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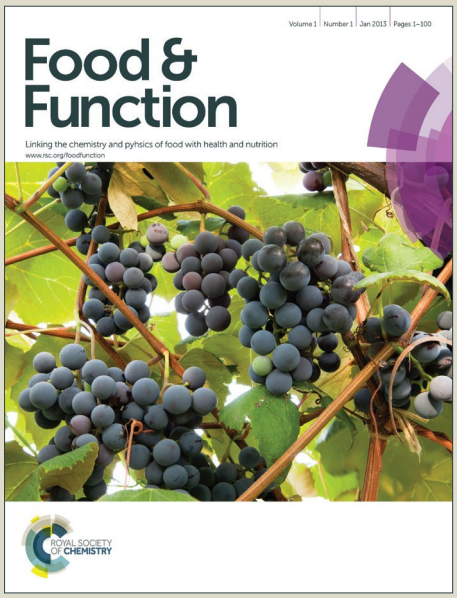

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Chemical characterization and bioactive properties of aqueous and organic extracts of *Geranium robertianum* L..

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Abstract

Geranium robertianum L. has been used in folk medicine and herbalism practice for the treatment of various conditions, but the study of its bioactivity has being barely addressed. Although its phytochemical composition has received some attention, contributions to nutritional composition are practically unknown. Herein, *G. robertianum* gathered in Trás-os-Montes, Northeastern Portugal, was chemically characterized regarding nutritional parameters, and the antioxidant activity and the cytotoxicity against several human tumor cell lines and non-tumor porcine liver primary cells of several aqueous and organic extracts were evaluated. *G. robertianum* showed to be an equilibrated valuable herb, rich in carbohydrates and proteins, and poor in fat, providing sugars, tocopherols, organic and essential fatty acids. Amongst the extracts, the acetone one showed the highest total phenols and total flavonoids contents, as well as the greatest antioxidant and cytotoxic activities. This extract showed to contain hydrolysable tannins (*e.g.* geraniin and castalagin/veascalagin), as the main phenolic compounds.

Keywords: *Geranium robertianum* L.; Nutritional chemical composition; Phenolic compounds; Antioxidant activity; Antitumor activity.

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1. Introduction

Geranium robertianum L., commonly known as Herb Robert or Red Robin, is an annual or biennial herbaceous plant belonging to the Geraniaceae family, being native from Central Europe, Mediterranean and Asia. It is common in Europe, with the exception of the Far North, in temperate parts of Asia, North Africa, Atlantic area of North America, and temperate parts of South America.[1] It grows spontaneously, especially in cool and moist places, and it is found most commonly in shaded or partly shaded habitats, such as woodlands, waste lands, roadsides, hedge banks or old walls.[2]

This plant has been used for a long time in folk medicine and herbalism practice to prepare decoctions and infusions, which are claimed to be effective for the treatment of a variety of ailments such as influenza, headaches, gastritis, liver problems, tonsillitis, diarrhea, diabetes, oropharyngeal inflammation, gallbladder, kidney and bladder inflammations, calculosis, sinuses diseases, nose bleeding, gout, sciatica, rheumatic, hypercholesterolemia, hypertension, and cancer. Externally, it has been used as vulnerary and to treat mosquito bites, mild rashes, osteoarticular diseases, parasitosis of the scalp, labial herpes, sciatica and ovine, cattle and horses scab.[3]

Although the therapeutic properties of *G. robertianum* have long been recognized, and this species is very appreciated in herbal medicine, the systematic study of its phytochemical composition and bioactivity has been barely addressed. Some bioactive properties of *G. robertianum* have been investigated such as the antioxidant,[4-10] antimicrobial,[5,11,12] anti-inflammatory[13-15] and anti-hyperglycaemic activities.[16] Extracts of *G. robertianum* have shown enzymes' inhibitory activity against urease, α-chymotrypsin and acetylcholinesterase.[5,10] Even though *G. robertianum* has being used in some folk medicines for the treatment of cancer,[17] the evaluation of its toxicity against cancer cells has been poorly investigated. Only two recent reports on the antitumor

