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Bio-guided fractionation of extracts of *Geranium robertianum* L.: Relationship between phenolic profile and biological activity

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ABSTRACT

Geranium robertianum L. is used in folk medicine and herbalism practice for the treatment of a variety of ailments. Recently, we studied the bioactivity of several aqueous and organic extracts of this plant. In this work, the more active extracts were fractionated and the fractions evaluated for their antioxidant activity and cytotoxicity against several human tumor cell lines and non-tumor porcine liver primary cells. Some of the fractions from the acetone extract consistently displayed low EC_{50} and GI_{50} values and presented the higher contents of total phenolic compounds in comparison to other fractions. The phenolic compounds profile of the fractions was determined. The bio-guided fractionation of the extracts resulted in several fractions with improved bioactivity relative to the corresponding extracts. Their lower compositional complexity allowed the identification of more than two dozen compounds, to the best of our knowledge, so far not reported in *G. robertianum*.

1. Introduction

Geranium robertianum L., commonly known as Herb Robert or Red Robin, belongs to Geraniaceae family and is commonly found in woodlands, waste lands, roadsides, hedge banks or old walls (Cunha et al., 2012). Widely spread across Europe, it can also found in Asia, Africa and America (Allen and Hatfield, 2004). This plant has been used in folk medicine and herbalism practice for the treatment of a variety of digestive system disorders, and also as anti-inflammatory, haemostatic, antidiabetic, antibacterial, antiallergic, anti-cancer and diuretic (Graça et al., 2016a).

As a consequence of the lifestyle, the contemporary society is subjected to continuous exposure to various sources of aggressors which can provoke an increase in the production of reactive oxygen species (ROS). Free radicals have been implicated in the onset of various diseases such as cardiovascular diseases, cancer, cataracts, age-related decline in the immune system, and degenerative diseases. Antioxidants have the ability to neutralize these species, protecting cells (Aprioku, 2013; Carocho and Ferreira, 2013; Rubió et al., 2013).

G. robertianum contains many biologically active compounds, mostly polyphenols (Amaral et al., 2009; Fodorea et al., 2005; Ivancheva and Petrova, 2000; Kartnig and Bucar-Stachel, 1991; Kobakhidze and Alaniya, 2004; Neagu et al., 2013; Paun et al., 2011, 2012, 2014).

The antioxidant effect of phenolic compounds is mainly due to their redox properties and their capacity to block the production of ROS such as superoxide, hydrogen peroxide and hydroxyl, preventing, for example, DNA oxidative damage and/or lipid peroxidation (Rubió et al., 2013). Flavonoids are the main phenolic compounds found in *G. robertianum* (Graça et al., 2016a), and their effects on human health are very often ascribed to their potential ability to act by diminishing free radical steady-state concentration in biological systems and so providing antioxidant protection (Galleano et al., 2010).

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 of *Geranium robertianum* L. were recently evaluated by our research group (Graça et al., 2016b). In the present work, the more active extracts − ethyl acetate, acetone and methanol − were fractionated by gradient elution column chromatography on silica gel and the resulting fractions were evaluated for their bioactive properties, namely the antioxidant and antiproliferative properties. The profile of phenolic compounds of the different fractions was determined and correlated with their bioactivity.

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2. Materials and methods

2.1. Sample

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 (ETBO 62 May 2015) are deposited at the herbarium of the Escola Superior Agrária de Bragança (BRESA). The ethyl acetate, acetone and methanol extracts were obtained by sequential extraction of the whole plant, as described previously (Graça et al., 2016b).

2.2. Standards and reagents

Silica gel 0.060–0.200 mm, 60 A was obtained from Acros Organics (Geel, Belgium). Acetonitrile was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), acetic acid, formic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris was purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic compound standards were purchased from Extrasynthèse (Genay, France). 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, UT, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Fractionation of extracts

2.3.1. Ethyl acetate extract

The extract (0.7 g) was dissolved in a minimum of CH_2Cl_2, mixed with silica gel, and the mixture evaporated to dryness and placed on the top of silica gel column. The dry-loaded extract was fractionated by gradient elution column chromatography using: CH_2Cl_2; CH_2Cl_2/EtOAc − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); EtOAc, EtOAc/acetone − (90:10), (80:20), (70:30), (60:40), (50:50), (60:40), (70:30), (80:20), (90:10); acetone; acetone/MeOH − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); MeOH; MeOH/formic acid (99:1), (97:3), (95:5). Three hundred and eighty two samples (~23 mL each) were collected and grouped in eleven fractions (FEA1-11) according to the similarity of their TLC profiles. The solvent of these final fractions was removed under reduced pressure at 40 °C.

2.3.2. Acetone extract

The extract (3 gr) was dissolved in acetone, mixed with silica gel, and the mixture evaporated to dryness and placed on the top of a silica gel column. The dry-loaded extract was fractionated by gradient elution column chromatography using: CH_3Cl; CH_3Cl/acetone − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); acetone; acetone/MeOH − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:7orated to dryness and placed on the top of a silica gel column. The dry-loaded extract was fractionated by gradient elution column chromatography using: CH_3Cl; CH_3Cl/acetone − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); acetone; acetone/MeOH − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:7orated to dryness and placed on the top of a silica gel column. The dry-loaded extract was fractionated by gradient elution column chromatography using: CH_3Cl; CH_3Cl/acetone − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); acetone; acetone/MeOH − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); MeOH; MeOH/formic acid (99:1), (97:3), (95:5). Six hundred and seventy-three fractions (~23 mL each) were collected and combined on the basis of their TLC profiles to yield eleven final fractions (FA1-11) which were evaporated to dryness under reduced pressure at 40 °C.

2.3.3. Methanol extract

The extract (4 gr) was dissolved in MeOH, mixed with silica gel, and the mixture evaporated to dryness and placed on the top of a silica gel column. The dry-loaded extract was fractionated by gradient elution column chromatography using: CH_2Cl_2; CH_2Cl_2/EtOAc − (80:20), (40:60), (20:80); EtOAc, EtOAc/acetone − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (20:80), (10:90); acetone; acetone/MeOH − (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90); MeOH; MeOH/formic acid (99:1), (97:3), (95:5). Three hundred and forty three fractions (~23 mL each) were obtained, checked by TLC and those with similar band patterns were combined, yielding thirteen final fractions (FM1-13). The solvent was removed at 40 °C under reduced pressure.

2.4. Phenolic profile of the fractions

The fractions were re-dissolved 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 (Dias et al., 2013; Roriz et al., 2014). Double online detection was carried out with a DAD using 280 nm and 370 nm as preferred wavelengths and with a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV–vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with the available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard (2 − 100 µg/mL) was constructed based on the UV signal. The available phenolic standards are: chlorogenic acid (y = –161172 + 168823x, r^2 = 0.9999); protocatechuic acid (y = 27102 + 214168x, r^2 = 0.9999); ferulic acid (y = –185462 + 633126x, r^2 = 0.999); *p*-hydroxibenzoic acid (y = 173056 + 208604x, r^2 = 0.9995); vannilic acid (y = –28661 + 29751x, r^2 = 0.9999); caffeic acid (y = 406369 + 388345x, r^2 = 0.9939); quercetin-3-*O*-glucoside (y = –160173 + 34843x, r^2 = 0.9998); ellagic acid (y = –317255 + 26719x, r^2 = 0.9986); *p*-coumaric acid (y = 6966.7 + 301950x, r^2 = 0.9999); quercetin-3-*O*-rutinoside (y = 76751 + 13343x, r^2 = 0.9998); kaempferol-3-*O*-rutinoside (y = 30861 + 11117x, r^2 = 0.9999); naringenin (y = 78903 + 18433x, r^2 = 0.9998). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results are expressed in mg per g of extract.

2.5. Evaluation of bioactivity of the fractions

2.5.1. Antioxidant activity

Three 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) and inhibition of β-carotene bleaching as previously described (Dias et al., 2013; Roriz et al., 2014)

2.5.2. 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 (Dias et al., 2013). The hepatotoxicity was evaluated against a non-tumor porcine liver primary cells (PLP2), as described earlier (Barros et al., 2013; Dias et al., 2013).

2.6. Statistical analysis

For all the experiments, three samples were analysed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD). All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 23.0. (IBM Corp., Armonk, NY, USA). The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals (data not shown) and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively. Furthermore, a Pearson's correlation analysis between the antioxidant activity and all the analysed compounds was carried out, with a 95% confidence level.

