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Development of novel techniques to extract phenolic compounds from Romanian cultivars of Prunus domestica L. and their biological properties

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ABSTRACT

In the present work, fourteen cultivars of Prunus domestica were analyzed to investigate their phenolic pattern with the purpose of using the leaves as potential resources of bioactive compounds in the pharmaceutical and food industry. Microwave-assisted extraction (MAE), dispersive liquid–liquid microextraction and sugaring-out liquid–liquid extraction techniques were optimized in order to obtain an exhaustive multi-component panel of phenolic compounds. The best phenolic-enriched recovery was achieved using MAE in water:methanol (30:70), and this procedure was further applied for quantitative analysis of phenolic compounds in real samples. In order to prove the safeness of these extracts, the biological potential of the Prunus cultivars was tested by several in vitro antioxidant and enzyme inhibitory assays. Moreover, their cytotoxicity was evaluated on human gingival fibroblasts (HGFs), and in most of the cases the treatment with different concentrations of extracts didn't show cytotoxicity up to 500 μg/mL. Only ‘Carpatin’ and ‘Minerva’ cultivars, at 250 and 500 μg/mL, reduced partially cell viability of HGFs population. Noteworthy, Centenar cultivar was the most active for the α-glucosidase inhibition (6.77 mmolACAE/g extract), whereas Ialomița cultivar showed the best antityrosinase activity (23.07 mgKAE/g extract). Overall, leaves of P. domestica represent a rich alternative source of bioactive compounds.

1. Introduction

Despite the large number of synthetic pharmaceuticals, interest to traditional herbal medicine has still not disappeared. Herbal products contain high amount of biologically active compounds with a less harmful effect on human body than their synthetic counterparts. Prunus domestica L. is a very popular edible species cultivated in Europe. Depending on local climatic and soil conditions, different cultivars with various content of bioactive compounds could be grown (Walkowiak-Tomczak and Regu, 2008). Moreover, P. domestica fruits (plums) are known for their nutritional value and therapeutic properties (Mitic et al.,

2016). Plums (P. domestica fruits) are a rich source of polyphenols, carotenoids and anthocyanins (Jaiswal et al., 2013), but the phenolic pattern of leaves is insufficiently known. Hence, the leaves could be a cheap and available resource of phenolic compounds and naturally-occurring antioxidants which would be able to find their application for the development of herbal medicines and food supplements (Routray and Orsat, 2014). Phenolic compounds are widespread secondary plant metabolites comprising a wide variety of molecules and characterized by a classic phenol ring structure (and several hydroxyl groups linked to the aromatic rings) (Chen et al., 2018, 2017). Nowadays, polyphenols are used and considered as functional ingredients in the composition of different phytopharmaceuticals and dietary supplements (Chen et al., 2018; Xiao, 2017) due to a wide panel of demonstrated biological properties, acting as antioxidants, modulators of inflammation, antivirals, antimicrobials, or showing antiangiogenic and antithrombogenic effects (Khan et al., 2018; Xiao et al., 2016). Moreover, epidemiological studies provide convincing evidence of their role in the prevention of cancer and other progressive diseases, such as metabolic syndrome, Alzheimer's and cardiovascular disease (Chen et al., 2018). iendly extraction techniques are required (Ameer et al., 2017; Zengin et al., 2018). For this reason, microwave-assisted extraction (MAE) was utilized for the characterization of phenolic constituents of leaves (Du et al., 2009; Proestos and Komaitis, 2008; Švarc-Gajić et al., 2013). In comparison with traditional extraction, MAE is time-saving, provides high sample throughput and employs less organic solvent (Armenta et al., 2008; Mollica et al., 2016). Additionally, dispersive liquid–liquid microextraction (DLLME), among other liquid phase microextraction techniques has been established as a ‘green methodology’, which allows to isolate a great number of compounds (Lima et al., 2017; Yilmaz and Soylak, 2016). Nevertheless, sugaring-out liquid–liquid extraction (SULLE) based on addition of the sugars (monosaccharides or disaccharides) at high concentration for phase separation (De Brito Cardoso et al., 2013) is compatible with the analytical equipment, such as high performance liquid chromatography (Anthemidis and Ioannou, 2009; Ebrahimpour and Yamini, 2014; McConvey et al., 2012; Timofeeva et al., 2017). In current study, the phenolic pattern of 14 old local cultivars of P. domestica L. were investigated, using ‘green’ extraction techniques, such as MAE, DLLME and SULLE followed by HPLC-PDA identification and quantification. Moreover, their biological properties were investigated by assessing their cytotoxicity on human dermal gingival fibroblasts as well as their inhibitory properties on selected enzymes such as αamylase and α-glucosidase, tyrosinase and human recombinant monoamine oxidases (MAO-A and MAO-B).

2. Experimental part

2.1. Materials

Chemical standards, such as gallic acid, catechin, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, p-coumaric acid, rutin, sinapinic acid, t-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, o-coumaric acid, quercetin, harpagoside, tcinnamic acid, naringenin, and carvacrol (purity > 98%) were purchased from Sigma-Aldrich (Milan, Italy). Solvents: ethyl acetate (≥99%), acetonitrile (HPLC-grade), methanol (HPLC-grade), acetic acid (≥99%), as well as sugars: D (+)-saccharose, D(+)-glucose and D(−)-fructose (≥99%) were otained from Sigma Aldrich (Milan, Italy). Sodium chloride (≥99%) was obtained from Honeywell (Seelze, Germany). β-Cyclodextrin (≥97%) was purchased from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA).

2.2. Sampling and sample preparation

Leaf samples of the 14 cultivars (‘Tuleu gras’, ‘Centenar’, ‘Tita’, ‘Minerva’, ‘Tuleu timpuriu’, ‘Record’, ‘Diana’, ‘Carpatin’, ‘Alutus’, ‘Silvia’, ‘Alina’, ‘Flora’, ‘Ialomiţa’, ‘Andreea’) of Prunus domestica L. cultivars were offered by Fruit Growing Research Station (SCDP) Valcea, which belongs to the University of Craiova (UCv). UCv-SCDP Valcea is responsible for a part of the national Prunus germplasm collection. The leaves were dried in a shaded place at room temperature and ground using a laboratory mill. The powdered material was kept in plastic boxes in a dark place at room temperature.

2.3. Equipment

2.3.1. Microwave-assisted extractor An automatic Biotage Initiator™ 2.0 (Uppsala, Sweden), characterized by 2.45 GHz high-frequency microwaves and power range 0–300 W, was used for MAE procedure. An IR sensor probe controlled the internal vial temperature. 2.3.2. HPLC-PDA equipment The quali-quantitative analysis of phenolic compounds was performed according to the reported method (Zengin et al., 2016). The chromatographic system consisted of HPLC Waters liquid chromatograph instrument (model 600 solvent pump, 2996 PDA; Waters Spa, Milford, MA, USA). Mobile phase was directly degassed on-line by using a Biotech 4CH DEGASI Compact (Onsala, Sweden). For separation of twenty-two compounds, C18 reversed-phase column (Prodigy ODS(3), 4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA), thermostated at 30 °C (± 1 °C) was used. The collection and analysis of the data were performed by Empower v.2 software (Waters Spa, Milford, MA, USA). The elution mode was applied with a mobile phase solution A (3% solution of acetic acid in water) and B (3% solution of acetic acid in acetonitrile) in a ratio 93:7 (v:v). The total separation was completed in 1 h. In Supplementary materials section S1 were reported the HPLC experimental conditions used for the herein performed analyses and the chromatographic separation of the considered twenty-two compounds. 2.3.3. Auxiliary equipment Centrifuge model NF048 (Nuve, Ankara, Turkey), vortex (VELP Scientifica Srl, Usmate, Italy), and ultrasonic bath (Falc Instruments, Treviglio, Italy) were used to assist the extraction procedures. Rotary evaporator model Laborota 4000 (Heidolph, Schwabach, Germany) and freeze dryer model (VirTis lyophilizer) were used for obtaining the dry extracts.

