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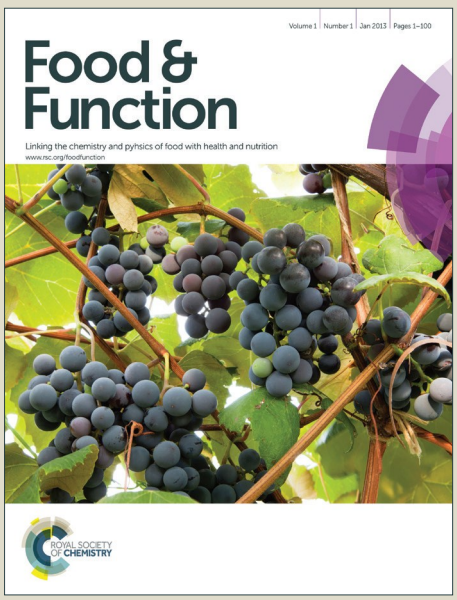

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Chemical characterization and bioactive properties of *Geranium molle* L.: from the plant to the most active extract and its phytochemicals

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Abstract

After a period of indifference, in which synthetic compounds were favored, there is an increasing interest in the study of the biological properties of plants and the active principles responsible for their therapeutic properties. *Geranium molle* L. has been used in the Portuguese folk medicine for the treatment of various ailments including cancer but, unlike many of the species from the *Geranium* genus, its phytochemical characterization and biological activity are virtually unexplored. In this study a *G. molle* sample from Trás-os-Montes, north-eastern Portugal, was chemically characterized regarding nutritional value, free sugars, organic acids, fatty acids and tocopherols, and several aqueous (decoction, infusion) and organic (*n*-hexane, dichloromethane, ethyl acetate, acetone, methanol) extracts of the plant were assessed for their bioactive properties. The antioxidant activity was evaluated by means of the free radicals scavenging activity, reducing power and inhibition of lipid peroxidation. The cytotoxicity of the different extracts was assessed *in vitro* against several human cancer cell lines (breast, lung, cervical and hepatocellular carcinomas) and, additionally, their hepatotoxicity was evaluated using a porcine liver primary cell culture. *G. molle* was shown to be rich in carbohydrates and proteins, providing tocopherols and essential fatty acids. Amongst the various extracts, the acetone extract was found to have the highest content of phenolic compounds (mainly ellagitannins, but also some flavone and flavonol glycosides) as well as the highest antioxidant and cytotoxic activities. To the best of our knowledge, this is the first report on the chemical composition and bioactive properties of *G. molle*.

Keywords: *Geranium molle* L.; Nutrients; Phytochemicals; Antioxidant; Cytotoxicity.

1. Introduction

The use of medicinal plants to improve health was highly valued in ancient civilizations. Until the mid-nineteenth century, plants were the main therapeutic agents used by humans.[1,2] After a period of indifference, in which synthetic compounds were favored, in recent years it has been observed an increasing interest of researchers in the study of the biological activity of plants and the active principles responsible for their therapeutic properties.[3,4]

Geranium molle L., commonly known as Dove's-foot Crane's-bill or Dovesfoot Geranium, is an annual or biennial herb that belongs to the Geraniaceae family. This plant is native of Europe, northern Africa, and western Asia. It was introduced in southern Africa, the Americas, eastern Asia, Australia, and New Zealand.[5,6] *Geranium molle* L. grows spontaneously in cultivated and waste places, open habitats, dunes, and dry grassland or roadsides, between 0 and 1500 m in altitude.[7,8]

Unlike other species of *Geranium* genus, namely the closely related *Geranium robertianum* L., there seems to be an almost complete absence of references to the use of *Geranium molle* in folk medicine. Exception is made to an ethnopharmacological study carried out by Neves et al.[9] in some regions of Trás-os-Montes (north east Portugal), where the flowering aerial parts and roots of the wild plant are traditionally used to prepare decoctions and infusions for stomach acidity and stomach ache, gingivitis, eye inflammation and cuts, uterus inflammation and cancer treatment.

