PERIODICUM BIOLOGORUM
VOL. 124, No 1–2, 41–54, 2022
DOI: 10.18054/pb.v124i1-2.20513

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original Research Article

Polyphenol bioavailability and modulatory potential on brain antioxidative markers in C57BL/6 mouse

DOMAGOJ ĐIKIĆ[1]*
VEDRAN BALTA[1]
SANDRA PEDISIĆ[2]
ZORAN ZORIĆ[2]
JASNA PADOVAN[3]
DRAŽAN BUTORAC[4]
ASTRID MILIĆ[3]
DRAGAN JURI[1]
IRENA LANDEKA JURČEVIĆ[2]

[1] Faculty of Science, University of Zagreb, Zagreb, Croatia

[2] Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia

[3] Fidelta Ltd. Research and Development, Zagreb, Croatia

[4] Department of Gynecology and Obstetrics, Sestre milosrdnice University Hospital Centre, Zagreb, Croatia

***Correspondence:**
Domagoj Đikić
e-mail: magistar_djikic1@yahoo.com

Keywords: *Prunus spinosa* extract; polyphenols; bioapsorption; neuroprotection

Abstract

Background and purpose: Prunus spinose *L. is a traditionally consumed, recently scientifically reexamined plant. Brain bioavailability and functionality of polyphenols (PPH) of blackthorn flower extract (PSE) was investigated.*

Materials and methods: *C57BL/6 mice received oral daily repeated doses of 25* mg/kg *body weight of total PSE polyphenols for 28 days. Brain concentrations of individual polyphenols from PSE were determined by UPLC/MS on 1st,7th, 14th, 21st, and 28th day. Brain antioxidative defense markers were examined as indicators of functionality after bioaccumulation.*

Results: *A total of 68.7% PPH present in PSE were detected in the brain. Higher (*p≤*0.05) Cmax/AUClast in the PSE treatment vs. control group was recorded for 59.1% of brain detected compounds, indicating relatively good bioaccumulation in the brain. The highest present compounds in PSE were not necessarily the ones mostly bioapsorbed in the brain. Kaempherols were not significantly distributed, opposite to phenolic acids, quercetins or epicgaloatechin-3-gallate. The compounds with the highest concentrations on 28th day were 4-p-coumaroylquinonic acid, (-)-epicatechin, quercetin-3-O-rutinoside, quercetin–rhamnoside, kaempherol-3-rutinoside and quercetin-3-gucoside. Brain lipid peroxidation (MDA) decreased (p<0.05) on the 21st and 28th day in the PSE group. Increase (p<0.05) in GSH concentration was observed on the 21st and 28th and SOD activity on the 28th day. Catalase activity was unchanged. It could be hypothesized that highest PPH concentration-ratios, caused reduction of lipid peroxidation by radical scavenging and simultaneous induction of glutathione and SOD pathways.*

Conclusions: *Screened compounds could be candidates for examining or creation of brain targeted "neuro-nutriceuticals" polyphenol mixtures.*

Received January 4, 2022
Revised February 16, 2022
Accepted February 17, 2022

INTRODUCTION

Polyphenols are a large group of secondary herbal biomolecules that have potential physiological properties *(1)*. They have been investigated for a range of (mostly) beneficial biological effects in the prevention of various diseases *(2-9)*. Some of described molecular mechanisms of action in the human and animal cells include the interaction with transcriptional factors, enzymes, receptors, hormones, neurotransmitters or cellular redox balance by diminishing free radicals or modulating redox status of transitional metals (iron or copper) in oxidation processes *(6,7,10,11)*. In the brain they are studied as compounds that might reduce oxidative stress, decrease neuroinflammation, mediate neuroplas-

ticity of neurodegeneration and aging, influence cognition and protect against the development of age-related cognitive decline. Mechanisms by which they alter brain function outside the CNS include physiological increase of blood flow (the best known compounds are ginkgo-based remedies, with ginkgolide polyphenols) or include modulating signaling pathways from peripheral organs to the brain by altering multi-drug resistant protein-dependent influx and efflux mechanisms at blood brain barrier (BBB) of various biomolecules. Some polyphenols can act by directly influencing the work of neurons and glial cells *(10,12-14)*.

However, polyphenols are usually described by low brain bioavailability after exposing the organism to relatively high doses *(15)*. The research of physiological effects of plant polyphenols needs to be supplemented with data on brain bioavailability, not only in experiments with application of particular individual isolated polyphenol compounds, but also in experiments applying natural plant polyphenol extracts which are mixtures of many phenolic compounds. Knowledge on pharmacokinetics and bioavailability may determine the real impact of the daily intake of these components on the protection and improvement of the health status of the organism *(10,16-18)*. In this model study of brain polyphenol bioavailability, we used the blackthorn flowers polyphenol extract as a model polyphenol mixture to determine the *in vivo* model of absorption, distribution of particular groups of polyphenolic molecules that can enter the brain of C57BL/6 mice. Blackthorne (*Prunus spinosa* L) is a rich source of polyphenols such as kaempferols, quercetin, rutine and others *(19,20)*. This plant is traditionally consumed and reexamined in recent years as a medicinal plant for functional food *(19-26)*. Flowers contain relatively high polyphenol content, and some authors mention effective antioxidant activity of its extract *(1)*. We wanted to determine the biologically available concentrations reached by a particular groups of polyphenols from *Prunus spinosa* flower extract (PSE) and evaluate the polyphenol brain bioavailability and the changes of brain oxidative stress biomarkers (MDA, CAT, GSH, SOD). Screening out the particular polyphenol molecules that most successfully enter the brain or alter the antioxidative status might serve as a guidance toward design of pharmacognosy or functional food nutraceutical polypehenol mixtures for targeted brain delivery.

MATERIALS AND METHODS

Study design in vivo: animals and diets

For this experiment, 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 hours 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, soybean hulled, corn gluten feed, fishmeal, dicalciumphosphate, sodium chloride, whey powder, soybean oil, yeast and analytical components and supplements 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), E6 (Zn).

Maintenance and care of all experimental animals was performed according to guidelines in the Republic of Croatia *(27)*. The experimental procedures were approved by the Bioethics Committee of the Faculty of Science, University of Zagreb *(28)* and were conducted according to Guidelines on *in vivo* experiments and accepted and international standards *(29)*. Animals were randomly divided according to treatment (C – Control group and PSE group – "*Prunus spinosa* Extract" group), as well as based on the time of sacrifice post treatment. Animals from either C or PSE groups following subchronic treatment were sacrificed on the 1st, 7th, 14th, 21st and 28th day. Each group contained 5 animals in both the control treatment and in the PSE treatment groups. The treatments (saline for control and PSE in the treatment group), were administered as a daily single oral gavage dose. Treatment of all animals took place between 8 and 10 o'clock in the morning to equalize circadian differences between treatments. Control (saline) and PSE groups were dosed in a volume of 0.3 mL PBS, administered orally by gavage. The PSE treated groups were dosed at 25 mg of total phenols in a solution/kg of mice body weight (bw). This dose was derived from pilot experiments (lower doses were scarcely detectable by used method and higher doses were causing disturbed behaviors in animals). Additionally, in our previous experiments, we have employed similar dose regimes *(5-7)*. Individual doses of particular polyphenol molecules in administered PSE solution used in this study are given in Table 1.

PSE extract and determination of total phenolics content in PSE

The preparation of the PSE extract, the source of plant material, the process of microwave extraction, the measurement of total polyphenolic content and the list of individual polyphenol compounds are described in detail in Elez Garofulić et al. *(30)*, Lovrić et al. *(31)* and Dragović Uzelac et al. *(32)*. Briefly, for the analysis of total polyphenol content (TP) a volume of 250 µL (2.5-fold diluted) of each extract was mixed with 0.25 mL of 1 g/L HCl and 4.55 mL of 2 g/L HCl. The absorbance of the mixture was measured at 360 nm for total flavonols. Concentration measurements of individual polyphenol compounds were assayed by sophisticated method Ultra High-Performance Liquid-Chromatography Tandem Mass Spectrometry (UPLC-MS), was used for quantification of polyphenol

Table 1. *The estimated intake doses of polyphenols from in Prunus spinosa extract (PSE) and Mucedola mouse standard feed pellets applied to C57BL/6 mice.*