2.4. Extraction procedures

2.4.1. Maceration preliminary test For preliminary determination of the multi-component pattern extracted from P. domestica leaves, a maceration of 10 mg of dry plant material was carried out in 1 mL of methanol, water or a mixture of water:methanol in the ratio 30:70 (v:v) for 14 h at the room temperature (20 ± 2 °C). For separation of solid particles, the extract was centrifuged for 5 min at 12000×g and then directly injected to HPLC system. 2.4.2. Microwave-assisted extraction (MAE) 10 mg of dry leaves were placed into the 5-mL vessel for an automatic single-mode microwave reactor. Then, 1 mL of solvent was added thus obtaining a final green suspension with the same solid-to-liquid ratio reported above. After 5 s of pre-stirring, the sample was sealed and heated promptly by the microwave source at 80 °C (± 1 °C) for 13 min and 8 s (which are comparable to 14 h of maceration at 20 °C according to Arrhenius equation), and then cooled steeply with pressurized air. The resulting suspension was filtered through a 0.2 µm syringe filter (Sigma-Aldrich, Milan, Italy), and directly injected to HPLC system. 2.4.3. Dispersive liquid–liquid microextraction (DLLME) For DLLME, 10 mg of dry leaves were placed into the Eppendorf tube and 1 mL of water or other extraction medium was added. Then 200 µL of ethyl acetate and 100 µL of acetonitrile were rapidly injected to the Eppendorf tube for formation of cloudy solution. Subsequently, the solution was injected for 30 s. After 1 min, needed for distribution of analytes in the extraction solvent, the sample solution was centrifuged for 5 min at 12000×g. 100 µL of the top layer were transferred to a new Eppendorf tube and evaporated under a gentle stream of N2. The dry residue was re-dissolved in 50 µL of mobile phase solution, ultrasonicated for 5 min and 20 µL of the obtained solution were injected to HPLC system. 2.4.4. Sugaring-out liquid–liquid extraction (SULLE) For SULLE, 10 mg of dry leaves were placed into the Eppendorf tube. Then, 200 µL of water and 400 µL of acetonitrile were successively added. After 30 s of gentle shaking, 200 µL of the corresponding sugar solution were rapidly injected, and the solution was vortexed for 1 min. Phase separation is ensured by centrifugation for 5 min at 12000×g. To avoid pick split, 250 µL of the top layer containing analytes were collected and transferred to the new Eppendorf tube and dried under a gently stream of N2. Then, the dry residue was re-dissolved in 50 µL of mobile phase, ultrasonicated for 5 min and 20 µL of this solution were injected into the HPLC system (Diuzheva et al., 2018).

2.5. Total phenolic and flavonoid content

The total phenolic content (TPC) was determined using the FolinCiocâlteu method as previously reported (Mocan et al., 2017). For a high throughput analysis of the samples, a SPECTROstar Nano microplate reader with 96-wells plates (BMG Labtech, Offenburg, Germany) was used. Briefly, a mixture containing 25 µL of sample extract, 125 µL of Folin-Ciocâlteu reagent (10-fold freshly diluted from the stock solution), and 100 µL of sodium carbonate (Na2CO3) solution (7.5% w/v) was homogenized and incubated at room temperature in the dark for 30 min. The absorbance of the samples and blank were measured at 760 nm. Gallic acid was used as a standard reference, and the TPC was expressed as gallic acid equivalents (GAE) in mg/g dry weight (dw) of herbal extract. The total flavonoid content (TFC) was measured according to the method described previously by Mocan et al. (2017). Briefly, an 100 µL aliquot of 2% AlCl3 ethanolic solution was added to 100 µL of the sample and mixed. After incubating for 15 min at room temperature, the absorbance was measured at 420 nm. Results were expressed as quercetin equivalents (QE) in mg/g dry herbal extract weight (dw).

2.6. Biological evaluation

2.6.1. Antioxidant capacity evaluation 2.6.1.1. DPPH radical scavenging assay. The capacity to scavenge the "stable" free radical DPPH, monitored according to the method described by Martins et al. (2015) with some modifications, was performed by using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). The reaction mixture in each of the 96- wells consisted of 30 µL of sample solution (in an appropriated dilution) and a 0.004% methanolic solution of DPPH. The mixture was further incubated for 30 min in the dark, and the reduction of the DPPH radical was determined by measuring the absorption of the sample at 515 nm. Trolox was used as reference, and the results were expressed as milimoles of trolox equivalents per g of dry herbal extract weight (mmolTE/g extract dw). 2.6.1.2. Trolox equivalent antioxidant capacity (TEAC) assay. The radical scavenging activity of the different Prunus cultivars against the stable synthetic ABTS radical cation was measured using the method previously described by Mocan et al. (2016a). A Trolox calibration curve was plotted as a function of the percentage of ABTS radical scavenging activity. The final results were expressed as milligrams of Trolox equivalents (TE) per gram of herbal extract dry weight (mgTE/g extract dw). 2.6.1.3. Total antioxidant capacity by phosphomolybdenum assay. The total antioxidant capacity of the samples was evaluated using the phosphomolybdenum assay according to the protocol previously described by Mocan et al. (2016b). Briefly, 0.3 mL of samples solution was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the sample was read at 695 nm after a 90 min incubation at 95 °C. Trolox was used as reference. Total antioxidant capacity was expressed as millimoles of trolox equivalents per gram of dry herbal extract weight (mmol TE/g extract dw). 2.6.1.4. Metal chelating assay. The metal chelating ability of the samples was investigated using the protocol previously described by Savran et al. (2016) Briefly, 2 mL of sample solution were added to 0.05 mL FeCl2 solution (2 mM), and the reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine. In a similar manner, a blank was prepared by adding the same reagents without ferrozine (solvent instead). The sample and blank absorbances were read at 562 nm after 10 min of incubation at room temperature. The metal chelating activity was expressed as milligrams of EDTA (disodium edetate) equivalents per gram of dry herbal extract weight (mg EDTAE/g extract dw). 2.6.2. Isolation of human gingival fibroblasts (HGFs) Ten (10) donors, periodontally and systemically healthy, subjected to the extraction of the third molar, signed informed consent according to the Italian Legislation and in accordance with the code of Ethical Principles for Medical Research comprising Human Subjects of the World Medical Association (Declaration of Helsinki). The project obtained the approval of the Local Ethical Committee of the University of Chieti (approval number 1173, approved on 31/03/2016). The tissue fragments were immediately placed in Dulbecco's modified Eagle's medium (DMEM) for at least 1 h, rinsed thrice in phosphate-buffered saline solution (PBS), minced into small tissue pieces and cultured in DMEM, containing 10% foetal bovine serum (FBS), 1% penicillin/ streptomycin and 1% fungizone. Cells were maintained at 37 °C in a humidified atmosphere of 5% (v/v) CO2. After 1 week, fungizone was removed from culture medium. Cells were used after 7–14