3. Results and discussion

3.1. Analysis of phenolic profile of the fractions

Peak characteristics and tentative identities are presented in Table 1 and the quantification of each individual compound present in each fraction is presented in Tables 2–4.

The most abundant compounds were assigned to hydrolyzable tannins, being compounds **2**, **9, 12, 17, 21, 23, 27** and **35** identified as gallotannins composed by two, three, four or five galloyl moieties linked to glucose. The mass spectra characteristics of these compounds consisted of the deprotonated molecule ($[M-H]^-$ ions at *m/z* 483 (compound **2**), 635 (compounds **9**, **12** and **17**), 787 (compounds **21**, **23** and **27**), and 939 (compound **35**), with the loss of one or more galloyl groups (152 u) and/or gallic acid (170 u). Compounds **14**, **15**, **18** ($[M-H]^-$ ion at *m/z* 951) and **30** ($[M-H]^-$ ion at *m/z* 933), which were abundantly present in the *G. robertianum* acetone and MeOH extracts, were tentatively identified as ellagitannins: three isomers of geraniin and castalagin/vescalagin (both with the same molecular weight), respectively. The different chromatographic retention times of compounds **14**, **15** and **18**, presenting the same molecular mass, strongly suggests that they correspond to distinct isomers. These compounds have also been reported in different *Geranium* species (Okuda et al., 2000; Tuominen, 2013; Tuominen et al., 2013) and previously reported by us in *G. robertianum* (Graça et al., 2016b). Compound **8** ($[M-H]^-$ at *m/z* 633) was identified as galloyl-HHDP-glucose, while peaks **19** and **26** ($[M-H]^-$ at *m/z* 785) were coherent with two digalloyl-HHDP-glucose isomers. The pseudomolecular ion $[M-H]^-$ of compound **32** (*m/z* at 935) might point to a galloyl-bis-HHDP-glucose, although a different fragmentation pattern was described (Dias et al., 2015; Hanhineva et al., 2008). However, the presence of two HHDP moieties in its structure was supported by the loss of an HHDP fragment (302 Da, from the transition 935 > 633), together with the observation of a product ion at *m/z* 301. All of these mentioned compounds have already been described in the previously study *G. robertianum* (Graça et al., 2016b).

Peaks **6** and **38** were tentatively associated to ellagitannins, based on their UV spectra and on the observation of an MS^2 fragment ion at *m/z* 301 ($[HHDP-H]^-$). Nevertheless, a full identification of these compounds was not possible being identified as unknown ellagitannins.

Seventeen flavonoid glycosides derivatives were detected nine of which were kaempferol derivatives, seven quercetin derivatives and a narigenin derivative. Peaks **25** (quercetin-3-*O*-rutinoside), **34** (kaempferol-3-*O*-rutinoside), **36** (quercetin-3-*O*-glucoside) and **39** (narigenin-7-*O*-glucoside), were positively identified according to their retention time and UV and mass spectra, in comparison with commercial standards.

Compounds **28**, **29**, **33**, **43**, **47**, **48**, **49** and **50** were identified as kaempferol glycoside derivatives owing to the product ion observed at *m/z* 285 and UV spectra (λ_{max} around 348 nm). Compound **28** ($[M-H]^-$ at *m/z* 765) presented two MS^2 fragments, revealing the alternative loss of acetylglucuronyl (*m/z* at 547; −42–176 u) and malonylglucuronyl (*m/z* at 285; −86–176 u) residues, indicating location of each residue on different positions of the aglycone. For compound **29** ($[M-H]^-$ at *m/z* 739) only one MS^2 fragment was present (*m/z* at 285; −146-146–162 u) suggesting that the three sugars were linked together. No information about the identity of the sugar moieties and location onto the aglycone could be obtained, so, these compounds were tentatively identified as kaempferol-*O*-acetylglucuronyl-*O*-malonylglucuronide and kaempferol-*O*-deoxyhexosyl-(deoxyhexosyl-hexoside), respectively. Mass characteristics of peak **47** ($[M-H]^-$ at *m/z* 609), **43** and **49** ($[M-H]^-$ at *m/z* 637) indicated that these compounds corresponds to kaempferol derivatives bearing two hexosyl (-162–162 u) and glucuronyl (-176–176 u) residues, respectively. The observation of only one MS^2 fragment at *m/z* 285, also indicated that the residue moieties were linked together. Thus, these compounds were tentatively identified as kaempferol-*O*-dihexoside and kaempferol-*O*-diglucuronide. Similarly, compounds **48** ($[M-H]^-$ at *m/z* 607) and **50** ($[M-H]^-$ at *m/z* 591), also released one fragment at *m/z* 285, from the respective losses of deoxyhexosyl-glucuronide (322 u) and acetylpentosyl-pentoside (306 u) moieties, thus being tentatively assigned as kaempferol-*O*-deoxyhexosyl-glucuronide and kaempferol-*O*-acetylpentosyl-pentoside. Compound **33** presented the same molecular weight as compound **34** (kaempferol-3-*O*-rutinoside), but with an earlier retention time, thus being assigned to kaempferol-*O*-deoxyhexosyl-hexoside.

Compounds **20**, **24**, **31**, **37** and **41** were identified as quercetin glycosides derivatives based on their UV spectra (λ_{max} around 350 nm) and the production of a MS^2 fragment ion at *m/z* 301. Tentative identities of these compounds were assigned based on their pseudomolecular ions using a similar reasoning as for kaempferol derivatives. Thus, compound **20** ($[M-H]^-$ at *m/z* 755), **24** and **31** ($[M-H]^-$ at *m/z* 609) could correspond to quercetin-*O*-dideoxyhexosyl-hexoside and quercetin-*O*-deoxyhexosyl-hexoside, respectively, whereas peaks **37** ($[M-H]^-$ at *m/z* 597) and **41** ($[M-H]^-$ at *m/z* 623) should correspond to quercetin-*O*-deoxyhexosyl-pentoside and quercetin-*O*-deoxyhexosyl-glucuronide, respectively.

Peaks **20**, **25**, **29**, **33**, **34** and **36**, correspond to different quercetin and kaempferol glycosides that have been previously described in *G. robertianum* (Fodorea et al., 2005; Graça et al., 2016b; Ivancheva and Petrova, 2000; Kartnig and Bucar-Stachel, 1991; Kobakhidze and Alaniya, 2004).

Twelve phenolic acids were detected, five hydroxybenzoic acids (compounds **3**, **11, 13, 40** and **42**) and seven hydroxycinnamic acid and derivatives (compounds **1**, **4**, **5**, **7**, **10**, **16** and **22**). Protocatechuic acid (compound **3**), 5-*O*-caffeoylquinic acid (compound **5**), *p*-hydroxybenzoic acid (compound **11**), vanillic acid (compound **13**), *p*-coumaric acid (compound **22**) were positively identified according to their retention time and UV and mass spectra, in comparison with commercial standards. 3-*O*-Caffeoylquinic acid (compound **1**), 3-*p*-coumaroylquinic acid (compound **4**), 3-*O*- and 4-*O*-feruloylquinic acids (compounds **7** and **10**, respectively), were tentatively identified taking into account their fragmentation pattern and relative intensities similar to those reported by Clifford et al. (2003) and Clifford et al. (2005). Compound **16** ($[M-H]^-$ at *m/z* 335) was tentatively identified as a caffeoylshikimic acid due to its similar fragmentation pattern described by Al-Ayed (2015) and Bastos et al. (2007). Compounds **40** ($[M-H]^-$ at *m/z* 625) and **42** ($[M-H]^-$ at *m/z* 591), both produced a major MS^2 fragment ion at *m/z* 301, which can be interpreted as corresponding to deprotonated ellagic acid; in the case of compound **40**, it lost two hexosyl moieties (–324 u), which was tentatively identified as an ellagic acid dihexoside. No full identity was obtained for compound **42**, being assigned as ellagic acid derivative. Moreover, no tentative identification was possible to achieve for compounds **44**, **45** and **46**, remaining as