	TP in *P.spinosa* dry flower (mg/100 g dw)*				Dose of TP in PSE (mg/kg bw of C57BL/6 mouse)	Mucedola feed pellet
	2508.6				25.00	intake *ad libitum*
	Analysed polyphenols					
No.	Compound name	RT	m/z	m/z (prod.)	Dose of individual polyphenolic compounds (µg/kg bw mice)*	Concentration of individual polyphenolic compounds in feed pellet (µg/100 mg dw of pellet)
1	Caffeic acid	4.387	179	135	342.0 ± 5.1	0.175 ± 006
2	3-O-caffeoylquinic acid (neo-chlorogenic acid)	3.979	353	191	1913.4 ± 52.4	<LOD
3	4-O-caffeoquinic acid	4.444	353	173	239.6 ± 0.9	0.070 ±0.001
4	Chlorogenic acid	3.776	353	191	552.8 ± 9.2	0.018 ±0.005
5	p-coumaric acid	5.764	163	119	235.9 ± 2.6	0.055 ±0.010
6	3-p-coumaroylquinic acid	3.507	337	163	2152.6 ± 49.6	<LOD
7	4-p-coumaroylquinic acid	5.181	337	173	613.2 ± 7.0	0.077 ±0.021
8	Ferullic acid	6.427	193	134	86.6 ± 1.4	0.098 ±0.090
9	3-O-feruloylquinic acid	4.043	367	193	1317.4 ± 36.4	<LOD
10	Gallic acid	1.245	169	125	17.4 ± 0.2	0.133 ±0.027
11	(+)-Catechin	3.796	291	139	853.7 ± 15.2	0.026 ±0.001
12	(-)-Epicatechin	4.829	291	139	699.1 ± 11.3	0.068 ±0.004
13	(-)-Epicatechin gallate	6.583	443	139	5.0 ± 0.1	0.073 ±0.012
14	(-)-Epigallocatechin gallate	4.98	459	139	1.5 ± 0.1	0.013 ±0.002
15	Isorhamnetin-rutinoside	7.355	625	317	46.6 ± 0.9	2.501 ± 0.781
16	Kaempferol-3-rutinoside	6.216	595	287	516.6 ± 9.7	0.005 ± 0.000
17	Kaempherol-acetylrutinoside	9.402	637	287	6.7 ± 0.5	<LOD
18	Kaempferol-pentosylhexoside	7.291	581	287	500.9 ± 10.5	0.024 ± 0.008
19	Kaempferol-pentoside	8.234	419	287	4932.4 ± 87.9	0.111 ±0.078
20	Kaempferol-rhamnoside	7.166	433	287	4351.2 ± 79.2	<LOD
21	Kaempherol-acetylhexoside	11.290	491	287	9.1 ± 0.4	0.010 ± 0.000
22	Kaempferol rhamnosyl-hexoside	8.064	595	287	496. ± 10.4	0.029 ± 0.003
23	Kaempherol-3-glucoside	7.489	449	287	5.9 ± 0.3	0.009 ± 0.001
24	Apigenin	11.16	271	153	32.2 ± 0.6	0.029 ± 0.005
25	Luteolin	9.8	287	153	66.5 ± 1.3	0.013 ±0.000
26	Quercetin-3-rutinoside (rutin)	6.448	611	303	820.6 ± 11.5	0.162 ± 0.002
27	Quercetin acetylrutinoside	8.596	653	303	31.7 ± 0.4	<LOD
28	Quercetin-3-glucoside	6.737	465	303	311.8 ± 4.1	0.015 ± 0.001
29	Quercetin-pentoside	7.396	435	303	2259.5 ± 57.1	0.069 ± 0.08
30	Quercetin-acetylhexoside	5.096	507	303	23.3 ± 0.2	0.138 ± 0.059
31	Quercetin-rhamnoside	7.557	449	303	808.7 ± 12.3	0.057 ±0.050
32	Quercetin-pentosyl-hexoside	6.605	597	303	566.1 ± 11.8	0.013 ± 0.001

PSE – *P.spinosa* extract; TP – Total polyphenols; dw – dry weight, bw – body weight; <LOD – bellow limit of detection;
* Complete list of PSE polyphenol in extract and methods of analysis are given in references *(30-32)*.

in tissues, by protocols recommended by Ganguly et al. *(33)*. The doses of each individual polyphenol compound in PSE and administered per mg/kg bw of mice are presented as a list in Table 1. which also contains data of analyzed polyphenol content of standard diet Mucedola® standard diet feed pellets.

Reagents and standards

Formic acid and acetonitrile were HPLC grade, purchased from BDH Prolabo, VWR (Lutterworth, England). Commercial phenolic compound standards: quercetin-3-glucoside, kaempferol-3-rutinoside, caffeic acid, gallic acid, ferrulic acid, chlorogenic acid and p-coumaric acid were purchased from Sigma–Aldrich (Steinheim, Germany). Epicatechin, catechin, epigallocatechin gallate, epicatechin gallate, procyandine B1, apigenine and luteolin were purchased from Extrasynthese (Genay, France) and quercetin-3-rutinoside from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Deionized water Milli-Q quality (Millipore Corp., Bedford, USA) was used throughout the experiment.

Standard preparation for polyphenolic compound analysis

Standard stock solution was prepared by dissolving standards in methanol (1 mg/L) and a multicompound standard solution was prepared in methanol for external calibration of the UPLC–MS/MS system. Peak areas for each standard were used to make the respective standard curves reports *(30)*.

The UPLC-MS analysis

Chromatographic separation was carried out on a Zorbax Eclipse Plus C18 column (100 x 2.1 mm, 1.8 µm) (Agilent, Santa Clara, CA, USA) using an Agilent 1290 RRLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary gradient pump, autosampler and column compartment. The column oven was maintained at 35 °C and the flow rate was at 0.35 mL/min. Gradient conditions previously reported by Serra et al. *(34)* were used with minor modifications. The mobile phase consisted of redistilled water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was 0.35 mL/min. A QQQ 6430 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) was used for detection, with Agilent MassHunter Workstation Software used for data acquisition and analysis. The mass spectrometer was used in the dynamic multiple reaction monitoring mode (dMRM) in ESI-positive and negative modes and operated with the following source parameters: capillary voltage, +4000/-3500 V, nitrogen drying gas temperature maintained at 300 °C with a flow rate of 11 L/h and the pressure of nebulizer set at 40 psi. The total analytical time was 12.5 min. The analytes were identified by comparing retention times and mass spectra for individual polyphenol compounds with mass spectra of corresponding standards analyzed under the same conditions. The calibration curves obtained in the dMRM mode were used for quantification of all analytes. For unavailable standards, the structural identification of phenolic compounds was carried out by comparing the mass fragments 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, by injecting a series of dilute solutions of known concentrations *(30,34)*.

Brain tissue preparations

Animals were anesthetized by isoflourane prior to experimental procedure of brain collection. Animals were perfused with 10 mL PBS as recommended by Schaffer and Halliwell *(10)*. Animals were sacrificed by cervical dislocation. Brains were extracted and immediately frozen at -80 °C until analysis. Rationale for analysis of whole mice brain instead of separate regions was that the amount of tissue in separate regions would not be sufficient for the quantification of polyphenols, as proven in pilot experiments during dose determination.

The analysis of polyphenols in brain samples

Enzymatic hydrolysis

The brain samples (200 µL of the supernatant of tissue homogenates) was mixed with 10 µL of a mixture of ß-glucuronidase (250 units) and sulfatase (20 units), and then incubated at 37 °C for 45 min. The reaction mixture was extracted by ethyl acetate twice. The combined ethyl acetate solutions were added to 10 µL of a 20% ascorbic acid solution, and then evaporated to dryness in a vacuum centrifuge concentrator. Prior to chromatographic analysis, the samples were dissolved in 300 µL of a 10% aqueous acetonitrile solution (v/v) and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to an injection vial and then an aliquot of 2.5 µL of the supernatant was injected for LC–MS/MS analysis *(33)*.

Markers of oxidative stress

Before analysis tissue samples for the determination of malondialdehyde (MDA), superoxide dismutase (SOD), catalaze (CAT) and glutathione (GSH) were placed in 50 mM phosphate buffer (pH = 7.4) and homogenized (10% of homogenate, by tissue mass per volume of PBS) with the ultrasonic homogenizer (SONOPLUS Bandelin HD2070, Bandelin Electronic GmbH & Co KG, Germany) using a probe MS73 (Bandelin, Electronic GmbH & Co KG Germany). The brains were sonicated on ice for 30 s in three 10-seconds s intervals and centrifuged at 20,000 × g for 15 min at 4 °C. The supernatant was further processed for polyphenol concentration and antioxidative defense system analysis similar as in the work of Ganguly et al. *(33)*. The detailed methods of antioxidative defense system were previously described in detail in Landeka et al. *(9)*. Here we bring brief descriptions:

Determination of protein in brain tissues

Protein concentration in the samples was estimated by the method of Lowry et al. *(35)* with bovine serum albu-

Table 2. *Polyphenol content and basic pharmacokinetic parameters in the brain of experimental animals after 28 days, daily repeated oral application of Prunus spinosa extract (PSE), in the dose of 25 mg of polyphenols/kg body weight.*

