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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.*

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Abstract:
Bioavailability, biotransformation, bioaccumulation, metabolic conversion, concentrations in organs determine the real impact of polyphenols (PPH) on health. Low number of such studies exists compared to the number of studies on their physiological effects. In this model (*in vivo*) study, polyphenolic water extract (PSE) from the flowers of the blackthorn (*Prunus spinosa L.*), a traditionally consumed plant, reexamined industrially and pharmacologically as a medicinal and nutriceutical plant, was used to investigate the plasma bioavailability of 32 PPF given to the C57BL/6 mice as a single acute dose of 25 mg/kg body weight of total PPH. Concentrations were measured at 15 min, 30 min, 1h, 2h, 4h, 6h and 24h post treatment by UPLC/MS. Approximately, 53.1% out of all compounds present in PSE were detected in mouse plasma. Significantly higher Cmax/AUClast value than in the control was found in 31.2% of PPH from PSE. This indicate moderate to low absorption potential. Phenolic acids were best bioapsorbed followed by flavonols (kaempherol PPH> quercetine PPH, apigenin and luteolin). Excretory or distributional phase towards organs occurred at most up to 2 hours post treatment. Results indicate good organ biodistribution and should be further studied in organ biodistribution experiment of acute and prolonged subchronic dosing.

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INTRODUCTION

Polyphenols (PPH) are large group of at least 8000 diverse molecules, present in food as secondary plant metabolites. They are abundant in medicinal and spice plants, honey and bee products, nuts and seeds, fruits and vegetables. Commonly occurring dietary polyphenols are flavonoids and phenolic acids.[1-6]
Pharmacological investigation of polyphenols is in a focus of research. Studies of polyphenols have been historically driven by epidemiological studies indicating that certain types of food might have beneficial health effects. An example is research on genistein, a soy isoflavone that emerged in the 1980s throughout epidemiological studies of Japanese population which shown lower incidence of prostate and breast cancer compared to the population in the United States. Such observation brought analysis of the differences in diet, among other epidemiological factors. Genistein, an isoflavone was screened as a common molecule mostly present in the Japanese diet and for the most part absent in American diets. Later experimental biomedical research showed that genistein is one of the first (natural) non-cytotoxic tyrosine kinase inhibitors that can suppress growth of cancer cell. Only afterward some modern drugs were developed whose mechanism of action is inhibition of tyrosine kinase. Similar reasons lead scientists to study polyphenolic physiological impacts. Such plant derived

molecules are available, affordable, and possibly less toxic than designed drugs and at certain concentrations they can hit desired targets.[7] Current research reports that polyphenols are known to inhibit specific enzymes, influence redox balance in the cell, stimulate transcriptional factors, hormones, neurotransmitters or mediate transitional metals (copper and iron), modulate receptors, interfere with cellular signal pathways and neutralize oxidation processes of free radicals in human and animal cells.[4-9]

Such biological effects depend on their bioavailability after intake i.e. intestinal absorption, metabolism, blood distribution and secretion from the organism. Thus, knowledge on pharmacokinetics and bioavailability may determine the real impact of the daily intake of these components on the protection and enhancement of the health status of the organism.[9] The number of reports on the bioavailability is extremely low compared with the increase in published studies on polyphenolic physiological effects that appear in recent years. Little information is available on the bioavailability (and/or bioaccumulation) with the exception of quercetin and a few other flavonoids such as catechins. Lack of information on bioavailability, biotransformation, bioaccumulation, the significance of the metabolic conversion, effective concentration in the organism should be complemented with more additional data.[11]

Biomedical studies of polyphenolic bioapsorption, metabolism and bioavailability employ natural plant polyphenolic extracts and examine the bioavailability in vitro and in model animals such as rat, mice, pigs etc. We designed this *in vivo* model-study to evaluate the bioapsorption potential and plasma entry of major polyphenolic molecules (flavones, flavonoles, phlenolic acids and flavonoides) from a natural complex polyphenolic mixture i.e. natural plant extracts. We used polyphenolic water extract from the flowers of the blackthorn (*Prunus spinosa L.*) as a model-mixture. Blackthorn is a rich source of polyphenolic molecules and presents a good model polyphenolic mixture to explore the affinity of intestinal bioapsorption in mice. The fruits of the blackthorn were consumed traditionally in Croatia usually among village population, during fall - winter months. Fruits were usually thermally processed to reduce bitterness and consumed. The consumption in the second half of 20 century declined, and in the 21 century it was completely abandoned, probably as a consequence of post war urbanization and decline of elderly village population that was accustomed on its consumption. In other Mediterranean countries it is still consumed in various food products. In recent years all parts of plant are reexamined industrially and pharmacologically as a medicinal plant, nutriceutical, as potential pharmacological source of polyphenols and as functional food.[8, 12-15]

MATERIAL AND METHODS

Study design in vivo: animals and diets

Inbred C57BL/6 mice, weighing 30±1.5 g were obtained from the rodent breeding facility of the Department of Animal Physiology, Faculty of Science, University of Zagreb, Croatia. Animals were fed a standard laboratory diet, received tap water *ad libitum* and kept under regime of 12 hours of light per day. The standardized diet was 4 RF 21, Mucedola, Settimo Milanese, Italy. Composition of standardized pellet mouse feed contained wheat, wheat straw, hazelnut skins, maize, soy bean hulled, corn gluten feed, fishmeal, dicalciumphosphate, sodium chloride, whey powder, soy bean oil, yeasts, 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 the valid guidelines in the Republic of Croatia Act on Animal Welfare.[16] The experimental procedure was approved by the Bioethics Committee of the Faculty of Science, University of Zagreb.[17] The procedures were conducted according to Guidelines on in vivo experiments and accepted and international standards.[18]

The inbred C57BL/6 mice were randomly divided according to treatment (C-Control group and PSE group - *Prunus spinosa* Extract group) and time of sacrifice post treatment. Animals from both C or PSE group were sacrificed at 0h, 0.25h (15min), 0.5h (30min), 1h, 2h, 3h, 6h, 24h (1st day) post treatment. Each time/sacrifice 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 acutely as single oral dose.