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potential of aqueous and aqueous/ethanolic extracts of plants native from Romania against human epidermoid laryngeal carcinoma cells (Hep-2p) can be found in the literature.[7,9]

Phytochemical studies of this species have been mainly focused on phenolic compounds from alcoholic or hydroalcoholic extracts, more particularly flavonoids.[7-10,13,18-21] The chemical composition of its essential oils has received much less attention.[11,22]

In the present work, a *G. robertianum* wild sample was chemically characterized regarding nutritional composition -proteins, fat, carbohydrates and ash- and free sugars, organic acids, fatty acids and tocopherols. The antioxidant and antitumor activities, as well as the hepatotoxicity, of infusions and decoctions (common forms of consumption) and of different organic extracts of the plant, obtained by sequential extraction of the raw material with solvents of increasing polarity, were assessed and compared. The later extracts were obtained in order to evaluate the most suitable solvent to achieve the highest yield of bioactive compounds.

2. Materials and methods

2.1. Plant material

Geranium robertianum L. was collected in França, Serra de Montesinho, Bragança, Northeastern Portugal, in May 2015. The botanical identification was confirmed by Ana Maria Carvalho from the Department of Biology and Biotechnology of the School of Agriculture, Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). Voucher specimens are deposited at the herbarium of the Escola Superior Agrária de Bragança (BRESA). The collected sample (~1400 g) was lyophilized, so that its chemical composition was preserved the most possible, until further analysis. Afterwards, the plant

was reduced to a fine dried powder (~20 mesh), mixed to obtain a homogeneous sample and stored in a refrigerator at -20 °C.

2.2. Standards and Reagents

Acetonitrile, *n*-hexane and ethyl acetate were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U) were purchased from Sigma (St. Louis, MO, USA), as also were L-ascorbic acid, sugar and organic acid standards, acetic acid, formic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic compound standards were purchased from Extrasynthèse (Genay, France). Racemic tocol (50 mg/mL) and individual tocopherols were purchased from Matreya (Pleasant Gap, PA, 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).

2.3. Chemical characterization

2.3.1. Macronutrient composition and energetic value

A sample of the crude plant was analysed for its nutritional chemical composition (proteins, fat, carbohydrates and ash) and energetic value by standard procedures[23] as previously described.[24]

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118 *2.3.2. Hydrophilic compounds*

119 Free sugars were determined by high performance liquid chromatography using a
120 refraction index detector (HPLC-RI), after an extraction procedure previously described
121 by the authors,[25] as reported before.[24] Organic acids were determined by ultra-fast liquid
122 chromatography (UFLC), following a procedure previously optimized and described by
123 the authors.[25]

124

125 *2.3.3. Lipophilic compounds*

126 Fatty acids were determined after transesterification by gas chromatography (GC) using
127 a flame ionization detector (FID), according to the procedure previously described.[25]
128 Tocopherols were determined by HPLC-RI, following a procedure previously
129 described.[25]

130

131 *2.4. Preparation of organic and aqueous extracts*

132 The organic (*n*-hexane, dichloromethane, ethyl acetate, acetone and methanol) and
133 aqueous (infusion and decoction) extracts were prepared as reported elsewhere.[24]

134

135 *2.5. Total Phenols and total Flavonoids*

136 For total phenols determination, the organic extracts were redissolved in methanol and
137 the aqueous extracts in water to obtain stock solutions with a concentration of 10 mg/mL.
138 The solutions obtained were further diluted to different suitable concentrations (625-78
139 µg/mL). The total phenols content was determined by the *Folin-Ciocalteu* method as
140 previously described.[24] The total flavonoid content was determined by the aluminium
141 chloride colorimetric method following the procedure described in Graça et al.[24]

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2.6. Evaluation of bioactivity of the extracts

For the assessment of the antioxidant activity of the different extracts, stock solutions were prepared as described in section 2.5.1. Each of the stock solutions was diluted to different working concentrations (1250-9.75 μg/mL). 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.