Brain bioavailability		1-28 day, subchronic dose*		
Compound	Group	Cmax (ug/g)	Tmax (h)*	AUClast (h*ug/g)
Phenolic acids				
4-O-Caffeoylquinic acid	Control	1.562±0.043	672±0.0	745.700±126.421
	PSE	2.011±0.115#	168±0.0	1072.000±43.323#
4-p- Coumaroylquinic acid	Control	0.692±0.250	24±3	356.302±153.67
	PSE	2.142±0.050#	504±0.0	1166.354±47.9#
Ferullic acid	Control	1.529±0.001	168±0.0	856.468±0.001
	PSE	1.64±0.003	504±0.0	929.631±0.002
3-O-feruloylquinic acid	Control	0.488±0.050	168±19	202.000±114.921
	PSE	0.791±0.001#	168±0.0	232.400±53.703
Gallic acid	Control	1.879±0.470	24±0.0	185.8106±143.800
	PSE	2.502±0.463	24±0.0	685.642±26.007#
Flavones				
Apigenin	Control	0.199±0.070	672±83	116.120±21.301
	PSE	0.154±0.001	168±0.0	83.800±4.324
Luteolin	Control	0.333±0.030	504±83	27.900±3.421
	PSE	0.371±0.001	168±0.0	109.854±0.001#
Flavanols				
(+)-Catechin	Control	0.129±0.050	24±374	80.400±1.172
	PSE	0.138±0.000	168±37	90.301±1.70
(-)-Epicatechin	Control	<LOD	/	/
	PSE	0.180±0.001#	504±0.0	39.925±0.00#
(-)-Epicatechin-3-gallate	Control	1.892±0.001	168±0.0	346.2000±0.003
	PSE	2.238±0.004	168±0.0	461.604±0.004
(-)-Epigallocatechin-3-gallate	Control	0.353±0.090	336±0.0	207.700±0.000
	PSE	2.137±0.005#	168±0.0	567.600±0.000#
Flavonols				
Isorhamnetin-3-rutinoside	Control	0.397±0.060	672±0.0	258.000±1.570
	PSE	0.430±0.020	168±0.0	265.701±3.523
Kaempherol-3-glucoside	Control	0.243±0.060	168±0.0	108.269±11.610
	PSE	0.196±0.000	168±0.0	89.605±0.521
Kaempherol-3-rutinoside	Control	0.0640±0.010	24±0.0	33.00±5.302
	PSE	0.287±0.001#	672±0.0	147±103.300#
Kaempherol pentoside	Control	2.586±0.0400	168±83	1295.609±22.825
	PSE	2.646±0.050	168±0.0	1326.701±17.601
Kaempherol rhamnosyl hexoside	Control	0.651±0.042	24±8	294.600±15.710
	PSE	0.731±0.016	336±0.0	416.703±5.520
Quercetin-3-O-glucoside	Control	0.146±0.010	336±0.0	62.000±6.821
	PSE	0.247±0.030#	672±0.0	106.821±5.501#
Quercetin-3-rutinoside	Control	1.277±0.150	24±3.0	393.710±55.080
	PSE	2.841±0.042#	336±0.0	1306.212±74.082#
Quercetin acetyl hexoside	Control	0.636±0.631	504±97	372.700±15.821
	PSE	0.631±0.011	24±0.0	381.811±17.020
Quercetin-pentoside	Control	1.508±0.220	24±8	691.774±146.822
	PSE	2.094±0.263#	24±0.0	829.854±28.805#
Quercetin-pentosyl-hexoside	Control	0.328±0.037	24±8	158.541±78.665
	PSE	1.959±0.040#	168±83	979.135±8.314#
Qurercetin-rhamnoside	Control	0.678±0.020	504±83	425.915±4.000
	PSE	2.916±0.010#	672±0.0	1136.421±0.580#

values are statistically significantly different ($p \leq 0.05$) between control and PSE treated group; <LOD – bellow limit of detection. *Samples of brain were taken on 1st, 7th, 14th, 21st and 28th day, and Tmyx was estimated by pharmacokinetic software in hours from intake. In the pharmakinetic analysis software, the days of sampling are represented by hours from the begining of the experiment 1st day correspond to 24h, 7th day to 168 h, 14th day to 336 h, 21st day to 504 h and 28th day to 672 h.

min (BSA) as the standard. Protein measurements were used to express the values of measured oxidative stress parameters.

Malonyldialdehide concentration (MDA) assay in brain tissues

Lipid peroxidation was determined by measuring the concentration of MDA using a modified method of Ohkawa et al. *(36)*. A sample of 200 µL of homogenized tissue was mixed with 200 µL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL 20% acetic acid (pH = 3.5) and 1.5 mL of 0.81% thiobarbituric acid, and incubated for 60 min at 95 °C. 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: $A_{total}=A_{532\ nm}-A_{600\ nm}$

MDA levels were determined using molar absorption coefficient for malondialdehyde-thiobarbiturate (MDA–TBA) complex of $1.56\times10^5\ M^{-1}cm^{-1}$.

Superoxide dismutase (SOD) activity assay in brain tissues

The SOD assay is a modification of the method by Flohé and Ötting *(37)*. The SOD activity was calculated from the percentage of inhibition of the reaction of xanthine oxidation (ΔA/min≈0.025), which creates a superoxide anion as a substrate for the SOD present in the samples. The superoxide anion not used by the enzyme oxidizes the cytochrome. An undiluted sample (25 µL) was mixed with 1.45 mL of reaction solution (cytochrome C, 0.05 mM; Xanthine, 1 m*M* mixed in a 10:1 ratio with DTNB). A volume of 20 µL of xantin oxidase (0.4 U/mL) was added to start the reaction. The reaction was measured over 3 min at 550 nm with a Libro S22 spectrophotometer (Biochrom). One unit of total SOD activity was defined as the amount of enzyme required to achieve 50% inhibition in the typical calibration curve obtained with standard SOD. Horse heart cytochrome C (type VI), human blood SOD (type I, lyophilized powder, 2400 U/mg protein), xanthine and xanthine oxidase (200 U/mL) were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Catalase (CAT) activity assay in brain tissues

CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm with Libro S22 spectrophotometer (Biochrom) *(38)*. The reaction mixture contained 33 mM H_2O_2 in 50 mM phosphate buffer pH = 7.0 and CAT activity was calculated using the molar absorption coefficient of 43.6 $M^{-1}cm^{-1}$for H_2O_2. Hydrogen peroxide (30%) for preparation of working solution of 33 mM H_2O_2 was purchased from Sigma, St. Louis, MO, USA.

Concentration of total reduced glutathione (GSH) assay in brain tissues

GSH assay was measured in a 96-well plate, 40 µL of 10 mM 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's Reagent) was mixed with 20 µL of sample supernatant pretreated with 40 µL of 0.035M HCL, incubated for 10 min and measured at 412 nm in an ELISA plate reader (Biorad Laboratories, Hercules CA, USA). Then, 100 µL of reaction solution: 9980 µL 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. GSH levels were determined by using molar absorption coefficient for DTNB of 8.22 $M^{-1}cm^{-1}$. DTNB, and NADPH, glutathione reductase were purchased from Sigma, St. Louis, MO, USA.

Statistical analysis

Data on the concentration of particular polyphenolic compounds and pharmacokinetic parameters (Cmax/AUClast) and oxidative stress markers (MDA, SOD, CAT, GSH) are presented as mean values and standard deviation. The pharmacokinetic data was analyzed by Phoenix 64 software, SERTAA version f.0.03176. The data of PK analysis and antioxidative defense system were compared for statistical significance ($p\leq0.05$) between the PSE and control groups by t-test and analyzed by SPSS Statistics for Windows, Version 17.0 *(39)*. The data of PK analysis were compared for statistically significant differences ($p\leq0.05$) by t-test between the control and PSE treated group (Table 2). The compounds that showed significant difference between the control and PSE treated animals was also shown in Figures 1 and 2. The Kruskal-Wallis ANOVA was used for testing the statistically significant differences ($p\leq0.05$) in brain antioxidative parameters (SOD, CAT, GSH) between days of sampling within the control and PSE group, respectively.

RESULTS

The UPLC-MS analysis of polyphenol compounds in PSE extract, mouse feed pellets

A total of 32 polyphenol compounds were analyzed in *Prunus spinosa* extract (PSE) by UPLC/MS. Within the extract, the highest polyphenol concentrations were detected for 3-O-caffeoylquinic, 3-p-coumaroylquinic and 3-O-feruloylquinic acid among phenolic acids. Then, (+)-catechin and (-)-epicatechin among flavan 3-ols. Among flavonoids the highest detected were kaempferol–pentoside and kaempferol –rhamnoside in kaempherol group and quercetin-3-rutinoside, quercetine-pentoside and quercetin-rhamnoside in the quercetin group. The same 32 polyphenols were analyzed in Mucedola standard diet mouse feed and the analysis revealed that only 26 compounds were detected and in markedly lower concentrations than in the PSE extract (Table 1). According to the treatment of mice with 25 mg/kg bw of total polyphenols (TP) from PSE per dose, the Table 1. also summarizes individual doses of each polyphenol compound administered to mice (mg/kg bw per single dose).

