory organs after ingestion and application of the blackthorn flower extract in mice, but mainly after three to four weeks of daily extract consumption.

The results also prove that the blackthorn flower extract has a similarly significant oxidation-inhibiting potential (Figure 2 and 3) as other plants that are rich sources of phenolics and were previously verified as good antioxidants. Similar properties as in our work are shown for other numerous plants, for example *Aronia melanocarpa*, for whom it was demonstrated that the 8-week consumption of its extract by animals lowered the serum MDA concentration in the experimental model of the metabolic syndrome [Broncel *et al.,* 2010]. Those results obtained in mice were later confirmed in humans. For example, the consumption of *Aronia melanocarpa* juice prior to an ergonomic test in humans [Pilaczynska-Szczesniak *et al.,* 2005], caused a significant reduction in DNA damage and reactive oxidative species (ROS) concentration and significant improvement of oxidative stress markers, antioxidant enzyme activity, and mitochondrial performance. Therefore, we expect the *P. spinosa* flower extract to be equally efficient in humans as tested in this model. Similar effects, noticed first in animals and then in humans, were recorded for many nutritional products and fruits such as tea, black wine, apple juice and goji berries, *etc.*, plants which are known to contain large amounts of phenolics [Ganguly *et al.,* 2016; Jin & Yin, 2012; Landeka Jurčević *et al.,* 2017; Nardi *et al.,* 2016; Olszewska & Wolbis, 2001; Teng & Chen *et al.,* 2019].

Definitely, based on the presented results, we can confirm that this major finding of antioxidative properties *in vivo*, supports the *in vitro* properties of antioxidant effects of blackthorn extract earlier reported by Marchelak *et al.* [2017] and other authors [Elez-Garofulić *et al.,* 2018; Lovrić *et al.,* 2017; Meschini *et al*., 2017; Mikulic-Petkovsek *et al*., 2016; Olszewska & Wolbis, 2001; Pinacho *et al*., 2015]. However, the prolonged intake of at least three weeks is necessary to achieve first observable antioxidative bioactivity in healthy (not pathologically challenged) mice at the dose of 25 mg of TP/kg bw. This result can serve as a guideline for future mechanistic studies in mice and based on estimations and literature it remains to be seen if a dose calculated to human equivalent dose (HED) would achieve a similar effect in humans.