The PSE treated group received the dose of total polyphenol in the administered PSE concentration of 25 mg/kg body weight (bw) of total polyphenols (Table 1). Correspondingly, the doses of individual polyphenol molecules followed in this study are also given in Table 1.

Prunus spinosa extract (PSE)

The preparation of the PSE extract and details on polyphenolic content and individual polyphenolic compounds are is described in detail in Lovric et al.[19] and Dragovic Uzelac et al.[20] However, the measurement of concentration of individual polyphenolic compounds were described in named reports assayed with HPLC method. Within this research, we employed the more sophisticated (than HPLC) method of Ultra-Performance Liquid-Chromatography Tandem Mass Spectrometry (UPLC-MS), which is recommended by most recent literature[21] as more accurate for organ analysis. We also analyzed the polyphenolic content of Mucedola feed pellet and

PSE solution that was given to mice (Table 1). Details on the method are covered in sections: Enzymatic hydrolysis as a preparation for the UPLC-MS Analysis and section Determination of polyphenolic component in PSE and plasma by UPLC-MS Analysis.

Blood samples

Blood samples were collected from the heart of halothane anesthetized mice into heparinized tubes and animals were sacrificed by decapitation. The blood samples were mixed thoroughly to prevent blood clotting and were centrifuged at 2000 g for 10 minutes. The resultant plasma was mixed with one-tenth the volume of an ascorbate-EDTA preservation solution (20% ascorbic acid and 0.01% Na_2-EDTA dissolved in a 0.4 M sodium phosphate buffer, final pH 3.6), and the mixture was stored at -80°C until use.

Enzymatic hydrolysis as a preparation for the UPLC-MS Analysis

The sample (300 μL of PSE extract or 100 μL of plasma) was mixed with 10 μL of a mixture of β-glucuronidase (250 units/mL) and sulfatase (20 units/mL), 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 UPLC-MS analysis. The method was adopted from Ganguly et al.[21]

Determination of polyphenolic component in PSE and plasma by UPLC-MS analysis

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, 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

To prepare the standard stock solution, the standards were dissolved in methanol (1 mg/L) and a multi compound standard solutions were prepared in methanol for external calibration of the UPLC-MS/MS system. The areas of the peaks of each standard were used to make the respective standard curves.

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 a Agilent 1290 RRLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary gradient pump, auto-sampler and column compartment. The column oven was maintained at 35°C and flow rate was at 0.35 mL/min. The gradient conditions were according to method reported by Serra et al. and Ganguly et al.[21, 22] with minor modifications. The mobile phase consisting of redistilled water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Flow rate was at 0.35 mL/min. The mass spectrometry was performed on a triple quadrupole mass spectrometer (QQQ 6430, Agilent, Santa Clara, CA, USA). Agilent MassHunter Workstation Software was used for data acquisition and analysis. The mass spectrometer was used in the dynamic multiple reaction monitoring mode (dMRM) in the ESI-positive and negative mode 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 was set at 40 psi. The total analytical time was 12.5min. The analytes were identified by comparing retention times and mass spectra with the mass spectra of the corresponding standards tested under the same conditions. The calibration curves obtained in the dMRM mode were used for quantification of all analytes. In case of unavailability of 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.[21, 22]

Statistical analysis

The main pharmacokinetic parameters (Tmax, Cmax, AUClast) are expressed as mean value of each group respectively. The pharmacokinetic data was analyzed by Phoenix 64 software, SERTAA version f.0.03176. The data were compared for statistical significance ($p \leq 0.05$) between the groups by t-test and analyzed by SPSS version 17.0 software.[23]

RESULTS

The UPLC-MS analysis of polyphenolic compounds in applied PSE extract, mouse feed pellets and doses of individual polyphenolic compounds

There were a total of 32 polyphenolic compounds analysed by UPLC/MS in *Prunus spinosa* extract (PSE) and 26 in Mucedola standard mouse feed (Table 1). Within the extract, the highest concentrations were 3-O-caffeoylquinic and 3-p-coumaroylquinic acid, (+)-catechin and (-)-epicatechin among flavan 3-ols, kaempferol- and quercetin-pentoside and kaempferol-rhamnoside. The Table 1 also shows individual doses of each polyphenolic compound applied to mouse (mg/kg bw per single dose).

The UPLC-MS analysis of polyphenolic component in the plasma of C57BL/6 mice

Only 17 polyphenols (53.1 %) out of 32 (in PSE) were detected in the plasma of PSE treated animals (Table 2). Only 10 polyphenols (out of 32 present in PSE and out of 17 detected in plasma), had significantly different ($p \leq 0.05$) plasma AUClast and/or Cmax in PSE treated animals, compared to the control animals (Table 2).

Major detected groups were phenolic acids (N=5 detected), flavan-3-ols (N=2 detected), kaempherol polyphenols (4 detected), quercetin polyphenols (N=4 detected) and apigenin and luteolin.