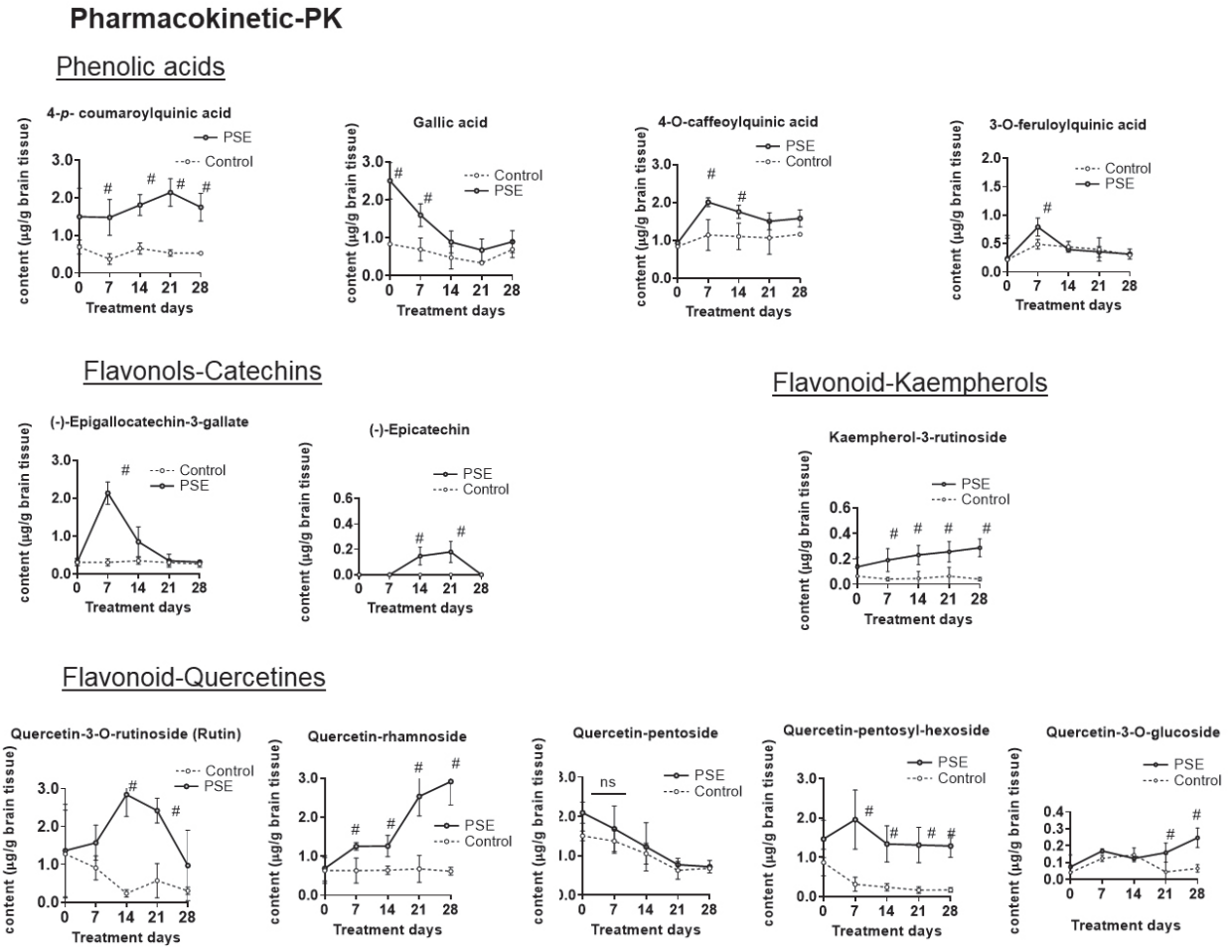

Figure 1. *The brain pharmacokinetic curves of polyphenols bioavailable after subchronic/daily repeated (0-28th day) intake of PSE and control C57BL/6 mice. Graphs show the compounds whose levels were statistically different (#=p<0.05) between control and treated animals (PSE). The results are presented as average ± SD values; CO – control; PSE – Prunus spinosa flower water extract.*

Polyphenols in the brain after PSE treatment for 28 days

From 32 different polyphenol compounds analyzed in PSE (Table 1), following the subchronic dosing (day 1 to day 28), a 68.7% of polyphenols measured in PSE, or 22 in number, were above detection limit in the brain (Table 2). However, it is visible (Table 1) that a significantly different (p≤0.05) and higher Cmax or AUClast in the PSE treatment vs. control group was recorded for 13 compounds in the brain (40.6% of total N in PSE, 59.1% of brain detected). The polyphenol compounds detected in the brain, could be divided in range groups according to concentrations and described as relatively high (in the range of 2-3 µg/g brain tissue), moderate (in the range of 1-2 µg/g brain tissue), low (0.3-1 µg/g brain tissue), or very low (0-0.3 µg/g brain tissue) concentrations. The brain concentrations of polyphenols up to 2.92 (range 2-3) of µg/g tissue respectively, prove a good bioaccumulation potential after 28 daily treatment of PSE polyphenol mixture water extracts.

Among phenolic acids bioaccumulated in the brain, gallic, 4-p-coumaroylquinic, 4-O-caffeoylquinic and 3-O-feruloylquinic acid were the ones with significantly (p≤0.05) higher Cmax/AUClast during 28 days of PSE treatment compared to the control. Among them the range of gallic and 4-p-coumaroylquinic acid could be regarded as high, while others significant ones were in moderate concentration range in PSE treated animals (Figure 1 and 2). The 4-p-coumaroylquinic acid was the only one whose Cmax was reached between days 21 and 28 (Figure 1).

Among the group of flavan-3-ol compounds (catechin group), based on the significantly (p≤0.05) higher AUClast/Cmax compared to control animals, the brain had preference for epicatehin-3-gallate epigallocatehin-3-gallate bioaccumulation. Flavan-3-ols (Figure 1 and 2), rather stayed at medium level (Figure 1 and 2). Epicatechin-3-gallate and epigalloctocatechin-3-gallate were the most prominent catechin compounds in such medium concentration range, while epicatechin and catechin were

