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Proceeding Paper

Metabolomic Fingerprinting of Phenolic Compounds in Blood Serum from Rats Treated with Chestnut Shells Extract †

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Abstract: The intake of dietary polyphenols has clearly boosted in the last decades owing to their health-promoting benefits. Food by-products have been recognized as promising sources of polyphenols. Chestnut shells (CS) are one of these by-products. This study explores the targeted metabolomic profile of polyphenols in rat blood serum by LC-ESI-LTQ-Orbitrap-MS, following the acute intake of two doses of CS extract (50 and 100 mg/kg body weight). Results attested the presence of phases I (hydrogenation and hydroxylation) and II (glucuronidation, methylation and sulfation) metabolites mostly derived from phenolic acids. Outcomes enlighten the metabolomic mechanisms underlying the acute intake of phenolics-enriched CS extract.

Keywords: *Castanea sativa* shells; subcritical water extraction; phenolic compounds; nutraceutical; in vivo study; metabolomic

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1. Introduction

Nowadays, the increasing consumption of food supplements and nutraceuticals as complementary approach to the diet embraces a broad range of health-promoting benefits through delivering micronutrients and phytochemicals to the human body, such as polyphenols. Besides fruits and vegetables, food by-products have also been recognized as promising sources of polyphenols. Chestnut shells (CS) are one of these agro-industrial by-products [1,2]. Chestnut industry has strengthened the Portuguese fruit economy, producing more than 42 thousand tons in 2020. Notwithstanding, the shells produced from chestnut peeling are an abundant and undervalued by-product [1]. Most studies demonstrated the in vitro bioactivity and phytochemical composition of CS extracts, mostly prepared by eco-friendly technologies [1–3]. A deep insight on the bioavailability of polyphenols is crucial to unveil the mechanisms behind their bioactivity [4]. Thus, metabolomic studies in animals may be an useful tool to attempt a more profound understanding of the absorption and distribution profile of bioactive compounds and their metabolites in tissues and biological fluids.

The present study aims to investigate the targeted metabolomic profile of polyphenols in blood serum from rats orally treated with CS extract by liquid chromatography coupled to Orbitrap-mass spectrometry (LC-ESI-LTQ-Orbitrap-MS). Notably, this is the first study that proposes the metabolomic fingerprinting of polyphenols in animal models administered with a chestnut by-product.

2. Materials and Methods

2.1. Chemicals

The following standards were used for the metabolomic analysis: 2,5-dihydroxybenzoic, 2,6-dihydroxybenzoic, 3,5-dihydroxybenzoic, 3,4-dihydroxyhydrocinnamic, 3-(2,4-dihydroxyphenyl)propionic, 4-hydroxybenzoic, 3-hydroxyphenylacetic, 3-(4-hydroxyphenyl)propionic, caffeic, catechol, cinnamic, *o*-coumaric, *m*-coumaric, ellagic, gallic, hippuric, homovanillic, phenylacetic, protocatechuic, sinapic and vanillic acids, enterodiol, enterolactone, pyrogallol, secoisolariciresinol, vanillin, urolithin A and B purchased from Sigma-Aldrich (Steinhemin, Germany); (-)-epicatechin, 3-hydroxybenzoic, *p*-coumaric, ferulic and syringic acids supplied by Fluka (St. Louis, MI, USA); methyl gallate acquired from Phytolab (Vestenbergsgreuth, Germany); and 3-(4-hydroxy-3-methoxyphenyl)propionic acid obtained from Alfa Aesar (Haverhill, MA, USA). Acetonitrile, formic acid, and methanol were acquired from Sigma-Aldrich (Steinhemin, Germany).

2.2. Subcritical Water Extraction of Chestnut Shells

C. sativa shells were kindly supplied by Sortegel (Bragança, Portugal) in October 2018. Shells were first dehydrated at 40 °C/24 h, ground to a particle size of 1 mm and then kept at room temperature in the dark until further extraction. SWE was performed as described by Pinto, Vieira, et al. [2] that conducted an optimization study for CS extraction using the same technology. Extraction was conducted at 220 °C, 40 bar, and for 30 min, at a solid-to-liquid ratio of 1 g: 10 mL using distilled water.