Table 1. The UPLC-MS analysis of polyphenolic component in *Prunus spinosa* extract (PSE) and Mucedola mouse standard feed pellets and doses of the total and individual estimated doses of polyphenols applied to C57BL/6 mice.

	Polyphenols in PSE				TP in *P.spinosa* dry flower (mg/100 g dw)		Dose of TP in PSE (mg/kg bw of C57BL/6 mouse)	
					2508.6		25.000	
No.	Compound name	**RT**	**m/z**	**m/z (prod.)**	**Concentration of individual polyphenolic compounds in *P.spinosa* dry flower (mg/100 g dw)**	**Concentration of individual polyphenolic compounds in PSE (µg/mg TP)**	**Dose of individual polyphenolic compounds from PSE (µg/kg bw of C57BL/6 mouse)**	**Concentration of individual polyphenolic compounds in Mucedola feed pellet (µg/100 mg dw)**
1	Caffeic acid	4.387	179	135	34.32	13.681	342.023	0.175
2	3-O-caffeoylquinic acid (neochlorogenic acid)	3.979	353	191	192.00	76.537	1913.418	<LOD
3	4-O-Caffeoquinic Acid	4.444	353	173	24.04	9.583	239.576	0.070
4	Chlorogenic acid	3.776	353	191	55.47	22.112	552.798	0.018
5	p-coumaric acid	5.764	163	119	23.67	9.436	235.889	0.055
6	3-p-coumaroylquinic acid	3.507	337	163	216.00	86.104	2152.595	<LOD
7	4-p-coumaroylquinic acid	5.181	337	173	61.53	24.528	613.191	0.077
8	Ferullic acid	6.427	193	134	8.69	3.464	86.602	0.098
9	3-O-feruloylquinic acid	4.043	367	193	132.2	52.699	1317.468	<LOD
10	Gallic acid	1.245	169	125	1.75	0.698	17.440	0.133
11	(+)-Catechin	3.796	291	139	85.67	34.151	853.763	0.026
12	(-)-Epicatechin	4.829	291	139	70.16	27.968	699.195	0.068
13	(-)-Epicatechin gallate	6.583	443	139	0.51	0.203	5.083	0.073
14	(-)-Epigallocatechin gallate	4.98	459	139	0.15	0.060	1.495	0.013
15	Isorhamnetin-rutinoside	7.355	625	317	4.68	1.866	46.640	2.501
16	Kaempferol-3-rutinoside	6.216	595	287	51.84	20.665	516.623	0.005
17	Kaempherol-acetylrutinoside	9.402	637	287	0.68	0.271	6.777	<LOD
18	Kaempferol-pentosylhexoside	7.291	581	287	50.27	20.039	500.977	0.024
19	Kaempferol-pentoside	8.234	419	287	494.94	197.297	4932.432	0.111
20	Kaempferol-rhamnoside	7.166	433	287	436.62	174.049	4351.232	<LOD
21	Kaempherol-acetylhexoside	11.29	491	287	0.92	0.367	9.168	0.010
22	Kaempferol rhamnosyl-hexoside	8.064	595	287	49.79	19.848	496.193	0.029
23	Kaempherol-3-glucoside	7.489	449	287	0.60	0.239	5.979	0.009
24	Apigenin	11.16	271	153	3.23	1.288	32.189	0.029
25	Luteolin	9.8	287	153	6.68	2.663	66.571	0.013
26	Quercetin-3-rutinoside (rutin)	6.448	611	303	82.35	32.827	820.677	0.162
27	Quercetin acetylrutinoside	8.596	653	303	3.18	1.268	31.691	<LOD
28	Quercetin-3-glucoside	6.737	465	303	31.29	12.473	311.827	0.015
29	Quercetin-pentoside	7.396	435	303	226.75	90.389	2259.727	0.069
30	Quercetin-acetylhexoside	5.096	507	303	2.34	0.933	23.320	0.138
31	Quercetin-rhamnoside	7.557	449	303	81.15	32.349	808.718	0.057
32	Quercetin-pentosyl-hexoside	6.605	597	303	56.81	22.646	566.152	0.013

Legend: PSE- *Prunus spinosa* extract; TP - Total polyphenols; dw - dry weight, bw - body weight; <LOD - bellow limit of detection

Table 2. The UPLC-MS analysis of polyphenolic content and basic pharmacokinetic parameters of plasma absorbed polyphenolic compounds in the period of 24 hours, compared between control C57BL/6 mice (PBS saline) and mice that received single acute oral dose of *Prunus spinosa* extract (PSE), applied as 25 mg/kg bw of total polyphenolic water soluble content. Measurements were made in times 15 min, 30min, 1h, 2h, 3h, 6h and 24h post application.

