Plant extracts with anti-inflammatory properties—A new approach for characterization of their bioactive compounds and establishment of structure–antioxidant activity relationships

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ABSTRACT

Geranium robertianum L. (Geraniacea) and *Uncaria tomentosa* (Willd.) DC. (Rubiaceae) plant extracts, frequently used in traditional medicine for treatment of inflammatory and cancer diseases, were studied to identify potential bioactive compounds that may justify their therapeutic use and their underlying mechanisms of action. Since some of the pharmacological properties of these plant extracts may be linked to their antioxidant potential, the antioxidant activity, in relation to free radical scavenging, was measured by the ABTS/HRP and DPPH· assays, presenting *U. tomentosa* the higher activity. The antioxidant activity was also evaluated by scavenging of HOCl, the major strong oxidant produced by neutrophils and a potent pro-inflammatory agent. *U. tomentosa* was found to be a better protector against HOCl, which may justify its effectiveness against inflammatory diseases. SPE/LC-DAD was used for separation/purification purposes and ESI-MS/MS for identification/characterization of the major non-volatile components, mainly flavonoids and phenolic acids. The ESI-MS/MS methodology proposed can be used as a model procedure for identification/characterization of unknowns without the prerequisite for standard compounds analysis. The ESI-MS/MS data obtained were consistent with the antioxidant activity results and structure–activity relationships for the compounds identified were discussed.

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1. Introduction

Medicinal plants have long been used in traditional medicine for therapeutic purposes and their healing effects have well been recognized since ancient times. *Geranium robertianum* L. and *Uncaria tomentosa* (Willd.) DC. are medicinal plants with many therapeutic applications, being used in the treatment of anti-inflammatory and anti-cancer diseases.

G. robertianum L. belongs to the family of Geraniacea and is native from central and meridian Europe and Asia. *U. tomentosa* (Willd.) DC. is a climbing liana, belonging to the Rubiaceae family, which grows in the Amazon forests of Peru, and is locally known as 'Uña de Gato'.[1] The aqueous extract and decoction of the bark of this plant are widely used in traditional Peruvian medicine for the treatment of cancer[2–4], relief of arthritis pain and gastritis, probably due to its antioxidant and anti-inflammatory properties, throughout the inhibition of TNF-α production and prostaglandin modulation.[5,6]

The healing properties of medicinal plants have been typically attributed to their phenolic content, mostly flavonoids and phenolic acids, and their probable role in the prevention of diseases associated with oxidative stress.[7] In this context several pharmacological effects of these compounds have been related to their antioxidant properties, which can be due to their ability to scavenge free radicals[8,9] and other very reactive non-radical species, such as hypochlorous acid (HOCl)[10] and peroxynitrite (ONOO−).[11] Flavonoids can also chelate metal ions[12] and thus prevent their participation in free radical generation reactions.[13] The potential health-promoting effects of flavonoids, based on their antioxidant properties, have recently been challenged, because plasma concentrations achieved are low and flavonoid metabolites tend to have lower antioxidant activity.[14,15] Nevertheless, it has been suggested that the antioxidant protection of phenolic compounds may occur before absorption, that is, within the stomach, intestines and colon. They can thus play an important role in protecting the gastrointestinal tract from oxidative damage, and in delaying the development of stomach, colon and rectal cancer.[16,17] This protection can be attributed to the scavenging of reactive nitrogen, chlorine, and oxidative species, generated by the gastrointestinal tract itself or by chemical reactions of dietary

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components (iron, ascorbate, heme proteins, lipid peroxides, and nitrite) in the stomach.[17] Moreover, some biological effects of flavonoids, that only require small concentrations, have also been linked to modulatory actions in the cell, by influencing the cellular processes of signal transduction mediated by oxidants.[18] To this regard oxidants can activate the important transcription factor NF-kB, which regulates expression of key cytokines and chemokines that further modulate the inflammatory response.[19] Therefore, as result of the involvement of oxidants in the activation and nuclear transport of NF-kB, several inhibitors that are primarily antioxidants have been reported.[20,21] In addition, it has been shown that those antioxidant inhibitors act by scavenging free radicals.[21] A similar effect can also be attributed to polyphenol compounds, an assumption that gains support from numerous studies showing the ability of certain flavonoids to inhibit pro-inflammatory mediators .[22–24] Therefore, the therapeutic outcome mediated by flavonoids will result from a complex and interactive network of effects, whose prediction requires a deep and integrated knowledge of their properties.