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of phenolic compounds of the extracts of *Geranium robertianum* L.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Ethyl Acetate	Acetone	Metanol
1	4.88	328	353	191(64),179(32),161(3),135(40)	3-*O*-Caffeoylquinic acid	d	d	d
2	4.57	276	483	331(13),313(23),271(11),169(6)	Digalloyl-glucose	nd	d	nd
3	5.76	260,sh294	153	109(100)	Protocatechuic acid[a]	d	nd	d
4	6.52	312	337	191(4),173(5),163(100),119(50)	3-*p*-Coumaroylquinic acid[a]	nd	d	d
5	7.01	320	353	191(100),179(5),173(6), 161(5), 135(5)	5-*O*-Caffeoylquinic acid	d	nd	d
6	7.37	224/sh286	815	797(25),753(8),725(6),301(60)	Unknown ellagitannin	nd	d	nd
7	7.56	323	367	193(100),191(3),173(3),148(6),134(4),107(50)	3-*O*-Feruloylquinic acid[a]	d	d	d
8	8.01	274	633	463(34),301(100)	Galloyl-HHDP-glucose	nd	d	d
9	8.16	278	635	483(6),465(100),313(26),295(5),169(5)	Trigalloyl-glucose	nd	d	nd
10	8.66	328	367	193(22),191(21),173(3),161(100),135(13)	4-*O*-Feruloylquinic acid[a]	nd	nd	d
11	8.69	256	137	93(100)	*p*-Hydroxybenzoic acid[a]	d	nd	nd
12	9.63	278	635	483(6),465(100),313(26),295(5),169(5)	Trigalloyl-glucose	nd	nd	d
13	10.35	261,sh292	167	108(100)	Vanillic acid[a]	d	nd	nd
14	11.42	270	951	933(70),633(3),481(3),451(4),301(39)	Geraniin isomer 1	nd	d	d
15	12.53	270	951	933(70),633(3),481(3),451(4),301(39)	Geraniin isomer 2[a]	nd	d	nd
16	12.76	326	335	179(100),135(16)	Caffeoylshikimic acid	d	nd	nd
17	12.81	284	635	483(11),465(35),343(17),295(5),169(20)	Trigalloyl-glucose	nd	d	nd
18	13.12	270	951	933(70),633(3),481(3),451(4),301(39)	Geraniin isomer 3[a]	nd	d	nd
19	14.31	280	785	483(12),301(100)	Digalloyl-HHDP-glucose	nd	d	nd
20	14.83	356	755	301(100)	Quercetin-*O*-dideoxyhexosyl-hexoside	d	d	d
21	14.88	280	787	635(22),617(12),465(4),169(13)	Tetragalloyl-glucose	d	d	d
22	15.18	310	163	119(100)	*p*-Coumaric acid	d	nd	d
23	15.77	280	787	635(22),617(12),465(4),169(13)	Tetragalloyl-glucose[a]	nd	d	nd
24	15.97	350	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside[a]	nd	d	d
25	16.37	354	609	301(100)	Quercetin-3-*O*-rutinoside	d	d	d
26	16.39	278	785	633(5),615(5),483(15),301(84),275(6)	Digalloyl-HHDP-glucose	nd	d	nd
27	16.82	280	787	635(5),617(100),465(7),169(5)	Tetragalloyl-glucose	nd	d	d
28	16.95	348	765	547(26),285(100)	Kaempferol-*O*-acetylglucuronyl-*O*-malonylglucuronide[a]	d	nd	nd
29	17.19	350	739	285(100)	Kaempferol-*O*-dideoxyhexosyl-hexoside	d	d	d
30	18.08	226/sh280	933	915(12),765(10),631(3),613(3),463(10),301(42)	Castalagin/Vescalagin	nd	d	nd
31	18.15	353	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside[a]	d	d	d
32	18.18	277	935	633(6),301(21)	Galloyl-bis-HHDP-glucose[a]	nd	d	nd
33	18.77	350	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside	d	d	d
34	18.82	350	593	285(100)	Kaempferol-3-*O*-rutinoside	d	d	nd
35	19.3	269/sh365	939	787(3),769(76),617(9),465(3)	Pentagalloyl glucose[a]	nd	d	nd
36	19.32	358	463	301(100)	Quercetin-3-*O*-glucoside	nd	nd	d
37	19.63	347	579	301(100)	Quercetin-*O*-deoxyhexosyl-pentoside[a]	nd	nd	d
38	20.64	280	935	917(16),767(21),749(11),465(8),301(23)	Unknown ellagitannin	nd	d	nd
39	21.3	283	433	271(100)	Naringenin − 7-*O*-glucoside[a]	d	nd	nd
40	21.85	370	625	301(100)	Ellagic acid dihexoside[a]	nd	nd	d
41	23.09	355	623	301(100)	Quercetin-*O*-deoxyhexosyl-glucuronide[a]	d	nd	d
42	23.26	365	591	301(100),179(50),151(30)	Ellagic acid derivative	nd	d	nd
43	24.34	340	637	285(100)	Kaempferol-*O*-diglucuronide[a]	nd	nd	d
44	24.69	312	433	307(100)	Unknown	d	nd	d
45	24.87	259/sh305	579	459(13),441(73),289(100),169(86)	Unknown	nd	nd	d
46	25.42	307	433	307(100)	Unknown	d	nd	nd
47	25.85	351	609	285(100)	Kaempferol-*O*-dihexoside[a]	nd	nd	d
48	27.25	351	607	285(100)	Kaempferol-*O*-deoxyhexosyl-glucuronide[a]	d	nd	d
49	30.94	349	637	285(100)	Kaempferol-*O*-diglucuronide[a]	nd	nd	d
50	31.48	348	591	285(100)	Kaempferol-*O*-acetylpentosyl-pentoside[a]	nd	nd	d

d − detected. nd − not detected.
[a] Hitherto unreported in *G. robertianum*.

unknown compounds. 5-*O*-Caffeoylquinic acid, 3-*O*-caffeoylquinic acid, *p*-coumaric acid have been reported in some *Geranium* species Paun et al., 2011, 2012; Tuominen, 2013; Tuominen et al., 2013).

In this work, the lower compositional complexity of the fractions obtained compared with the whole extracts turned possible the identification of more than two dozens of compounds, mostly quercetin and kampferol glycosides, phenolic acids and galloyl tannins (Table 1), which, to the best of our knowlege, have not hitherto been reported in *G. robertianum*.

3.2. Evaluation of bioactivity of the fractions

The antioxidant properties of the fractions obtained from the most bioactive extracts of *G. robertianum* (EtOAc, acetone and MeOH) were evaluated by three different assays: DPPH radical scavenging capacity, reducing power and β-carotene bleaching inhibition. The results are shown in Table 5. The cytotoxic activities of the same fractions were tested against four human tumor cell lines (i.e., MCF-7, NCI-H460, HeLa and HepG2) and in a non-tumor primary culture (porcine liver primary cell culture). The results are presented in Table 5.

Table 2
Quantification of phenolic compounds present in the fractions of ethyl acetate extract of *Geranium robertianum* L.[a].

Peak	Tentative identification	FEA5 mg/g	FEA6	FEA7	FEA8	FEA9	FEA10
1	3-*O*-Caffeoylquinic acid	–	–	–	–	–	1.335 ± 0.006
3	Protocatechuic acid	–	0.137 ± 0.002	0.23 ± 0.01	0.1302 ± 0.0007	–	–
5	5-*O*-Caffeoylquinic acid	–	–	–	0.259 ± 0.003	–	–
7	3-*O*-Ferulouylquinic acid	–	–	–	0.2425 ± 0.0009	–	–
11	*p*-Hydroxybenzoic acid	2.67 ± 0.02	0.399 ± 0.009	0.173 ± 0.008	–	–	–
13	Vanillic acid	0.348 ± 0.007	–	–	–	–	–
16	Caffeoylshikimic acid	–	tr	–	–	–	–
20	Quercetin-*O*-dideoxyhexosyl-hexoside	–	–	–	0.8924 ± 0.0001	–	–
21	Tetragalloyl-glucose	–	–	–	–	2.636 ± 0.001	–
22	*p*-Coumaric acid	0.65 ± 0.01	tr	–	–	–	–
25	Quercetin-3-*O*-rutinoside	–	–	–	tr	tr	–
28	Kaempferol-*O*-acylglucuronyl-*O*-malonylglucuronide	–	tr	–	–	–	–
29	Kaempferol-*O*-dideoxyhexosyl-hexoside	–	–	–	–	tr	–
31	Quercetin-*O*-deoxyhexosyl-hexoside	–	–	–	0.8916 ± 0.0001	–	–
33	Kaempferol-*O*-deoxyhexosyl-hexoside	–	–	–		tr	–
34	Kaempferol-3-*O*-rutinoside	–	–	–	–	tr	–
39	Naringenin-7-*O*-glucoside	–	tr	–	–	–	–
41	Quercetin-*O*-deoxyhexosyl-glucuronide	–	–	–	0.9250 ± 0.0009	0.9421 ± 0.0004	–
44	Unknown	–	–	–	–	–	–
46	Unknown	–	–	–	–	–	–
48	Kaempferol-*O*-deoxyhexosyl-glucuronide	–	2.32 ± 0.05	–	tr	tr	1.28 ± 0.02
TPA		3.66 ± 0.04	0.536 ± 0.007	0.404 ± 0.004	0.631 ± 0.003	–	1.335 ± 0.006
TED		–	–	–	–	2.6365 ± 0.0008	–
TF		–	2.32 ± 0.05	–	2.7090 ± 0.0009	0.9421 ± 0.0004	1.28 ± 0.02
TPC		3.66 ± 0.04	2.85 ± 0.04	0.483 ± 0.002	3.341 ± 0.004	3.579 ± 0.001	2.61 ± 0.03