Plasma 0-24 h Acute dose Compound	**Control Group**			**PSE Group**		
	Tmax (h)	**Cmax** (µg/mL)	**AUClast** (h*µg/mL)	**Tmax** (h)	**Cmax** (µg/mL)	**AUClast** (h*µg/mL)
Phenolic acids						
3-O-Caffeoylquinic acid	-	<LOD	-	2	**0.004**#	**0.010**#
4-p- Coumaroylquinic acid	0.25	0.097	1.347	0.5	0.105	**2.342**#
Caffeic acid	0.25	0.004	0.089	0.5	**0.011**#	0.010
Ferulic acid	0.5	0.157	2.385	0.5	0.157	2.654
p-Coumaric acid	0.25	0.010	0.004	2	0.098	**0.121**#
Flavan-3-ols						
(+)-Catechin	0.25	0.014	0.311	0.25	0.013	0.310
(-)-Epigallocatechin-3-gallate	2	0.031	0.715	2	0.030	0.715
Flavonols						
Kaempherol pentoside	0.25	0.004	0.089	2	0.045	0.059
Kaempherol rhamnoside	0.5	0.006	0.136	2	**0.088**#	**0.261**#
Kaempherol rhamnosyl-hexoside	-	<LOD	-	0.25	**0.005**#	**0.014**#
Kaempherol-3-rutinoside	-	<LOD	-	0.25	**0.006**	**0.057**#
Apigenin	0.5	0.028	0.556	0.25	**0.080**#	**0.911**#
Luteolin	0.5	0.022	0.363	0.25	0.044	**0.671**#
Quercetin-3-glucoside	-	<LOD	-	2	**0.004**#	**0.010**#
Quercetin-3-rutinoside (rutin)	0.5	0.144	3.404	0.25	0.142	3.330
Quercetin-pentoside	-	<LOD	-	2	0.008	0.004
Quercetin-pentosyl-hexoside	0.5	0.014	0.326	2	0.014	0.333

Legend: # - the values are statistically different ($p \leq 0.05$) between control and PSE treated group.
PSE - *Prunus spinosa* extract; bw - body weight; <LOD - bellow limit of detection; Cmax - maximal concentration of the compound in plasma; Tmax - average time when compound reach Cmax; AUClast - Area under the curve, a pharmacokinetic parameter.

Phenolic acids were the group of polyphenolic molecules that was absorbed and detected the most (Table 2, Figure 1). Among phenolic acids, the caffeic acid and p-coumaric acid were present in plasma only up to 3 hours post application of PSE extract. The 3-O-caffeoylquinic acid, although in low concentration, was present up to 6 hours after treatment (Figure 1).
Kaempherol compounds were the second best bioapsorbed group after phenolic acids (Table 2, Figure 1). The polyphenols from the flavan-3-ols group ((+)-catechin and (-)-epigallocatechin-3-gallate), although detected in plasma of PSE animals did not have significantly ($p \leq 0.05$) higher concentration than control in the plasma after PSE treatment.
Among 7 different quercetin polyphenols present in the PSE (Table 1), only 4 different quercetin polyphenols detected in the plasma (Table 2). Quercetin-3-glucoside was the only quercetine compound that was significantly different in PSE group compared to the the control animals by measured concentration and AUClast.
None of the detected polyphenols were held longer than 6 hours in plasma of PSE animals, and 5 of 11 were down to baseline levels within 3 hours of PSA treatment (Figure 1).
Maximal concentrations of bioapsorbed polyphenolic compounds did not exceed 0.1 µg/mL. All other significantly present polyphenols were lower in concentration.

DISCUSSION

Within this study we monitored a total number of 32 polyphenols detected in PSE. Out of these compounds, 53.1% was detected in the plasma of PSE treated animals. However, only 31.2% of PSE monitored polyphenols had significantly higher Cmax and/or AUClast than in the control animals.
Only these significantly different polyphenols, with a certain degree of confidence, could be declared to be a consequence of PSE treatment. For other detected polyphenols we can not be sure whether their level is a consequence of compound present in feed pellets of mice. Nevertheless, the results of total number of detected polyphenols (and compounds with significantly higher Cmax / AUClast), together with the concentration maximums of up to 0.1 µg/mL or lower, indicate relatively moderate to low bioabsorption potential of polyphenols in the study.
Such findings were somewhat expected, since majority of the bioavailability studies or reviews mention low plasma bioavailability, especially in the acute designed studies.[11, 24, 25]. Most similar studies employing natural plant extracts which estimated polyphenolic intake