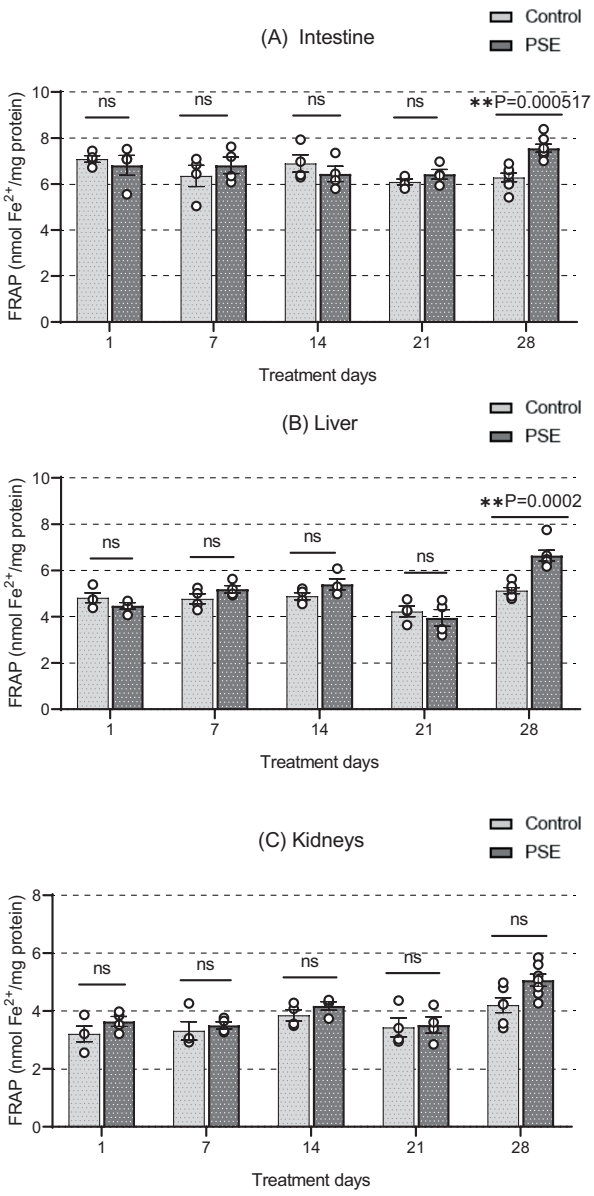

FIGURE 2. The antioxidant capacity of the tissue homogenates as ferric reducing antioxidant power (FRAP) of the (A) intestine, (B) liver, and (C) kidneys of mice treated with blackthorn flower extract and untreated control animals (Control). PSE – blackthorn flower extract-treated group. The values that are statistically different between control and PSE-treated group are marked with p value of t-test. ns – no statistically significant differences (p>0.05).

What are the mechanistic and molecular explanations of the observed antioxidative properties of a blackthorn flower extract in mice tissues which might bring innovation to this and similar future studies?

We believe that the blackthorn flower extract in mice caused the boosting of beneficial physiological or antioxidant effects in the target tissues by molecular mechanism described previously [Bao *et al.,* 2018; Dominko & Đikić, 2018; Dominko *et al.,* 2020; Squillaro *et al.,* 2018]. Based on the literature, we provide two major mechanistic explanations and hypotheses that can explain the observed inhibition of lipid peroxidation and increased antioxidative activity in mice tissues after three to four weeks of consumption.

The first presumption and explanation is that the lowered lipid peroxidation (TBARS) is a consequence of the direct ROS scavenging properties of the bioabsorbed and bioavailable phenolics that were accumulated in the tissues (as listed for each tissue in Tables 2–4 and discussed in the next section). The second mechanistic explanation of the antioxidative effects could be that those phenolics all together synergistically indirectly induced the transcriptional mechanism (and other cellular mechanisms) for the production of in-

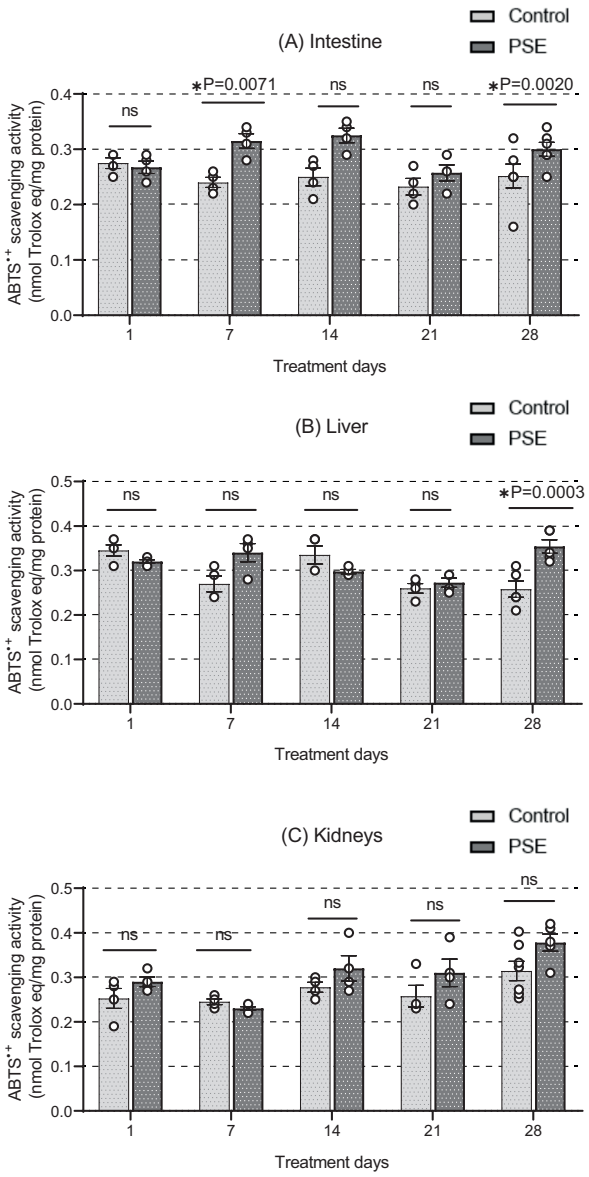

FIGURE 3. The ABTS$^{\bullet+}$ scavenging activity of the tissue homogenates (A) intestine, (B) liver and, (C) kidneys of mice treated with blackthorn flower extract and untreated control animals (Control). PSE – blackthorn flower extract-treated group. The values that are statistically different between control and PSE-treated group are marked with p value of t-test. ns – no statistically significant differences ($p>0.05$).