For cytotoxicity activity evaluation, the aqueous and organic extracts were redissolved in water and 20% ethanol, respectively, to obtain stock solutions with a concentration of 8 mg/mL. Each of the stock solutions was further diluted to different working concentrations (400-1.56 μg/mL). The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.6.1. Antioxidant activity

Four different in vitro assays were performed using solutions prepared by serial dilution of the stock solutions: scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, reducing power (measured by ferricyanide Prussian blue assay), inhibition of β-carotene bleaching and inhibition of lipid peroxidation in brain cell homogenates by TBARS (thiobarbituric acid reactive substances) as previously described.[26,27]

2.6.2. Cytotoxicity in human tumor cell lines and hepatotoxicity in non-tumor cells

Four human tumor cell lines were tested using solutions prepared by serial dilution of the stock solutions: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma), as previously

described.[26] The hepatotoxicity was evaluated against a non-tumor porcine liver primary cells (PLP2), as described earlier.[25,26]

2.7. Phenolic profile of the acetone extract

The acetone extract was redissolved in water/methanol 80:20 (v/v) (final concentration 5 mg/mL). Phenolic compounds were determined by high performance liquid chromatography with a diode array detector, coupled to mass spectrometry using the electrospray ionization interface (HPLC-DAD-ESI/MS) as previously described.[26,27]

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 (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Nutritional characterization of Geranium robertianum L.

The results obtained for macronutrients, sugars, organic acids, fatty acids and tocopherols of *G. robertianum* are presented in **Table 1**. Carbohydrates were the major macronutrients found (52 g/100 g dw), followed by proteins, fat and ash. The plant showed high levels of moisture (84.4 g/100 g fw) and an energetic value of 439 kcal/100 g dw.

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The main sugar found in this plant material was glucose, closely followed by fructose. Sucrose was present in minor amounts. Regarding organic acids, oxalic acid was the most abundant one, followed by shikimic and malic acids.

Twenty-eight fatty acids were identified. Saturated fatty acids (SFA) predominated over monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Oleic acid (C18:1n9) was the major fatty acid present in the sample, followed by palmitic acid (C16:0) and stearic acid (C18:0).

All the isoforms of tocopherols were found in *G. robertianum*. α-Tocopherol was the main isoform (26 mg/100 g dw), whereas β-tocopherol and γ-tocopherol were present in similar amounts (0.94 mg/100 g dw and 1.15 mg/100 g dw, respectively). δ-Tocopherol was detected in very low amounts (0.06 mg/100 g dw).

To the best of our knowledge there are no previous reports on the nutritional characterization of *G. robertianum*.

3.2. Bioactive compounds in the Geranium robertianum L. *extracts*

The total phenols (Folin-Ciocalteu assay) and total flavonoids contents were determined in the different *G. robertianum* extracts and the results are presented in **Table 2**. The highest yield in total phenols (347 mg GAE/g extract) and total flavonoids (53 mg CE/g extract) extraction was obtained with acetone, confirming the suitability of this organic solvent for polyphenol extraction.[28] The infusion and the decoction presented similar amounts of total flavonoids but different contents of total phenols, the infusion being richer in the later. Both dichloromethane and *n*-hexane extracts showed to be poor solvents for this kind of compounds. The colour of these extracts did not allow the determination of the total flavonoids content most probably due to the presence of chlorophylls or other pigments.

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3.3. Evaluation of bioactivity of the Geranium robertianum L. *extracts*

3.3.1. Antioxidant activity

As there is no single universal method to accurately assess the antioxidant capacity, the antioxidant properties of the *G. robertianum* extracts were evaluated by four different tests: DPPH radical scavenging capacity, reducing power, β-carotene bleaching inhibition and TBARS assay in brain homogenates. Results are shown in **Table 3**. Acetone, methanol and aqueous extracts showed similar antioxidant abilities in the DPPH assay. The acetone extract displayed the highest antioxidant activity in reducing power and TBARS assays, as well as in the β-carotene bleaching inhibition assay together with the decoction. The dichloromethane and the *n*-hexane extracts were those with lower antioxidant capacity. In general, a relationship existed between the antioxidant values and the contents of total phenols as determined by the Folin-Ciocalteu assay, which seems logical as this reagent determines total reducing compounds. Trolox was used as positive control in the antioxidant activity assays. However, as this is an individual compound, it should not be considered as standard and the direct comparison with the results obtained for the extracts/oral preparations should be avoided since the synergistic and additive effects of the bioactive compounds present in natural extracts can provide higher antioxidant values than those of the individual molecules.[29,30]