In order to contribute for understanding the mechanisms underlying the beneficial effects of G. robertianum L. and U. tomentosa (Willd.) DC extracts, we decided to study the chemical composition of these plants and the antioxidant potential conferred by the hydrophilic compounds (mainly phenolic compounds). The knowledge of the individual constituents of the extracts will enable to further identify bioactive compounds that may justify the health effects associated as well as the underlying mechanisms of action. The evaluation of the antioxidant potential may justify in part the use of these extracts in the treatment of inflammatory and cancer diseases.

The antioxidant potential was evaluated in relation to the scavenging of two stable nitrogen-centered radicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$)[25] and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical (ABTS$^{\cdot+}$).[26] The scavenging of hypochlorous acid (HOCl), the major strong oxidant produced by neutrophils and that plays an important role in inflammation, was also investigated. The determination of the total content in flavonoids complements the antioxidant activity characterization.

The chemical characterization of the non-volatile fraction was achieved by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) after separation by solid phase extraction followed by high performance liquid chromatography with diode array detection (SPE/LC-DAD). This methodology can be used as a model procedure for identification/characterization of unknowns without the prerequisite for standard compounds analysis, hampered by endless possibilities for appropriate standards selection.

2. Results and discussion

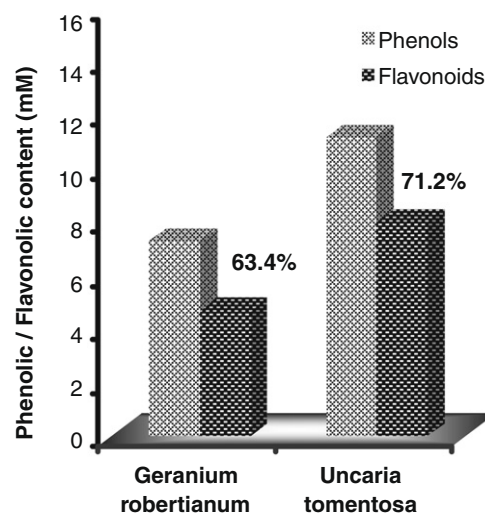### 2.1. Phenolic and flavonoid content

G. robertianum and U. tomentosa liquid extracts, showed to possess phenolic groups, with different concentrations expressed in catechin equivalents. The phenolic content was found to be 7.34 ± 0.35 mM for G. robertianum and 11.22 ± 0.74 mM for U. tomentosa. In terms of flavonoid content, the results obtained exhibited a similar hierarchy, that is, the amount of flavonoids present in G. robertianum (4.65 ± 0.03 mM) is smaller than the amount of flavonoids present in U. tomentosa (7.99 ± 0.03 mM). Since the quantity of solid plant material used for the preparation of the water/ethanol (50:50) mixtures is unknown, in order to establish a hierarchy for their antioxidant potential the same amount of phenol groups in each extract was used as reference for comparative purposes. The amount of phenolic groups and the amount of flavonoids for each extract are depicted in