The phytochemistry of *Geranium* genus is reasonably well-known and clearly dominated by phenolic constituents,[10] the most studied classes of compounds being tannins, flavonoids and phenolic acids. The phenolic compounds, especially the flavonoids, have been cited as the main biologically active components among those found in *Geranium* species.[11] This class of compounds from *Geranium* spp. were reported to exhibit antiviral,

antitumor, antithrombotic, hepatoprotective, anti-inflammatory, antiallergic, antiproliferative, anticancer and immune stimulant effects.[11-13]

Although the *Geranium* genus phytochemistry is relatively well-known, reports on the chemical composition and biological properties of *Geranium molle* L. cannot be found in the literature. Therefore, in the present study, a wild sample of *Geranium molle* L. was analysed for its nutritional composition (proteins, fat, carbohydrates and ash) and chemically characterized regarding hydrophilic (sugars, organic acids) and lipophilic (fatty acids and tocopherols) molecules. An infusion and a decoction (common forms of consumption) and different organic extracts were evaluated for their bioactive properties, namely the antioxidant and antitumor properties, being the most active extract characterized in terms of phenolic compounds.

2. Materials and methods

2.1. Standards and Reagents

Acetonitrile (99.9%), *n*-hexane (97%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also, L-ascorbic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), organic acids, sugar standards, acetic acid, formic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris. Phenolic compound standards were purchased from Extrasynthèse (Genay, France). Tocol (50 mg/mL) and individual tocopherols were purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin

solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Plant material

Geranium molle L. specimens in blossom (including thin roots, basal leaves, ascending stems, upper hairy leaves and flowers) were collected in Serra da Nogueira, Bragança, north-eastern Portugal, in March 2015, and subsequently cleaned out all dirt and dried parts. The amount of plant material collected was around 780 g. Voucher specimens are deposited at the herbarium of the Escola Superior Agrária de Bragança (BRESA). The botanical identification was confirmed by the agronomist Dra. Ana Maria Carvalho of the School of Agriculture, Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). The sample was lyophilized, reduced to a fine dried powder (~ 20 mesh), mixed to obtain a homogenous sample and stored in a refrigerator at –20 ºC, protected from light.

2.3. Chemical characterization

2.3.1. Macronutrients composition of the crude plant material

The sample was analysed for its nutritional chemical composition (proteins, fat, carbohydrates and ash) through standard procedures.[14] The crude protein content (N × 6.25) of the sample was estimated by the macro-Kjeldahl method. The crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus. The ash content was determined by incineration at 600 ± 15 ºC. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Hydrophilic compounds

Free sugars. Free sugars were determined via high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by the authors[15] using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), a degasser system (Smart line manager 5000) and an auto-sampler (AS-2057 Jasco, Easton, MD, USA) coupled to a refraction index (RI) detector (Knauer Smartline 2300). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (5 µm, 4.6 × 250 mm, Knauer) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v), at a flow rate of 1 mL/min. Sugars identification was performed by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex, Podohradska, Czech Republic). Quantification was based on the RI signal response of each standard, using the IS (melezitose) method and by using calibration curves obtained for the commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Organic acids. Organic acids were determined following a procedure previously optimized and described by the authors.[16] The analysis was performed using a Shimadzu 20A series ultra-fast liquid chromatograph (UFLC) (Shimadzu Coperation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 µm, 4.6 × 250 mm) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out with a diode array detector (DAD), using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison

of the area of their peaks, recorded at 215 or 245 nm, with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.3. Lipophilic compounds

Fatty acids. Fatty acids were determined after transesterification according to the procedure previously described by the authors.[15] The fatty acids profile was analyzed with a DANI 1000 gas chromatograph (GC) equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acids identification was performed by comparing the relative retention times of the sample's FAME peaks with standards. The results were recorded and processed using Clarity 4.0.1.7 Software (DataApex, Podohradska, Czech Republic) and expressed in relative percentage of each fatty acid.

Tocopherols. Tocopherols were determined following a procedure previously described by the authors.[15] The analysis was performed by HPLC (equipment described in Section 2.3.2. Free sugars), using a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (YMC Waters, Milford, MA, USA) normal-phase column (5 µm, 4.6 mm × 250 mm), operating at 35 ºC. The mobile phase used was

a mixture of *n*-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparison with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.4. Preparation of organic and aqueous extracts

The organic (hexane, dichloromethane, ethyl acetate, acetone and methanol) and aqueous (obtained by infusion and decoction) extracts were prepared from the lyophilized plant. For the preparation of the organic extracts a sample (100 g) was extracted with 500 mL of *n*-hexane and the mixture was stirred vigorously at room temperature, 150 rpm, for 48 h, and then filtered under reduced pressure successively through a Whatman No. 541 paper and a sintered glass funnel. The solid residue was extracted with an additional 500 mL of *n*-hexane under the same conditions. The combined extracts were evaporated to dryness at 40 ºC under reduced pressure. The resulting residue was further extracted sequentially with dichloromethane, ethyl acetate, acetone and finally methanol, according to the procedure described above.