ternal cellular antioxidative defense enzymes and molecules (CAT, GSH, SOD, and others). There is also a possibility that both proposed mechanisms took place in parallel, probably by the early onset (third week) of scavenging properties and later (fourth week) jointly supported by induction. In support of the first mechanical explanation of the direct scavenging activities by organ-bioavailable phenolics, we hypothesized that the phenolic compounds bioabsorbed in tissues would increase the overall antioxidant capacity of tissue homogenates. However, both FRAP and ABTS$^{\bullet+}$ scavenging activity method did not yield similar positive results for all tissues – an increase in the antioxidative capacity was recorded only in liver and/or intestine after the fourth week of extract consumption. These results could support the proposed hypothesis only to the limited extent. Perhaps, the use of other antioxidative tests would give positive results in the kidneys, as in the case of Jin & Yin [2012] who investigated the antioxidant effect of phenolics from leaves of bamboo plants in mice tissues based on the oxygen radical absorbance capacity (ORAC). Thus, one important guideline for future studies on the similar model is to incorporate diverse antioxidative measurements in tissues.

The second explanation of the changes taking place in the tissues of mice after blackthorn flower extract administration may be the indirect effect of phenolics on cellular and molecular mechanisms and activation of SOD, GSH, or CAT cellular pathways of the antioxidative defense. Those antioxidative effects achieved by molecular mechanisms were described in the literature [Dominko & Đikić, 2018; Dominko *et al.,* 2020; Squillaro *et al.,* 2018]. Phenolics boost the activation of transcription factors Erk-Nrf2-HO1, GCLM, and TrxR1 signal pathway and enhance the antioxidant enzymes such as heme oxygenase-1, phase II detoxification enzymes, and enzymes involved in GSH metabolism [Bao *et al.,* 2018; Dominko & Đikić, 2018; Dominko *et al.,* 2020; Nardi *et al.,* 2016; Squillaro *et al.,* 2018]. Expressions of phase II detoxification and antioxidant enzyme genes are controlled by the antioxidant response element (ARE), which contains genes that are regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2). Thus, after activation, Nrf-2 dissociates from Keap1 and is transferred into the nucleus to activate the translation of antioxidant genes and phase II detoxification genes, such as HO-1, NAD(P)H quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase modifier (GCLM) [Dominko & Đikić, 2018; Dominko *et al.,* 2020]. Reduced glutathione (GSH) can efficiently eliminate electrophiles and ROS that are generated during the chemical metabolism within cells. It is known that the enhancement of endogenous antioxidant defense by flavonoids is associated with the direct elimination of reactive oxygen species, inhibition of lipid peroxidation, reduction of oxidized glutathione level, increase of reduced glutathione level, and restoration of activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione *S*-transferase, and glucose 6-phosphate dehydrogenase) [Dominko & Đikić, 2018; Dominko *et al.,* 2020]. Many studies have reported that herbal extracts modulate the expression of glutamate-cysteine ligase (GCL) which consists of catalytic (GCLC) and modifier (GCLM) subunits, which is a rate-limiting enzyme of GSH synthesis. The secondary mechanism could refer to the neutralization of oxidative species, and inhibition of the activation of the nuclear transcription factor-κB (NF-κB) signaling pathways [Bao *et al.,* 2018; Nardi *et al.,* 2016; Squillaro *et al.,* 2018].

In this study, the activation of SOD, CAT, and GSH in the group receiving the blackthorn flower extract was tissue-specific. In all organs, there was an increased SOD activity but it occurred on different days of the experiment. Such an increase in kidneys was observed on the 28th day but not earlier, whereas in the liver a slight (although still statistically not significant) increase was recorded already on the 14th day of the experiment that became significant between 21st-28th day. Therefore, the accumulation of superoxide anion was a consequence of mitochondrial activity

TABLE 2. Phenolic compound profile in the mice intestine after 28 days of daily oral administration of the blackthorn flower extract compared to untreated control group expressed as area under the curve of phenolic content in the tissue *vs*. treatment period (AUClast), maximal concentration (C_{max}), and day of the experiment when it was achieved (T_{max}).