2.3. In Vivo Studies Using Rat as Animal Model

2.3.1. Experimental Design

Male Wistar rats (200 ± 50 g, 5–6 weeks old) acquired from Jackson Laboratory (Bar Harbor, ME, USA). After an acclimatization of one week, animals were housed in polypropylene cages under standard laboratory conditions (21 ± 2 °C temperature, 45–55% relative humidity, 12 h/12 h light/dark cycle) and fed *ad libitum* water and standard pellet diet. The animals were then randomly clustered into three groups (n = 6 per group): a normal control group administered with water (4 mL/kg body weight (b.w.)); and two treatment groups administered with two doses of CS extract (50 and 100 mg/kg b.w.). All solutions were administered by gastric gavage, once daily for 7 days. In vivo studies were accomplished following the European Directive 2010/63/EU under approval of the Local Ethical Committee. Blood was collected by cardiac puncture. Serum was recovered after centrifugation (2000 g, 15 min, 4 °C) and stored at −80 °C. At the end of experiments, a pentobarbital overdose (50 mg/kg b.w.) was employed to euthanize the animals.

2.3.2. Targeted Metabolomic Profile by LC-ESI-LTQ-Orbitrap-MS

The first step comprises the sample pre-treatment. The protein precipitation was performed according to López-Yerena et al. [5]. The metabolomic analysis was accomplished using LC-ESI-LTQ-Orbitrap-MS equipment equipped of Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) with a photodiode array detector, a quaternary pump, and a thermostated autosampler attached to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) containing an ESI source in negative mode. The chromatographic separation of compounds was performed using an Acquity™ UPLC® BEH C18 Column (2.1 × 100 mm, i.d., 1.7 μm particle size) (Waters Corporation, Milford, MA, USA). Mobile phases were water with 0.1% formic acid (A) and acetonitrile

with 0.1% formic acid (B), while solvent gradient (*v*/*v*) of B (t (min), %B) was set as: (0, 0); (2, 0); (3, 30); (4, 100); (5, 100); (6, 0); (9, 0). The injection volume was 5 μL, whereas column temperature and flow rate were fixed, respectively, at 30 °C and 0.450 mL/min. All samples were analyzed in a full scan mode at a resolving power of 30,000 at *m*/*z* 600, with data-dependent MS/MS events being conducted at a resolving power of 15,000. Mass in FTMS mode ranged from *m*/*z* 100 to 600. Polyphenols were identified using commercial standards, while their metabolites were identified by elution time, MS/MS fragments, chemical structure, and comparing with similar compounds. Calibration curves were prepared using gallic, ellagic, and protocatechuic acids, pyrogallol and methyl gallate (concentration range = 0.1–3 μg/mL, $R^2 > 0.994$) which were previously identified in CS extract prepared using the same extraction technology and conditions [2]. Human metabolome database (https://hmdb.ca) and literature data were consulted for the putative identification of polyphenols and metabolites. XCalibur 3.0 software (ThermoFisher Scientific, Hemel Hempstead, UK) was used for system control and data treatment. Results were presented in nmol of each polyphenol equivalents per mL of blood serum.

3. Results and Discussion

Targeted Metabolomic Profiling in Rat Blood Serum

Polyphenols, which are bioactive compounds naturally present fruits and vegetables, have been indicated as the most active ingredients to ameliorate metabolic diseases through in vitro and in vivo assays. Notwithstanding, the absorption and distribution of polyphenols in tissues and biofluids relies on their bioavailability which is directly affected by their metabolism, including phase I and II reactions that contribute to stabilization, enhance the water solubility, amend their distribution and excretion and, accordingly, deliver metabolites equally or more biologically active than their parent compounds [4,6,7]. Table 1 summarizes the polyphenols and their metabolites detected in the rat blood serum for CS extract treatment groups.