TPA − Total phenolic acids. TED − Total ellagitannins derivate. TF − Total flavonoids. TPC − Total phenolic compounds. FEA − fractions of ethyl acetate extract.
[a] Fractions FEA1, FEA2, FEA3 and FEA4 did not present phenolic compounds.

Table 3
Quantification of phenolic compounds present in the fractions of acetone extract of *Geranium robertianum* L.[a].

Peak	Tentative identification	FA4 mg/g	FA5	FA6	FA7	FA8	FA9	FA10	FA11
1	3-*O*-Caffeoylquinic acid	–	–	0.36 ± 0.01	3.3 ± 0.05	11.5 ± 0.5	1.98 ± 0.05	0.60 ± 0.01	–
2	Digalloyl-glucose	2.84 ± 0.02	4.22 ± 0.03	–	–	–	–	–	–
4	3-*p*-Coumarouylquinic acid	–	–	–	0.40 ± 0.01	–	–	–	–
6	Unknown ellagitannin	4.3 ± 0.1	2.77 ± 0.04	2.57 ± 0.04	–	–	–	–	–
7	3-*O*-Ferulouylquinic acid	–	–	–	0.352 ± 0.001	1.4 ± 0.0	–	–	–
8	Galloyl-HHDP-glucose	–	–	–	–	–	–	–	4.09 ± 0.03
9	Trigalloyl-glucose	5.82 ± 0.06	3.1 ± 0.1	–	–	–	–	–	–
14	Geraniin isomer 1	9.3 ± 0.2	28.6 ± 0.4	7.68 ± 0.07	7.2 ± 0.1	15.1 ± 0.6	–	–	7.69 ± 0.25
15	Geraniin isomer 2	–	–	4.94 ± 0.01	4.89 ± 0.07	–	–	–	–
17	Trigalloyl-glucose	3.37 ± 0.03	–	–	–	–	–	–	–
18	Geraniin isomer 3	–	2.68 ± 0.05	3.51 ± 0.04	3.4 ± 0.1	–	–	–	–
19	Digalloyl-HHDP-glucose	4.44 ± 0.03	2.68 ± 0.04	–	–	–	–	–	–
20	Quercetin-*O*-dideoxyhexosyl-hexoside	–	–	–	–	2.39 ± 0.03	–	–	–
21	Tetragalloyl-glucose	6.13 ± 0.05	3.15 ± 0.02	–	–	–	–	–	–
23	Tetragalloyl-glucose	4.08 ± 0.07	3.96 ± 0.04	–	–	–	–	–	–
24	Quercetin-*O*-deoxyhexosyl-hexoside	–	–	–	1.16 ± 0.01	1.085 ± 0.004	–	–	–
25	Quercetin-3-*O*-rutinoside	–	–	–	3.39 ± 0.06	tr	–	–	–
26	Digalloyl-HHDP-glucose	3.81 ± 0.02	–	–	–	–	–	–	–
27	Tetragalloyl-glucose	7.6 ± 0.3	4.10 ± 0.04	–	–	–	–	–	–
29	Kaempferol-*O*-dideoxyhexosyl-hexoside	–	–	–	0.04 ± 0.00	1.054 ± 0.001	–	–	–
30	Castalagin/Vescalagin	23.6 ± 0.6	31.5 ± 0.1	7.0 ± 0.2	–	–	–	–	–
31	Quercetin-*O*-deoxyhexosyl-hexoside	–	–	–	1.96 ± 0.04	–	–	–	–
32	Galloyl-bis-HHDP-glucose	–	–	–	–	15.5 ± 0.3	–	–	7.58 ± 0.03
33	Kaempferol-*O*-deoxyhexosyl-hexoside	–	–	–	2.61 ± 0.07	–	–	–	–
34	Kaempferol-3-*O*-rutinoside	–	–	–	1.63 ± 0.02	–	–	–	–
35	Pentagalloyl glucose	3.4 ± 0.1	7.03 ± 0.08	–	–	–	–	–	–
38	Unknown ellagitannin	5.2 ± 0.1	3.48 ± 0.02	–	–	–	–	–	–
42	Ellagic acid derivative	7.3 ± 0.2	–	–	–	–	–	–	–
TPA		–	–	0.36 ± 0.01	4.1 ± 0.1	12.9 ± 0.5	1.98 ± 0.05	0.60 ± 0.01	–
TED		91 ± 1	97.3 ± 0.3	25.67 ± 0.02	15.5 ± 0.3	30.7 ± 0.3	–	–	19.35 ± 0.31
TF		–	–	–	10.8 ± 0.2	4.53 ± 0.03	–	–	–
TPC		91 ± 1	97.3 ± 0.3	26.03 ± 0.03	30.4 ± 0.5	48.1 ± 0.9	1.98 ± 0.05	0.60 ± 0.01	19.35 ± 0.3

TPA − Total phenolic acids. TED − Total ellagitannins derivate. TF − Total flavonoids. TPC − Total phenolic compounds. FA − fractions of acetone extract.
[a] Fractions FA1, FA2 and FA3 did not present phenolic compounds.

Table 4
Quantification of phenolic compounds present in the fractions of methanol extract of *Geranium robertianum* L.[a].