Phenolic compound		AUClast (h·μg/g)#	C_{max} (μg/g) #	T_{max} (day)
		Phenolic acids		
Caffeic acid	Control	41.6±17.4	0.20±0.05	7
	PSE	111.8±13.9	0.37±0.02	7
p-Coumaric acid	Control	186.7±37.4	0.47±0.14	21
	PSE	444.9±6.3	1.66±0.94	1
3-*O*-*p*-Coumaroylquinic acid	Control	/	/	
	PSE	71.8±14.4	0.19±0.02	7
4-*O*-*p*-Coumaroylquinic acid	Control	627.7±4.2	1.22±0.05	1
	PSE	1095.0±27.8	2.16±1.27	21
Ferulic acid	Control	368.3±61.5	1.03±0.36	21
	PSE	740.9±9.7	1.68±0.95 ns	1
3-*O*-Feruloylquinic acid	Control	211.9±37.3	0.62±0.13	7
	PSE	768.4±6.3	4.60±0.42	7
Gallic acid	Control	215.1±96.7	1.27±0.67	1
	PSE	575.2±12.6	2.20±1.27 ns	7
		Flavones		
Luteolin	Control	89.5±19.2	0.19±0.10	7
	PSE	171.8±30.6	0.64±0.28 ns	1
Apigenin	Control	188.3±17.8	0.88±0.14	1
	PSE	523.9±72.5	2.5±0.25	1
		Flavan-3-ols		
(–)-Epigallocatechin 3-gallate	Control	6.3±0.8	0.02±0.01	21
	PSE	14.5±3.9	0.17±0.01	28
		Flavonols		
Kaempferol 3-*O*-glucoside	Control	93.5±2.1	0.16±0.00	7
	PSE	146.1±3.8	0.29±0.17 ns	7
Kaempferol pentoside	Control	1344.3±6.1	2.74±0.01	7
	PSE	1559.4±74.7	5.29±2.90 ns	1
Kaempferol rhamnoside	Control	/	/	
	PSE	9.9±0.01	0.05±0.02	28
Kaempferol pentosyl-hexoside	Control	260.6±13.9	0.51±0.01	7
	PSE	329.4±10.2	0.85±0.45	1
Quercetin 3-*O*-glucoside	Control	63.9±10.2	0.16±0.01	7
	PSE	151.0±12.5	0.55±0.05	21
Quercetin 3-*O*-rutinoside	Control	270.3±4.29	0.77±0.01	7
	PSE	952.0±12.1	1.73±0.21	1
Quercetin acetyl-hexoside	Control	219.2±23.8	0.51±0.01	28
	PSE	429.9±0.8	0.67±39.00 ns	28
Quercetin rhamnoside	Control	387.7±11.8	0.95±0.10	14
	PSE	723.7±17.3	1.67±0.94 ns	1

#The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group for each individual phenolic compound were determined excluding values marked with ns – no statistically significant differences ($p > 0.05$). PSE – blackthorn flower extract-treated group; / – not detected. AUClast – area under the curve, C_{max} – maximal detected concentration, T_{max} – day of experiment when maximal concentration was detected.

TABLE 3. Phenolic compound profile in the mice liver after 28 days of daily oral administration of the blackthorn flower extract compared to untreated control group expressed as area under the curve of phenolic content in the tissue *vs*. treatment period (AUClast), maximal concentration (C_{max}), and day of the experiment when it was achieved (T_{max}).