passages (Tete et al., 2014). 2.6.2.1. MTT assay. The metabolic activity of the HGFs was evaluated after 48 h of treatment with different extracts of Prunus domestica L. at 125, 250 and 500 µg/mL on 96-well polystyrene plates through the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Sigma Aldrich, Saint Louis, MO, USA), based on the capability of viable cells to reduce MTT into a colored formazan product. At the established time points the medium was replaced by a new one containing 0.5 mg/mL MTT and probed with cells for 4 h at 37 °C. The plate was incubated in DMSO (dimethylsulfoxide) solution for 30 min at 37 °C to solubilize salts and then read at 570 nm. Optical density was measured by means of spectrophotometric reading performed at 570 nm with a spectrophotometer Anthos (Anthos Labtec Instruments, Salzburg, Austria). Values obtained in the absence of cells were considered as background. 2.6.2.2. HGFs treatment. For each extract a stock solution of 100 µg/mL dissolved in DMSO was prepared. The stock solution was then diluted to obtain extract concentrations of 125, 250 and 500 µg/mL. To exclude DMSO cytotoxicity, in all the incubation media, the final concentration of DMSO was maintained at 0.5%. The HGFs were seeded at 8550 cells/ well in 96 well plate and once they reached the confluence the medium was replaced by a fresh one containing 125, 250 and 500 µg/mL of extracts. The samples were incubated for 48 h in a humidified atmosphere of 5% (v/v) CO2 at 37 °C. 2.6.3. Enzyme inhibitory assays 2.6.3.1. Inhibitory activity against selected carbohydrate hydrolases 2.6.3.1.1. α-Amylase inhibitory assay. The α-amylase inhibitory activity was assessed using the Caraway-Somogyi iodine/potassium iodide (IKI) method (Mocan et al., 2017). Briefly, 25 µL of sample solution (2 mg/mL) were mixed with 50 µL of α-amylase solution (porcine pancreas, EC 3.2.1.1, Sigma, Saint Louis, MO) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-wells microplate and incubated for 10 min at 37 °C. After the preincubation, the reaction was initiated with the addition of 50 µL starch solution (0.05%). Similarly, a blank was prepared by adding sample solution to all reaction reagents without the enzyme solution. The reaction mixture was again incubated 10 min at 37 °C, then stopped with the addition of 25 µL HCl (1 M). Following this, 100 µL of the iodine–potassium iodidesolution were added. The sample and blank absorbances were recorded at 630 nm. Acarbose was used as reference. The α-amylase inhibitory activity was expressed as millimoles of acarbose equivalents per gram of dry herbal extract weight (mmol ACAE/g extract dw). 2.6.3.1.2. α-Glucosidase inhibitory assay. The α-glucosidase inhibitory activity was tested using the protocol previously described by Mocan et al. (2016b). Briefly, 50 µL sample solution (2 mg/mL) were mixed with 50 µL glutathione (0.5 mg/mL), 50 µL α-glucosidase solution (from Saccharomyces cerevisiae, EC 3.2.1.20, Sigma, Darmstadt, Germany) in phosphate buffer (pH 6.8), and 50 µL of 10 mM PNPG (p-nitrophenyl-β-D-glucuronide) (Sigma-Aldrich, Schnelldorf, Germany) solution in a 96-wells microplate and incubated for 15 min at 37 °C. In a similar manner, the blank was prepared by adding sample solution to all reagents without the enzyme solution. Finally, the reaction was stopped with the addition of 50 µL of 0.2 M sodium carbonate. Sample and blank absorbances were recordedat 400 nm, and the α-glucosidase inhibitory activity was expressed as millimoles of acarbose equivalents per dry of herbal extract weight (mmol ACAE/g extract dw). 2.6.3.2. Tyrosinase inhibitory activity. The tyrosinase inhibitory activity was measured using the modified dopachrome method using L-DOPA as substrate, as previously reported by Mocan et al. (2017). 25 µL sample solution (2 mg/mL) were mixed with 40 µL tyrosinase solution and 100 µL phosphate buffer (pH 6.8) in a 96-wells microplate, and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 40 µL L-DOPA. A blank was prepared by adding sample solution to all reaction reagents without enzyme (tyrosinase). The sample and blank absorbances were recorded at 492 nm after incubating 10 min at 25 °C, and the tyrosinase inhibitory activity was expressed as equivalents of kojic acid per gram of dry herbal extract weight (mg KAE/g extract dw).

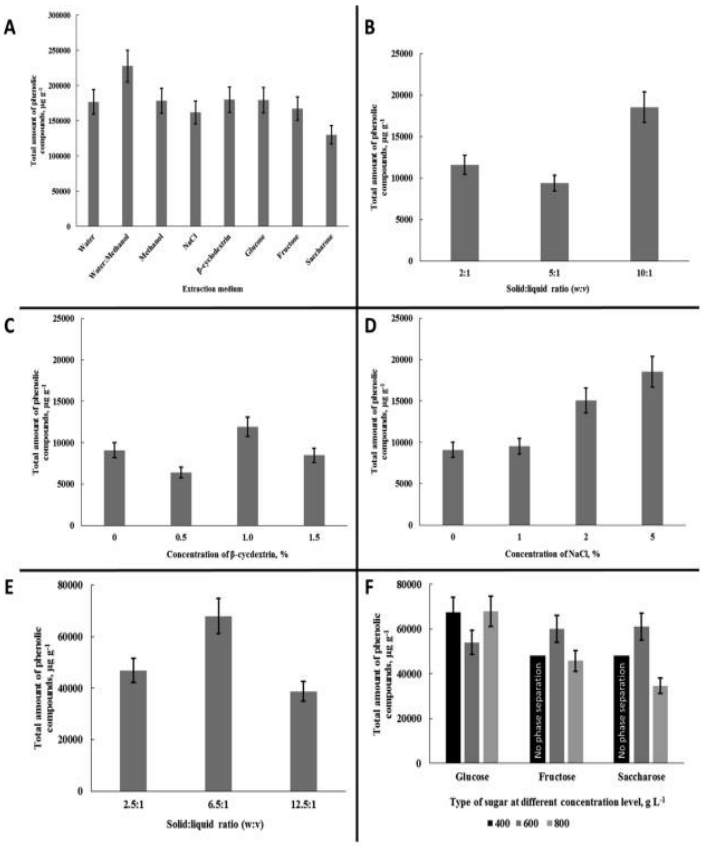

Fig. 1. Optimization of MAE, DLLME, and SULLE conditions.

2.6.3.3. Inhibition of MAO. To determine IC50 values for the inhibition of human MAO-A and MAO-B by the extracts obtained by MAE, the commercially available recombinant human enzymes expressed in insect cells were used (Sigma-Aldrich) (Mostert et al., 2015). The enzyme reactions were conducted in 96-well microtiter plates (white) in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) to a final volume of 200 µL, and contained kynuramine (50 µM) and the extracts at concentrations of 0.0006–20 µg/mL. The extracts were dissolved in DMSO and added to the enzyme reactions to yield a final DMSO concentration of 4%. After the addition of MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL), the microtiter plates were incubated for 20 min at 37 °C. Sodium hydroxide (80 µL of 2 N) was added to terminate the reactions and the microtiter plates were analyzed by fluorescence spectrophotometry (λex = 310; λem = 400 nm). For each experiment, a linear calibration curve constructed with 4-hydroxyquinoline (0.047–1.56 µM) was included. To determine the IC50 values, the inhibition data were fitted to the one site competition model incorporated into the Prism 5 software package (GraphPad). IC50 values were reported as the mean ± standard deviation (SD) of triplicate measurements.

3. Results and discussion

After the preliminary maceration test, eight phenolic compounds (catechin, chlorogenic acid, 3-OH benzoic acid, rutin, naringin, o-coumaric acid, t-cinnamic acid, naringenin) were detected and identified in the extracts, chlorogenic acid being the main compound. The presence of water as the extraction solvent allowed quantifying a 2-fold higher amount of total content of phenolic compounds by HPLC in comparison to alcoholic and hydroalcoholic solvents (data not shown). Based on our previous research (Diuzheva et al., 2018), the potential of the following techniques MAE, DLLME, SULLE for the characterization of the phenolic pattern of P. domestica L. leaves was investigated and optimized. Optimization of extraction procedures was carried out using 'Tuleu gras' cultivar of P. domestica L. (Fig. 1) and the best extraction conditions were applied for other cultivars as well.