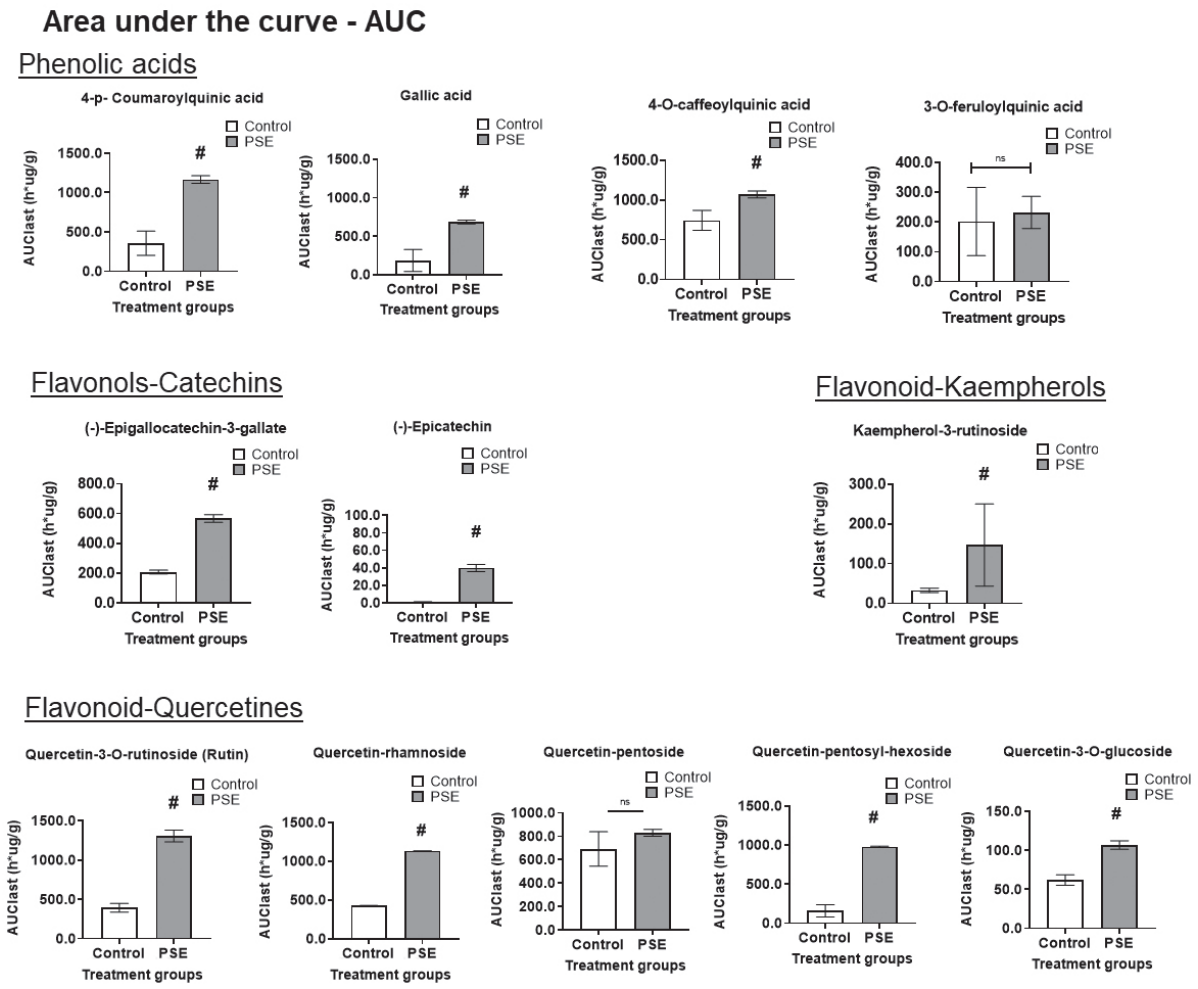

Figure 2. *The brain area under the curve-AUC values of polyphenols bioavailable after subchronic/daily repeated (0-28th day) intake of PSE and control C57BL/6 mice. Graphs show the compounds whose levels were statistically different (#=p<0.05) between control and treated animals (PSE). The results are presented as average ± SD values; CO – control; PSE – Prunus spinosa flower water extract.*

at low concentration range (Figure 1 and 2). Catechin concentration was not different in PSE treated animals and control animals and was given in Figures as an example of similar compounds (listed in Table 2), that were detected in both PSE and control, but their PK curves were similar. Epicatechin-3-gallate and epigallocatechin-3-gallate had most prominent levels between 1st and 14th day of the experiment while epicatechin (although in lower range than two previously mentioned compounds) reached its peaks only after 7th-14th day of treatment.

The brain did not readily accumulate kaempherol compounds, compared to other investigated polyphenol groups since the only significantly bioacumulated kaempherol in PSE animals was the kaempherol-3-rutinoside, but it was present in rather low concentration range (Table 2, Figure 1 and 2).

Quercetin compounds group showed best bioavailable levels since all detected quercetin compounds (5 out of 7 present in PSE) had significantly higher ($p \leq 0.05$) Cmax or AUClast value in PPSE treated animals compared to control, except quercetin acetyl hexoside. In the brain quercitin-rhamnnosid had the highest reached bioaccumulated concentration (Cmax PSE=2.92 μg/g tissue) from all analyzed polyphenol compounds, and this peak was reached on the 28th day in PSE treated animals. However, compared to the quercitin-rhamnnosid, the epigallocatechin-3-gallate, with approximately similar concentration (Cmax PSE=2.13 μg/g tissue) had the highest fold difference in both AUClast/Cmax of control and PSE treated animals. Beside quercitin-rhamnnosid, also the quercetin-rutinoside (Figure 1 and 2), had the highest reached concentration but it reached its high levels between 7-14th days of repeated treatment. Quercetin-pentosyl hexoside reached medium high concentrations between 1-7th day, but it stayed in the medium concentration range. Quercetin-3-glucoside was present in very low concentrations, although significantly higher in PSE animals compared to control.

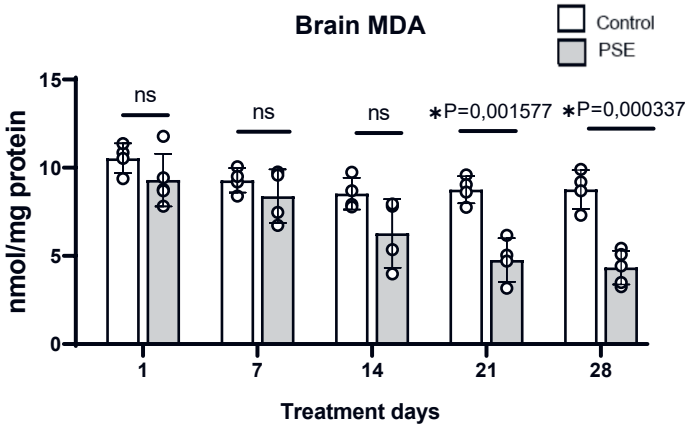

Figure 3. *The level of malondialdehyde (MDA) in the brain tissue of experimental animals after 28 days of daily repeated oral application of PSE compared to the Control mice. The results are presented as average ± SD values; dots represent each individual measurement from single animal, whiskers represent SD - standard deviation; *statistically significantly different (p<0.05) in relation to the control group on the day of sacrifice; PSE – Prunus spinosa flower water extract.*

Table 3. *The levels of major cellular antoxidative defense molecules superoxide dismutase, catalase, and total glutathione in the brain tissue of experimental animals after 28 days of daily repeated oral application of PSE compared to the control mice.*

Brain												
Day of sampling	SOD (U/mg protein)				Catalase (U/mg protein)				GSH (µmol/mg protein)			
	Control		PSE		Control		PSE		Control		PSE	
	Average	±SD	Average	±SD	Average	±SD	Average	±SD	Average	±SD	Average	±SD
1.	8.35	±1.09^{d}	9.41	±2.00^{d}	3.61	±3.12	5.63	±4.25	45.15	±6.23^{e}	46.78	±6.62^{e}
7.	9.74	±2.72^{d}	10.24	±1.18^{d}	4.20	±2.63	5.32	±1.07	39.56	±22.7^{e}	37.47	±4.70^{e}
14.	9.31	±3.58^{d}	9.57	±1.83^{d}	6.05	±2.92	5.46	±1.66	44.29	±18.5^{e}	42.09	±8.47^{e}
21	4.69	±1.09^{abc}	6.34	±2.09^{abce}	3.76	±1.18	5.53	±0.73	36.84	±3.61^{e}	51.47	±4.22$^{\#e}$
28.	8.22	±2.85^{d}	10.62	±1.62$^{\#d}$	3.90	±1.65	3.39	±1.05	60.37	±3.59^{abcd}	72.19	±3.35$^{\#abcd}$

$^{\#}$ statistically significantly different (p<0.05) in relation to the control group on the day of sacrifice. a,b,c,d,e – superscript letters show significantly differences (p≤0.05) between days of sampling in columns of the Control or PSE groups, respectively.

Antioxidative effects in the brain after subchronic PSE tratement

Lipid peroxidation (MDA concentration) after PSE treatment for 28 days

The levels of malonyldialdehide (MDA) concentration were lowered due to PSE treatment compared to controls. In Figure 3., the significantly (p≤0.05) lower concentrations of MDA were recorded on days 21 and 28 of the treatment, while on other sampling days there was no difference in MDA level between control and PSE animals.

Enzymatic activity of superoxide dismutase (SOD) in the brain after PSE treatment for 28 days

A statistically significant increase (p<0.05) of the SOD enzyme activity was observed in the group receiving PSE compared to the control group on the 28th day treatment (Table 3).

Enzymatic activity of catalase (CAT) in the brain after PSE treatment for 28 days

Analysis of enzymatic activity of the catalase of brain showed no statistically significant differences between the PSE group and the control group (Table 3).

Concentration of total reduced glutathione (GSH) in the brain after PSE treatment for 28 days

A statistically significant increase (p<0.05) of the GSH concentration was observed in the group receiving PSE compared to the control group on the 21st and on the 28th day of treatment (Table 3).