Phenolic compound		AUClast (h·μg/g)#	C_{max} (μg/g) #	T_{max} (day)
Phenolic acids				
Caffeic acid	Control	54.5±0.2	0.23±0.01	21
	PSE	479.4±39.1	1.78±0.15	21
p-Coumaric acid	Control	/	/	
	PSE	38.4±0.1	0.16±0.03	7
3-*O*-*p*-Coumaroylquinic acid	Control	8.3±0.7	0.03±0.02	1
	PSE	68.5±0.1	0.25±0.01	7
4-*O*-*p*-Coumaroylquinic acid	Control	317.5±2.8	0.53±0.01	1
	PSE	798.1±0.1	1.42±0.00	21
Ferulic acid	Control	494.6±0.4	0.77±0.02	14
	PSE	835.8±3.6	1.64±0.02	28
Gallic acid	Control	76.9±88.1	0.58±0.04	28
	PSE	110.1±16.2	1.31±0.19	28
Flavones				
Luteolin	Control	51.3±3.3	0.18±0.01	7
	PSE	78.7±1.0	0.33±0.02	7
Flavonols				
(–)-Epicatechin 3-gallate	Control	169.2±2.4	0.70±0.05	14
	PSE	265.9±0.1	1.40±0.01	14
(–)-Epigallocatechin 3-gallate	Control	256.6±0.1	0.87±0.00	14
	PSE	400.0±61.5	1.06±0.25 ns	7
Flavonols				
Kaempferol 3-*O*-glucoside	Control	71.6±0.4	0.15±0.01	1
	PSE	108.3±0.3	0.25±0.01	7
Kaempferol rhamnoside	Control	8.0±1.1	0.02±0.04	7
	PSE	26.2±0.5	0.12±0.02	7
Kaempferol acetyl-hexoside	Control	4.1±0.8	0.02±0.05	7
	PSE	13.4±0.1	0.07±0.01 ns	7
Quercetin 3-*O*-glucoside	Control	70.6±0.8	0.14±0.09	7
	PSE	168.6±0.1	0.39±0.03	1
Quercetin 3-*O*-rutinoside	Control	715.1±78.9	1.55±0.03	14
	PSE	1113.1±0.6	2.57±0.03	14
Quercetin pentoside	Control	84.2±1.1	0.13±0.01	7
	PSE	670.0±1.6	1.44±0.02	7

The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group for each individual phenolic compound were determined excluding values marked with ns – no statistically significant differences ($p > 0.05$). PSE- blackthorn flower extract-treated group; / – not detected. AUClast – area under the curve, C_{max} – maximal detected concentration, T_{max} – day of experiment when maximal concentration was detected.

and boosted metabolism. For example, the liver was the only organ that from the earlier onset of experiment (7th day) had increased CAT activity and the only organ whose catalase antioxidative pathway was induced. Other organs did not show CAT activation due to the blackthorn flower extract treatment. The liver was the only organ with increases in the values of all three parameters and the only organ that had increased CAT activity within the time of extract administration (but only on the 28th day). Liver, opposite to other organs, has normally higher physiological expression of catalase, and is the organ with a high metabolic rate (biotransformation activity). Therefore, we are firmly convinced that,

TABLE 4. Phenolic compound profile in the mice kidneys after 28 days of daily oral administration of the blackthorn flower extract compared to untreated control group expressed as area under the curve of phenolic content in the tissue *vs.* treatment period (AUClast), maximal concentration (C_{max}), and day of the experiment when it was achieved (T_{max}).

Phenolic compound		AUClast (h·μg/g)#	C_{max} (μg/g) #	T_{max} (day)
Phenolic acids				
4-*O*-*p*-Coumaroylquinic acid	Control	412.3±11.1	0.64±0.01	28
	PSE	1143.1±2.6	2.06±0.04	28
Ferulic acid	Control	261.6±2.8	0.84±0.56	21
	PSE	1354.5±0.3	5.45±0.00	21
Flavones				
Luteolin	Control	11.3±3.5	0.13±0.15	28
	PSE	131.2±13.7	0.52±0.19	1
Apigenin	Control	128.2±1.0	0.45±0.01	1
	PSE	349.2±2.6	0.96±0.02	21
Flavan-3-ols				
(+)-Catechin	Control	92.9±0.1	0.21±0.00	1
	PSE	776.2±1.4	1.41±0.01	7
(–)-Epicatechin	Control	0.3±0.0	0.02±0.03	1
	PSE	848.4±0.4	1.55±0.00	7
Flavonols				
Quercetin 3-*O*-glucoside	Control	85.1±1.0	0.15±0.00	7
	PSE	121.0±0.6	0.19±0.00 ns	1
Quercetin 3-*O*-rutinoside	Control	240.1±1.0	0.64±0.35	7
	PSE	1764.0±6.7	2.96±0.02	14
Quercetin pentosyl-hexoside	Control	86.8±1.2	0.13±0.00	1
	PSE	193.2±1.10	0.40±0.00	1

The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group for each individual phenolic compound were determined excluding values marked with ns – no statistically significant differences ($p > 0.05$). PSE – blackthorn flower extract-treated group; / – not detected AUClast – area under the curve, C_{max} – maximal detected concentration, T_{max} – day of experiment when maximal concentration was detected.