3.1. Optimization of extraction procedures 3.1.1. Optimization of MAE MAE was performed in different solvents (water, water:methanol (30:70), 5% sodium chloride, 1% β-cyclodextrin, 400–800 g L−1 solutions of glucose, fructose or saccharose). It was found that the additives (sodium chloride, β-cyclodextrin, glucose, fructose, saccharose) did not have a strong influence on the performance of the extraction (Fig. 1A), but the application of the water:methanol mixture (30:70, v:v) allowed to extract a higher amount of phenolic compounds expressed as μg g−1 of dry weight. 3.1.2. Optimization of DLLME The extractions were carried out with a series of different solid:liquid ratios (2:1, 5:1, 10:1, w:v) in 5% solution of NaCl. The total extracted content of phenolic compounds was the highest at the ratio 10:1 (w:v). As a result, the ratio 10:1 was selected for further analyses (Fig. 1B). In order to select the appropriate solvent for better extraction efficiency, DLLME was carried in water, 1% solution of sodium chloride and β-cyclodextrin. Both additives caused a positive influence on the extraction procedure in comparison with conventional DLLME, whereas NaCl increased the ionic strength of the solution, and the molecule of β-cyclodextrin could include some phenolics within its conical cavity (Fig. 1C and D). Therefore, we decided to optimize the concentration of each medium. To keep all the other parameters constant, the DLLME was performed in 0–5% solutions of NaCl and 0–1.5% solutions of β-cyclodextrin. The 5% solution of NaCl and 1% solution of β-cyclodextrin showed better extraction yield in comparison with the other concentrations of additives. 3.1.3. Optimization of SULLE Acetonitrile was selected as extraction solvent due to a good extraction efficiency and simple phase separation, after the addition of 400 g L−1 glucose solution. Various solid:liquid ratios, 2.5:1, 6.5:1, 12.5:1 (w:v), were tested for the achievement of a higher extraction of phenolic compounds. The maximum extraction was achieved at the ratio 6.5:1, and it slightly decreased with the increasing of the sample amount (Fig. 1E). Getting the best separation is the main point for the selection of the 'sugaring-out' agent. Monosaccharides – glucose and fructose, and the disaccharide – saccharose were tested. At the concentration level of 400 g L−1 with fructose and saccharose the phase separation was not observed. Therefore, the three sugars were further used at the concentrations of 600 and 800 g L−1 . Results showed that the best extraction was obtained with glucose at the concentration level of 400 g L−1 (Fig. 1F). As seen from the optimization procedures, the highest amount of phenolic compounds was obtained using MAE procedure in water:- methanol (30:70, v:v). The obtained total content of phenolic compounds by HPLC analysis is 227490.1 μg g−1 , in comparison with 18546.2 μg g−1 using DLLME in 5% of NaCl, 11923.3 μg g−1 using DLLME in 1% of β-cyclodextrin, and 67541.0 μg g−1 using SULLE. Fig. 2 shows that following all extraction procedures eight phenolic compounds were identified and quantified such as catechin, chlorogenic acid, epicatechin, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, rutin, 2,3-dimethoxybenzoic acid and benzoic acid. Using DLLME, additionally, syringic acid, p-coumaric acid and naringin were extracted. Therefore, depending on purpose, the proposed extraction techniques could be applied for the determination of phenolic compounds in P. domestica L. leaves. In our case, the best quantitative extraction procedure, MAE in water:methanol (30:70), was further selected for future analyses. 3.2. Application of extraction techniques Under the optimum experimental conditions, MAE in water:- methanol (30:70) was applied for the analysis of 14 cultivars of P. domestica L. leaves (Supplementary materials section S2 for the chromatograms of each sample). Table 1 shows their total amounts as μg g−1 . The standard deviations were calculated from the results of three independent

measurements. 3.3. Total phenolic (TPC) and flavonoid (TFC) content Phenolic compounds are considered as the most abundant antioxidants in the human diet, especially in the Western world. These metabolites, that can delay or inhibit the effects of oxidation, have been considered as anti-oxidants, including compounds that either inhibit specific oxidizing enzymes or react with oxidants before they damage critical biomolecules (Abeywickrama et al., 2016). Besides the identified compounds, several other phenolics can be present in the leaves of the P. domestica L. cultivars, and could be responsible for their biological properties. Therefore, the investigated samples were compared in terms of their total phenolic and flavonoid contents using the classical Folin-Ciocâlteu and AlCl3 assays, and the results are presented in Table 2. Phenolic compounds from Prunus leaves reduced the Folin-Ciocâlteu reagent and formed a blue complex that was read at 760 nm. Ialomița (139.67 mg GAE/g extract) and Alutus (139.15 mg GAE/g extract) showed the highest total phenolic content followed by Tita (135.31 mg GAE/g extract) and Alina (134.15 mg GAE/g extract) cultivars, while Minerva (84.08 mg GAE/g extract) and Carpatin (82.84 mg GAE/g extract) showed the lowest contents in terms of total phenolic content. Moreover, the results indicated that the TPC was higher than 100 mg GAE/g extract for 10 genotypes, while only two genotypes presented lower values than 90 mg GAE/g extract. Furthermore, according to Zieliński and Kozłowska (2000) compounds such as simple carbohydrates and/or amino acids which may be present in the crude extracts could interfere with determinations of TPC by the Folin-Ciocâlteu assay, leading to discrepancies. Nonetheless, another important fact could be that the total amount of phenolics in Prunus leaves varies among different genotypes and depends on the cultivar/clone, preharvest practices, environmental conditions, and maturity stage at harvest. All of these control the accumulation of phenolics by biosynthesizing different quantities and types of polyphenols (Abeywickrama et al., 2016). Flavonoids display a variety of biological activities both in in vitro and in vivo systems (Faggio et al., 2017; Pietta, 2000; Uriarte-Pueyo and Calvo, 2011). In this study, among the investigated genotypes, thehighest flavonoid fraction was detected in Ialomița (60.32 mg QE/g extract), while the lowest value in terms of TFC was obtained for Tuleu gras cultivar. It was also noted that the highest TFC value was obtained for the same cultivar which presented the highest value in terms of TPC, while the lowest for Tuleu gras cultivar (36.60 mg QE/g extract).

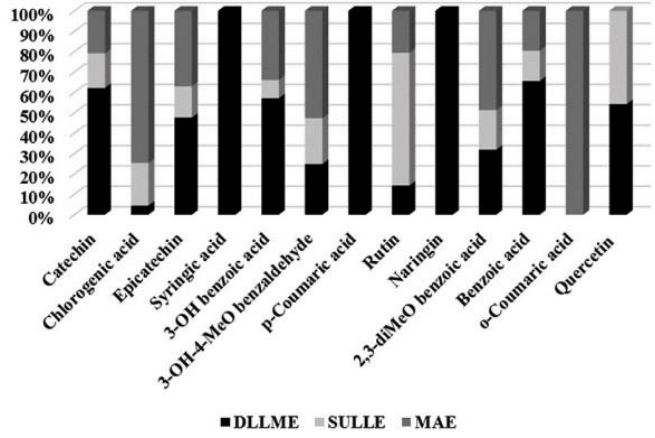

Fig. 2. Phenolics pattern obtained after the extraction optimization.

Table 1
Total amount (μg g^{-1} dry extract obtained by optimized MAE) of twenty-two compounds in *Prunus domestica* L. samples.

Cultivar	Tuleu gras	Centenar	Tita	Minerva	Tuleu timpuriu	Record	Diana
Conc. (μg g^{-1})							
Catechin	4087.2 ± 121.1	3215.7 ± 378.0	6783.3 ± 88.7	1935.5 ± 277.5	5104.9 ± 703.4	3985.3 ± 48.0	1542.6 ± 281.9
Chlorogenic acid	408555.8 ± 3618.1	312561.2 ± 19514.8	362487.6 ± 239.6	95275.4 ± 6216.3	229852.4 ± 13282.9	254672.8 ± 9977.4	222983.6 ± 3961.1
Epicatechin	513.8 ± 78.7	178.5 ± 78.5	688.4 ± 256.6	185.7 ± 41.3	940.2 ± 273.4	781.5 ± 55.4	299.0 ± 87.5
Syringic acid			210.6 ± 32.5				80.8 ± 1.1
3-OH-4-MeO benzaldehyde	276.5 ± 42.0	196.8 ± 21.8	200.7 ± 61.1	94.6 ± 11.9	154.4 ± 35.8	112.6 ± 46.2	124.9 ± 36.7
Rutin	1401.6 ± 17.1	968.3 ± 53.8	2409.9 ± 5.8	3920.5 ± 361.1	1918.2 ± 246.1	1690.2 ± 83.8	2036.4 ± 47.4
2,3-diMeO benzoic acid	403.3 ± 278.6	359.3 ± 6.3				158.1 ± 3.9	
Benzoic acid	464.7 ± 37.5	238.8 ± 1.6	147.1 ± 35.3		839.3 ± 66.2		
o-Coumaric acid	69.5 ± 14.2						
Quercetin			131.6 ± 7.3				
Total (μg g^{-1})	415772.3	317718.5	373059.3	101411.8	238809.3	261400.6	227067.4