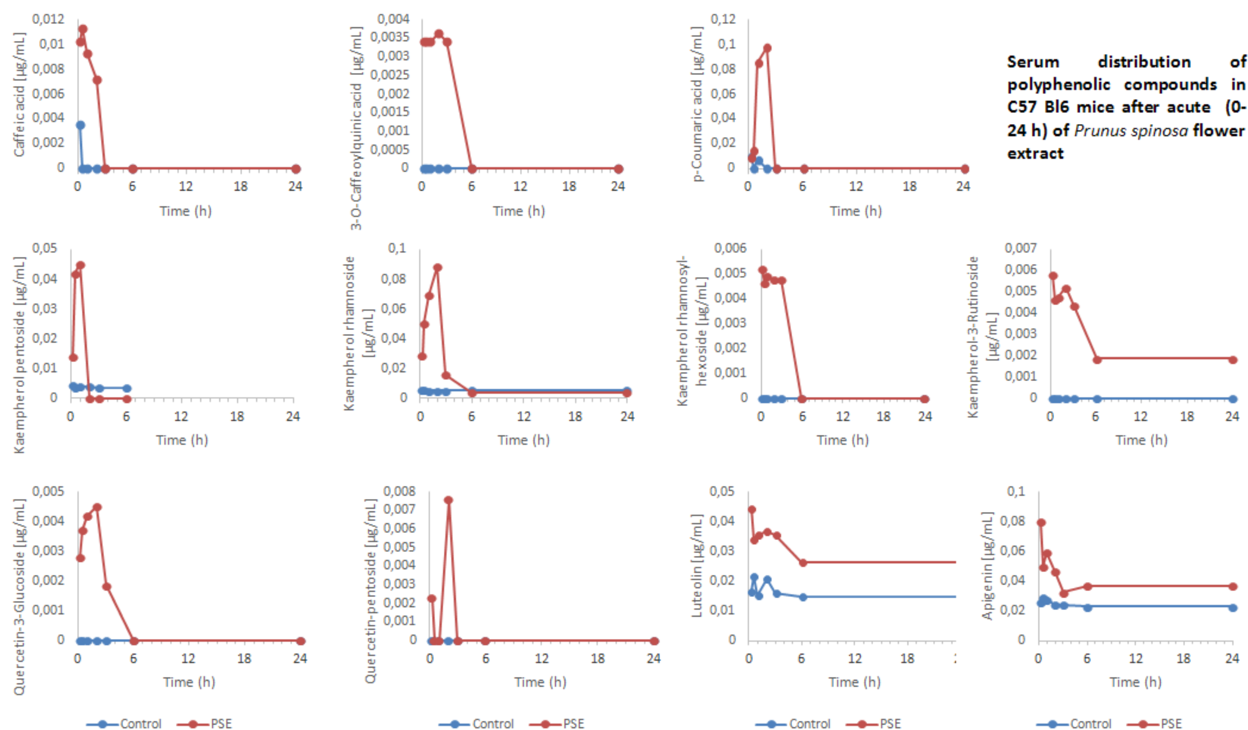

Figure 1. Plasma pharmacokinetics (0-24 h) of selected polypehenolic compounds in C57BL/6 mice after acute single dose of Prunus spinosa flower water extract.

describe the bioapsorption of flavonols (kaempferol, myricetin and quercetin), flavones (apigenin and luteolin), flavan-3-ols which include (-)-catechin, (+)-epicatechin (EC), (+)-epicatechin-3-gallate (ECG), (+)-epigallocatechin (EGC) and (+)-epigallocatechin-3-gallate (EGCG).[24] The results further show that phenolic acids were the best bioapsorbed, followed by flavonols (kaempherol and quercetine, apigenin and luteolin). The flavan-3-ols (catechin compounds) were poorly absorbed and distributed in the plasma of both control and PSE treated animals. Among detected groups, there were 5 detected phenolic acids, 2 detected flavan-3-ols, 4 detected kaempherol, 4 detected quercetin compounds, apigenin and luteolin. Polyphenols from the flavan-3-ols group, although detected in plasma of PSE animals, did not have significantly ($p \leq 0.05$) higher concentration than control or high concentration in the plasma of PSE animals.

Within literature, UPLC-MS analysis in the experiment of oral administration of two types of black tea given as 5% infusion to guinea pigs for 14 days, showed detectable accumulation of all major polyphenols in the plasma of the treated animals. The mean levels of epicatechin-gallate in the plasma of the first type of tea in animals was found to be approximately 12.9 ng/mL while that of epicatechin, epigallocatechin gallate and epicatechin gallate were 11.6 ng/mL, 130.8 ng/mL and 34.6 ng/mL respectively. In plasma of animals consuming other tea type approximately 8.4 ng/mL of epicatechin gallate, 7.6 ng/mL epicatechin, 40.2 ng/mL of epigallocatechin gallate and 19.3 ng/mL of epicatechin gallate were detected.[21] Furthermore, for example, Harada et al.[26] using Wistar rats treated with epicatechin aglycon in the acute gavage dose of 100mg/kg bw, determined Cmax (nmol/l) for epicatechin to be 1090, for epicatechin glucuronide to be 15560 and for 3'-o-methyl-epicatechin to be 3760. Silva et al.[27] treated Wistar rats epicatechin and catechin aglycon with acute gavage in the dose of 50mg/200g bw determined Cmax (nmol/l) after 1st and 6th hour to be for epicatechin-sulfate 13.3 (1h), epicatechin glucuronide 3.6 (6h), epicatechin sulfoglucuronide 52.1 (1h), methyl epicatechin 5.4 (6h). In the experiment of Abd el Mohsen et al.[28] using Wistar rats were treated with epicatechin aglycon in the acute gavage dose of 100 mg/kg bw and whose blood was taken 2h after treatment (experiment with one time point only), and polyphenols were enzymatic determined, high AUC (nmol*h/l) were recorded. Namely: for the epicatechin AUC=4500, for the 3-O-methyl epicatechin AUC=1220, for the epicatechin-glucuronide AUC=44640, for the 3-o-methyl epicatechin-glucuronide AUC=14400 and for the 4-o-methyl-picatechin- glucuronide AUC=1160.

Comparison of our results to the cited literature data leads to the conclusion that the percentual concentrations absorbed were similar but the number of absorbed catechins was lower probably as a consequence of low catechin presence in the PSE extract compared to other natural plant extracts such as tea. After absorption, tea catechins which are in abundance undergo extensive bioconversion *in vivo* mainly through glucuronidation, sulfation and methylation. Such metabolic conversions are catalyzed

by diverse enzymes like UDP-glucuronosyltransferases (UGT), catechol-O-methyltransferases (COMT), cytosolic sulfotransferases (SULT). Liver is the primary location of such bio-transformation reactions. Moreover, both glucuronidation and sulfation have been reported in other tissues like lung and kidney. Besides, (-)-epicatechin is rapidly decomposed and metabolized into galloylated epicatechins, epicatechin gallate and epigallocatechin gallate through enterocytes or hepatocytes.[21, 25] When plasma presence of flavan-3-ols is relatively poor, they are known to bind macromolecules and nutritional substances such as proteins and polysaccharides, thereby escaping being measured.[25] Beside lower presence in PSE than in other water extracts such as tea, molecular interactions with proteins and polysaccharides are the other probable reason for low bioavailability in the plasma detected in this study.