physiologically, it is possible that after 28 days of continuous intake of xenobiotic molecules in a higher concentration, peroxides were accumulating in hepatocytes and the expression and activity of catalase was increased. The GSH content also increased in all organs predominantly on the 28th day. This significant change, although minor compared to the control animals, indicates that phenolics present in organs probably enhance the metabolic activity. Presumably, the metabolic processes of conjugation activated the elimination of xenobiotic molecules (phenolics) as normal physiological characteristics, since kidneys are the major organs where conjugated glutathione-xenobiotic complexes (in this case polyphenol residue conjugates) enter the excretory pathway, cleaved as mercapturic acids [Dominko & Đikić, 2018]. The present study results are consistent with literature data, for example with Nardi *et al*. [2016] who investigated and compared the anti-inflammatory and antioxidative effects of goji berry, blueberry, and cranberry extracts in mice. Mice were treated with the extract at 50 and 200 mg/kg bw for 10 days. The antioxidant activity was determined by testing the GSH content and CAT activity, which were the highest in the group that received goji berries extract and the lowest in the group that obtained the blueberries extract. Similarly, Jin & Yin [2012] investigated the antioxidant effect of phenolics from leaves of bamboo plants on the aging process of mice. Three groups of mice were treated with the leaf extract at doses of 20, 40, and 80 mg/kg bw. The authors reported that the CAT and SOD activity and the GSH content in the serum and liver decreased while the MDA levels increased significantly in the groups treated with the leaf extract, compared with the control group.

Content of phenolic compounds in individual tissue following four-week administration of blackthorn flower extract in mice

The selective and specific tissue content was observed for specific subgroups of phenolic compounds after the intake of the blackthorn flower extract (Tables 2–4). In the intestine