Peak	Tentative identification	FM2	FM3	FM4	FM5	FM6	FM7	FM8	FM9	FM10	FM11	FM12	FM13
		mg/g											
1	3-*O*-Caffeoylquinic acid	–	–	–	–	–	20.4 ± 0.2	4.614 ± 0.106	2.341 ± 0.031	1.078 ± 0.038	1.26 ± 0.00	1.07 ± 0.04	–
3	Protocatechuic acid	0.66 ± 0.01	–	–	–	–	–	–	–	–	–	–	–
4	3-*p*-Coumarouylquinic acid	–	–	–	–	–	1.2 ± 0.1	–	–	–	–	–	–
5	5-*O*-Caffeoylquinic acid	–	–	–	–	–	2.2 ± 0.0	–	–	–	–	–	–
7	3-*O*-Ferulouylquinic acid	–	–	–	–	–	0.8 ± 0.0	–	–	–	–	–	–
8	Galloyl-HHDP-glucose	–	–	4.01 ± 0.06	3.33 ± 0.05	–	5.1 ± 0.1	4.103 ± 0.051	–	–	4.41 ± 0.14	6.55 ± 0.09	3.86 ± 0.06
10	4-*O*-Ferulouylquinic acid	0.37 ± 0.02	0.55 ± 0.01	–	–	–	–	–	–	–	–	–	–
12	Trigalloyl-glucose	–	3.4 ± 0.1	4.00 ± 0.03	–	–	–	–	–	–	–	–	–
14	Geraniin isomer 1	2.48 ± 0.04	5.1 ± 0.1	38.7 ± 0.4	9.74 ± 0.04	–	9.70 ± 0.18	5.643 ± 0.038	–	–	10.53 ± 0.08	22.46 ± 0.02	5.71 ± 0.05
20	Quercetin-*O*-dideoxyhexosyl-hexoside	–	–	–	1.77 ± 0.01	1.23 ± 0.01	1.16 ± 0.01	–	–	–	–	–	–
21	Tetragalloyl-glucose	–	–	4.8 ± 0.2	–	–	–	–	–	–	–	–	–
22	*p*-Coumaric acid	0.35 ± 0.01	–	–	–	–	–	–	–	–	–	–	–
24	Quercetin-*O*-deoxyhexosyl-hexoside	–	–	1.02 ± 0.02	1.01 ± 0.01	–	–	–	–	–	–	–	–
25	Quercetin-3-*O*-rutinoside	–	–	tr	tr	tr	–	–	–	–	–	–	–
27	Tetragalloyl-glucose	2.87 ± 0.02	4.59 ± 0.04	6.3 ± 0.2	–	–	–	–	–	–	–	–	–
29	Kaempferol-*O*-dideoxyhexosyl-hexoside	–	–	–	0.03 ± 0.00	tr	–	–	–	–	–	–	–
31	Quercetin-*O*-deoxyhexosyl-hexoside	–	–	1.71 ± 0.02	1.279 ± 0.005	1.08 ± 0.01	–	–	–	–	–	–	–
33	Kaempferol-*O*-deoxyhexosyl-hexoside	–	tr	0.39 ± 0.02	tr	–	–	–	–	–	–	–	–
36	Quercetin-3-*O*-glucoside	–	1.331 ± 0.001	1.185 ± 0.002	1.02 ± 0.01	–	–	–	–	–	–	–	–
37	Quercetin-*O*-deoxyhexosyl-pentoside	–	1.14 ± 0.01	1.038 ± 0.002	1.00 ± 0.01	–	–	–	–	–	–	–	–
40	Ellagic acid dihexoside	2.56 ± 0.04	–	–	–	–	–	–	–	–	–	–	–
41	Quercetin-*O*-deoxyhexosyl-glucuronide	1.03 ± 0.01	0.95 ± 0.01	–	–	1.020 ± 0.003	–	–	–	–	–	–	–
43	Kaempferol-*O*-diglucuronide	0.40 ± 0.01	–	–	–	–	–	–	–	–	–	–	–
44	Unknown	–	–	–	–	–	–	–	–	–	–	–	–
45	Unknown	–	–	–	–	–	–	–	–	–	–	–	–
47	Kaempferol-*O*-dihexoside	0.60 ± 0.02	–	–	–	–	–	–	–	–	–	–	–
48	Kaempferol-*O*-deoxyhexosyl-glucuronide	4.78 ± 0.11	0.49 ± 0.01	0.711 ± 0.02	tr	tr	–	–	–	–	–	–	–

(continued on next page)

Table 4 *(continued)*

Peak	Tentative identification	FM2	FM3	FM4	FM5	FM6	FM7	FM8	FM9	FM10	FM11	FM12	FM13
		mg/g											
49	Kaempferol-*O*-diglucuronide	1.74 ± 0.01	–	–	–	–	–	–	–	–	–	–	–
50	Kaempferol-*O*-acetylpentosyl-pentoside	0.76 ± 0.02	–	–	–	–	–	–	–	–	–	–	–
TPA		2.23 ± 0.06	0.55 ± 0.01	–	–	–	24.65 ± 0.31	4.6 ± 0.1	2.34 ± 0.03	1.08 ± 0.04	1.26 ± 0.00	1.07 ± 0.04	–
TED		11.43 ± 0.13	13.1 ± 0.2	57.8 ± 0.8	13.08 ± 0.09	–	14.83 ± 0.09	9.747 ± 0.089	–	–	14.94 ± 0.22	29.01 ± 0.11	9.57 ± 0.12
TF		9.30 ± 0.14	3.91 ± 0.01	6.06 ± 0.04	6.107 ± 0.002	3.32 ± 0.00	1.16 ± 0.01	–	–	–	–	–	–
TPC		22.97 ± 0.06	17.5 ± 0.2	63.9 ± 0.7	19.2 ± 0.1	3.32 ± 0.00	40.65 ± 0.21	14.4 ± 0.2	2.34 ± 0.03	1.08 ± 0.04	16.2 ± 0.2	30.1 ± 0.1	9.6 ± 0.1

TF – Total flavonoids. TPC – Total phenolic compounds. FM – fractions of methanol extract. TPA – Total phenolic acids. TED – Total ellagitannins derivate.

[a] Fractions FM1 did not present phenolic compounds.

In general all fractions resulting from the acetone extract (FA1-FA11) exhibited bioactivity. The most bioactive ones were FA4, FA5 and FA8, which also presented the higher contents of total phenolic compounds (91.0, 97.3 and 48.1 mg/g, respectively, Table 3) when compared to the other fractions. Amongst those, fraction FA4 displayed particularly low hepatotoxicity (IC_{50} > 400 μg/mL).

Fractions FA4 and FA5 showed to contain only ellagitannins and the higher amount of these group of compounds among all the tested fractions. Compound **30**, which was tentatively identified as castalagin or vescalagin, was the main compound of these fractions (23.6 mg/g in FA4 and 31.5 mg/g in FA5, Table 3). The antioxidant and cytotoxic properties of both castalagin and vescalagin have been previously described (Fernandes et al., 2009; Fridrich et al., 2008; Jordão et al., 2012). In each of the fractions FA4, FA5 and FA8 compound **14**, an isomer of geraniin, was the second most abundant compound present (9.3, 26.6 and 15.1 mg/g, respectively, Table 3). Geraniin has been described as the main phenolic compound in various *Geranium* species (Harborne and Williams, 2002; Okuda et al., 1980, 1979, 2000; Piwowarski et al., 2014; Tuominen, 2013; Tuominen et al., 2013; Wu et al., 2011) and is endowed with several beneficial biological activities (Cheng et al., 2017). Apart from the identified ellagitannins, FA8 showed to contain also some phenolic acids and flavonoids. In this fraction, compound **32**, tentatively identified as galloyl-bis-HHDP-glucose, was the main compound present (15.5 mg/g, Table 3).

The MeOH extract fractionation resulted in thirteen fractions (FM1-13). In general, all of them possessed biological activity. FM3 and FM4 were the fractions that exhibited, simultaneously, the best antioxidant and cytotoxic activities. FM2 also exhibited good cytotoxic activity but poor/moderate antioxidant activity, except for the DPPH assay. FM4 showed additionally the highest content of total phenolic compounds (63.9 mg/g, Table 4), but this direct relation activity/content of phenolic compounds was not observed for the remaining more bioactive fractions.

Fraction FM2 possessed approximate amounts of ellagitannins and flavonoids (11.43 and 9.30 mg/g, respectively, Table 4), and also some phenolic acids (2.23 mg/g, Table 4). Compound **48**, which was tentatively identified as kaempferol-*O*-deoxyhexosyl-glucuronide, was the main compound detected (4.78 mg/g, Table 4). Several biological properties, including antioxidant and antiproliferative activities, of different kaempferol glycosides has been previously reported (Calderón-Montaño et al., 2011; Xiao et al., 2014). The second most abundant compound in this fraction was tetragalloyl-glucose (compound **27**) The biological activity of this compound has been described (Sugimoto et al., 2009; Xiang et al., 2010; Zhang et al., 2009). FM3 showed to be constituted by phenolic acids, flavonoids and ellagitannin derivatives, this later group being the most abundant (13.1 mg/g, Table 4). Fraction FM4 consisted mainly of ellagitannin derivatives (57.8 mg/g, Table 4), together with a small amount of flavonoids (6.06 mg/g, Table 4). Compound **14** (geraniin isomer 1) was the most abundant in both fractions FM3 and FM4 (5.1 and 38.7 mg/g, respectively, Table 4).

Fractions FM7, FM8, FM11 and FM12 displayed high antioxidant activity but no relevant cytotoxic activity. With the exception of FM7, for which phenolic acids were the major constituents, in all the other fractions ellagitannin derivatives were the most abundant compounds. The major compound found in FM7 was tentatively identified as 3-*O*-caffeoylquiic acid (compound **1**), while for the remaining fractions geraniin isomer 1 (compound **14**) was the most abundant compound (Table 4).

The EtOAc extract yielded, after fractionation, eleven fractions (FEA1-11). However, the color of fractions FEA1-FEA4 did not allow the study of their bioactivity, most probably due to the presence of chlorophylls or other pigments. The remaining fractions of this extract possessed antioxidant activity much lower than that of the fractions resulting from the acetone and MeOH extracts. Fractions FEA5, FEA6 and FEA8 showed moderate cytotoxicity. Fraction FEA5 showed to

Table 5
Bioactive properties of different fractions of ethyl acetate, acetone and methanol extracts of *Geranium robertianum* L.