3.3.2. Cytotoxic activity

The effects of *G. robertianum* extracts on the growth of four human tumor cell lines (i.e., MCF-7, NCI-H460, HeLa and HepG2) are presented in **Table 3**. All extracts revealed some cytotoxic ability. Unlike the remaining ones, the acetone extract displayed low GI_{50} values consistently against all tumor cell lines, presenting the lowest one against the HeLa cells. Conversely, this extract also presented the highest toxicity against normal primary

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cells from porcine liver (PLP2) (GI_{50} ~176 μg/mL). However, the concentration required to reach 50% of growth inhibition of PLP2 cells is about 2-3 times higher than the concentration required to achieve the same percentage of growth inhibition of the human tumor cell lines tested. The behavior of the acetone extract of *G. robertianum* in relation to the other extracts parallels that of the acetone extract of *G. molle* in a similar study carried out by our research group.[24]

The dichloromethane extract showed to be particularly active against NCI-H460 cells, with a GI_{50} value similar to that of the acetone extract. The aqueous extracts (infusion and decoctions) exhibited greater cytotoxic effect against the HepG2 and MCF-7 cell lines, although their GI_{50} values on those lines were not significantly different from those showed by the acetone extract; however, the aqueous extracts did not reveal hepatotoxicity against PLP2 cells up to the maximal tested concentration (GI_{50} >400 μg/mL). Selective cytotoxicity of aqueous and aqueous/ethanolic extracts of *G. robertianum* against human epidermoid laryngeal carcinoma cells (Hep-2p) over primary cells (normal monkey kidney cells) was also previously observed.[9] Also, no hepatotoxicity against PLP2 cells was found for the dichloromethane and *n*-hexane extracts, whereas the methanol and the ethyl acetate extracts presented similar toxicity against this cell line (GI_{50} ~290 μg/mL). Ellipticine was used as positive control in the antitumor activity assays. However, by the same reason pointed out for Trolox, comparison with the results obtained for the various extracts should be avoided.

3.4. Analysis of phenolic compounds in the acetone extract

The acetone extract of *G. robertianum* was chosen to characterize individual phenolic compounds as it presented the highest levels of total phenols and of the antioxidant and cytotoxic values among the assayed extracts. As mentioned in the previous section, even

though this extract revealed some toxicity against porcine liver primary cells (PLP2), it presented much higher GI_{50} values for these cells when compared to the human tumor cell lines. Peak characteristics and tentative identities are presented in **Table 4**. Fourteen phenolic acid derivatives, mostly hydrolysable tannins, and six flavonoid glycosides were detected. Among them peaks **11 (**quercetin-3-*O*-rutinoside), **17 (**quercetin-3-*O*-glucoside), **18 (**kaempferol-3-*O*-rutinoside) and **19** (ellagic acid) were positively identified according to their retention and UV and mass spectra characteristics in comparison with commercial standards. Ellagic acid has been often detected, sometimes in considerable amounts, in *G. robertianum* extracts.[7,10,21,31]

The phenolic profile of *G. robertianum* revealed important differences with that of *G. molle*, previously characterized by our research group.[24] Only four common compounds, *i.e.*, peaks **8, 11**, **17** and **18**, corresponding to different quercetin and kaempferol glycosides, were observed between these two *Geranium* species. These same flavonols have also been previously described in *G. robertianum*.[7,18-21]