The infusions were prepared by adding the sample (1 g) to 100 mL of boiling distilled water, left to stand at room temperature for 5 min, and then filtered under reduced pressure successively through a Whatman No. 541 paper and a sintered glass funnel. The obtained infusions were frozen and lyophilized.

The decoctions were also prepared by adding the sample (1 g) to 100 mL of distilled water and boiled for 5 min. The mixture was left to stand for 5 min at room temperature and then filtered under reduced pressure successively through a Whatman No. 541 paper and a sintered glass funnel. The obtained decoctions were frozen and lyophilized.

2.5. Bioactive compounds in the extracts

For total phenolics determination, an aliquot of the different extracts (1 mL) were mixed separately, with *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10 *v/v*) and sodium carbonate (75 g/L, 4 mL). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for color development. Absorbance was then measured at 765 nm.[17] Gallic acid was used to calculate the standard curve (0.1-1 mM) and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

For total flavonoids determination, an aliquot of the different extracts (0.5 mL) were mixed separately, with distilled water (2 mL) and subsequently with $NaNO_2$ solution (5%, 0.15 mL). After 6 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to stand further 6 min, thereafter, NaOH solution (4%, 2 mL) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. The intensity of pink color was measured at 510 nm.[18] Catechin was used to calculate the standard curve (0.3-1 mM) and the results were expressed as mg of catechin equivalents (CE) per g of extract.

2.6. Evaluation of bioactive properties of the extracts

2.6.1. Antioxidant activity assays

The organic extracts were redissolved in methanol (final concentration 10 mg/mL). The aqueous extracts were redissolved in water (final concentration 10 mg/mL). The final solutions obtained were further diluted to different concentrations to be submitted to distinct evaluation assays of the antioxidant activity.

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH

discoloration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S and A_{DPPH} are, respectively, the absorbance of the sample solution and that of the DPPH solution at 515 nm. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS); the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B are the absorbance of the control and the sample solution, respectively.[16,19]

The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as positive control.

2.6.2. Cytotoxicity in human tumor cell lines

The aqueous and organic extracts were redissolved in water and ethanol 20%, respectively, in order to obtain a final concentration 8 mg/mL. The final solution was further diluted to different concentrations (400 to 1.5 µL/mL) to be submitted to *in vitro* cytotoxicity evaluation. Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). The cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7 and

NCI-H460) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. The cells were then treated for 48 h with the different diluted sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% TCA (100 μL) and incubated for 60 min at 4 ºC. Plates were then washed with deionized water and dried; SRB solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the bound SRB was solubilised with 10 mM Tris (200 μL, pH 7.4) and the absorbance was measured at 540 nm in the microplate reader mentioned above.[16] The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.6.3. Hepatotoxicity in non-tumor cells

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin, 100 μg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC under a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were

277 subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated
278 in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.[15,16]
279 Cells were treated for 48 h with the different diluted sample solutions and the same
280 procedure described in the previous section for SRB assay was followed. The results were
281 expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth).
282 Ellipticine was used as positive control.
283
284 *2.7. Phenolic composition of the acetone extract*
285 The acetone extract was redissolved in water/methanol 80:20 (v/v) (final concentration 5
286 mg/mL). Phenolic compounds were determined by HPLC (Hewlett-Packard 1100
287 chromatographer, Agilent Technologies, Santa Clara, CA, USA) as previously described
288 by the authors.[16,19] Double online detection was carried out with a DAD using 280 nm
289 and 370 nm as preferred wavelengths and with a mass spectrometer (MS) connected to
290 the HPLC system via the DAD cell outlet. The phenolic compounds were identified by
291 comparing their retention time, UV-vis and mass spectra with those obtained from
292 standard compounds, when available. Otherwise, peaks were tentatively identified
293 comparing the obtained information with available data reported in the literature. For
294 quantitative analysis, a calibration curve for each available phenolic standard was
295 constructed based on the UV signal. For the identified phenolic compounds for which a
296 commercial standard was not available, the quantification was performed through the
297 calibration curve of other compound from the same phenolic group. The results were
298 expressed in mg per g of extract.
299
300 *2.8. Statistical analysis*

For all the experiments, three samples were analyzed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD). The differences between the different samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. This treatment was carried out using the SPSS v. 22.0 program.