Table 1. Identification of polyphenols and their metabolites in blood serum from rats treated with extract by LC-ESI-LTQ-Orbitrap-MS.

Compound	Neutral Molecular Formula	Rt (min)	Ion Mass [M-H]$^-$		Error (ppm)	MS^2 Fragment Ions [M-H]$^-$
			Theoretical	*Experimental*		
Methyl-pyrogallol-*O*-sulfate	$C_7H_8O_6S$	0.73	218.9958	218.9967	0.336	125.02416, 138.97908
Dihydrogallic acid	$C_7H_8O_5$	0.74	171.0288	171.0265	−2.828	127.03718, 141.01816
Protocatechuic acid-*O*-sulfate	$C_7H_6O_7S$	4.34	232.9751	232.9756	0.012	109.02917, 153.01849, 188.98633
Dimethyl-pyrogallol-*O*-glucuronide	$C_{14}H_{18}O_9$	4.44	329.0867	329.0870	−0.247	125.02511, 139.03949, 153.01916
Catechol-*O*-sulfate	$C_6H_6O_5S$	4.47	188.9852	188.9860	0.800	109.02921
Protocatechuic acid	$C_7H_6O_4$	4.48	153.0182	153.0192	0.366	109.02860, 125.02344
Urolithin A-*O*-sulfate	$C_{13}H_8O_7S$	4.50	306.9907	306.9909	−0.308	227.03806
Pyrogallol-*O*-sulfate	$C_6H_6O_6S$	4.53	204.9801	204.9806	−0.083	123.00834, 125.02385, 162.96728
Hydroxybenzoic acid-*O*-sulfate	$C_7H_6O_6S$	4.56	216.9801	216.9798	−0.903	121.02944, 137.02446
Dimethyl-pyrogallol-*O*-sulfate	$C_8H_{10}O_6S$	4.59	233.0114	233.0121	0.146	125.02511, 139.03949,153.01931, 204.98111
Methyl-protocatechuic acid-*O*-sulfate	$C_8H_8O_7S$	4.62	246.9907	246.9914	0.192	109.02931, 153.01921, 167.03576, 232.97561
Dihydrocaffeic acid-*O*-sulfate	$C_9H_{10}O_7S$	4.64	261.0064	261.0071	0.790	113.06058, 137.06062, 181.05060
Pyrogallol-*O*-glucuronide	$C_{12}H_{14}O_9$	4.65	301.0554	301.0591	3.163	125.02433