	Antioxidant activity (EC_{50}, μg/mL)			Antiproliferative activity (GI_{50}, μg/mL)				Hepatotoxicity (GI_{50}, μg/mL)
Fractions	DPPH	Reducing power	β-Carotene/linoleic acid	MCF-7	NCI-H460	HeLa	HepG2	PLP2
FEA1	nd	nd	nd	nd	nd	nd	nd	nd
FEA2	nd	nd	nd	nd	nd	nd	nd	nd
FEA3	nd	nd	nd	nd	nd	nd	nd	nd
FEA4	nd	nd	nd	nd	nd	nd	nd	nd
FEA5	119.0 ± 0.3e	974 ± 2c	945 ± 61c	92 ± 1d	51 ± 4e	96 ± 7d	92 ± 10e	128 ± 5c
FEA6	302 ± 5d	232 ± 6e	448 ± 43c	54 ± 2f	119 ± 2d	34 ± 3e	72 ± 3e	238 ± 20b
FEA7	614 ± 3c	396 ± 2d	509 ± 43c	90 ± 5d	243 ± 17b	89 ± 2d	237 ± 26a	> 400
FEA8	2777 ± 35a	3617 ± 77a	1628 ± 146b	82 ± 6e	169 ± 6c	88 ± 2d	83 ± 5e	235 ± 9b
FEA9	1516 ± 128b	1591 ± 6b	15128 ± 943a	110 ± 7c	232 ± 19b	115 ± 8c	125 ± 15d	302 ± 20a
FEA10	260 ± 12d	179 ± 8f	529 ± 59c	188 ± 1b	236 ± 23b	186.4 ± 0.4b	153 ± 5c	> 400
FEA11	271 ± 7d	171 ± 9f	610 ± 16c	234 ± 2a	304 ± 18a	236 ± 6a	207 ± 11b	> 400
p-values (n = 21)								
Homoscedasticity[1]	< 0.001	< 0.001	< 0.001	0.246	0.337	0.256	0.252	0.071
1-way ANOVA[2]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
FA1	4062 ± 75b	690 ± 12b	1577 ± 67a	87 ± 4 cd	240 ± 15a	87 ± 4c	93 ± 9 cd	286 ± 15a
FA2	9903 ± 18a	774 ± 7a	1530 ± 52a	195 ± 22b	251 ± 17a	223 ± 12a	164 ± 13b	> 400
FA3	1152 ± 17c	648 ± 9c	734 ± 57b	91 ± 8c	98 ± 1bc	71 ± 1d	251 ± 26a	196 ± 16b
FA4	52 ± 3g	31.2 ± 0.1j	61 ± 5e	237 ± 7a	72 ± 7 cd	39 ± 4d	56 ± 3e	> 400
FA5	44 ± 1g	32.1 ± 0.2j	67.4 ± 0.2e	48 ± 9d	61.9 ± 0.4d	67 ± 3d	50 ± 2e	127 ± 12e
FA6	57 ± 2g	179 ± 6f	226 ± 9c	48 ± 8d	231 ± 16a	90 ± 3c	74 ± 4de	172 ± 18bcd
FA7	119 ± 1f	89 ± 5h	125 ± 7d	67 ± 6 cd	92 ± 6bc	61 ± 1d	60 ± 4e	151 ± 10de
FA8	112 ± 3f	46.9 ± 0.3i	35 ± 1e	62 ± 3 cd	117 ± 2b	60 ± 6d	63 ± 2e	167 ± 16 cd
FA9	553 ± 14d	269 ± 9e	195 ± 5c	191 ± 18b	244 ± 18a	171 ± 10b	170 ± 17b	> 400
FA10	439 ± 37e	285 ± 2d	239 ± 12c	264 ± 8a	247 ± 20a	215 ± 11a	267 ± 23a	> 400
FA11	139 ± 2f	108 ± 2g	155 ± 1d	77 ± 7 cd	259 ± 21a	102 ± 12c	101 ± 4c	191 ± 12bc
p-values (n = 33)								
Homoscedasticity[1]	< 0.001	< 0.001	< 0.001	0.089	0.242	0.236	0.110	0.072
1-way ANOVA[2]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
FM1	13471 ± 916a	1595 ± 16a	1590 ± 53a	95 ± 6c	269 ± 16d	138 ± 13e	116 ± 11d	322 ± 33b
FM2	63 ± 2c	147 ± 8e	913 ± 65b	> 400	59 ± 4g	53 ± 6g	52 ± 4e	> 400
FM3	23 ± 1c	51.5 ± 0.1ij	23 ± 1hi	74 ± 1c	170 ± 2e	77 ± 1f	67 ± 7e	207 ± 10c
FM4	17.2 ± 0.3c	44.6 ± 0.7j	7.0 ± 0.5i	78 ± 1c	123 ± 8f	78 ± 1f	65 ± 6e	166 ± 13d
FM5	53 ± 1c	95 ± 1f	207 ± 11e	247 ± 9a	323 ± 27ab	258 ± 1ab	215 ± 8c	> 400
FM6	98 ± 6c	188 ± 3c	281 ± 12d	213 ± 12ab	311 ± 23bc	233 ± 4c	241 ± 12b	> 400
FM7	26 ± 2c	63 ± 8gh	101 ± 10f	189 ± 24b	279 ± 18 cd	203 ± 20d	103 ± 8d	> 400
FM8	49 ± 1c	65.7 ± 0.4g	85 ± 1fg	188 ± 30b	353 ± 11a	241 ± 5bc	205 ± 9c	> 400
FM9	82 ± 1c	171 ± 3d	89 ± 9fg	223 ± 17ab	> 400	270 ± 3a	259 ± 15b	> 400
FM10	132 ± 1c	232 ± 4b	509 ± 4c	250 ± 1a	332 ± 22ab	265 ± 15a	252 ± 14b	> 400
FM11	26.0 ± 0.5c	33 ± 1k	69 ± 4fg	222 ± 12ab	264 ± 7d	255 ± 18ab	103 ± 5d	> 400
FM12	36.5 ± 0.4c	51 ± 1ij	52.9 ± 0.5gh	197 ± 9b	305 ± 22bc	204 ± 10d	104 ± 12d	367 ± 27a
FM13	569 ± 9b	56 ± 1hi	213 ± 13e	178 ± 15b	353 ± 21a	200 ± 5d	293 ± 16a	> 400
p-values (n = 39)								
Homoscedasticity[1]	< 0.001	< 0.001	< 0.001	0.016	0.324	0.112	0.874	0.006
1-way ANOVA[2]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

FEA − fractions of ethyl acetate extract; FA − fractions of acetone extract; FM − fractions of methanol extract; 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) and 18 μg/mL (β-carotene bleaching inhibition). GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor 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).

[1] Homoscedasticity among fractions of each exctract were tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

[2] $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each fraction, means within a column with different letters differ significantly ($p < 0.05$).

contain only phenolic acids and, at the same time, the higher amount of this phenolic group among all the tested fractions. The major compound found in this fraction was *p*-hydroxybenzoic acid (compound **11**, Table 2). Fractions FEA6 and FEA8 showed to contain, apart from phenolic acids, flavonoids as the main phenolic compounds. In both fractions, the most abundant compounds were derivatives of quercetin and kaempferol (Table 2).

The bioactivity of several fractions was found to be higher, in some cases considerably, than that of the corresponding extract. However, for the majority of fractions the bioactivity was lower what seems to point to a synergistic effect of the mixture of compounds present in the extract. In a number of fractions the hepatotoxicity displayed GI_{50} values higher than 400 mg/mL, while for the whole extracts GI_{50} values ranged from 176 to 290 mg/mL.

3.3. Correlation between the phenolic profile and the bioactivity of the fractions

The relationship between the sample's cytotoxic and antioxidant properties and the phenolic composition was established based on the correlation factors between total phenolic acids, total ellagitannin derivatives, total flavonoids and total phenolic compounds, and the EC_{50} and GI_{50} values found, respectively, for the antioxidant and cytotoxic activities (Table 6). In what concerns the fractions obtained from the EtOAc extract, there seems to exist no relevant correlation between phenolic composition and antioxidant activity. However, the cytotoxic activity of these fractions was correlated significantly with total flavonoids ($r^2 = -0.814$, -0.834 and -0.739, for the NCI-H460, HepG2 and PLP2 cell lines, respectively). The total phenolic compounds of

Table 6
Correlation coefficients of phenolic compounds with bioactivity of the fractions of *Geranium robertianum* L. extracts.