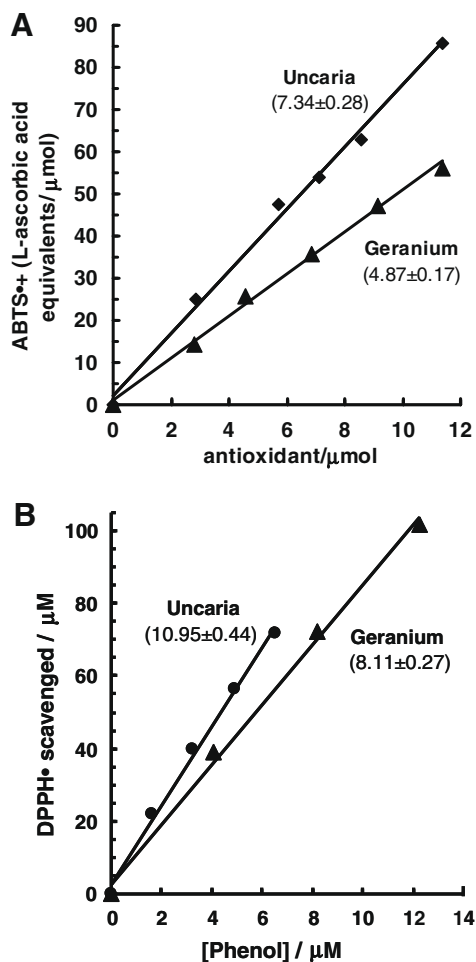Figure 1. In addition, the % of flavonoids with respect to phenols is also shown.

2.2. Antioxidant activity

The ABTS$^{\cdot+}$ and DPPH$^{\cdot}$ scavenging activities obtained for G. robertianum and U. Tomentosa are depicted in Figure 2. The extracts scavenged ABTS$^{\cdot+}$ and DPPH$^{\cdot}$ in a phenol concentration-dependent manner. The antioxidant activity of the plant extracts according to the ABTS$^{\cdot+}$ assay, expressed in terms of L-ascorbic acid equivalents (μmol) per μmol of phenol, was 7.34 ± 0.28 for Uncaria and 4.87 ± 0.17 for geranium. According to the DPPH$^{\cdot}$ assay, the values expressed in terms of μmol of DPPH$^{\cdot}$ scavenged per μmol of phenol were 10.95 ± 0.44 for Uncaria and 8.11 ± 0.27 for Geranium. These results showed that U. tomentosa extract presented higher antioxidant activity than G. robertianum, as shown in Figure 2. Moreover, these data suggest that the U. tomentosa extract contains phenolic compounds with an antioxidant activity higher than the G. robertianum extract. The high antioxidant activity found for U. tomentosa is in agreement with previous literature data.[27] It should also be noted that, although the DPPH and ABTS methods are based on the capacity of compounds to donate hydrogen atoms, the values obtained by the former assay are higher than the ones obtained by the latter. The explanation may be due to the fact that the DPPH method is performed in an ethanol medium, whereas the ABTS occurs in an aqueous buffer. In ethanol, the antioxidant capacity is conferred by all phenolic compounds including the volatile components. To note, however, that since the extracts are water ethanol mixtures, the contribution from the compounds with antioxidant activity in the volatile fraction can only be small. Data concerning the compounds with antioxidant activity in the volatile fractions are presented as Supplementary data (Table 1).

Protection of the extracts against HOCl-mediated TNB oxidation was also evaluated by determining the IC50 (concentration able to inhibit 50% of TNB oxidation). The results obtained are shown in Figure 3. U. tomentosa presented significantly lower IC50 value (55.22 ± 1.41 μM) than G. robertianum (111.94 ± 2.53 μM). Quercetin, a flavonol considered to be an effective antioxidant[9], was used as positive control, presenting an IC$_{50}$ of 34.22 ± 0.72 μM. The for-

Figure 1. Amount of phenolic groups and flavonoids in Geranium robertianum and Uncaria tomentosa extracts and their relation (%). The phenolic content was found to be 7.34 ± 0.35 mM for Geranium robertianum and 11.22 ± 0.74 mM for Uncaria tomentosa. In terms of flavonoid content, the amount of flavonoids was found to be in Geranium robertianum 4.65 ± 0.03 mM and in Uncaria tomentosa 7.99 ± 0.03 mM. For each extract the values presented are the average and standard deviation of three independent experiments and each assay was carried out in triplicate.