3. Results and discussion

3.1. Chemical characterization of Geranium molle *L.*

The results of the nutritional characterization of *G. molle,* namely, macronutrients, sugars, organic acids and fatty acids, are presented in **Table 1**. Carbohydrates were the most abundant macronutrients found in the studied sample, followed by proteins, fat and ash. This plant presented 72.2% of moisture and the energetic contribution was ~ 436 kcal/100 g dw.

Fructose, glucose and sucrose were the free sugars detected in this sample. The total sugars content was ~ 9 g/100 g dw, glucose being present in a much larger amount (~ 6 g/100 g dw) than the two other sugars.

A total of six different organic acids were detected in the plant, namely, oxalic, quinic, malic, ascorbic, citric and fumaric acids (**Table 1**). Malic acid was the most abundant (~ 2.8 g/100 g dw), followed by quinic acid (~ 2.6 g/100 g dw).

Twenty-eight fatty acids (FA) were determined in *G. molle* (**Table 1**). Highest percentages were found for oleic (C18:1n9), palmitic (C16:0) and stearic acids (C18:0). Saturated fatty acids (SFA) predominated over monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

Regarding tocopherols, α-tocopherol (18.9 mg/100 g dw) was the most abundant isoform present in *G. molle*. β-Tocopherol and γ-tocopherol were present but in very small amounts. δ-Tocopherol was not detected in this plant.

3.2. Bioactive compounds in different Geranium molle *L. extracts.*

Concentrations of total polyphenols and total flavonoids, as determined by photometrical methods in different *G. molle* extracts are presented in **Table 2**. The acetone extract displayed the highest content of both total polyphenols (497 mg GAE/g extract) and total flavonoids (112 mg CE/g extract). Acetone has been reported as a good solvent for the extraction of phenolic compounds and flavonoids.[20,21] The infusion and the decoction presented similar concentrations of total flavonoids but somewhat different content of total polyphenols, the extract resulting from the infusion being richer in this group of compounds. The dichloromethane and the *n*-hexane extracts showed very low concentrations of both total polyphenols and total flavonoids.

3.3. Bioactive properties of different Geranium molle *L. extracts.*

The *in vitro* antioxidant and cytotoxic properties of different extracts of *G. molle* were evaluated, and the results are presented in **Table 3**. The antioxidant activity was determined by free radical (DPPH) scavenging activity, reducing power, inhibition of lipid peroxidation in brain cell homogenates, and TBARS assays. The cytotoxicity was tested against human tumor cell lines (breast, lung, cervical and hepatocellular carcinomas) and the hepatotoxicity was evaluated using a porcine liver primary cell culture.

In general, all the extracts revealed antioxidant potential. The acetone extract displayed the highest antioxidant activity in all the assays. This was probably related to the higher

content of total phenols and total flavonoids found in this extract, compared to the other prepared extracts (**Table 2**). The antioxidant properties of many plants are closely related to the presence of phenolic compounds, which constitute the most abundant secondary metabolites of plants.[22]

Regarding the cytotoxic properties, almost all of the extracts showed activity. The aqueous extract obtained by infusion, and methanol and ethyl acetate extracts showed similar cytotoxic effect against MCF-7, NCI-H460 and HeLa. The dichloromethane extract was ineffective against the HepG2 cell line even at the maximum concentration assayed (400 µg/mL). The *n*-hexane extract showed activity only against HeLa cells. The acetone extract displayed the highest cytotoxic effect, being significantly more potent than the remaining extracts (GI_{50} values approximately four times lower) against all cell lines. Unfortunately, this extract also presented the highest toxicity against normal primary cells from porcine liver (PLP2) (GI_{50} ~ 190 µg/mL). However, the concentration required to reach 50% of growth inhibition of PLP2 is about 3-4 times higher than the concentration required to achieve 50% of growth inhibition of the human tumor cell lines tested. Therefore, it would be recommended a dosage of 85 µg/mL of *G. molle* acetone extract to guarantee 50% of growth inhibition of the human tumor cell lines tested, without presenting toxicity effects for non-tumor cells.

The aqueous, dichloromethane and *n*-hexane extracts did not show hepatotoxicity against PLP2 cells up to the maximal tested concentration (GI_{50} > 400 µg/mL). The methanol and ethyl acetate extracts presented similar hepatotoxicity against this cell line (GI_{50} ~ 330 µg/mL).