Compounds **12** $([M-H]^-$ ion at *m/z* 739) and **16** $([M-H]^-$ ion at *m/z* 593) were also related to kaempferol glycosides owing to their λ_{max} around 348 nm and the production of a fragment ion at *m/z* 285. Peak **16** presented the same molecular weight as compound **18** (kaempferol-3-*O*-rutinoside) but an earlier retention time, thus being assigned to a different kaempferol-*O*-deoxyhexoside-hexoside. The molecular ion of peak **12** pointed to a kaempferol derivative bearing two deoxyhexosyl and one hexosyl residues. The fact that only one MS^2 fragment was released corresponding to the aglycone suggests that the three sugars constituted a trisaccharide, thus being tentatively identified as kaempferol-*O*-dideoxyhexoside-hexoside, as for peak **8** associated to the equivalent quercetin derivative glycoside.

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Most of the remaining compounds were assigned to hydrolyzable tannins. Compounds **2**, **4**, **6**, **9** and **13** were identified as gallotannins composed by two, three or four galloyl moieties linked to glucose. The mass spectra characteristics of these compounds consisted of the deprotonated molecule ($[M-H]^-$ ions at *m/z* 483, 635 and 787), with the loss of one or more galloyl groups (152 u) and/or gallic acid (170 u). These compounds have also been reported in different *Geranium* species.[32-34] Compounds **5** ($[M - H]^-$ ion at *m/z* 951) and **15** ($[M - H]^-$ ion at *m/z* 933) were the most abundant compounds present in *G. robertianum* and were tentatively identified as the ellagitannins geraniin and castalagin/vescalagin (both with the same molecular weight), respectively. Geraniin has been previously described as the main phenolic compound in various *Geranium* species.[15,32-38] However, to the best of our knowledge catalagin/vescalagin have not been previously reported.

Peaks **7** and **10** showed the same pseudomolecular ion ($[M-H]^-$ at *m/z* 785) coherent with digalloyl-HHDP-glucose isomers. Two compounds with the same molecular mass were also detected in *G. molle*[24] although they possessed different chromatographic retention times, suggesting that they correspond to distinct isomers.

Peaks **3** and **20** were tentatively associated to unknown ellagitannins, based on their UV spectra and the observation of an MS2 fragment ion at *m/z* 301 ($[HHDP-H]^-$). The pseudomolecular ion $[M-H]^-$ of peak **20** (*m/z* at 935) might point to a galloyl-bis-HHDP-glucose isomer, although a different fragmentation pattern was described for this latter.[29,39] The presence in its structure of HHDP moieties was, however, supported by the observation of the loss of an HHDP fragment (302 Da, from the transition 767 > 465) besides the product ion at *m/z* 301.

Finally, peaks **1** and **14** were identified as 3-*O*-caffeoylquinic acid and ellagic acid pentoside. These phenolic acid derivatives have been previously reported in other *Geranium* species.[33,34]

4. Conclusions

Geranium robertianum L. showed to be a valuable balanced herb, rich in carbohydrates and proteins, and poor in fat, providing sugars, organic acids, tocopherols and essential fatty acids (C18:2n6 and C18:3n3). All the aqueous and organic extracts revealed antioxidant activity and were found to be toxic against the different human tumour cell lines tested. The acetone extract was the only to display consistently low EC_{50} values in all antioxidant activity assays, which should be related to its higher content of total phenols and flavonoids compared to the other extracts, and low GI_{50} values against all tumor cell lines. Although the acetone extract also presented the highest toxicity against porcine liver primary cells (PLP2), its GI_{50} value for PLP2 was about 2-3 times higher than those for the tumor cell lines tested. For the NCI-H460 cell line, the dichloromethane extract presented the lower GI_{50} value, without hepatotoxicity against PLP2 cells up to the maximal tested concentration (400 µg/mL). The aqueous extracts (infusion and decoctions) displayed similar cytotoxic effect against HepG2 and MCF-7 cell lines as the acetone extract, but they did not reveal hepatotoxicity in primary cell lines. The phenolic profile of the acetone extract was analyzed by HPLC-DAD-ESI/MS and showed to be constituted mainly by hydrolysable tannins. The results obtained herein corroborate the bioactive properties of *G. robertianum*, namely the anticancer properties, claimed by the folk medicine and herbalism.