Cultivar	Carpatin	Alutus	Silvia	Alina	Flora	Ialomița	Andreea
Conc. (μg g^{-1})							
Catechin	2844.5 ± 415.9	3879.6 ± 420.5	4257.2 ± 637.1	3737.5 ± 140.8	2765.1 ± 227.6	5132.0 ± 459.9	3998.1 ± 102.7
Chlorogenic acid	101801.2 ± 3079.0	328724.0 ± 10985.9	142180.6 ± 2564.2	329109.1 ± 3063.1	207061.3 ± 900.5	389802.9 ± 5661.2	217631.1 ± 966.3
Epicatechin	360.5 ± 15.6	4901.8 ± 331.4	1445.7 ± 337.4	9382.8 ± 123.2	344.5 ± 20.2	870.3 ± 13.4	844.3 ± 27.4
Syringic acid		103.7 ± 18.8			80.8 ± 8.1	120.7 ± 18.9	99.4 ± 9.9
3-OH-4-MeO benzaldehyde	81.7 ± 6.6	208.9 ± 37.0	121.4 ± 12.8	324.8 ± 57.8	182.9 ± 18.8		58.1 ± 17.4
Rutin	4293.4 ± 245.7	1112.5 ± 67.4	4035.2 ± 137.4	2836.9 ± 56.7	3798.1 ± 17.0	1356.0 ± 43.1	2615.9 ± 6.6
2,3-diMeO benzoic acid						385.8 ± 126.2	
Benzoic acid				192.7 ± 48.0	124.6 ± 2.1		277.2 ± 3.9
Quercetin						117.7 ± 7.8	
Total (μg g^{-1})	109381.2	338930.4	152040.0	345583.7	214316.7	397785.3	225474.4

Data are reported as mean ± standard deviation of three independent measurements (n = 3). Following compounds were not found: gallic acid, *p*-OH benzoic acid, vanillic acid, 3-OH benzoic acid, *p*-coumaric acid, sinapinic acid, *t*-ferulic acid, naringin, harpagoside, *t*-cinnamic acid, naringenin, carvacrol.

3.4. Biological activity evaluation 3.4.1. Antioxidant capacity evaluation As antioxidants, phenolic compounds prevent the formation of free radicals, which have deleterious health effects, or neutralize them once formed, and are therefore important in disease risk reduction (Abeywickrama et al., 2016; Shahidi and Ambigaipalan, 2015). DPPH and TEAC assays measure the ability of Prunus extracts to reduce the in vitro formed radicals (Table 2). In this study, Tuleu timpuriu cultivar exhibited the highest TEAC value (540.80 mg TE/g extract) followed by Alutus (520.75 mg TE/g extract), both presenting values above 500 mg TE/g extract, while the lowest TEAC value was registered for Andreea cultivar (371.58 mg TE/g extract). Moreover, the DPPH scavenging activity of the Prunus samples ranged from 3.50 mmol TE/g extract –Tuleu gras cultivar to 1.83 mmol TE/g extract for Carpatin cultivar, following a slightly different trend compared to TPC and TFC measurements. This demonstrates that the contents of total phenolics and flavonoids may not sufficiently explain the observed antioxidant activity of plant phenolic extracts, which are mixtures of different compounds with various activities in the tested samples. Furthermore, the obtained values for a tested extract represent the sum of antioxidant compounds, which depend on the solvent used to extract them from their source matrix (Abeywickrama et al., 2016; Naczk and Shahidi, 2004; Shahidi and Ambigaipalan, 2015; Shahidi and Naczk, 2004).

Table 2
Variation of total phenolic and flavonoid content, DPPH and ABTS scavenging capacity, total antioxidant capacity by phosphomolybdenum assay and metal chelating ability of leaves of *Prunus domestica* L. cultivars after microwave-assisted extraction.

Cultivar name	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	DPPH scavenging (mmol TE/g extract)	ABTS scavenging (mg TE/g extract)	Phosphomolybdenum (mmol TE/g extract)	Metal chelating ability (mg EDTAE/g extract)
Tuleu gras	112.36 ± 2.46	36.60 ± 2.90	3.50 ± 0.02	452.69 ± 3.95	1.85 ± 0.08	10.86 ± 0.36
Centenar	131.21 ± 2.46	41.93 ± 3.00	3.39 ± 0.04	446.63 ± 16.48	1.77 ± 0.02	11.60 ± 0.41
Tita	135.31 ± 0.35	50.93 ± 2.72	3.04 ± 0.12	490.45 ± 19.11	1.72 ± 0.06	12.62 ± 0.91
Minerva	84.08 ± 0.79	49.67 ± 2.45	2.06 ± 0.05	372.05 ± 3.29	1.43 ± 0.05	12.75 ± 0.93
Tuleu timpuriu	111.85 ± 2.61	46.57 ± 2.45	2.83 ± 0.05	540.80 ± 1.97	1.57 ± 0.05	14.18 ± 0.07
Record	127.92 ± 4.77	49.23 ± 2.64	2.98 ± 0.07	512.83 ± 5.93	1.79 ± 0.06	9.15 ± 0.41
Diana	102.79 ± 3.61	44.17 ± 3.49	2.60 ± 0.03	490.92 ± 1.31	1.67 ± 0.03	8.43 ± 0.54
Carpatin	82.84 ± 3.02	47.28 ± 2.88	1.83 ± 0.03	394.42 ± 16.48	1.34 ± 0.04	12.49 ± 0.42
Alutus	139.15 ± 6.02	40.54 ± 3.56	2.96 ± 0.04	520.75 ± 19.77	1.67 ± 0.02	11.26 ± 0.39
Silvia	99.38 ± 4.63	51.05 ± 3.79	2.15 ± 0.03	415.40 ± 32.96	1.44 ± 0.06	15.76 ± 0.22
Alina	134.15 ± 2.54	47.33 ± 3.23	2.63 ± 0.12	460.62 ± 38.89	1.64 ± 0.01	6.59 ± 0.09
Flora	113.60 ± 0.88	54.54 ± 3.22	2.74 ± 0.02	430.78 ± 1.97	1.64 ± 0.02	7.11 ± 0.17
Ialomița	139.67 ± 3.61	60.32 ± 4.12	3.42 ± 0.05	481.60 ± 7.91	1.90 ± 0.07	8.50 ± 0.19
Andreea	96.13 ± 2.18	45.54 ± 4.81	2.69 ± 0.03	371.58 ± 1.31	1.64 ± 0.11	14.76 ± 0.15

QE: Quercetin equivalents; GAE: Gallic acid equivalents; TE: Trolox equivalents; EDTAE: Ethylenediaminetetraacetic acid equivalents; Data are reported as mean ± standard deviation (n = 3).

Table 4
MTT assay in HGFs treated with 125, 250 and 500 µg/mL extracts for 48 h.

Cultivar	% cell viability (µg/mL)		
	125	250	500
Tuleu gras	93.4	76.6	73.4
Centenar	86.5	73.9	82.6
Tita	108.0	85.6	79.1
Minerva	84.9	69.1	66.4
Tuleu timpuriu	88.7	80.9	83.9
Record	113.1	94.0	88.5
Diana	112.2	99.4	82.3
Carpatin	67.0	55.1	50.3
Alutus	110.0	88.4	94.1
Silvia	86.8	70.0	85.0
Alina	117.5	91.2	93.3
Flora	104.8	81.2	76.8
Ialomiţa	98.7	78.8	80.1
Andreea	91.9	75.4	82.4

Cell viability was evaluated using MTT and data are presented as a percentage of viable cells compared to untreated control (DMSO).

Table 3
Variation of α-amylase, α-glucosidase, tyrosinase and MAO (A and B isozymes) inhibitory effects of *Prunus domestica* L. cultivars extracts after microwave-assisted extraction.