Trolox and ellipticine were used as positive controls in the antioxidant and cytotoxic activity assays, respectively. However, as these are individual compounds, they should not be considered as standards and the comparison with the results obtained for the

375 extracts/oral preparations should be avoided since an eventual synergistic effect of the
376 mixtures cannot be precluded.
377
378 *3.4. Analysis of phenolic compounds in the acetone extract*
379 Amongst the several *G. molle* extracts prepared, the acetone extract was found to exhibit
380 the highest amount of total phenolic compounds, as well as the highest antioxidant and
381 cytotoxic activity, so that its detailed phenolic composition was also analysed. The HPLC
382 phenolic profile of that extract recorded at 280 and 370 nm is shown in **Figure 1**. The
383 peak characteristics and tentative identities are presented in **Table 4**. Sixteen phenolic
384 compounds were detected, five of which were ellagitannins, one phenolic acid and ten
385 flavonoids.
386 (+)-Catechin (compound 3), 5-*O*-caffeoylquinic acid (compound 4), luteolin-6-*C*-
387 glucoside (compound 9), quercetin-3-*O*-rutinoside (compound 10), apigenin-6-*C*-
388 glucoside (compound 12), quercetin-3-*O*-glucoside (compound 13), kaempferol-3-*O*-
389 rutinoside (compound 14) and kaempferol-3-*O*-glucoside (compound 16) were positively
390 identified according to their retention, mass and UV-vis characteristics by comparison
391 with commercial standards.
392 Compounds 1, 2 and 5-7 were identified as hydrolysable tannins (ellagitannin derivatives)
393 and assigned as different hexahydroxydiphenoyl (HHDP)-glucose esters based on their
394 pseudomolecular ions and fragmentation patterns. Thus, mass characteristics of
395 compound 1 ($[M-H]^-$ at *m/z* 633) pointed to a galloyl-HHDP-glucose isomer, whereas
396 those of compounds 2 and 5 ($[M-H]^-$ at *m/z* 785) were consistent with digalloyl-HHDP-
397 glucose isomers, and compounds 6 and 7 with bis-HHDP-glucose isomers.[23,24] Similar
398 compounds have been described in other *Geranium* species;[10,25-31] actually HHDP esters
399 are considered the main hydrolysable tannins and majority phenolic compounds in most

species of *Geranium*.[10,25,27,32,33] However, to the best of our knowledge, they have never been reported in *G. molle*.

The remaining compounds would correspond to flavonoids. Compounds 8 ($[M-H]^-$ at *m/z* 755) and 15 ($[M-H]^-$ at *m/z* 447) were associated to quercetin glycosides based on their absorption spectra and the production of an ion fragment at *m/z* 301. Their molecular weights pointed to a quercetin-*O*-pentoside (peak 15) and a quercetin derivative bearing two deoxyhexosyl and one hexosyl residues (peak 8). In this latter, the fact that only one MS^2 fragment was released corresponding to the aglycone suggests that the three sugars constituted a trisaccharide. Flavonol derivatives have been previously described in other Geranium sp. pl., namely different quercetin and kaempferol aglycones and glycoside derivatives.[34-40]

Finally compound 11 ($[M-H]^-$ at *m/z* 563) was tentatively assigned as apigenin 2''-*O*-pentosyl-6-*C*-hexoside according to its pseudomolecular ion and fragmentation pattern. A compound with the same characteristics had been previously found by our group in *Arenaria montana*[41] and identified based on the fragmentation patterns described by Ferreres et al.[42] and Ferreres et al.[43] for *O,C*-glycosyl flavones. As far as we know this type of compound is reported herein for the first time in *Geranium* species, although some flavone *O*-glycosides and aglycones, i.e., luteolin and apigenin, have been reported in *Geranium* sp. pl..[35,38-40]

Digalloyl-HHDP-glucoside (compound 5) was the most abundant ellagitannin present, while luteolin-6-C-glucoside (compound 9) was the best represented flavonoid. Chlorogenic acid (i.e., 5-*O*-caffeoylquinic acid) was the only hydroxycinnamoyl derivative found in the analysed sample.