In the experiment of Goldberg et al.[29] in the human subjects, where quercetin was measured after treatment with in solution v-8, wine or juice in acute oral dose of 10 mg/70kg bw of quercetin concentration (free and conjugated), authors reported: AUC (nmol*h/l) of 0.53 (0-4h) and Cmax (nmol/L) of 0.21 for tested substance v-8, Cmax (nmol/L) of 0.42and AUC (nmol*h/l) of 0.88 (0-4h) for wine and AUC (nmol*h/l) of 0.0033 (0-4h) and Cmax (nmol/L) of 0.16 for juice. Three polyphenols were present in plasma and urine predominantly as glucuronide and sulfate conjugates, reaching peak concentrations in the former around 30min after consumption. The free polyphenols accounted for 1.7 to 1.9% (trans-resveratrol), 1.1 to 6.5% ([+]-catechin) and 17.2 to 26.9% (quercetin) of the peak plasma concentrations. In that study, similar as in ours the [+]-catechin was the poorest by distributed by the criteria of urine 24-h excretion (and accounted for the 1.2%-3.0% of the consumed dose). By same criteria the quercetin was intermediate (urine 24-h excretion 2.9%-7.0% of the consumed dose). Within our study, we concluded that flavonols (kaempherol and quercetines) were second-best (intermediately) bioapsorbed because out of 7 different quercetin compounds that were present in the PSE, 4 quercetin compounds (57.1 %) were detected in the plasma even though only quercetin-3-glucoside was the most significant one by measured concentration. Graefe et al.[31] treated human subjects with quercetin in the acute oral dose of 100 mg quercetin (free and conjugated) and determent plasma quercetin 4'-glucoside AUC (nmol*h/l) of 27.8 (0-24h) and Cmax (nmol/L) of 7. Such concentrations slightly resemble the results in our mice model. Considering differences in the species and the amount of tested compound it is well to say that the quercetine-3-glucoside entry into plasma has similar biological properties when being absorbed regardless of the species.

Literature reference quote that quercetine glucosylation boost its bioavailability by uplifting the accessibility to the absorptive cells. Prenylation improve bioaccumulation at the target site by escalating the cellular uptake. The concentration of the total conjugated metabolites usually reaches μM level within a few hours after the intake of quercetin, but rarely exceed 10 μM, even though an extremely high quantity of quercetin is orally administered. Its half-life is approximately 4h in mammalian organism. Quercetin supplementation in the doses of 500 or 1000 mg/day over 12 weeks caused a significant increase in plasma quercetin, with a net raise of 332 ± 21.0 and 516 ± 30.8 μg/L (analogous to 1.10 ± 0.07 and 1.71 ± 0.10 μM), respectively.[11] Literature data on absorption rate for the quercetine-glycosides demonstrate that it is 52%, more present than aglycone forms (24%) in humans, and intake of quercetin glycoside express further rapid and larger increase of quercetin levels in plasma than intake of for example rutinoside forms of quercetin.[11, 25, 31-33] Flavonoids can be transferred into the blood circulation and subjected to metabolic conversion through phase II enzymes during the absorption process, resulting in conjugated metabolites in the circulation. Terao et al. Scholz and Wiliamson, Gonzales et al.[25, 31, 32] further explain that glucose-bound quercetin is deglucosylated to aglycone by lactase-phlorizin hydrolase (LPH) or cytosolic b-glucosidase (CBG) at the absorptive intestinal cells, and then conjugated into glucuronide/sulfate conjugates before its absorption. Food matrix plays an essential role in the bioavailability of quercetin from plant foods. Dietary flavonoids are mostly excreted to the feces without intestinal absorption or decomposed to ring-scission products by the action of enterobacteria in the large intestine. According to such data the best absorption recorded for quercetin-3-glucoside was in concordance with literature. However, once polyphenolic compounds or their conjugated metabolites manage to pass through the intestinal barrier, they are transported through the circulatory system to their target tissues. Blood concentrations and bioactivity of these compounds may be influenced by their affinity to proteins inherent in the blood. As with food proteins flavonoids have been shown to interact with blood proteins mostly human plasma albumin (HSA).[11, 25, 30-32] Therefore, low concentrations detected in plasma might also be a consequence of such interactions.

None of the absorbed polyphenols were held longer than 6 hours in plasma of PSE animals, and 5 of 11 were down to baseline levels within 3 hours of PSA treatment (Figure 1). Also, our results indicate that all compounds entered to excretory or bioaccumulation phase towards organs up to at most 2 hours post treatment (based on downwards direction of PK concentration slope). Eight polyphenols had Tmax at 2 h post treatment and declined in concentration, 3 had decline in plasma concentration 0.5 h (30 minutes) after treatment and 6 had decline after 0.25 (15 minutes) after treatment.