Methyl-syringic acid-*O*-sulfate	$C_{10}H_{12}O_8S$	4.66	291.0169	291.0178	0.287	137.06066, 197.04301, 211.06108
Methyl-catechol	$C_{12}H_{14}O_8$	4.68	123.0441	123.0448	0.754	109.02926
Methyl-protocatechuic acid	$C_8H_8O_4$	4.69	167.0339	167.0348	0.366	109.02923, 153.01918
Caffeic acid-*O*-sulfate	$C_9H_8O_7S$	4.69	258.9907	258.9920	0.850	135.04504, 179.03489, 215.00110
Dihydroferulic acid-*O*-glucuronide	$C_{16}H_{20}O_{10}$	4.70	371.0973	371.0979	0.597	151.03982, 195.06628
Hydroxyphenylacetic acid-*O*-sulfate	$C_8H_8O_6S$	4.72	230.9958	230.9965	0.665	107.05001, 151.03999, 187.00711, 213.00527
Dimethyl-syringic acid-*O*-sulfate	$C_{11}H_{14}O_8S$	4.75	305.0326	305.0330	0.426	153.05548, 197.04302, 225.07685,277.00170
Catechol-*O*-glucuronide	$C_{12}H_{14}O_8$	4.77	285.0605	285.0608	0.286	109.02917
Methyl-ferulic acid-*O*-glucuronide	$C_{17}H_{20}O_{10}$	4.82	383.0973	383.0982	0.947	163.02670, 174.98718, 193.05078, 207.05070, 369.08253
Dihydroferulic acid-*O*-sulfate	$C_{10}H_{12}O_7S$	4.84	275.0220	275.0229	0.850	195.06596
Hydroxyphenylpropionic acid-*O*-sulfate	$C_9H_{10}O_6S$	4.86	245.0114	245.0122	0.795	121.02935, 165.05573, 201.07681
Methyl-coumaric acid-*O*-sulfate	$C_{10}H_{10}O_6S$	4.87	257.0114	257.0158	4.405	119.04989, 163.03987, 177.05564, 199.00630
Methyl-catechol-*O*-sulfate	$C_7H_8O_5S$	4.88	203.0009	203.0018	0.879	123.04492
Dihydrocaffeic acid	$C_9H_{10}O_4$	4.89	181.0495	181.0505	0.366	109.04048, 137.06017
Hippuric acid	$C_9H_9NO_3$	4.92	178.0499	178.0506	0.172	134.06059
Syringic acid	$C_9H_{10}O_5$	4.95	197.0444	197.0451	0.072	137.02383, 153.05535
Ellagic acid	$C_{14}H_6O_8$	4.96	300.9979	300.9981	−0.372	201.01796, 229.01404, 257.00763, 283.99559
Methyl-dihydroferulic acid-*O*-sulfate	$C_{11}H_{14}O_7S$	4.97	289.0376	289.0384	−1.792	151.07650, 165.01923, 195.06564, 209.08016
3-Hydroxybenzoic acid	$C_7H_6O_3$	4.98	137.0233	137.0242	0.321	93.03386
Hydroxyphenylpropionic acid	$C_9H_{10}O_3$	5.05	165.0546	165.0553	0.171	121.02926
Hydroxyferulic acid	$C_{10}H_{10}O_5$	5.06	209.0444	209.0455	1.030	133.99126, 149.02384, 165.05574, 178.05046
Methyl-syringic acid	$C_{10}H_{12}O_5$	5.06	211.0601	211.0611	0.462	137.06030, 153.05548, 197.04174
ρ-Coumaric acid	$C_9H_8O_3$	5.07	163.0390	163.0399	0.351	119.04975
Dimethyl-hydroxyphenylpropionic acid-*O*-sulfate	$C_{11}H_{14}O_6S$	5.07	273.0427	273.0434	0.675	121.02919, 165.05538, 193.08687
(Epi)catechin-*O*-sulfate	$C_{15}H_{14}O_9S$	5.07	369.0275	369.0269	−0.629	245.01231, 289.07153, 303.01690
Cinnamic acid-*O*-glucuronide	$C_{15}H_{16}O_8$	5.09	323.0761	323.0770	0.806	147.04501
Dihydroferulic acid	$C_{10}H_{12}O_4$	5.10	195.0652	195.0662	0.426	135.04459, 151.07562
Dimethyl-ellagic acid	$C_{16}H_{10}O_8$	5.11	329.0292	329.0275	−2.292	113.06061, 174.98718, 300.99997
Methyl-ferulic acid-*O*-sulfate	$C_{11}H_{12}O_7S$	5.13	287.0220	287.0224	0.430	149.06051, 193.05064, 207.06135, 273.00712
Dihydrocaffeic acid-*O*-glucuronide	$C_{15}H_{18}O_{10}$	5.14	357.0816	357.0823	0.717	113.06061, 137.06066, 174.98715, 181.05055
m-Coumaric acid	$C_9H_8O_3$	5.15	163.0390	163.0398	0.301	119.04967
Sinapic acid-*O*-sulfate	$C_{11}H_{12}O_8S$	5.15	303.0169	303.0168	−0.074	223.06104