DISCUSSION

Based on the results and differences in the number of detected compounds in the brain and subchronic repeated treatment it can be noted that the brain has a moderate bioaccumulation potential for polyphenols. This is evident from the results showing that out of the 32 compounds monitored and present in the model extract of PSE, 40.6% had significantly higher concentration values than control animals during chronic repeated dosing for the period of 28 days. Such results together with the concentration maximums of up to approximately 2-3 μg/g brain tissue of particular compounds (although in only few), indicate relatively low-moderate absorption potential of brain as an organ despite the blood brain barrier (BBB). Particularly is interesting that kaempherols were not readily distributed in the brain, compared to phenolic acids or quercetins or epicgaloatechin-3-gallate. The results are instructive since majority of the bioavailability studies or reviews mention low serum or other organ bioavailability in general *(40)*. Factors such as food matrix interactions, food processing, host (human)-related factors (e.g., age, occurrence of certain diseases and lifestyle) and the flavonoids' chemical structure contribute to polyphenol poor intestinal bioavailability, according to some authors *(15)*. Approximately up to 8000 different plant polyphenol types have been identified so far but most studies estimate flavonoid gut absorption or organ distribution based on individual flavonols (kaempferol, myricetin and quercetin) and flavones (apigenin and luteolin), flavan-3-ols including (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-gallate (EGCG) *(41)*. In the colon polyphenols undergo microbial catabolism, leading to the formation of small polyphenols that are able to reach the liver, be subjected to metabolic conversion through phase II enzymes, resulting in conjugated metabolites in the circulation *(42)*. Frequently, polyphenols are excreted in feces without intestinal absorption, or decomposed to ring-excision products by the action of enterobacteria in the large intestine adding to low bioapsorption *(41)*. In comparison, it is believed that bioaccumulation efficacy of polyphenols is much lower than that of antioxidative vitamins/pro-vitamins, even when polyphenol consumed concentration are higher than that of such vitamins/pro-vitamins in food. Some researchers provide reports that the polyphenols as xenobiotic in excess amounts in mammalian organism exhibit adverse effects (prooxidative properties) and that this is the reason that their absorption is limited as an evolutionary adaptation to avoid plant xenobiotic (adverse) effects *(42)*. Despite such premises, the results here show that 40% of PSE present polyphenols can reach brain tissue, although in moderate to low concentrations ranges. Literature sources mention the main efflux transporters involved in blood brain barrier transport of polyphenols to be ATP-binding cassette transporters which include p-glycoprotein, multidrug resistance-associated proteins, and breast cancer resistance protein *(41)*. For example, by using a young piglet model, Chen *(41)* determined plasma and brain pharmacokinetics of polyphenol metabolites using high (82.5 mg/kg bw) or physiological (27.5 mg/kg bw) doses of apple/grape seed and bilberry (*Vaccinium*) extracts. Chen *(41)* found that in a physiological dose achievable by supplementation, polyphenol metabolites were able to cross BBB to deposit in the brain. He found a difference in regional brain deposition with cerebellum being a preferred site for accumulation. Within our study, we were limited by mouse brain size and analysis by brain sections as in Chens piglet brain. The amount of mouse brain tissue per section would be too small to reach detectable levels of majority of shown polyphenols. Furthermore, Chen *(41)* showed that the flavan-3-ol metabolites had the highest brain levels at 200 nM/mg tissue compared to other metabolites. Similarly, our results show that in subchronic dosing the (-)-epicatechin-3-gallate had the approximately 6-fold higher Cmax than in the control animals. This was the compound which showed highest fold-elevation in Cmax value than any other detected compound. For example, although in the brain quercitin-rhamnnosid had the highest reached bioaccumulated concentration (Cmax PSE=2.92 μg/g tissue) from all analyzed polyphenol compounds, the epigallocatechin-3-gallate, with approximately similar concentration (Cmax PSE=2.13 μg/g tissue) had the highest fold difference in both AUClast/Cmax of control and PSE treated animals. In addition, Prasain et al. *(44)* in SD rats treated with the grape seed extract in the dose of 300 mg/kg bw 2x/day for 3d by gavage determined, 4h post treatment the catechin concentration to be 53.16 ng/g in perfused brain tissue (corresponding to 0.053 μg/g tissue). Our results showed Cmax concentrations to be even higher (in case of catechin 3 times higher) but we believe it was because of the interspecies metabolic differences and different time of exposure. Similar ranges of levels as in our experiment were detected by UPLC-MS/MS analysis in the experiment of oral administration of two types of black tea as 5% infusion to guinea pigs for 14 days by Ganguly et al. *(15)*. The mean levels of epicatechin, epicatechin-3-gallate and epigallocatechin-3-gallate in the plasma or organs (lung, liver, kidney) of animals in the ranges approximately from 0.0129 - 0.144.6 μg/g, with epicatechin-gallate 0.1 being the highest *(15)*.

Some studies have shown that although fruits or berries that are traditionally consumed in other plant parts (leaves or flowers) there is a higher polyphenol content. For example, a study of polyphenols in samples of blueberries and lingo berries established that the leaves have a much higher antioxidant capacity than the fruits with biological effects *(18)*. We used the blackthorn polyphenol extract from flowers as a model polyphenol mixture. Elez Garofulić, Dragović-Uzelac et al. and Lovrić et al. *(30-32)* screened the blackthorn (*Prunus spinosa* L) flowers among

other investigated plants as a rich source of polyphenols (although berries are traditionally consumed in Europe). Analysis confirmed the abundance of flavan-3-ols (312.08-645 mg/kg dried flower), neochlorogenic acid (420.5-735 mg/kg dried flower), and quercetin-3-rutinoside (89.54-168.11 mg/kg dried flower). Interestingly, such results corroborate the entry of quercetine compounds and flavan-3-ols (catechins) in the brain, but on the opposite neochlorogenic acid, although high in extract, did not accumulate due to the subchronic dosing in the same way. Thus, the polyphenols that have the highest concentration in the extract are not necessarily the ones present in the brain, with kaempherols being another example of such observation. On the opposite, the abundance of quercetin compounds in the plant material reflected the brain entry of quercetin compounds. Some of the reported data on quercetin deposition in the brain tissues were summarized by Chen *(41)*, reporting that de Boer et al. *(45)* in Fisher 344 rat, fed quercetin aglycon 1% (500-800 mg/kg bw) for 11wks in the diet, detected the maximal concentration of 0.06 nmol/g of quercetin aglycon in the brain tissue. In addition, Ho et al. *(46)* used SD rats and fed them with Cabernet sauvignon 150 mg total polyphenols/kg bw for 10 days by gavage. In the perfused brain tissue authors detected the concentration of quercertin-3-O-glucuronide of 0.91 pmol/g brain tissue. Further, Huebbe et al. *(47)* showed results of experiment in C57BL/6J mice, as in our experiment, that were treated with quercetin aglycon in the dose of 2 g/kg in diet for 6 weeks and measured quercetin-aglycon to be 0.28 and methyl-qercetin to be 0.08 nmol/g in the brain tissue. Such values correspond to average Cmax values of quercetin compounds recorded in our study.

Beside previous analytical evaluation of the polyphenol content of wild fruits Elez Garofulić, Dragović-Uzelac and Lovrić and coauthors *(30-32)*, their studies measured the antioxidant capacity of various plants and screened the blackthorn (*Prunus spinosa* L) flower extract to have potent antioxidant capacity *in vitro*. Extract was examined by 2, 2-diphenylpicrylhydrazyl method and showed significant linear correlation of antioxidant capacity and flavonols concentrations. Similarly, Marchelak et al. *(1)* report that in *in vitro, P. Spinosa* extract, with the contents of total phenolics (TPC) of 584.07 mg/g dry weight (dw), the flavonoids of 490.63 mg/g dw, proanthocyanidins of 109.43 mg/g dw and phenolic acids of 66.77 mg/g dw, displayed potent antioxidant properties by FRAP, TBARS and enzyme (lipoxygenase and hyaluronidase) inhibition assays. Moreover, Marchelak et al. *(1)*, showed at *ex vivo*-relevant concentrations of 1-5 μg/mL, that the extracts effectively protected the human plasma components against peroxynitrite-induced damage (reduced the levels of oxidative stress biomarkers: 3-nitrotyrosine, lipid hydroperoxides, and thiobarbituric acid-reactive substances) and enhanced the total antioxidant status of isolated plasma. The authors further reported that among various extraction methods, the defatted methanol-water (7:3, v/v) extract of *P- spinosa* and its diethyl ether and ethyl acetate fractions emerged as most beneficial for biological applications. Similar finding was reported by Olszewska and Wolbi *(20)*. In this *in vivo* research, however, an aqueous flower extract was used to avoid potentially toxic traces of organic solvents in animals. In addition, the advantages of using water liquid extracts were the same ratio of the active compounds in the starting material, easier handling and dosing, retention of volatile components and avoidance of the organic solvents in the extract. Additionally, studies have shown that flavonoids or flavonoid matrices with high aqueous solubility promote intestinal absorption *in vivo (15)*. As above mentioned examples of antioxidant capacity of PSE measured *in vitro*, it seems that *in vivo,* in the brain tissue, the polyphenol mixture and absorbed detected compounds had similar effect. In support to this thesis, the recorded levels of malondialdehyde in the brain tissue (MDA used as a marker of lipid peroxidation of cellular lipids), showed decreased MDA concentrations in PSE treated animals. Such effects were most prominent on the 21st and the 28th days (but not on earlier examined day). By observation of PK curves of significantly ($p \leq 0.05$) bioaccumulated compounds, it can be observed that only a portion of measured compounds reach their maximum levels after 14th, (that is between the 21st and 28th day) when the reduced lipid peroxidation was noted. The compounds with the highest concentrations on the 21st and 28th days were 4-p-coumaroylquinonic acid, (-)-epicatechin, quercetin-3-O-rutinoside and quercetin–rhamnoside and in the lower concentration ranges: the kaempherol-3-rutinoside and quercetin-3-gucoside. We have reasons to believe that the lowered MDA concentration and consequently lipid peroxidation might be directly linked to the increase in Cmax and bioaccumulation of the named compounds from 14th up to 28th day and to specific concentration ratios among them. Furthermore, since the results showed that the antioxidative defense system enzyme the superoxide dismutase increased in activity on the 28th sampled day it could also be responsible for the diminished MDA levels. Total glutathione concentration, measured as well as cellular biomarker of antioxidative defense, was the only parameter that slightly increased in PSE animals between 21st and 28th day. Thus, it can be hypothesized that the highest absorbed named polyphenol compounds and their concentration ratios, caused two possible mechanisms of reduction of lipid peroxidation. Firstly, by molecular properties of polyphenol molecules to act as radical scavengers that directly lowers lipid peroxidation or secondly by directly influencing glutathione synthesis which indirectly contributed to MDA decrease. Similar observations were noted in some of our previous research with wine lees polyphenol extracts *(9)*, where the mechanisms of polyphenol scavenging within tissues occurred as described. Detailed physiological mechanisms of glutathione level regulation by foreign molecules (also poly-

phenols) and their control of transcriptional factors are well known *(48-51)*.