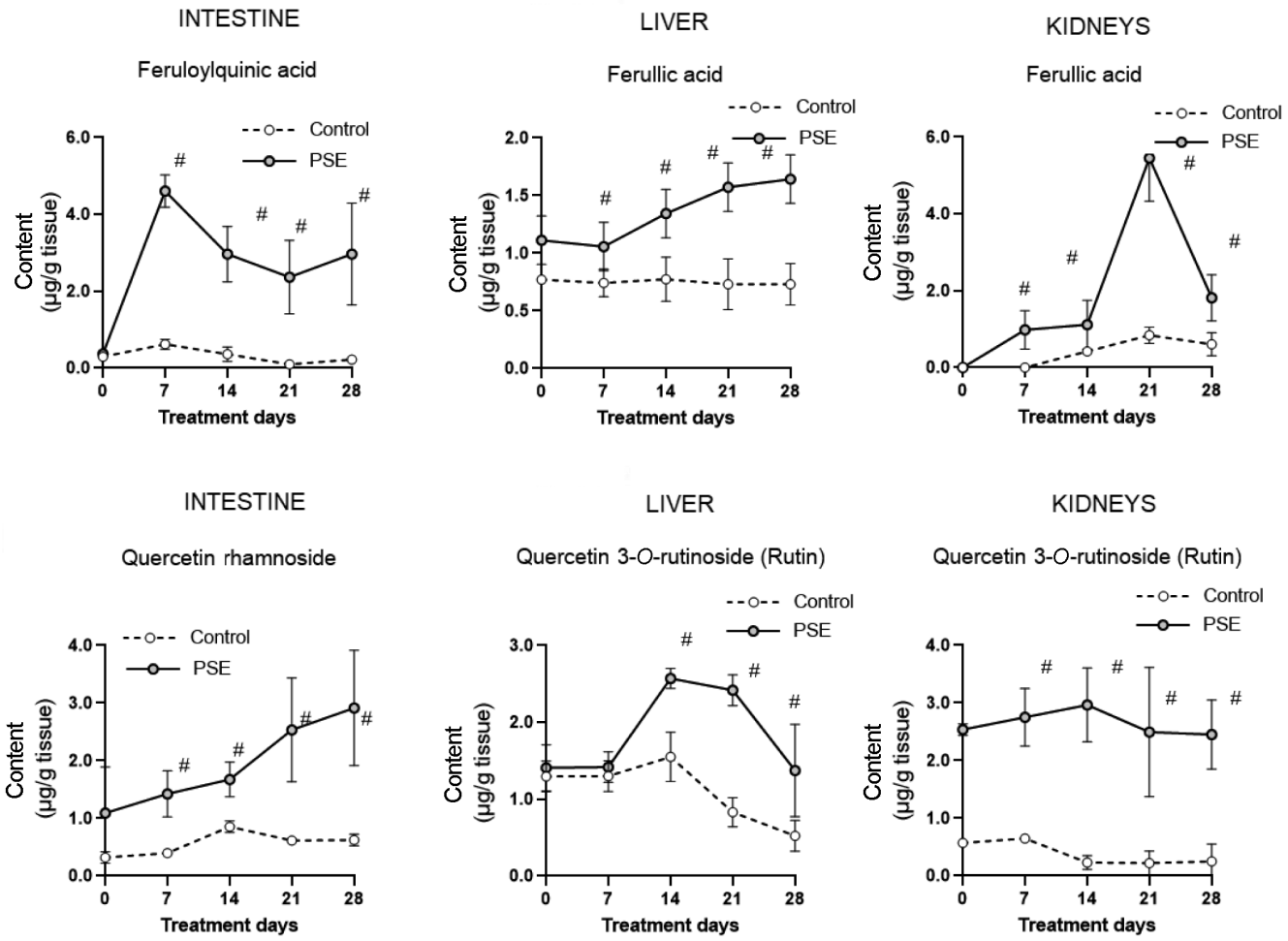

FIGURE 4. Individual phenolic content in the intestine, liver, and kidneys of mice treated with blackthorn flower extract and untreated control animals (control). PSE – blackthorn flower extract-treated group. #The values are statistically different ($p \leq 0.05$) between untreated control and PSE-treated groups.

and the liver, more phenolics were detected than in the kidneys and their composition was generally similar.

Among the seven phenolic acids detected in the intestine, 3-*O*-feruloylquinic acid was the only one absent in the liver. Opposite to those tissues, only two phenolic acids, namely 4-*O*-*p*-coumaroylquinic acid and ferulic acid, were determined in the kidneys. Ferulic acid was, therefore, the only one present in all three organs and its AUClast after 28 days was the highest in the kidneys of the treated animals.

Among the flavan-3-ols, (+)-catechin and (–)-epicatechin were found only in the kidneys. (–)-Epicatechin 3-gallate was present only in the liver and (–)-epigallocatechin 3-gallate was detected in the intestine and liver. Among the flavonols, the intestine contained four kaempferol glycosides, while three and four quercetin glycosides were present in the liver and intestine, respectively. The least varied phenolic composition was observed in the kidneys, with no detected kaempferol glycosides and only three quercetin glycosides. Interestingly, quercetin 3-*O*-glucoside and quercetin 3-*O*-rutinoside were present in all three organs.

The specific distribution of phenolic compounds in each tissue was observed (Tables 2–4) Namely, in the intestine, 3-*O*-feruloylquinic acid was present with most prominently different C_{max} that was approximately 4 times higher in the tissue of the treated animals than in the control ones. Intestine contained also other compounds in high quantities (either AUClast or C_{max}), such as 4-*O*-*p*-coumaroylqiunic acid, kaempferol pentoside, quercetin rhamnoside, and quercetin 3-*O*-rutinoside, but their difference from the control was less prominent than in 3-*O*-feruloylquinic acid (Table 2, Figure 4). In the liver, the highest AUClast values were determined for quercetin 3-*O*-rutinoside (1113.1 h·µg/g) followed by ferulic acid (835.8 h·µg/g) (Table 3, Figure 4). The content of phenolics in kidneys was generally lower. The highest C_{max} was determined for ferulic acid and quercetin 3-*O*-rutinoside followed by 4-*O*-*p*-coumaroylquinic acid (Table 4, Figure 4).

Does the distribution of phenolics occur in tissues after 28-day consumption of blackthorn flower extract?

We further hypothesized that the differences in the antioxidative defense activation and specific organ patterns were a consequence of differences in the bioaccumulation of various phenolic compounds from the extract. Therefore, we decided to screen major phenolics in the analyzed organs (Tables 2–4, Figure 4). Probably, they were jointly and synergistically responsible for the observed antioxidative effects

and no individual compound among detected ones could be, with certainty, proclaimed to individually cause the bioactive effects measured in each analyzed organ.

The selective tissue content and distribution was observed for specific subgroups of phenolic compounds of the blackthorn flower extract. Since mouse has a faster metabolism, in humans the time at which similar concentrations can be reached could probably be longer. However, current scientific methodology does not have the means to estimate concentrations of absorbed phenolics in human tissues in a similar manner of time dynamics as shown here in the animal model.