		Fractions of EtOAc extract				Fractions of Acetone extract				Fractions of MeOH extract			
		TPA	TED	TF	TPC	TPA	TED	TF	TPC	TPA	TED	TF	TPC
Antioxidant activity													
DPPH	r^2	−0.397	–	0.397	0.257	−0.383	−0.778	–	−0.71	−0.299	−0.278	0.141	−0.34
	p-values	0.143	–	0.202	0.303	0.159	< 0.001	–	< 0.001	0.156	0.161	0.576	0.043
Reducing Power	r^2	−0.091	–	0.466	0.437	−0.784	−0.672	–	−0.842	−0.215	−0.334	0.225	−0.583
	p-values	0.748	–	0.127	0.07	0.001	0.002	–	< 0.001	0.312	0.089	0.37	< 0.001
β-Carotene/ linoleic acid	r^2	0.112	–	−0.652	0.375	−0.959	−0.545	–	−0.801	−0.159	−0.324	0.693	−0.264
	p-values	0.69	–	0.022	0.125	< 0.001	0.019	–	< 0.001	0.459	0.099	0.001	0.12
Cytotoxic activity													
NCI-H460	r^2	−0.686	–	−0.814	−0.514	−0.704	−0.645	–	−0.853	0.075	−0.376	−0.652	−0.576
	p-values	0.005	–	0.001	0.029	0.003	0.004	–	< 0.001	0.727	0.053	0.003	< 0.001
HeLa	r^2	0.21	–	−0.648	0.032	−0.585	−0.556	–	−0.755	0.066	−0.366	−0.497	−0.563
	p-values	0.453	–	0.023	0.899	0.022	0.016	–	< 0.001	0.76	0.06	0.036	< 0.001
HepG2	r^2	−0.286	–	−0.834	−0.863	−0.501	−0.616	–	−0.698	−0.135	−0.419	−0.28	−0.699
	p-values	0.301	–	0.001	< 0.001	0.057	0.007	–	< 0.001	0.529	0.03	0.26	< 0.001
MCF-7	r^2	0.151	–	−0.658	−0.011	−0.439	0.491	–	−0.192	−0.094	−0.454	0.552	−0.362
	p-values	0.592	–	0.02	0.966	0.101	0.039	–	0.37	0.663	0.017	0.018	0.03
PLP2	r^2	−0.621	–	−0.739	−0.698	−0.473	0.435	–	−0.258	0.24	−0.7	−0.031	−0.613
	p-values	0.013	–	0.006	0.001	0.075	0.071	–	0.224	0.259	< 0.001	0.903	< 0.001

TPA – Total phenolic acids. TED – Total ellagitannins derivate. TF – Total flavonoids. TPC – Total phenolic compounds. r^2 – Correlation coefficient.

these fractions also showed significant correlation ($r^2 = -0.863$) with the toxicity against the HepG2 cell line.

Acetone fractions showed the highest correlation between total phenolic acids and the antioxidant activity determined by the β-carotene/linoleic acid assay ($r^2 = -0.959$), and a significant correlation ($r^2 = -0.784$) between the total phenolic acids and the antioxidant activity assessed by the reducing power assay. The total ellagitannin derivatives showed good correlation with the results of the DPPH assay ($r^2 = -0.778$). The total phenolic compounds and the antioxidant activity of the acetone fractions showed to be significantly correlated ($r^2 = -0.710$, −0.842 and −0.801, for DPPH, reducing power and β-carotene/linoleic acid assays, respectively). The cytotoxic effects of these fractions and the respective phenolic composition also presented relatively good correlation factors, being the cytotoxicity displayed against the NCI-H460 cell line correlated with total phenolic acids ($r^2 = -0.704$) and the cytotoxicity observed against the NCI-H460, HeLa and HepG2 cell lines correlated with total phenolic compounds ($r^2 = -0.853$, −0.755 and −0.698, respectively). The activities of the fractions obtained from the MeOH extract revealed to be poorly correlated with the phenolic composition. The best correlation was found between total phenolic compounds and the cytotoxicity observed on the HepG2 cell line ($r^2 = -0.69$).

4. Conclusions

Following a recent work conducted by our research group in which the bioactivity of different organic extracts of *G. robertianum* was studied, in this work the most bioactive extracts (EtOAC, acetone and MeOH) were fractionated by gradient elution column chromatography. The phenolic profile of the resulting fractions was determinated and the antioxidant and cytotoxic activities were evaluated and correlated with the respective phenolic composition. In addition to hydrolyzable tannins, the most abundant compounds found in this plant, were also identified flavonoid glycosides derivatives – mainly kaempferol and quercetin derivatives – and phenolic acids. To the best of our knowledge, about two dozen of the compounds found were identified *in G. robertianum* for the first time. In general, the fractions from the acetone extract were those exhibiting higher bioactivity when compared with those from the other extracts. Three fractions of acetone extract (FA4, FA5 and FA8) displayed consistently low EC_{50} and GI_{50} values. These fractions also presented the higher contents of total phenolic compounds when compared with the other fractions. Fraction FA4 displayed particularly low hepatotoxicity (IC_{50} > 400 μg/mL). Generally, the fractions resulting from the MeOH extract possessed moderate biological activity. The EtOAc fractions presented antioxidant activity much lower than that of the fractions resulting from the acetone and MeOH extracts. Some of these fractions showed moderate cytotoxicity. The acetone fractions were the ones that presented the highest correlation factors between biological activity and phenolic composition.

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References

Al-Ayed, A.S., 2015. Integrated mass spectrometry approach to screening of phenolic molecules in *Hyphaene thebiaca* fruits with their antiradical activity by thin-layer chromatography. Indian J. Chem. Technol. 22, 155–161.
Allen, D.E., Hatfield, G., 2004. Medicinal Plants in Folk Tradition: An Ethnobotany of Britain & Ireland. Timber Press, Portland, Cambridge.
Amaral, S., Mira, L., Nogueira, J.M.F., Silva, A.P., Florêncio, M.H., 2009. Plant extracts with anti-inflammatory properties? A new approach for characterization of their bioactive compounds and establishment of structure–antioxidant activity relationships. Bioorg. Med. Chem. 17, 1876–1883.
Aprioku, J.S., 2013. Pharmacology of free radicals and the impact of reactive oxygen species on the testis. J. Reprod. Infertil. 14, 158–172.
Barros, L., Pereira, E., Calhelha, R.C., Dueñas, M., Carvalho, A.M., Santos-Buelga, C., Ferreira, I.C.F.R., 2013. Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. J. Funct. Foods 5, 1732–1740.
Bastos, D.H., Saldanha, L.A., Catharino, R.R., Sawaya, A., Cunha, I.B., Carvalho, P.O., Eberlin, M.N., 2007. Phenolic antioxidants identified by ESI–MS from yerba maté (*Ilex paraguariensis*) and green tea (*Camelia sinensis*) extracts. Molecules 12, 423–432.
Calderón-Montaño, J.M., Burgos-Morón, E., Pérez-Guerrero, C., López-Lázaro, M., 2011. A Review on the dietary flavonoid kaempferol. Mini Rev. Med. Chem. 11, 298–344.
Carocho, M., Ferreira, I.C.F.R., 2013. A review on antioxidants, prooxidants and related controversy Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem. Toxicol. 51, 15–25.
Cheng, H.S., Ton, S.H., Kadir, K.A., 2017. Ellagitannin geraniin: a review of the natural sources, biosynthesis, pharmacokinetics and biological effects. Phytochem. Rev. 16, 159–193.
Clifford, M.N., Johnston, K.L., Knight, S., Kuhnert, N., 2003. Hierarchical scheme for LC-