4. Conclusions

425 *Geranium molle* L. has shown to be rich in carbohydrates and proteins, providing
426 tocopherols and essential fatty acids. In general, the various aqueous and organic extracts
427 showed antioxidant activity and cytotoxicity against the different human tumour cell lines
428 tested. The acetone extract presented the highest antioxidant potential in the different
429 assays, which is most probably related to its higher content of polyphenols and flavonoids
430 compared with the other prepared extracts. The acetone extract also displayed the highest
431 cytotoxic effect, being significantly more potent than the remaining extracts against all
432 cell lines (GI_{50} values approximately four times lower). Although that extract also
433 presented the highest toxicity against porcine liver primary cells (PLP2), the GI_{50} value
434 for PLP2 was about 3-4 times higher than those for the tumor cell lines tested. The
435 decoction and the infusion of the plant, which are the common forms of folk consumption,
436 did not show hepatotoxicity against PLP2 cells up to the maximal tested concentration
437 (400 µg/mL), but presented GI_{50} for the tumor cells 3-4 times higher than the acetone
438 extract. The phenolic profile of the acetone extract was determined by HPLC-MS and
439 shown to be constituted mainly by ellagitannins, as well as some flavone and flavonol
440 glycosides. All in all, the obtained results support the folk medicinal use of *G. molle*, and
441 its interest as a source of phytochemicals with bioactive properties to be explored in the
442 medicine and food industries.
443

444 **Acknowledgements**

445 This work is supported by national funds by FCT - Portuguese Foundation for Science
446 and Technology, under the project UID/AGR/04033/2013 and PEst-
447 OE/AGR/UI0690/2014. V.C. Graça, M.I. Dias, R.C. Calhelha and L. Barros thank FCT
448 for SFRH/BD/52542/2014, SFRH/BD/84485/2012, SFRH/BPD/68344/2010 and
449 SFRH/BPD/107855/2015 grants, respectively.

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526

Table 1. Chemical characterization of *Geranium molle* L. in terms of macronutrients, and hydrophilic and lipophilic compounds.

	Quantity		Quantity
Macronutrients		C14:1	0.14 ± 0.01
Moisture (g/100 g fw)	72.2± 0.3	C15:0	0.95 ± 0.01
Fat (g/100 g dw)	15.5 ± 0.5	C16:0	24.43 ± 0.01
Proteins (g/100 g dw)	20.9 ± 0.4	C16:1	0.69 ± 0.01
Ash (g/100 g dw)	10.5 ± 0.1	C17:0	1.21 ± 0.01
Carbohydrates (g/100 g dw)	53.1 ± 0.2	C18:0	17.2 ± 0.2
Energy (kcal/100 g dw)	436 ± 2	C18:1n9	26.1 ± 0.1
		C18:2n6	4.58 ± 0.07
Hydrophilic compounds		C18:3n3	5.56 ± 0.01
Fructose	1.62 ± 0.01	C20:0	0.72 ± 0.02
Glucose	6.32 ± 0.01	C20:1	0.08 ± 0.01
Sucrose	0.99 ± 0.01	C20:2	0.034 ± 0.002
Sum of sugars (g/100 g dw)	8.93 ± 0.02	C20:3n6	0.08 ± 0.01
Oxalic acid	0.71 ± 0.01	C20:4n6	0.39 ± 0.01
Quinic acid	2.6 ± 0.3	C20:3n3+C21:0	0.18 ± 0.01
Malic acid	2.821 ± 0.002	C20:5n3	0.20 ± 0.01
Ascorbic acid	0.362 ± 0.001	C22:0	0.47 ± 0.01
Citric acid	1.09 ± 0.05	C22:1n9	0.038 ± 0.001
Fumaric acid	0.0063 ± 0.0001	C22:6n3	0.22 ± 0.01
Sum of organic acids (g/100 g dw)	7.6 ± 0.3	C24:0	0.30 ± 0.01
		C24:1	0.043 ± 0.001
Lipophilic compounds		SFA (%)	61.64 ± 0.18
C6:0	2.11 ± 0.01	MUFA (%)	27.12 ± 0.09
C8:0	1.39 ± 0.01	PUFA (%)	11.24 ± 0.08
C10:0	3.35 ± 0.01	α-Tocopherol	18.9 ± 0.6
C11:0	0.022 ± 0.001	β-Tocopherol	0.33 ± 0.01
C12:0	1.98 ± 0.01	γ-Tocopherol	0.76 ± 0.01
C13:0	0.063 ± 0.004	Sum of tocopherols (mg/100 g dw)	19.99 ± 0.55
C14:0	7.39 ± 0.01		

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecylic acid (C11:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9c+t); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11, 14-Eicosadienoic acid (C20:2); Eicosatrienoic acid (C20:3n6); Arachidonic acid (C20:4n6); *cis*-11, 14, 17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3+C21:0); Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); Erucic acid (C22:1n9); Docosahexaenoic acid (C22:6n3); Lignoceric acid (C24:0); Nervonic acid (C24:1); SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; fw- fresh weight; dw- dry weight.