Secoisolariciresinol	$C_{20}H_{26}O_6$	5.16	361.1648	361.1649	−0.183	165.05508, 346.14081
Hydroxyphenylacetic acid-*O*-glucuronide	$C_{14}H_{16}O_9$	5.26	327.0711	327.0717	0.652	151.03983, 175.02472, 309.05656
Enterolactone-*O*-disulfate	$C_{18}H_{18}O_{10}S_2$	5.32	457.0258	457.0262	−0.123	297.11398, 377.06977
Methyl-urolithin A	$C_{14}H_{10}O_4$	5.32	241.0495	241.0493	−0.774	211.00470
Cinnamic acid	$C_9H_8O_2$	5.39	147.0441	147.0445	−0.074	102.94863, 129.04442
Enterodiol-*O*-glucuronide	$C_{24}H_{30}O_{10}$	6.79	477.1755	477.1774	1.298	175.02505, 301.12904, 459.15735

A total of 52 compounds were identified in the blood serum. Phenolic acids and their metabolites represent most of the polyphenols detected, whereas only one flavonoid metabolite ((epi)catechin-sulfate) and one lignan (secoisolariciresinol) along with two gut microbial metabolites (enterodiol-glucuronide and enterolactone-disulfate) were present. Considering the polyphenols previously reported in the CS extract prepared by the same extraction technology and conditions [2], metabolites from pyrogallol, ellagic, gallic, and protocatechuic acids were detected in the blood serum. Certain polyphenols are directly deactivated by phase II enzymes without undergoing phase I reactions, which are the cases of pyrogallol and protocatechuic acid [6]. Unmetabolized protocatechuic and ellagic acids reached the systemic circulation unchanged. Sulphated and methylated forms of urolithin A were detected as gut microbiota metabolites of ellagic acid. Gallic acid only underwent hydrogenation (phase I). Furthermore, metabolites of caffeic and ferulic acids followed different metabolic paths: (i) being directly metabolized by phase II enzymes and generating sulfated and methylated plus sulfated or glucuronidated metabolites; (ii) undergoing only phase I reactions namely hydrogenation and hydroxylation; and (iii) being metabolized via phase I (hydrogenation and hydroxylation) and then phase II reactions (methylation, sulfation and glucuronidation) [4]. Unmetabolized p-coumaric and m-coumaric acids reached the systemic circulation along with methyl-coumaric acid-sulfate. According to Li et al. [8], the metabolomic profile of Chinese water chestnut revealed caffeic, chlorogenic, cinnamic, and phenylacetic acids. Among hydroxybenzoic acids, syringic acid and three conjugates, as well as 3-hydroxybenzoic acid and its sulfated metabolite, were found. Unmetabolized hydroxyphenylpropionic acid was identified along with two phase II metabolites, while only two hydroxyphenylacetic acid metabolites resulting from sulfation and glucuronidation reached the blood. In a nutshell, most of the phenolic acids identified correspond to weak basic molecules with moderate lipophilicity (log P~0.9) and low molecular weight (<250 g/mol) which facilitates their absorption through passive transport [4,6]. The detection of phenolic acids in serum attested their absorption in unmetabolized form. Moreover, phase II metabolites were secreted into circulating blood due to their high polarity and molecular weight. Indeed, metabolites resulted from phase II reactions correspond to 80% of all the metabolites detected, whilst the remaining 20% derived from phase I reactions. Likewise, 11 compounds correspond to parent compounds and the remaining are their metabolites.

4. Conclusions

The current work attempt to validate for the first time a new nutraceutical ingredient extracted from chestnut shells through an eco-friendly technology and explore it as a promissory source of antioxidant compounds that embrace outstanding in vivo health-promoting properties. The metabolomic profile was useful for the fingerprinting of polyphenols and metabolites in blood serum, pointing out identical profiling for both doses. Notwithstanding, ongoing studies regarding the quantification of polyphenols and metabolites are still being attempted, while further studies should center in the exploitation of a potential causal relationship of metabolomic profile and their in vivo bioactivity, particularly concerning the antioxidant effects. A forthcoming exploitation of CS as a preventive measure against metabolic disorders should be also on the spotlight.

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