MSn identification of chlorogenic acids. J. Agric. Food Chem. 51, 2900–2911.
Clifford, M.N., Knight, S., Kuhnert, N., 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. J. Agric. Food Chem. 53, 3821–3832.
Cunha, A.P., Silva, A.P., Roque, A.R., 2012. Plantas E Produtos Vegetais Em Fitoterapia, 4th ed. Fundação Calouste Gulbenkian, Lisboa.
Dias, M.I., Barros, L., Dueñas, M., Pereira, E., Carvalho, A.M., Alves, R.C., Oliveira, M.B.P.P., Santos-Buelga, C., Ferreira, I.C.F.R., 2013. Chemical composition of wild and commercial *Achillea millefolium* L. and bioactivity of the methanolic extract infusion and decoction. Food Chem. 141, 4152–4160.
Dias, M.I., Barros, L., Oliveira, M.B.P.P., Santos-Buelga, C., Ferreira, I.C.F.R., 2015. Phenolic profile and antioxidant properties of commercial and wild *Fragaria vesca* L. roots: a comparison between hydromethanolic and aqueous extracts. Ind. Crops Prod. 63, 125–132.
Fernandes, A., Fernandes, I., Cruz, L., Mateus, N., Cabral, M., Freitas, V., 2009. Antioxidant and biological properties of bioactive phenolic compounds from *Quercus suber* L. J. Agric. Food Chem. 57, 11154–11160.
Fodorea, C.S., Vlase, L., Suciu, S., Tamas, M., Leucuta, S.E., 2005. Preliminary HPLC study on some polyphenols of *Geranium robertianum* L. (Geraniaceae). Rev. Med. Chir. Soc. Med. Nat. Iasi 109, 174–178.
Fridrich, D., Glabasnia, A., Fritz, J., Esselen, M., Pahlke, G., Hofmann, T., Marko, D., 2008. Oak ellagitannins suppress the phosphorylation of the epidermal growth factor receptor in human colon carcinoma cells. J. Agric. Food Chem. 56, 3010–3015.
Galleano, M., Verstraeten, S.V., Oteiza, P.I., Fraga, C.G., 2010. Antioxidant actions of flavonoids: thermodynamic and kinetic analysis. Arch. Biochem. Biophys. 501, 23–30.
Graça, V.C., Ferreira, I.C.F.R., Santos, P.F., 2016a. Phytochemical composition and biological activities of *Geranium robertianum* L.: a review. Ind. Crops Prod. 87, 363–378.
Graça, V.C., Barros, L., Calhelha, R.C., Dias, M.I., Carvalho, A.M., Santos-Buelga, C., Santos, P.F., Ferreira, I.C.F.R., 2016b. Chemical characterization and bioactive properties of aqueous and organic extracts of *Geranium robertianum* L. Food Funct. 7, 3807–3814.
Hanhineva, K., Rogachev, I., Kokko, H., Mintz-Oron, S., Venger, I., Kärenlampi, S., Aharoni, A., 2008. Non-targeted analysis of spatial metabolite composition in strawberry (*Fragaria* x *ananassa*) flowers. Phytochemistry 69 2463–2248.
Harborne, J.B., Williams, C.A., 2002. Phytochemistry of the genus *Geranium*. In: Lis-Balchin, M. (Ed.), Geranium and Pelargonium – The Genera *Geranium* and *Pelargonium*. Taylor & Francis, London, pp. 20–29.
Ivancheva, S., Petrova, A., 2000. A chemosystematic study of eleven *Geranium* species. Biochem. Syst. Ecol. 28, 255–260.
Jordão, A.M., Correia, A.C., DelCampo, R., SanJosé, M.L.G., 2012. Antioxidant capacity, scavenger activity, and ellagitannins content from commercial oak pieces used in winemaking. Eur. Food Res. Technol. 235, 817–825.
Kartnig, T., Bucar-Stachel, J., 1991. Flavonoide aus den oberirdischen Teilen von *Geranium robertianum* (Flavonoids from the aerial parts of *Geranium robertianum*). Planta Med. 57, 292–293.
Kobakhidze, K.B., Alaniya, M.D., 2004. Flavonoids from *Geranium robertianum*. Chem. Nat. Compd. 40, 89–90.
Neagu, E., Paun, G., Constantin, D., Radu, G.L., 2013. Cytostatic activity of Geranium robertianum L. extracts processed by membrane procedures. Arab. J. Chem. http://dx.doi.org/10.1016/j.arabjc.2013.09.028.
Okuda, T., Mori, K., Seno, K., Hatano, T., 1979. Constituents of *Geranium thunbergii* sieb. et zucc. VII. High-performance reversed-phase liquid chromatography of hydrolysable tannins and related polyphenols. J. Chromatogr. 171, 313–320.
Okuda, T., Mori, K., Hatano, T., 1980. The distribution of geraniin and mallotusinic acid in the order geraniales. Phytochemistry 19, 547–551.
Okuda, T., Yoshida, T., Hatano, T., 2000. Correlation of oxidative transformations of hydrolyzable tannins and plant evolution. Phytochemistry 55, 513–529.
Paun, G., Neagu, E., Tache, A., Radu, G.L., Parvulescu, V., 2011. Application of the nanofiltration process for concentration of polyphenolic compounds from *Geranium robertianum* and *Salvia officinalis* extracts. Chem. Biochem. Eng. Q 25, 453–460.
Paun, G., Neagu, E., Litescu, S.C., Rotinberg, P., Radu, G.L., 2012. Application of membrane processes for the concentration of *Symphytum officinale* and *Geranium robertianum* extracts to obtain compounds with high anti-oxidative activity. J. Serb. Chem. Soc. 77, 1191–1203.
Paun, G., Litescu, S.C., Neagu, E., Tache, A., Radu, G.L., 2014. Evaluation of *Geranium* spp., *Helleborus* spp. and *Hyssopus* spp. polyphenolic extracts inhibitory activity against urease and α-chymotrypsin. J. Enzyme Inhib. Med. Chem. 29, 28–34.
Piwowarski, J.P., Granica, S., Zwierzyńska, M., Stefańska, J., Schopohl, P., Melzig, M.F., Kiss, A.K., 2014. Role of human gut microbiota metabolism in the anti-inflammatory effect of traditionally used ellagitannin-rich plant materials. J. Ethnopharmacol. 155, 801–809.
Roriz, C.L., Barros, L., Carvalho, A.M., Santos-Buelga, C., Ferreira, I.C.F.R., 2014. *Pterospartum tridentatum*, Gomphrena globosa and Cymbopogon citratus: a phytochemical study focused on antioxidant compounds. Food Res. Int. 62, 684–693.
Rubió, L., Motilva, M.-J., Romero, M.-P., 2013. Recent advances in biologically active compounds in herbs and spices A review of the most effective antioxidant and anti-Inflammatory active principles. Crit. Rev. Food Sci. Nutr. 53, 943–953.
Sugimoto, K., Nakagawa, K., Hayashi, S., Amakura, Y., Yoshimura, M., Yoshida, T., Yamaji, R., Nakano, Y., Inui, H., 2009. Hydrolyzable tannins as antioxidants in the leaf extract of *Eucalyptus globulus* possessing tyrosinase and hyaluronidase inhibitory activities. Food Sci. Technol. Res. 15, 331–336.
Tuominen, A., Toivonen, E., Mutikainen, P., Salminen, J.-P., 2013. Defensive strategies in *Geranium sylvaticum*. Part 1: Organ-specific distribution of water-soluble tannins, flavonoids and phenolic acids. Phytochemistry 95, 394–407.
Tuominen, A., 2013. Defensive strategies in *Geranium sylvaticum*, Part 2: Roles of water-soluble tannins, flavonoids and phenolic acids against natural enemies. Phytochemistry 95, 408–420.
Wu, Q.-Y., Zhou, Y., Jin, X., Guan, Y., Xu, M., Liu, L.-F., 2011. Chromatographic fingerprint and the simultaneous determination of five bioactive components of *Geranium carolinianum* L. Water extract by high performance liquid chromatography. Int. J. Mol. Sci. 12, 8740–8749.
Xiang, Y-f., Ju, H.-Q., Li, S., Zhang, Y.-J., Yang, C.-R., Wang, Y.-F., 2010. Effects of 1,2,4,6-tetra-O-galloyl-β-D-glucose from P. emblica on HBsAg and HBeAg secretion in HepG2.2.15 cell culture. Virol. Sin. 25, 375–380.
Xiao, J., Chen, T., Cao, H., 2014. Flavonoid glycosylation and biological benefits. Biotechnol. Adv. http://dx.doi.org/10.1016/j.biotechadv.2014.05.004.
Zhang, J., Li, L., Kim, S.-H., Hagerman, A.E., Lü, J., 2009. Anti-cancer, anti-diabetic and other pharmacologic and biological activities of penta-galloyl-glucose. Pharm. Res. 26, 2066–2080.