Cultivar name	α-amylase inhibition (mmol ACAE/g extract)	α-glucosidase inhibition (mmol ACAE/g extract)	tyrosinase inhibition (mg KAE/g extract)	IC_{50} MAO-A (µg/mL)	IC_{50} MAO-B (µg/mL)
Tuleu gras	0.51 ± 0.01	6.73 ± 0.03	15.53 ± 1.92	> 20	> 20
Centenar	0.56 ± 0.01	6.77 ± 0.02	12.93 ± 1.68	> 20	> 20
Tita	0.48 ± 0.01	6.64 ± 0.13	13.52 ± 1.04	> 20	> 20
Minerva	0.51 ± 0.02	5.37 ± 0.37	8.90 ± 0.11	> 20	> 20
Tuleu timpuriu	0.52 ± 0.01	6.66 ± 0.12	14.78 ± 0.83	> 20	> 20
Record	0.49 ± 0.02	na	17.98 ± 1.66	> 20	> 20
Diana	0.57 ± 0.01	na	16.93 ± 1.72	> 20	> 20
Carpatin	0.48 ± 0.01	5.55 ± 0.82	12.19 ± 1.53	> 20	> 20
Alutus	0.48 ± 0.01	na	13.13 ± 1.78	> 20	> 20
Silvia	0.55 ± 0.01	6.65 ± 0.14	13.05 ± 1.62	> 20	> 20
Alina	0.59 ± 0.02	na	14.73 ± 1.45	> 20	> 20
Flora	0.47 ± 0.01	6.06 ± 0.31	9.93 ± 1.32	> 20	> 20
Ialomiţa	0.52 ± 0.01	6.69 ± 0.09	23.07 ± 1.56	> 20	> 20
Andreea	0.53 ± 0.01	4.14 ± 0.07	19.04 ± 0.53	> 20	> 20

ACAE: Acarbose equivalents; KAE: Kojic acid equivalents; na: not active; Data are reported as mean ± standard deviation (n = 3).

Concerning the total antioxidant capacity by phosphomolybdenum assay, the results followed a quite similar trend as in the DPPH and TPC assays, the highest values being obtained for Ialomiţa (1.90 mmol TE/g extract) and Tuleu gras cultivars (1.85 mmol TE/g extract), while the lowest for Carpatin (1.34 mmol TE/g extract). Chelating agents, which bind to prooxidant metals, are considered as effective secondary antioxidants (Shahidi and Zhong, 2015, 2007). The present study demonstrated significant chelating activity of investigated Prunus phenolic extracts against Fe2+ in all tested genotypes, the highest value being determined for Silvia cultivar (15.76 mg EDTAE/g extract). Nonetheless, it is worthy of mentioning that among tested cultivars, nine of them presented higher chelating abilities than 10 mg EDTAE/g extract. 3.4.2. α-Amylase and α-glucosidase inhibitory activities One strategy for development of a novel antidiabetic agent is to discover substances that are capable of retarding carbohydrate digestion by inhibiting intestinal α-glucosidase and α-amylase (Eom et al., 2012). Moreover, polyphenolic extracts or phenolic compounds from a number of plants have been found to inhibit intestinal α-glucosidaseand α-amylase activities (Hemalatha et al., 2016; Rahman et al., 2017; Zhang et al., 2013) as they possess specific

structural features which may bind to enzyme active sites through hydrogen bonding or other interaction mechanisms. Both enzymes are involved in the digestion of carbohydrates and their inhibitors are able to delay the breakdown of starch and lower the postprandial blood glucose levels (Hemalatha et al., 2016). Particularly, α-glucosidase is a membrane-bound enzyme located on the epithelium of the small intestine. Moreover, yeast α-glucosidase assay can be an inexpensive and rapid method to screen for potential αglucosidase inhibitors (Apostolidis et al., 2011), as reported here. Results obtained in the present study show that most of the investigated Prunus samples are efficient inhibitors of α-glucosidase – the highest results being obtained from Centenar and Tuleu gras cultivars (Table 3) – while all samples present a low inhibitory potential on α-amylase. According to Apostolidis et al. (2011), previous reports have indicated that phenolic compounds have lower α-amylase inhibitory activity and a stronger inhibition activity against yeast α-glucosidase. 3.4.3. Tyrosinase inhibitory activity Tyrosinase is a multifunctional, glycosylated, and copper-containing oxidase, which catalyzes the first two steps in mammalian melanogenesis and is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing (Chang, 2009; Fu et al., 2014). Tyrosinase inhibitors have shown to be effective ingredients towards skin lightening, and among the bioactive compounds found in plants and plant extracts, phenolics have been reported as the ones that contribute most significantly to their antityrosinase activity (Taofiq et al., 2017). The inhibitory activity against tyrosinase of the Prunus samples ranged from 23.07 mg KAE/g extract for Ialomița cultivar to 8.90 mg KAE/g extract for Minerva cultivar. Therefore, Prunus leaves extracts studied in the present work can be further exploited as tyrosinase inhibitors to reduce the severity of hyperpigmentation and decrease melanin biosynthesis, partly due to the registered amounts of phenolic compounds. 3.4.4. MAO inhibitory activity The monoamine oxidases (MAOs) consist of two isoforms: MAO-A and MAO-B. The function of the MAOs is to catalyze the α-carbon twoelectron oxidation of monoamine substrates in the peripheral tissues and brain (Gidaro et al., 2016). In the present study, in vitro assays were performed to evaluate, for the first time, the inhibition of the human MAOs (hMAO-A and hMAO-B) by extracts from selected P. domestica cultivars. However, as seen in Table 3, MAO inhibition was not observed at the maximal concentrations tested. 3.4.5. Cytotoxicity on human gingival fibroblasts (HGFs) In most of the cases the treatment with different concentrations of extracts didn't show cytotoxicity at any concentration until 500 µg/mL. Only ‘Carpatin’ and ‘Minerva’ cultivars of P. domestica L. at 250 and 500 µg/mL reduced partially cell viability of HGFs population. In particular, ’Carpatin’ at 250 and 500 µg/mL induced an increased cell death of HGFs population, as shown in Table 4. After treatment with most of the extracts, HGFs morphology was also observed at each concentration (125, 250 and 500 µg/mL) evidencing elongated adherent fibroblasts similarly to untreated cells (Fig. 3).

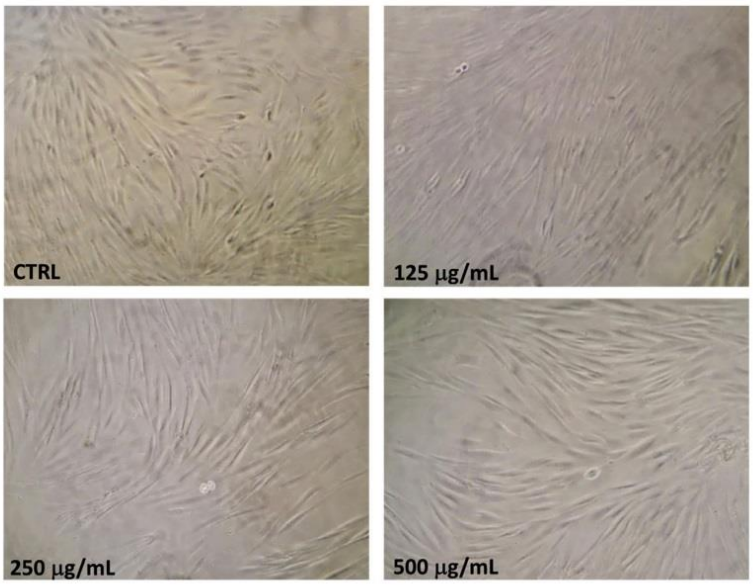

Fig. 3. Example of HGFs morphology observed at extract concentration of 125, 250 and 500 μg/mL in comparison with untreated cells.

4. Conclusions

The most suitable extraction method for the phenolic fraction of the dried leaves of P. domestica L. was demonstrated to be MAE in water:- methanol (30:70, v:v). It promoted to extract a high amount of phenolic compounds and is an environmental friendly procedure. Furthermore, the chemical composition using HPLC-PDA was investigated revealing chlorogenic acid as the dominant phenolic acid, and rutin as the most abundant flavonoid among all cultivars. The highest radical scavenging capacity was obtained for Tuleu timpuriu cultivar, in the ABTS assay, while the best metal chelating ability was showed by Silvia cultivar. Centenar cultivar was found to be the most active on α-glucosidase inhibition (6.77 mmol ACAE/g extract), while the best antityrosinase activity was shown by Ialomița cultivar (23.07 mg KAE/g extract). Nonetheless, in most of the cases the treatment with different concentrations of extracts didn't show cytotoxicity at any concentration until 500 μg/mL. Only 'Carpatin' and 'Minerva' cultivars of P. domestica, at 250 and 500 μg/mL, reduced partially cell viability of HGFs population. However, further evidence is needed in order to deeply elucidate the mechanisms of in vivo activities, bioavailability, and involved metabolic pathways.