Figure 2. ABTS$^{·+}$ (A) and DPPH$^·$ (B) scavenging activities for *Geranium robertianum* and *Uncaria tomentosa*. The slopes of the plots (between brackets), give the antioxidant activity of the plant extracts, expressed in L-ascorbic acid equivalents (μmol) per μmol of phenol for the ABTS assay and in DPPH$^·$ scavenged per μmol of phenol for the DPPH assay. For each extract the values presented are the average and standard deviation of three independent experiments and each assay (for each phenol concentration) was carried out in triplicate.

mer extract proved therefore to be good protector against HOCl oxidation. Indeed, under the present experimental conditions, with 40 μM of HOCl, a very low IC$_{50}$ was obtained for *U. tomentosa*. This observation together with the antioxidant activity data we obtained may justify its effectiveness in the protection towards inflammatory diseases and supports a previous literature report.[28]

2.3. Characterization of the non-volatile fraction

For purification/preparation purposes solid phase extraction (SPE) combined with LC-DAD was used.

In an attempt to identify the main non-volatile constituents of the extracts, a preliminary study using several flavonoids as standards, was performed by SPE/LC-DAD. Figure 4 depicts the chromatogram obtained for each extract by SPE/LC-DAD at 280 nm, the best wavelength for the purpose. The peak at 20.50 min retention time in *G. robertianum* chromatogram was identified as kaempferol whereas the peak at 17.50 min in *U. tomentosa* chromatogram was suspected to be quercetin. This preliminary procedure enabled, however, the identification of only two compounds, one of which not unequivocally. For this reason, a new approach was necessary for identification/characterization purposes. According to this methodology all peaks of each extract were iso-

lated and further studied by ESI-MS, either in the positive or in the negative ion mode, whenever more appropriate, followed by MS/MS for unequivocal identification. The fragmentation pattern established, based on the ESI-MS/MS data, sustain the identities proposed.

An example of the identification approach used is shown for the peak at 14.84 min, one of the major components in the *G. robertianum* extract chromatogram (Fig 4A). This peak was attributed to homoeriodictyol, based on the ESI/MS spectrum (Fig. 5A) as well as on the ESI/MS/MS of *m/z* 301 ions (Fig. 5B). The fragmentation pattern established sustains this proposal (Scheme 1). For this extract, the other components identified based on the same model were acetovanillione identified at 4.51 min and syringic acid and 3′,4′-dimethoxyflavone at 11.72 min. Furthermore, at 16.80, 19.66 and 20.51 min, homoeriodictyol, ferulic acid methyl ester, ferulic acid ethyl ester and kaempferol were identified, respectively. ESI in the negative ion mode was used for this extract, due to the presence of many interfering peaks in the positive ion mode.

Based on the same identification approach, for *U. tomentosa* extract, syringic acid, narirutin, 6-methoxyluteolin and quercetin were attributed to the chromatographic peaks at 11.83, 13.50, 16.87 and 17.50 min, respectively. For the peaks at 4.56, 13.74 and 15.00 min, a quinovic acid analogue and quercetagetin, β-sitosterol and quinovic acid, myricetin and astragalin, respectively, were identified, also arguing for co-elution to occur. Table 1 summarizes all non-volatile compounds identified in each extract by ESI-MS and MS/MS after SPE/LC-DAD, which include, among others, flavonoids and phenolic acids.

Flavonoids are a class of compounds which exhibit antioxidant activity and therefore the antioxidant potential of the extracts may be understood in terms of the activity of the existing flavonoids, determined by their structural features. As proposed by Bors et al.[29], three structural features are responsible for the radical scavenging effects of flavonoids (Fig. 6). One of these features is the *ortho*-dihydroxy or catechol group in the B-ring (3′,4′-OH), which confer a high stability to the radical formed. Another is the conjugation of the B-ring to the 4-oxo group via the C2–C3 double bond, which ensures the electron delocalization from the B-ring. The latter is the 3- and 5-OH groups with the 4-oxo group, which allow electron delocalization from the 4-oxo group to both substituents. In addition, when the B-ring possesses a third OH group (3′,4′,5′-OH), the central OH bond is the weakest, as the two other OH groups can form two hydrogen bonds with the flavonoid radical.[9] The combination of all these structural features enables a higher electron delocalization, conferring a high stability to the resulting radicals, thus favoring its formation on a thermodynamic base.[30]