This observation indicates that the absorbed polyphenols are readily distributed toward organs or eliminated from the body in rather quick time (considering 24h monitoring). The reasons to believe

that the first scenario is the more probable one, is because polyphenols (including flavonoids) move beyond systemic circulation to exert their bioactivities even if their bioavailabilities are so poor. Recent studies indicated the presence of charged flavonoid conjugates which readily move toward remote tissues as biologically active charged flavonoid molecules even in low concentrations.[7] The hydrophobic coplanar structure of flavonoids is likely to promote their affinity to the phospholipid bilayer of biomembranes.[11] Only around 30 % out of 32 polyphenols present in PSE, that were detected in plasma had significantly higher Cmax and/or AUClast value than the control animals. Such results indicate moderate to low absorption potential of polyphenolic compounds after PSE treatment. Nevertheless, literature data from other studies show that the bioaccumulation efficacy of polyphenols is much lower than that of antioxidative vitamins and pro-vitamins, although their consumption is higher or comparable to such vitamins/pro-vitamins in food and some researchers provide evidence that the polyphenols in excess amounts in mammalian organism can exhibit even adverse effects and as such their absorption is limited.[11] Literature data explains mechanism by which the polyphenolic compounds with a high degree of polymerization which cannot be readily absorbed at the small intestine, reach colon and undergo microbial catabolism. Microbiome causes the formation of small polyphenols that are able to reach the liver where they are subjected to conjugation and redistribution to circulatory system. Both liver phase-II enzymes metabolized polyphenols and microbial metabolites reach the systemic circulation and are distributed to different organs and tissues, later to be excreted through the urine. Consequently, although pharmacokinetics of these metabolites provide an useful information, estimates such as of AUC (area-under-the curve values) do not necessarily yield accurate quantitative data on absorption but are informative in observations of polyphenolic dynamics.[25] Such observations from the literature coincide with our results. Polyphenols that entered blood went to excretory or bioaccumulation phase towards organs at most up to 2 hours post treatment (decline of PK slopes after maximal concentration peaks, shown in Figure 1). Such quick clearance time from plasma (ending up to 3 h or at most 6 h), might indicate good systemic biodistribution and organ bioaccumulation (or otherwise quick metabolism and elimination). In order to confirm whether the microbial gut activation (post intake), biodistribution or excretion (recorded here for all substances to start up to 2h post intake) occur, further experimental monitoring of the concentrations in various organs (intestine, liver, kidney, brain etc.) and its biological function (antioxidative defense) is necessary.[11, 25, 31, 32, 34, 35] This preliminary research sets the direction for such further experiments and its design.

CONCLUSIONS

From the practical pharmacological point of view, it would be interesting to see whether the ratios of the screened compounds within the results of this study could be applied as a formulated mixture for antioxidant effects as “nutriceuticals” (perhaps with enhanced bioavailability by substitutive conjugations). This premise is just one of the contributions of this work beside reported concentrations levels, PK curves, Cmax and AUClast for individual polyphenol compounds, usually insufficiently covered by literature. These preliminary results indicate the need for detailed study of organ biodistribution not only in acute dosing but also in prolonged subchronic (daily repeated) dosing regimen.

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REFERENCES

1. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: Extraction Methods, Antioxidative action, bioavailability and anticarcinogenic effects. molecules. 2016;21(7). pii: E901. doi: 10.3390/molecules21070901
2. Sima IA, Sârbu C. Evaluation of polyphenolic fingerprints and antioxidant profiles of wild fruits. Food Science and Technology. 2016;51(6):1433-1440. doi:doi.org/10.1111/ijfs.13111
3. Oršolić N, Sirovina D, Gajski G, Garaj-Vrhovac V, Jazvinšćak Jembrek M, Kosalec I. Assessment of DNA damage and lipid peroxidation in diabetic mice: effects of propolis and epigallocatechin gallate (EGCG). Mutat Res. 2013;757(1):36-44. doi: 10.1016/j.mrgentox.2013.04.022.
4. a) Oršolić N, Skurić J, Dikić D, Stanić G. 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. 2014;93:18-30. doi: 10.1016/j.fitote.2013.12.007.
5. b) Oršolić N, Goluža E, Dikić D, Lisičić D, Sašilo K, Rođak E, Jeleč Z, Lazarus MV, Orct T. Role of flavonoids on oxidative stress and mineral contents in the retinoic acid-induced bone loss model of rat. Eur J Nutr. 2014;53(5):1217-27. doi: 10.1007/s00394-013-0622-7.
6. Landeka I, Jurčević, Dora M, Guberović I, Petras M, Rimac S, Brnčić, Đikić D. polyphenols from wine lees as a novel functional bioactive compound in the protection against oxidative stress and hyperlipidaemia. Food Technol Biotechnol. 2017;55(1):109-116. doi: 10.17113/ftb.55.01.17.4894.
7. Hu M, Wu B, Liu Z. Bioavailability of polyphenols and flavonoids in the era of precision medicine. Mol Pharm. 2017;14(9):2861-2863. doi: 10.1021/acs.molpharmaceut.7b00545.