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Appendix A. Supplementary data

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Transparency document

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References

Abeywickrama, G., Debnath, S.C., Ambigaipalan, P., Shahidi, F., 2016. Phenolics of selected cranberry genotypes (Vaccinium macrocarpon Ait.) and their antioxidant efficacy. J. Agric. Food Chem. 64http://dx.doi.org/10.1021/acs.jafc.6b04291. acs.jafc. 6b04291. Ameer, K., Shahbaz, H.M., Kwon, J.-H., 2017. Green extraction methods for polyphenols from plant matrices and their byproducts: a review. Compr. Rev. Food Sci. Food Saf. 16, 295–315. http://dx.doi.org/10.1111/1541-4337.12253. Anthemidis, A.N., Ioannou, K.G., 2009. Recent developments in homogeneous and dispersive liquid–liquid extraction for inorganic elements determination. A review. Talanta 80, 413–421. http://dx.doi.org/10.1016/j.talanta.2009.09.005. Apostolidis, E., Li, L., Lee, C., Seeram, N.P., 2011. In vitro evaluation of phenolic-enriched maple syrup extracts for inhibition of carbohydrate hydrolyzing enzymes relevant to type 2 diabetes management. J. Funct. Foods 3, 100–106. http://dx.doi.org/10. 1016/j.jff.2011.03.003. Armenta, S., Garrigues, S., de la Guardia, M., 2008. Green analytical chemistry. Trends Anal. Chem. 27, 497–511. http://dx.doi.org/10.1016/j.trac.2008.05.003. Chang, T.S., 2009. An updated review of tyrosinase inhibitors. Int. J. Mol. Sci. 10, 2440–2475. http://dx.doi.org/10.3390/ijms10062440. Chen, L., Teng, H., Jia, Z., Battino, M., Miron, A., Yu, Z., Cao, H., Xiao, J., 2017. Intracellular signaling pathways of inflammation modulated by dietary flavonoids: the most recent evidence. Crit. Rev. Food Sci. Nutr. 8398. http://dx.doi.org/10.1080/ 10408398.2017.1345853. Chen, L., Teng, H., Xie, Z., Cao, H., Cheang, W.S., Skalicka-Woniak, K., Georgiev, M.I., Xiao, J., 2018. Modifications of dietary flavonoids towards improved bioactivity: an update on structure–activity relationship. Crit. Rev. Food Sci. Nutr. 58, 513–527. http://dx.doi.org/10.1080/10408398.2016.1196334. De Brito Cardoso, G., Mourão, T., Pereira, F.M., Freire, M.G., Fricks, A.T., Soares, C.M.F., Lima, Á.S., 2013. Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. Separ. Purif. Technol. 104, 106–113. http://dx.doi.org/10.1016/j.seppur.2012.11.001. Diuzheva, A., Carradori, S., Andruch, V., Locatelli, M., De Luca, E., Tiecco, M., Germani, R., Menghini, L., Nocentini, A., Gratteri, P., Campestre, C., 2018. Use of innovative (Micro)Extraction techniques to characterise Harpagophytum procumbens root and its commercial food supplements. Phytochem. Anal. 29, 233–241. http://dx.doi.org/10. 1002/pca.2737. Du, F.Y., Xiao, X.H., Luo, X.J., Li, G.K., 2009. Application of ionic liquids in the microwave-assisted extraction of polyphenolic compounds from medicinal plants. Talanta 78, 1177–1184. http://dx.doi.org/10.1016/j.talanta.2009.01.040. Ebrahimpour, B., Yamini, Y., 2014. Homogeneous liquid-phase microextraction followed by filtration-based phase separation coupled to high-performance liquid chromatography. J. Separ. Sci. 37, 2002–2009. http://dx.doi.org/10.1002/jssc.201400257. Eom, S.H., Lee, S.H., Yoon, N.Y., Jung, W.K., Jeon, Y.J., Kim, S.K., Lee, M.S., Kim, Y.M., 2012. α-Glucosidase- and α-amylase-inhibitory activities of phlorotannins from Eisenia bicyclis. J. Sci. Food Agric. 92, 2084–2090. http://dx.doi.org/10.1002/jsfa. 5585. Faggio, C., Sureda, A., Morabito, S., Sanches Silva, A., Mocan, A., Nabavi, S.F., Nabavi, S.M., 2017. Flavonoids and platelet aggregation: a brief review. Eur. J. Pharmacol. 807, 91–101. http://dx.doi.org/10.1016/j.ejphar.2017.04.009. Fu, R., Zhang, Y., Guo, Y., Chen, F., 2014. Antioxidant and tyrosinase inhibition activities of the ethanol-insoluble fraction of water extract of Sapium sebiferum (L.) Roxb. leaves. South Afr. J. Bot. 93, 98–104. http://dx.doi.org/10.1016/j.sajb.2014.04.003. Gidaro, M.C., Astorino, C., Petzer, A., Carradori, S., Alcaro, F., Costa, G., Artese, A., Rafele, G., Russo, F.M., Petzer, J.P., Alcaro, S., 2016. Kaempferol as selective human MAO-A inhibitor: analytical detection in calabrian red wines, biological and molecular modeling studies. J. Agric. Food Chem. 64, 1394–1400. http://dx.doi.org/10. 1021/acs.jafc.5b06043. Hemalatha, P., Bomzan, D.P., Rao, B. V. Sathyendra, Sreerama, Y.N., 2016. Distribution of phenolic antioxidants in whole and milled fractions of quinoa and their inhibitory effects on α-amylase and α-glucosidase activities. Food Chem. 199, 330–338. http:// dx.doi.org/10.1016/j.foodchem.2015.12.025. Jaiswal, R., Karaköse, H., Rühmann, S., Goldner, K., Neumüller, M., Treutter, D., Kuhnert, N., 2013. Identification of phenolic compounds in plum fruits (Prunus