In *U. tomentosa* extract, the major compound found (Table 1) was the flavone myricetin which, according to the structural features above described, is the most efficient flavonoid antioxidant, followed by the flavone quercetin, a very effective antioxidant. Also found in this plant extract were quercetagetin, 6-methoxyluteolin, astragalin and narirutin. Quercetagetin is a flavonoid with a structure similar to quercetin (it possesses an additional 6-OH group), being thus a very good antioxidant; 6-methoxyluteolin has only two of the three mentioned structural features (the B-ring *ortho*-catechol group and B-ring conjugation with the 4-oxo group) and an O-methylated group on the A-ring, which does not influence its antioxidant activity. Astragalin and narirutin have only an OH group attached to the B-ring, thus exhibiting an antioxidant activity lower than the ones with a catechol group in the B-ring. The presence of quinovic acid and quinovic acid glycosides can also play a role in the antioxidant activity of this extract, as described by Ref. 31 Furthermore, syringic acid, a phenolic compound with moderate antioxidant activity (due to an OH group connected to a benzenic ring) was also found.
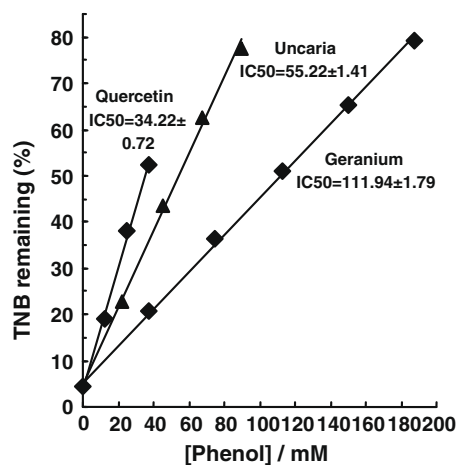
Table 1
Non-volatile compounds identified for *Geranium robertianum* and *Uncaria tomentosa* by ESI-MS and MS/MS after SPE/LC-DAD

Extract	rt[a] (min)	Compound identified	m/z	Ionization mode
Geranium robertianum	4.51	Acetovanillione	167[b]	(+)
	11.72	Syringic acid; 3′,4′-dimethoxyflavone	199[b]/197[c]; 282[b]	(+/−); (+)
	14.84	Homoeriodictyol	301[c]	(−)
	16.80	Ferulic acid methyl ester	209[b]/207[c]	(+/−)
	19.66	Ferulic acid ethyl ester	223[b]	(+)
	20.51	Kaempferol	287[b]	(+)
Uncaria tomentosa	4.56	Quinovic acid derivate; quercetagetin	515[b];319[b]	(+); (+)
	11.83	Syringic acid	199[b]/197[c]	(+/−)
	13.50	Narirutin	581[b]	(+)
	13.74	Quinovic acid, β-sitosterol	487[b]; 415[b]	(+); (+)
	15.00	Myricetin, astragalin	319[b]/317[c]; 447[c]	(+/−);(−)
	16.87	6-Methoxyluteolin	315[c]	(−)
	17.50	Quercetin	303[b]/301[c]	(+/−)