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

	Quantity		Quantity
Macronutrients		*Lipophilic compounds (cont.)*	
Moisture (g/100 g fw)	84.4 ± 0.4	C16:0	23.95 ± 0.01
Fat (g/100 g dw)	15.6 ± 0.5	C16:1	0.70 ± 0.01
Proteins (g/100 g dw)	22.5 ± 0.3	C17:0	1.19 ± 0.01
Ash (g/100 g dw)	9.8 ± 0.1	C18:0	16.8 ± 0.2
Carbohydrates (g/100 g dw)	52 ± 1	C18:1n9	26.2 ± 0.1
Energy (kcal/100 g dw)	439 ± 2	C18:2n6	4.2 ± 0.2
		C18:3n3	5.7 ± 0.2
Hydrophilic compounds		C20:0	0.70 ± 0.02
Fructose	2.72 ± 0.04	C20:1	0.08 ± 0.01
Glucose	2.98 ± 0.03	C20:2	0.030 ± 0.001
Sucrose	0.17 ± 0.02	C20:3n6	0.060 ± 0.001
Sum of sugars (g/100 g dw)	5.87 ± 0.01	C20:4n6	0.32 ± 0.03
Oxalic acid	3.4 ± 0.1	C20:3n3+C21:0	0.17 ± 0.01
Malic acid	1.8 ± 0.2	C20:5n3	0.21 ± 0.01
Shikimic acid	2.89 ± 0.01	C22:0	0.39 ± 0.02
Sum of organic acids (g/100 g dw)	8.1 ± 0.2	C22:1n9	0.040 ± 0.001
		C22:6n3	0.19 ± 0.02
Lipophilic compounds		C24:0	0.24 ± 0.01
C6:0	2.53 ± 0.18	C24:1	0.050 ± 0.001
C8:0	1.63 ± 0.09	SFA (%)	62 ± 0.4
C10:0	3.82 ± 0.13	MUFA (%)	27.2 ± 0.1
C11:0	0.030 ± 0.001	PUFA (%)	10.8 ± 0.4
C12:0	2.10 ± 0.05	α-tocopherol	26 ± 1
C13:0	0.070 ± 0.001	β-tocopherol	0.94 ± 0.04
C14:0	7.54 ± 0.18	γ-tocopherol	1.15 ± 0.01
C14:1	0.16 ± 0.01	δ-tocopherol	0.06 ± 0.01
C15:0	0.95 ± 0.02	Sum of tocopherols (mg/100 g dw)	28 ± 1

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 (C18:1n9); Linoleic acid (C18:2n6); 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. Levels of total phenols and total flavonoids in different *Geranium robertianum* L. extracts.

Extracts	Infusion	Decoction	*n*-Hexane	Dichloromethane	Ethyl Acetate	Acetone	Methanol
Total Phenols (mg GAE/g extract)	228 ± 5c	212 ± 4d	30.7 ± 0.5f	3.8 ± 0.1g	176 ± 3e	347 ± 4a	268 ± 8b
Total Flavonoids (mg CE/g extract)	35.9 ± 0.1c	34.57 ± 0.02c	-	-	50 ± 3ab	53 ± 4a	48 ± 1b

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 robertianum* L. extracts.