salicina L. and Prunus domestica L.) by high-performance liquid chromatography/tandem mass spectrometry and characterization of varieties by quantitative phenolic fingerprints. J. Agric. Food Chem. 61, 12020–12031. http://dx.doi.org/10.1021/jf402288j. Khan, H., Jawad, M., Kamal, M.A., Baldi, A., Xiao, J., Nabavi, S.M., Daglia, M., 2018. Evidence and prospective of plant derived flavonoids as antiplatelet agents: strong candidates to be drugs of future. Food Chem. Toxicol. http://dx.doi.org/10.1016/j. fct.2018.02.014. Lima, M.J.A., Nascimento, C.F., Rocha, F.R.P., 2017. Feasible photometric measurements in liquid–liquid extraction by exploiting smartphone-based digital images. Anal. Methods 9, 2220–2225. http://dx.doi.org/10.1039/C7AY00388A. Martins, N., Barros, L., Dueñas, M., Santos-Buelga, C., Ferreira, I.C.F.R., 2015. Characterization of phenolic compounds and antioxidant properties of Glycyrrhiza glabra L. rhizomes and roots. RSC Adv. 5, 26991–26997. http://dx.doi.org/10.1039/ C5RA03963K. McConvey, I.F., Woods, D., Lewis, M., Gan, Q., Nancarrow, P., 2012. The importance ofacetonitrile in the pharmaceutical industry and opportunities for its recovery from waste. Org. Process Res. Dev. 16, 612–624. http://dx.doi.org/10.1021/op2003503. Mitic, V., Ilic, M., Dimitrijevic, M., Cvetkovic, J., Ciric, S., Jovanovic, V.S., 2016. Chemometric characterization of peach, nectarine and plum cultivars according to fruit phenolic content and antioxidant activity. Fruits 71, 57–66. http://dx.doi.org/ 10.1051/fruits/2015042. Mocan, A., Schafberg, M., Crișan, G., Rohn, S., 2016a. Determination of lignans and phenolic components of Schisandra chinensis (Turcz.) Baill. using HPLC-ESI-ToF-MS and HPLC-online TEAC: contribution of individual components to overall antioxidant activity and comparison with traditional antioxidant assays. J. Funct. Foods 24, 579–594. http://dx.doi.org/10.1016/j.jff.2016.05.007. Mocan, A., Zengin, G., Crişan, G., Mollica, A., 2016b. Enzymatic assays and molecular modeling studies of Schisandra chinensis lignans and phenolics from fruit and leaf extracts. J. Enzym. Inhib. Med. Chem. 31, 200–210. http://dx.doi.org/10.1080/ 14756366.2016.1222585. Mocan, A., Zengin, G., Simirgiotis, M., Schafberg, M., Mollica, A., Vodnar, D.C., Crișan, G., Rohn, S., 2017. Functional constituents of wild and cultivated Goji (L. barbarum L.) leaves: phytochemical characterization, biological profile, and computational studies. J. Enzym. Inhib. Med. Chem. 32, 153–168. http://dx.doi.org/10.1080/ 02699931.2011.628301. Mollica, A., Locatelli, M., Macedonio, G., Carradori, S., Sobolev, A.P., De Salvador, R.F., Monti, S.M., Buonanno, M., Zengin, G., Angeli, A., Supuran, C.T., 2016. Microwaveassisted extraction, HPLC analysis, and inhibitory effects on carbonic anhydrase I, II, VA, and VII isoforms of 14 blueberry Italian cultivars. J. Enzym. Inhib. Med. Chem. 6366, 1–6. http://dx.doi.org/10.1080/14756366.2016.1214951. Mostert, S., Petzer, A., Petzer, J.P., 2015. Indanones as high-potency reversible inhibitors of monoamine oxidase. ChemMedChem 10, 862–873. http://dx.doi.org/10.1002/ cmdc.201500059. Naczk, M., Shahidi, F., 2004. Extraction and analysis of phenolics in food. J. Chromatogr. A 1054, 95–111. http://dx.doi.org/10.1016/j.chroma.2004.08.059. Pietta, P.G., 2000. Flavonoids as antioxidants. J. Nat. Prod. 63, 1035–1042. http://dx.doi. org/10.1021/np9904509. Proestos, C., Komaitis, M., 2008. Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds. LWT - Food Sci. Technol. 41, 652–659. http://dx.doi.org/10.1016/j.lwt.2007.04.013. Rahman, M.J., de Camargo, A.C., Shahidi, F., 2017. Phenolic and polyphenolic profiles of chia seeds and their in vitro biological activities. J. Funct. Foods 35, 622–634. http:// dx.doi.org/10.1016/j.jff.2017.06.044. Routray, W., Orsat, V., 2014. MAE of phenolic compounds from blueberry leaves and comparison with other extraction methods. Ind. Crop. Prod. 58, 36–45. http://dx.doi. org/10.1016/j.indcrop.2014.03.038. Savran, A., Zengin, G., Aktumsek, A., Mocan, A., Glamočlija, J., Ćirić, A., Soković, M., 2016. Phenolic compounds and biological effects of edible Rumex scutatus and Pseudosempervivum sempervivum: potential sources of natural agents with health benefits. Food Funct. 7, 3252–3262. http://dx.doi.org/10.1039/C6FO00695G. Shahidi, F., Ambigaipalan, P., 2015. Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects - a review. J. Funct. Foods 18, 820–897. http://dx.doi.org/10.1016/j.jff.2015.06.018. Shahidi, F., Naczk, M., 2004. Phenolics in Food and Nutraceuticals. CRC Press Taylor & Francis Group, New York. http://dx.doi.org/10.1017/CBO9781107415324.004. Shahidi, F., Zhong, Y., 2015. Measurement of antioxidant activity. J. Funct. Foods 18, 757–781. http://dx.doi.org/10.1016/j.jff.2015.01.047. Shahidi, F., Zhong, Y., 2007. Measurement of antioxidant activity in food and biological systems. In: Shahidi, F., Ho, C.-T.

(Eds.), Antioxidant Measurement and Applications. American Chemical Society, pp. 36–66. http://dx.doi.org/10.1021/bk-2007-0956. ch004. Švarc-Gajić, J., Stojanović, Z., Segura Carretero, A., Arráez Román, D., Borrás, I., Vasiljević, I., 2013. Development of a microwave-assisted extraction for the analysis of phenolic compounds from Rosmarinus officinalis. J. Food Eng. 119, 525–532. http://dx.doi.org/10.1016/j.jfoodeng.2013.06.030. Taofiq, O., Heleno, S.A., Calhelha, R.C., Alves, M.J., Barros, L., González-Paramás, A.M., Barreiro, M.F., Ferreira, I.C.F.R., 2017. The potential of Ganoderma lucidum extracts as bioactive ingredients in topical formulations, beyond its nutritional benefits. Food Chem. Toxicol. 108, 139–147. http://dx.doi.org/10.1016/j.fct.2017.07.051. Tete, S., Zizzari, V.L., Borelli, B., De Colli, M., Zara, S., Sorrentino, R., Scarano, A., Gherlone, E., Cataldi, A., Zarone, F., 2014. Proliferation and adhesion capability of human gingival fibroblasts onto zirconia, lithium disilicate and feldspathic veneering ceramic in vitro. Dent. Mater. J. 33, 7–15. http://dx.doi.org/10.4012/dmj.2013-185. Timofeeva, I., Shishov, A., Kanashina, D., Dzema, D., Bulatov, A., 2017. On-line in-syringe sugaring-out liquid-liquid extraction coupled with HPLC-MS/MS for the determination of pesticides in fruit and berry juices. Talanta 167, 761–767. http://dx.doi.org/ 10.1016/j.talanta.2017.01.008. Uriarte-Pueyo, I., Calvo, M.I., 2011. Flavonoids as acetylcholinesterase inhibitors. Curr. Med. Chem. 18, 5289–5302. http://dx.doi.org/10.2174/092986711798184325. Walkowiak-Tomczak, D., Regu, J., 2008. Physico-chemical properties and antioxidant activity of selected plum cultivars fruit. Acta Sci. Pol., Technol. Aliment. 7, 15–22. Xiao, J., 2017. Dietary flavonoid aglycones and their glycosides: which show better biological significance? Crit. Rev. Food Sci. Nutr. 57, 1874–1905. http://dx.doi.org/ 10.1080/10408398.2015.1032400. Xiao, J., Capanoglu, E., Jassbi, A.R., Miron, A., 2016. Advance on the flavonoid C-glycosides and health benefits. Crit. Rev. Food Sci. Nutr. 56, S29–S45. http://dx.doi.org/ 10.1080/10408398.2015.1067595. Yilmaz, E., Soylak, M., 2016. Latest trends, green aspects, and innovations in liquidphase–based microextraction techniques: a review. Turk. J. Chem. 40, 868–893. http://dx.doi.org/10.3906/kim-1605-26. Zengin, G., Menghini, L., Bellagamba, G., Fabiana, M., De Luca, E., Armellani, L., Cifelli, ., Granese, A., 2018. Analytical procedures for secondary metabolites determination: recent trends and future perspectives. Lett. Drug Des. Discov. 15, 281–293. Zengin, G., Menghini, L., Malatesta, L., De Luca, E., Bellagamba, G., Uysal, S., Aktumsek, A., Locatelli, M., 2016. Comparative study of biological activities and multicomponent pattern of two wild Turkish species: Asphodeline anatolica and Potentilla speciosa. J. Enzym. Inhib. Med. Chem. 31, 203–208. http://dx.doi.org/10.1080/ 14756366.2016.1178247. Zhang, Z., Luo, A., Zhong, K., Huang, Y., Gao, Y., Zhang, J., Gao, H., Xu, Z., Gao, X., 2013. α-Glucosidase inhibitory activity by the flower buds of Lonicera japonica Thunb. J. Funct. Foods 5, 1253–1259. http://dx.doi.org/10.1016/j.jff.2013.04.008. Zieliński, H., Kozłowska, H., 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. J. Agric. Food Chem. 48, 2008–2016. http://dx.doi.org/10.1021/jf990619o.