Table 2. Bioactive compounds in different *Geranium molle* L. extracts.

	Aqueous extracts		Organic extracts				
Extracts	Infusion	Decoction	Hexane	Dichloromethane	Ethyl Acetate	Acetone	Methanol
Total Polyphenols (mg GAE/g extract)	79 ± 1c	63 ± 1d	13 ± 1e	6.15 ± 0.03f	216 ± 2b	497 ± 8a	76 ± 5c
Total Flavonoids (mg CE/g extract)	27 ± 1d	25.1 ± 0.2d	4.5 ± 0.4e	2.1 ± 0.02e	74 ± 6b	112 ± 1a	53 ± 3c

GAE- gallic acid equivalents; CE- catechin equivalents. In each row different letters mean significant differences ($p<0.05$).

Table 3. Bioactive properties of different *Geranium molle* L. extracts.

	Aqueous extracts		Organic extracts				
	Infusion	Decoction	Hexane	Dichloromethane	Ethyl Acetate	Acetone	Methanol
Antioxidant activity (EC_{50}, µg/mL)							
DPPH scavenging activity	324 ± 9b	248 ± 4c	1816 ± 126a	>10000	128 ± 5d	18.9 ± 0.5e	135 ± 3d
Reducing power	141 ± 1c	170 ± 6b	266 ± 5a	265 ± 1a	51 ± 1e	20.3 ± 0.2f	105 ± 4d
β-Carotene bleaching inhibition	197 ± 8e	249 ± 9b	226 ± 4c	253 ± 11b	212 ± 5d	61 ± 3f	274 ± 6a
TBARS inhibition	54 ± 3d	144 ± 7a	98 ± 4c	130 ± 6b	34 ± 2e	6.5 ± 0.2f	38 ± 2e
Antitumor activity (GI_{50} values, µg/mL)							
MCF-7 (breast carcinoma)	225 ± 15b	187 ± 9c	>400	370 ± 20a	215 ± 14b	85 ± 13d	229 ± 17b
NCI-H460 (non-small lung cancer)	190 ± 16bc	172 ± 3c	>400	256 ± 17a	200 ± 14b	63 ± 4d	206 ± 8b
HeLa (cervical carcinoma)	226 ± 19b	267 ± 18a	211 ± 18b	234 ± 21b	232 ± 15b	56 ± 5c	204 ± 15b
HepG2 (hepatocellular carcinoma)	241 ± 14a	170 ± 13c	>400	>400	204 ± 16b	50 ± 4d	260 ± 13a
Hepatotoxicity (GI_{50} value, µg/mL)							
PLP2	>400	>400	>400	>400	338 ± 12a	191 ± 15b	332 ± 32a

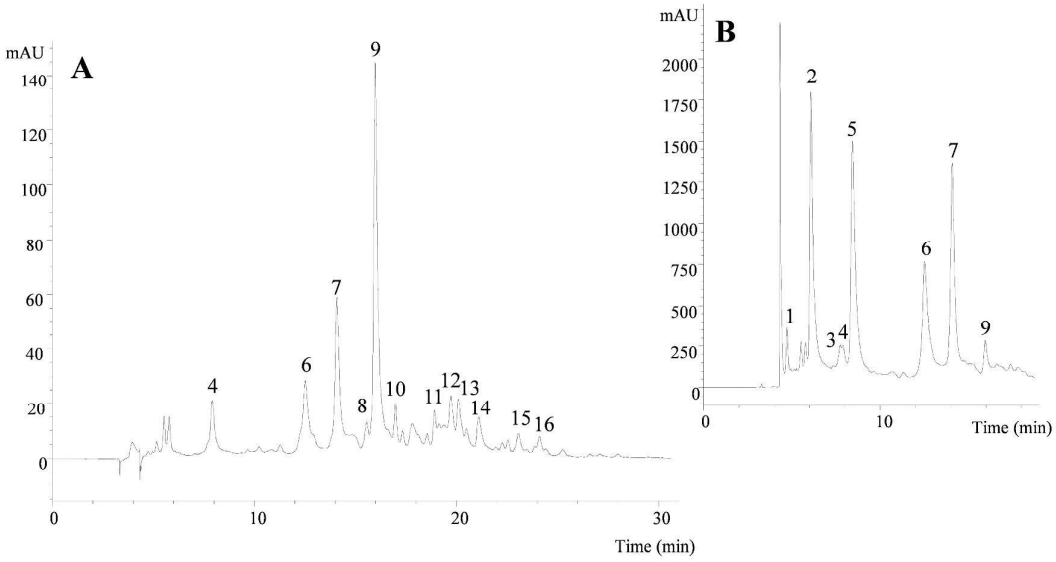
The antioxidant activity was expressed as EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 41 µg/mL (reducing power), 42 µg/mL (DPPH scavenging activity), 18 µg/mL (β-carotene bleaching inhibition) and 23 µg/mL (TBARS inhibition). GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. Ellipticine GI_{50} values: 1.21 µg/mL (MCF-7), 1.03 µg/mL (NCI-H460), 0.91 µg/mL (HeLa), 1.10 µg/mL (HepG2) and 2.29 µg/mL (PLP2). In each row different letters mean significant differences (p<0.05).