The lowest number of phenolics with a low concentration was observed in the kidneys. It was probably not only a consequence of lesser vascularization (than in the intestine and liver) or low absorption, but also a consequence of the enhanced polyphenol excretion and their removal with urine. Thus, we believe that the measured antioxidative effects and activation of SOD, CAT, or GSH, probably followed the specific phenolic bioacummulation pattern in each organ. *In vivo* research has shown that phenolic compounds are absorbed in larger amounts than previously thought and especially after long-term consumption they are able to increase plasma antioxidant capacity [Teng & Chen, 2019]. Our study supports this observation. This effect was also noticed for many nutritional products such as tea, black wine and apple juice, which are known to contain large amounts of phenolics [Teng & Chen, 2019]. However, the bioabsorption and organ distribution of only a small number of phenolics is well known, as described in, *e.g*., historic works on quercetin [Olthof *et al.,* 2000; Wang *et al.,* 2016]. In our previous publication, Đikić *et al.* [2018], in the same model but with the single acute dose (analyzed in different times within 24 h), we have concluded that the serum does not give a true picture of phenolics content and distribution in body. Similar conclusions can be found in literature [Gonzales *et al.,* 2015]. The number of compounds and their bioavailable concentrations were very low in the acute (2 h) experiment compared to this experiment here with 28 days of exposure, and the recorded antioxidant bioactivity in tissue confirming that the molecular bioabsorption actually happened. The exact analysis of tissues showed that the concentrations up to approximately 5 μg/g in the intestine were approximately 2-fold higher *vs*. concentrations in the liver, but liver and intestine shared a similar pattern of compound types distribution. This balance is probably the reflection of normal physiological enterohepatic recirculation. The phenolic contents in the kidneys were detected by UPLC-MS/MS in the experiment of Ganguly *et al.* [2016] following oral administration of black tea as the aqueous infusion and alcoholic extract to guinea pigs for 14 days. The authors reported the contents of (–)-epigallocatechin (49.1 and 34.2 ng/g tissue, respectively), (–)-epicatechin (47.8 and 22.3 ng/g tissue), (–)-epigallocatechin 3-gallate (91.8 and 45.0 ng/g tissue), and (–)-epicatechin 3-gallate (22.5 and 12.8 ng/g tissue, respectively). If the content of flavan 3-ols in mice kidneys determined in our study was expressed in ng/g tissue, the range would be between 210–1553 ng/g tissue, depending on the compound. The higher values are probably due to the difference in experimental animals used (guinea pig *vs*. mice in this study) but also a longer (2 times) dosing regime in this study. The model used in this study shows that the consumption could be within line with safety standards for the concentration of bioabsobred phenolics from food supplements and medicinal plants [Harwood *et al*., 2007; Peng *et al*., 2016] and it the first published work on the antioxidative activity of blackthorn *in vivo*. Aside from fundamental knowledge of particular phenolics bioaccumulation and bioavailability and correlation to antioxidative effects, there is a practical value of the study in the way that the aqueous infusion in the form of an alcoholic extract can be used for production and consumption.

CONCLUSION

The contents of phenolics increased significantly in mice tissues after at least three weeks of blackthorn flower extract consumption. The extract had significant bioactive properties *in vivo* and induced antioxidative defense pathways in the organ-specific manner. Thus, the study demonstrates that the administration of *Prunus spinosa* flower extract to mice partially inhibited the oxidative stress in tissues. Among individual phenolic compounds of the extract, the study screened these bioavailable and absorbed. In the intestine, it was mainly 3-*O*-feruloylquinic acid, 4-*O*-*p*-coumaroylqiunic acid, kaempherol pentoside, and quercetin rhamnoside, in the liver – ferulic acid and quercetin 3-*O*-rutinoside, and in the kidneys – quercetin 3-*O*-rutinoside, ferulic acid, and 4-*O*-*p*-coumaroylquinic acid. Possibly, these phenolics in mixtures but together with other that were in lower contents, were responsible for the antioxidant effects of the blackthorn flower extract. It must be emphasized that, to the best of our knowledge, this is the first study on the antioxidative activity of blackthorn *in vivo* and there are no studies on human consumption conducted with the blackthorn flower extract.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

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