As for the limitations of the study, one should mention that although there are differences in the speed of metabolism between mouse and humans, animal model is currently irreplaceable because we can't measure the individual polyphenol concentrations in human brain using current available methods. However, Cox and Scholey *(51)*, made a most comprehensive review of 52 human polyphenol consumption studies describing beneficial effects of polyphenol molecules on memory, cognition, protection from neurodegenerative disease. Studies in the nervous system indicated the presence of charged flavonoid conjugates in the brain and that these charged flavonoid molecules are biologically active. Research of polyphenol bioavailability shows that CNS diseases can be the ultimate target of conjugated polyphenols with proper targeted compound delivery to the brain. In addition, a potential solution lies in polymeric nanoparticle-based polyphenol delivery systems that are able to enhance their absorption across the gastrointestinal tract, improve their bioavailability, and transport them to target organs *(52)*.

From the practical pharmacological point of view, beside antioxidative properties, it would be interesting to see whether the ratios of the screened compounds with highest significant Cmax/AUClast values could be applied as a formulated mixture of "nutriceuticals" (perhaps with enhanced bioavailability by substitutive conjugations) to reach antioxidant effects in some neurodegenerative disease. This premise is just one of the contributions of this work beside subchronic brain distribution comparison, reported concentration levels, PK curves, Cmax, AUClast values for individual polyphenol compounds, which contribute to the knowledge on brain bioavailability that is usually insufficiently covered by literature.

Acknowledgements: *The authors are grateful for the donation of the plant material from Suban Ltd, Strmec, Croatia. Research was financed and supported by grant from the Croatian Science Foundation: Application of innovative technologies for production of plant extracts as ingredients for functional food (HRZZ IP-2013-11-3035 / IT-PE-FF).*

REFERENCES

1. MARCHELAK A, OWCZAREK A, MATCZAK M, PAWLAK A, KOLODZIEJCZYK-CZEPAS J, NOWAK P, OLSZEWSKA MA 2017 Bioactivity Potential of *Prunus spinosa* L. Flower extracts: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress in vitro. Front Pharmacol 8: 680. https://doi.org/10.3389/fphar.2017.00680
2. PASUPULETI VR, KUMARA TK, NAGUIB S, SIEW HG 2016 Biological and therapeutic effects of honey produced by honey bees and stigles bees: a comparative review. Rev Bras Farmacogn 26: 657-664. https://doi.org/10.1016/j.bjp.2016.01.012
3. BRGLEZ MOJZER E, KNEZ HRNČIČ M, ŠKERGET M, KNEZ Ž, BREN U 2016 Polyphenols: extraction methods, antioxidative action, bioavailability and anticarcinogenic effects. Molecules 21(7): 901. https://doi.org/10.3390/molecules21070901
4. SINA COSMULESCU S, TRANDAFIR I, NOUR V 2017 Phenolic acids and flavonoids profiles of extracts from edible wild fruits and their antioxidant properties, Int J Food Prop 20(12): 3124-3134. https://doi.org/10.1080/10942912.2016.1274906
5. ORŠOLIĆ N, SIROVINA D, GAJSKI G, GARAJ-VRHOVAC V, JAZVINŠĆAK JEMBREK M, KOSALEC I 2013 Assessment of DNA damage and lipid peroxidation in diabetic mice: effects of propolis and epigallocatechin gallate (EGCG). Mutat Res 757(1): 36-44. https://doi.org/10.1016/j.mrgentox.2013.04.022
6. ORŠOLIĆ N, SKURIĆ J, DIKIĆ D, STANIĆ G 2014 Inhibitory effect of a propolis on di-n-propyl disulfide or n-hexyl salycilate-induced skin irritation, oxidative stress and inflammatory responses in mice. Fitoterapia 93: 18-30. https://doi.org/10.1016/j.fitote.2013.12.007
7. ORŠOLIĆ N, GOLUŽA E, DIKIĆ D, LISIČIĆ D, SAŠILO K, ROĐAK E, JELEČ Z, LAZARUS MV, ORCT T 2013 Role of flavonoids on oxidative stress and mineral contents in the retinoic acid-induced bone loss model of rat. Eur J Nutr 53(5): 1217-27. https://doi.org/10.1007/s00394-013-0622-7
8. ORŠOLIĆ N, GAJSKI G, GARAJ-VRHOVAC V, DIKIĆ D, PRSKALO ZŠ, SIROVINA D 2011 DNA-protective effects of quercetin or naringenin in alloxan-induced diabetic mice. Eur J Pharmacol 656(1-3): 110-8. https://doi.org/10.1016/j.ejphar.2011.01.021
9. LANDEKA I, JURČEVIĆ, DORA M, GUBEROVIĆ I, PETRAS M, RIMAC S, BRNČIĆ, ĐIKIĆ D 2017 polyphenols from wine lees as a novel functional bioactive compound in the protection against oxidative stress and hyperlipidaemia Food Technol Biotechnol 55(1): 109-116. https://doi.org/10.17113/ftb.55.01.17.4894
10. SCHAFFER S, HALLIWELL B 2012 Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations. Genes Nutr 7(2): 99-109. https://doi.org/10.1007/s12263-011-0255-5
11. HU M, WU B, LIU Z 2017 Bioavailability of polyphenols and flavonoids in the era of precision medicine. Mol Pharm 14(9): 2861-2863. https://doi.org/10.1021/acs.molpharmaceut.7b00545
12. GILDAWIE KR, GALLI RL, SHUKITT-HALE B, CAREY AN 2018 Protective effects of foods containing flavonoids on age-related cognitive decline. Curr Nutr Rep 7(2): 39-48. https://doi.org/10.1007/s13668-018-0227-0
13. FLANAGAN E, MÜLLER M, HORNBERGER M, VAUZOUR D 2018 Impact of flavonoids on cellular and molecular mechanisms underlying age-related cognitive decline and neurodegeneration. Curr Nutr Rep 7(2): 49-57. https://doi.org/10.1007/s13668-018-0226-1
14. KUJAWSKA M, JODYNIS-LIEBERT J 2018 Polyphenols in Parkinson's Disease: A systematic review of in vivo studies. Nutrients 10(5): 642. https://doi.org/10.3390/nu10050642
15. GONZALES GB, SMAGGHE G, GROOTAERT C, ZOTTI M, RAES K, VAN CAMP J 2015 Flavonoid interactions during digestion, absorption, distribution and metabolism: a sequential structure-activity/property relationship-based approach in the study of bioavailability and bioactivity. Drug Metab Rev 47(2): 175-90. https://doi.org/10.3109/03602532.2014.1003649
16. PINACHO R, CAVERO YR, ASTIASARÁN I, ANSORENA D, CALVO IM 2015 Phenolic compounds of blackthorn (*Prunus spinosa* L.) and influence of in vitro digestion on their antioxidant

capacity. J Funct Food 19: 49-62. https://doi.org/10.1016/j.jff.2015.09.015

17. QIAO J, KONG X, KONG A, HAN M 2014 Pharmacokinetics and biotransformation of tea polyphenols. Curr Drug Metab 15 (1): 30-36. https://doi.org/10.2174/1389200214666131229111336

18. VYAS P, KALIDINDI S, CHIBRIKOVA L, IGAMBERDIEV AU, WEBER JT 2017 Chemical analysis and effect of blueberry and lingonberry fruits and leaves against glutamate-mediated excitotoxicity. J Agric Food Chem 61(32): 7769-76. https://doi.org/10.1021/jf401158a

19. YUKSEL AK 2015 The Effects of Blackthorn (*Prunus spinosa* L.) addition on certain quality characteristics of ice cream. J Food Quality 38(6): 413-421. https://doi.org/10.1111/jfq.1217015

20. OLSZEWSKA M, WOLBIŚ M 2001 Flavonoids from the flowers of *Prunus spinose* L. Acta Pol Pharm 58(5): 367–372.

21. EGEA T, MARIA SIGNORINI A, ONGARO L, D, DE CASTRO CO, BRUSCHI P 2016 Traditional alcoholic beverages and their value in the local culture of the Alta Valle del Reno, a mountain borderland between Tuscany and Emilia-Romagna (Italy) J Ethnobiology Ethnomedicine 12:27. https://doi.org/10.1186/s13002-016-0099-6