8. Meschini S, Pellegrini E, Condello M, Occhionero G, Delfine S, Condello G, Mastrodonato F. Cytotoxic and apoptotic activities of prunus spinosa trigno ecotype extract on human cancer cells. Molecules. 2017;22(9). pii: E1578. doi: 10.3390/molecules22091578.
9. Li Y, Zhang JJ, Xu DP, Zhou T, Zhou Y, Li S, Li HB. Bioactivities and health benefits of wild fruits. Int J Mol Sci. 2016;17(8). pii: E1258. doi: 10.3390/ijms17081258.
10. Gonzales GB, Smagghe G, Grootaert C, Zotti M, Raes K, Van Camp J. 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. 2015;47(2):175-190. doi: 10.3109/03602532.2014.1003649.
11. Terao J. Factors modulating bioavailability of quercetin-related flavonoids and the consequences of their vascular function. Biochem Pharmacol. 2017;139:15-23. doi: 10.1016/j.bcp.2017.03.021.
12. Alarcón R, Pardo-de-Santayana M, Priestley C, Morales R, Heinrich M. Medicinal and local food plants in the south of Alava (Basque Country, Spain). J Ethnopharmacol. 2015;24(176):207-224. doi: 10.1016/j.jep.2015.10.022.
13. Yuksel AK. The Effects of Blackthorn (Prunus Spinosa L.) Addition on certain quality characteristics of ice cream. J Food Quality. 2015;38(6):413-421. doi: 10.1111/jfq.1217015.
14. Mikulic-Petkovsek M, Stampar F, Veberic R, Sircelj H. Wild prunus fruit species as a rich source of bioactive compounds. J Food Sci. 2016;81(8):C1928-37. doi: 10.1111/1750-3841.13398.
15. Menendez-Baceta G, Aceituno-Mata L, Tardío J, Reyes-García V, Pardo de Santayana M. Wild edible plants traditionally gathered in Gorbeialdea (Biscay, Basque Country). Gen Res Crop Evolut. 2012;59:1329-1347. 10.1007/s10722-011-9760-z.
16. Act on Animal Welfare Zagreb, Croatia: Official Gazette of the Republic of Croatia NN 55/2013, (1129); (in Croatian).
17. Bioethic approval of the experimental procedure within the project, Document No. 251-58-10617-14-37, Faculty of Science, University of Zagreb, Croatia. 2014 (in Croatian)
18. Guide for the care and use of laboratory animals, Washington DC, USA: National Academies Press; 2011;86-123.
19. Lovrić V, Putnik P, Kovačević DB, Jukić M, Dragović-Uzelac V. Effect of microwave-assisted extraction on the phenolic compounds and antioxidant capacity of blackthorn flowers. Food Technol Biotechnol. 2017;55(2):243-250. doi: 10.17113/ftb.55.02.17.4687.19.
20. Dragović-Uzelac V, Bursać Kovačević D, Pedisić S, Levaj B, Elez Garofulić I, Zorić Z, Repajić M. Polyphenolic composition and antioxidant capacity of Croatian wild fruits. Book of Abstract, 7th Central European Congress on Food/Food Chain Integration / Kakurinov, Vladimir (ur.). - Skopje: Consulting and Training Center KEY, 2014. 94-94 (ISBN: 978-608-4565-05-5). 2014.
21. Ganguly S, G TK, Mantha S, Panda K. 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. 2016;11(10):e0163498. doi: 10.1371/journal.pone.0163498.
22. Serra A, Macià A, Romero MP, Piñol C, Motilva MJ. 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. 2011;879(19):1519-28. doi: 10.1016/j.jchromb.2011.03.042.
23. SPSS version 17.0 (SPSS Inc. Chicago. IL). Available at: https://www.hks.harvard.edu/.../A%20SPSS%20Manuals/SPSS%2
24. Chen RY. Brain bioavailability of polyphenols: implications for delivery of brain health benefits. Open Access. Purdue University e-pubs. Dissertations. pp194. 2013.
25. Teng H, Chen L. Polyphenols and bioavailability: an update. Crit Rev Food Sci Nutr. 2018:0. doi: 10.1080/10408398.2018.1437023.
26. Harada M, Kan Y, Naoki H, Fukui Y, Kageyama N, Nakai M, Miki W, Kiso Y. Identification of the major antioxidative metabolites in biological fluids of therat with ingested (+)-catechin and (-)-epicatechin. Biosci Biotechnol Biochem.1999;63(6):973-977.
27. Da Silva EL, Piskula M, Terao J. Enhancement of antioxidative ability of rat plasma by oral administration of (-)-epicatechin. Free Radic Biol Med. 1998;24(7-8):1209-1216.
28. Abd El Mohsen MM, Kuhnle G, Rechner AR, Schroeter H, Rose S, Jenner P, Rice-Evans CA. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. Free Radic Biol Med. 2002;33(12):1693-1702.
29. Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem. 2003;36(1):79-87.
30. Dominko K, Đikić D. Glutathionylation: a regulatory role of glutathione in physiological processes. Arh Hig Rada Toksikol. 2018;69(1):1-24. doi: 10.2478/aiht-2018-69-2966.
31. Graefe EU, Wittig J, Mueller S, Riethling AK, Uehleke B, Drewelow B, Pforte H, Jacobasch G, Derendorf H, Veit M. Pharmacokinetics and bioavailability of quercetin glycosides in humans. J Clin Pharmacol. 2001;41(5):492-499.
32. Scholz S, Williamson G. Interactions affecting the bioavailability of dietary polyphenols in vivo. Int J Vitam Nutr Res. 2007;77(3):224-235.
33. Gonzales GB, Smagghe G, Grootaert C, Zotti M, Raes K, Van Camp J. 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. 2015;47(2):175-190. doi: 10.3109/03602532.2014.1003649.
34. Dominko, K., Đikić, D. Glutathionylation: a regulatory role of glutathione in physiological processes. Arh Hig Radi Toks. 2018;69(1):1-24.
35. Dominko K, Dikic D, Hecimovic S. Enhanced activity of superoxide dismutase is a common response to dietary and genetically induced increased cholesterol levels. Nutrit Neurosci; 2018; online ahead of print. 1-13. doi: 10.1080/1028415X.2018.1511027.