Extracts	Infusion	Decoction	*n*-Hexane	Dichloromethane	Ethyl Acetate	Acetone	Methanol
Antioxidant activity (EC_{50}, µg/mL)							
DPPH scavenging activity	65 ± 1d	60 ± 1d	877 ± 9b	1304 ± 71a	231 ± 3c	54 ± 1d	58 ± 1d
Reducing power	52 ± 1e	61 ± 3d	234 ± 1b	544 ± 6a	125 ± 1c	40.4 ± 0.2g	48 ± 1f
β-Carotene bleaching inhibition	145 ± 8d	117 ± 4e	178 ± 10c	420 ± 36b	447 ± 19a	110 ± 1e	119 ± 1e
TBARS inhibition	7.24 ± 0.05d	7.3 ± 0.2d	24 ± 1c	262 ± 9a	37.2 ± 0.4b	0.36 ± 0.04e	11.0 ± 0.4d
Antitumor activity (GI_{50} values, µg/mL)							
MCF-7 (breast carcinoma)	74 ± 6cd	64 ± 7cd	179 ± 20a	127 ± 10b	80 ± 6c	60 ± 4d	83 ± 9c
NCI-H460 (non-small lung cancer)	185 ± 6a	181.3 ± 0.2a	151 ± 15b	66 ± 6d	88 ± 4c	71 ± 6d	190 ± 5a
HeLa (cervical carcinoma)	236 ± 17b	380 ± 3a	162 ± 10d	225 ± 12bc	217 ± 2c	57 ± 1f	96 ± 4e
HepG2 (hepatocellular carcinoma)	45.68 ± 0.01d	52.2 ± 0.3d	177 ± 16a	111 ± 10b	81 ± 5c	59 ± 1d	82 ± 5c
Hepatotoxicity (GI_{50} value, µg/mL)							
PLP2	>400	>400	>400	>400	282 ± 19a	176 ± 26b	290 ± 17a

nd - not determined. 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, tentative identification and quantification of phenolic compounds of the acetone extract of *Geranium robertianum* L..

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identity	Quantification (mg/g extract)
1	5.5	328	353	191(64),179(32),161(3),135(40)	3-*O*-Caffeoylquinic acid	4.45±0.1
2	6.0	276	483	331(13),313(23),271(11),169(6)	Digalloyl-glucose	2.0±0.2
3	7.9	224/sh286	815	797(25),753(8),725(6),301(60)	Unknown ellagitannin	1.5±0.2
4	10.4	278	635	483(6),465(100),313(26),295(5),169(5)	Trigalloyl-glucose	6.76±0.04
5	12.4	270	951	933(70),633(3),481(3),451(4),301(39)	Geraniin	45±1
6	13.4	284	635	483(11),465(35),343(17),295(5),169(20)	Trigalloyl-glucose	10.2±0.3
7	15.2	280	785	483(12),301(100)	Digalloyl-HHDP-glucose	5.7±0.3
8	15.6	356	755	301(100)	Quercetin-*O*-dideoxyhexoside-hexoside	0.69±0.03
9	15.9	280	787	635(22),617(12),465(4),169(13)	Tetragalloyl-glucose	5.8±0.4
10	16.9	278	785	633(5),615(5),483(15),301(84),275(6)	Digalloyl-HHDP-glucose	5.7±0.3
11	17.3	354	609	301(100)	Quercetin-3-*O*-rutinoside	0.97±0.01
12	17.7	350	739	285(100)	Kaempferol-*O*-dideoxyhexoside-hexoside	0.82±0.03
13	17.9	280	787	635(5),617(100),465(7),169(5)	Tetragalloyl-glucose	9.4±0.4
14	18.7	356/366	433	301(100)	Ellagic acid pentoside	0.13±0.01
15	19.1	226/sh280	933	915(12),765(10),631(3),613(3),463(10),301(42)	Castalagin/Vescalagin	49.3±0.4
16	19.3	350	593	285(100)	Kaempferol-*O*-deoxyhexoside-hexoside	1.29±0.04
17	19.9	358	463	301(100)	Quercetin-3-*O*-glucoside	0.36±0.01
18	20.5	350	593	285(100)	Kaempferol-3-*O*-rutinoside	1.7±0.1
19	20.9	356/370	301	284(16),257(13),229(17),185(9)	Ellagic acid	0.34±0.02
20	21.8	280	935	917(16),767(21),749(11),465(8),301(23)	Unknown ellagitannin	8.2±0.5
					Total phenolic acids	4.9±0.1

Total hydrolysable tannins	149±4
Total flavonoids	5.8±0.2
Total phenolic compounds	160±5

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The phytochemical characterization, antioxidant activity and *in vitro* cytotoxicity against human cancer cell lines of *Geranium robertianum* L. extracts are reported.