[a] Retention time of each compound observed in the chromatogram of the corresponding extract obtained after SPE/LC-DAD (Fig. 4).
[b] Protonated molecule (M+H)$^+$ obtained for each compound detected by ESI-MS.
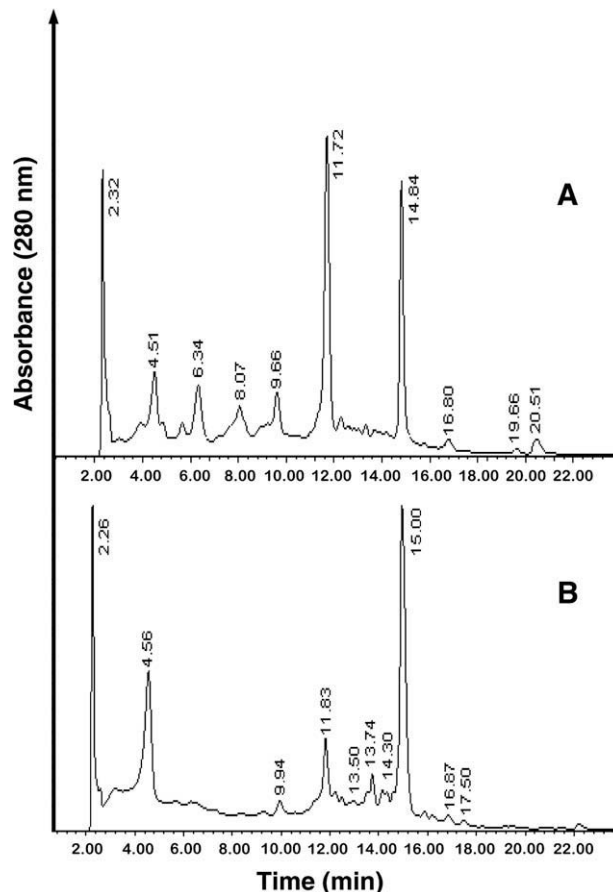[c] Deprotonated molecule (M−H)$^-$ obtained for each compound detected by ESI-MS.

Figure 3. Protection of *Geranium robertianum* and *Uncaria tomentosa* extracts against HOCl-mediated TNB oxidation. Quercetin, a flavonol considered to be an effective antioxidant, was used as positive control. The amount of TNB unchanged after incubation is calculated and expressed as percentage of the TNB initial concentration value. For each extract the values presented are the average and standard deviation of three independent experiments and each assay (for each phenol concentration) was carried out in triplicate.
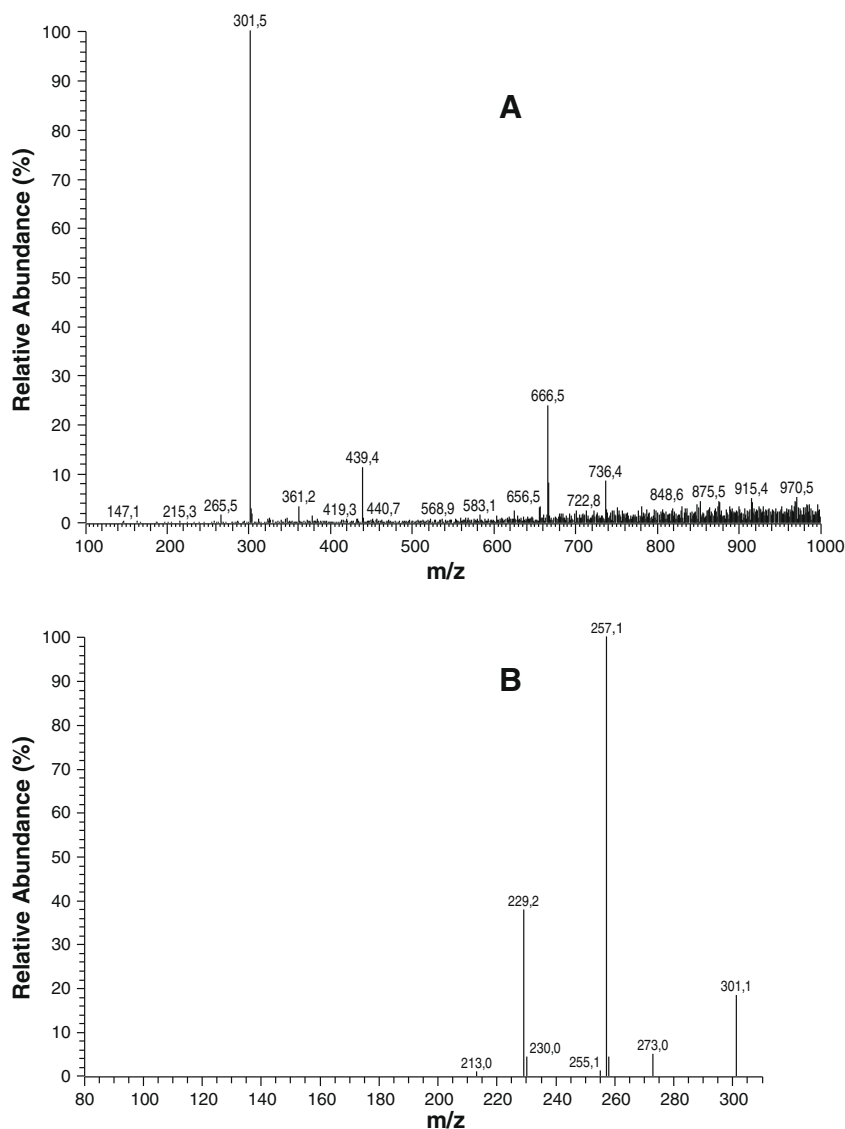
In *G. robertianum* extract three flavonoids have been identified: 3′,4′-dimethoxyflavone, homoeriodictyol and kaempferol (Table 1). The former two compounds (the major components identified) have the 4-oxo group and the C2–C3 double bond, but the *ortho*-catechol group has one OH group methylated (homoeriodictyol) or both (3,4-dimethoxyflavone), which may decrease significantly the antioxidant activity.[32] Kaempferol, although possessing just one OH group in the B-ring, is known to be a good antioxidant, since it exhibits the 3-OH and 5-OH groups with the 4-oxo group in the C-ring and the C2–C3 double bond. Syringic acid, acetovanillion, ferulic methyl ester and ferulic ethyl ester were also identified. The former two are phenolic compounds possessing some antioxidant potential and the ferulic derivatives also contribute with high antioxidant activity, due to their phenolic nucleus, besides an extended side chain conjugation which forms a resonance stabilized phenoxyl radical.