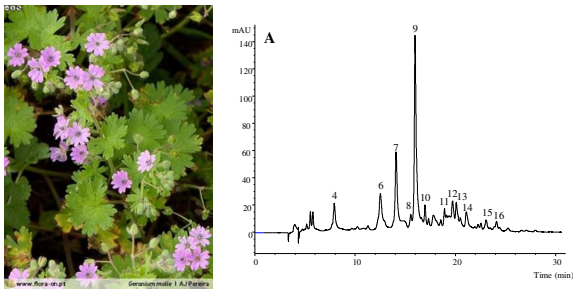
Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds of the acetone extract of *Geranium molle* L..

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g)
1	4.7	278	633	463(5),421(3),301(82),275(49),169(8)	Galloyl-HHDP-glucose	4.8 ± 0.1
2	6.1	276	785	633(9),615(10),483(15),301(64),275(30),169(7)	Digalloyl-HHDP-glucose	61 ± 4
3	7.7	278	289	245(40),203(30),187(40),161(30),137(20)	(+)-Catechin	16 ± 1
4	7.9	328	353	191(100),179(47),173(39),161(20),135(25)	5-*O*-Caffeoylquinic acid	7 ± 1
5	8.4	276	785	633(6),615(8),483(20),301(77),275(28),169(8)	Digalloyl-HHDP-glucose	66 ± 5
6	12.5	276	783	765(77),721(5),481(5),301(13),275(17)	Bis-HHDP-glucose	43 ± 3
7	14.1	278	783	765(89),721(3),481(3),301(17),275(15)	Bis-HHDP-glucose	58 ± 4
8	15.5	358	755	301(100)	Quercetin-*O*-dideoxyhexosyl-hexoside	0.18 ± 0.01
9	16.0	350	447	429(15),357(90),327(80),297(50),285(21)	Luteolin-6-*C*-glucoside	4.7 ± 0.3
10	17.0	358	609	301(100)	Quercetin-3-*O*-rutinoside	0.37 ± 0.03
11	18.9	340	563	443(17),413(33),311(67),293(33)	Apigenin 2''-*O*-pentosyl-6-*C*-hexoside	0.69 ± 0.04
12	19.7	336	431	413(5),341(40),311(100),283(53)	Apigenin-6-*C*-glucoside	1.53 ± 0.10
13	20.1	358	463	301(100)	Quercetin-3-*O*-glucoside	0.60 ± 0.02
14	21.1	354	593	285(100)	Kaempferol-3-*O*-rutinoside	0.89 ± 0.01
15	23.1	356	433	301(100)	Quercetin-*O*-pentoside	0.20 ± 0.02
16	24.1	348	447	285(100)	Kaempferol-3-*O*-glucoside	0.36 ± 0.02
					Total ellagitannins	234 ± 16
					Total hydroxycinnamoyl esters	7 ± 1
					Total flavonoids	25 ± 2
					Total phenolic compounds	265 ± 19

Figure Captions:

Figure 1. Phenolic profile of *Geranium molle* L. acetone extract recorded at 370 nm (A) and 280 nm (B).





The phytochemical characterization, antioxidant activity and *in vitro* cytotoxicity against human cancer cell lines of *Geranium molle* L. extracts are reported for the first time.