22. ALARCON R, PARDO-DE-SANTAYANA M, PRIESTLEY C, MORALES R, HEINRICH M 2015 Medicinal and local food plants in the south of Alava (Basque Country, Spain). J Ethnopharmacol 24(176): 207-224. https://doi.org/10.1016/j.jep.2015.10.022

23. ALEJANDRE M, ANSORENA D, CALVO MI, CAVERO RY, ASTIASARÁN I 2019 Influence of a gel emulsion containing microalgal oil and a blackthorn (*Prunus spinosa L.*) branch extract on the antioxidant capacity and acceptability of reduced-fat beef patties. Meat Sci 148: 219-222. https://doi.org/10.1016/j.meatsci.2018.05.022

24. MIKULIC-PETKOVSEK M, STAMPAR F, VEBERIC R, SIRCELJ H 2016 Wild Prunus fruit species as a rich source of bioactive compounds. J Food Sci 81(8):C1928-37. https://doi.org/10.1111/1750-3841.13398

25. MESCHINI S, PELLEGRINI E, CONDELLO M, OCCHIONERO G, DELFINE S, CONDELLO G, MASTRODONATO F 2017 Cytotoxic and apoptotic activities of *Prunus spinosa* trigno ecotype extract on human cancer cells. Molecules. 22(9): 1578. https://doi.org/10.3390/molecules22091578

26. MENENDEZ-BACETA G, ACEITUNO-MATA L, TARDÍO J, REYES-GARCÍA V, PARDO DE SANTAYANA M 2012 Wild edible plants traditionally gathered in Gorbeialdea (Biscay, Basque Country). Gen Res Crop Evolut 59: 1329-1347. https://doi.org/10.1007/s10722-011-9760-z

27. Act on Animal Welfare Zagreb, Croatia 2013 Official Gazette of the Republic of Croatia NN 55/2013, (1129) [in Croatian]

28. Bioethic approval of the experimental procedure within the project 2014 Document No. 251-58-10617-14-37, Faculty of Science, University of Zagreb, Croatia. [in Croatian]

29. Guide for the care and use of laboratory animals 2011 Washington DC, USA: National Academies Press: 86-123

30. ELEZ GAROFULIĆ I, ZORIĆ Z, PEDISIĆ S, BRNČIĆ M, DRAGOVIĆ-UZELAC V 2018 UPLC-MS profiling of blackthorn flower polyphenols isolated by ultrasound-assisted extraction. J Food Sci 83(11): 2782-2789. https://doi.org/10.1111/1750-3841.14368

31. LOVRIĆ V, PUTNIK P, KOVAČEVIĆ DB, JUKIĆ M, DRAGOVIĆ-UZELAC V 2017 Effect of microwave-assisted extraction on the phenolic compounds and antioxidant capacity of blackthorn flowers. Food Technol Biotechnol 55(2): 243-250. https://doi.org/10.17113/ftb.55.02.17.4687.19

32. DRAGOVIĆ-UZELAC V, BURSAĆ KOVAČEVIĆ D, PEDISIĆ S, LEVAJ B, ELEZ GAROFULIĆ I, ZORIĆ Z, REPAJIĆ M 2014 Polyphenol composition and antioxidant capacity of Croatian wild fruits. Book of Abstract, 7th Central European Congress on Food/ Food Chain Integration / Kakurinov, Vladimir (Ed.). – Skopje: Consulting and Training Center KEY, 2014. 94-94

33. GANGULY S, G TK, MANTHA S, PANDA K 2016 Simultaneous Determination of black tea-derived catechins and theaflavins in tissues of tea consuming animals using ultra-performance liquid-chromatography tandem mass spectrometry. PLoS One 11(10): e0163498. https://doi.org/10.1371/journal.pone.0163498

34. SERRA A, MACIÀ A, ROMERO MP, PIÑOL C, MOTILVA MJ 2011 Rapid methods to determine procyanidins, anthocyanins, theobromine and caffeine in rat tissues by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 879(19): 1519-28. https://doi.org/10.1016/j.jchromb.2011.03.042

35. LOWRY DH, ROSEBROUGH NJ, FARR AL 1951 Protein measurement with the Folin–phenol reagent. J Biol Chem 193: 265–75.

36. OHKAWA H, OHISHI N, YAGI K 1979 Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95(2): 351–358. https://doi.org//10.1016/0003-2697(79)90738-3

37. FLOHÉ L, ÖTTING F 1984 Superoxide dismutase assays. Methods Enzymol 105: 93-104. https://doi.org/10.1016/S0076-6879(84)05013-8

38. AEBI H. Catalase in vitro 1984 Methods Enzymol 105: 121–6. https://doi.org/10.1016/S0076-6879(84)05016-3

39. SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.

40. ĐIKIĆ D, BALTA V, KMETIĆ I, MURATI T, ORSOLIĆ N, DRAGOVIC UZELAC V, LANDEKA JURČEVIĆ I 2018 UPLC/MS analysis of plasma bioavailability of 32 polyphenols in C57BL/6 mice treated with single acute dose (24 h) of flower extract of the blackthorn *Prunus spinosa* L. Mol Exp Biol Med 2: 23-31.

41. CHEN RY 2013 Brain bioavailability of polyphenols: implications for delivery of brain health benefits. Open Access. Purdue University e-pubs. Dissertations. pp194. 2013. https://docs.lib.purdue.edu/cgi/viewcontent.cgi?article=1020&context=open_access_dissertations Last accessed 19-07-2022

42. TENG H, CHEN L 2018 Polyphenols and bioavailability: an update. Crit Rev Food Sci Nutr 59(13): 2040-2051. https://doi.org/10.1080/10408398.2018.1437023

43. TERAO J 2017 Factors modulating bioavailability of quercetin-related flavonoids and the consequences of their vascular function. Biochem Pharmacol. 139: 15-23. https://doi.org/10.1016/j.bcp.2017.03.021.

44. PRASAIN JK, PENG N, DAI Y, MOORE R, ARABSHAHI A, WILSON L, BARNES S, MICHAEL WYSS J, KIM H, WATTS RL 2009 Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. Phytomedicine 16(2-3): 233-43. https://doi.org/10.1016/j.phymed.2008.08.006

45. DE BOER VC, DIHAL AA, VAN DER WOUDE H, ARTS IC, WOLFFRAM S, ALINK GM, RIETJENS IM, KEIJER J, HOLLMAN PC 2005 Tissue distribution of quercetin in rats and pigs. J Nutr 135(7): 1718-25. https://doi.org/10.1093/jn/135.7.1718

46. HO L, FERRUZZI MG, JANLE EM, WANG J, GONG B, CHEN TY, L škola dugave OBO J, COOPER B, WU QL, TALCOTT ST, PERCIVAL SS, SIMON JE, PASINETTI GM 2013 Identification of brain-targeted bioactive dietary quercetin-3-O-glucuronide as a novel intervention for Alzheimer's disease. FASEB J 27(2): 769-81. https://doi.org/10.1096/fj.12-212118

47. HUEBBE P, WAGNER AE, BOESCH-SAADATMANDI C, SELLMER F, WOLFFRAM S, RIMBACH G 2010 Effect of dietary quercetin on brain quercetin levels and the expression of antioxidant and Alzheimer's disease relevant genes in mice. Pharmacol Res. 61(3): 242-6. https://doi.org/10.1016/j.phrs.2009.08.006

48. DOMINKO K, ĐIKIĆ D 2018 Glutathionylation: a regulatory role of glutathione in physiological processes. Arh Hig Rada Toksikol 69(1): 1-24. https://doi.org/10.2478/aiht-2018-69-2966

49. DOMINKO K, DIKIC D, HECIMOVIC S 2018 Enhanced activity of superoxide dismutase is a common response to dietary and genetically induced increased cholesterol levels. Nutr Neurosci 17: 1-13. https://doi.org/10.1080/1028415X.2018.151102 1511027

50. DIKIC D, JUTRIC D, DOMINKO K 2017 The dual nature of the antiepileptic drug valproic acid, with possible beneficial effects in alzheimer's disease. SEEMed J 1(1): 74-89. https://doi.org/10.26332/seemedj.v1i1.26

51. COX KHM, SCHOLEY A 2016 Polyphenols for Brain and Cognitive Health In: Recent Advances in Polyphenol Research. Editors(s): Kumi Yoshida, Véronique Cheynier, Stéphane Quideau. pp.59-288. https://doi.org/10.1002/9781118883303

52. SQUILLARO T, CIMINI A, PELUSO G, GIORDANO A, MELONE MAB 2018Nano-delivery systems for encapsulation of dietary polyphenols: An experimental approach for neurodegenerative diseases and brain tumors. Biochem Pharmacol 154: 303-317. https://doi.org/10.1016/j.bcp.2018.05.016