In summary, the ESI-MS/MS methodology, following SPE/LC-DAD separation/purification, applied to *G. robertianum* and *U. tomentosa* liquid extracts showed that their non-volatiles components

Figure 4. Chromatograms of *Geranium robertianum* (A) and *Uncaria tomentosa* (B) obtained by SPE/LC-DAD. All peaks in each chromatogram were isolated for identification purposes using electrospray ionization mass spectrometry and tandem mass spectrometry.

are mostly flavonoids as well as phenolic acids and esters. These results sustain the data from the antioxidant activity assays. In fact, compounds with recognized higher antioxidant activity have been identified in *U. tomentosa* extract, which showed the better antioxidant capacity, measured by the ABTS and DPPH methods.

Figure 5. ESI-MS, in the negative ion mode, of the peak at 14.84 min. in the chromatogram of *Geranium robertianum* (A); ESI-MS/MS of *m/z* 301 ion (homoeriodictyol deprotonated molecule) (B).

3. Conclusions

Identification of the major non-volatile components of *G. robertianum* and *U. tomentosa* extracts, by ESI-MS/MS of each peak isolated by LC-DAD, showed them to be composed by mostly flavonoids, besides phenolic acids and esters. These data sustain the antioxidant activity results obtained. The antioxidant activity is mostly due to the presence of the non-volatile components, mainly polyphenolic compounds, since the extracts are water/ethanol mixtures. Of the two extracts studied, *U. tomentosa* was the one that presented the higher antioxidant activity, in relation to radical scavenging, and also the one that presented a large set of components with high antioxidant activity, such as myricetin quercetin, and quinovic acid. Moreover *U. tomentosa* extract was found to be a better protector against hypochlorous acid, the major strong oxidant produced by neutrophils. The high antioxidant potential shown by this plant extract might justify its effectiveness in the treatment of inflammatory diseases and certain types of cancer the ones where inflammation is a component for its development. Indeed, because of the high concentrations present in gastrointestinal tract, phenolic compounds might exert direct
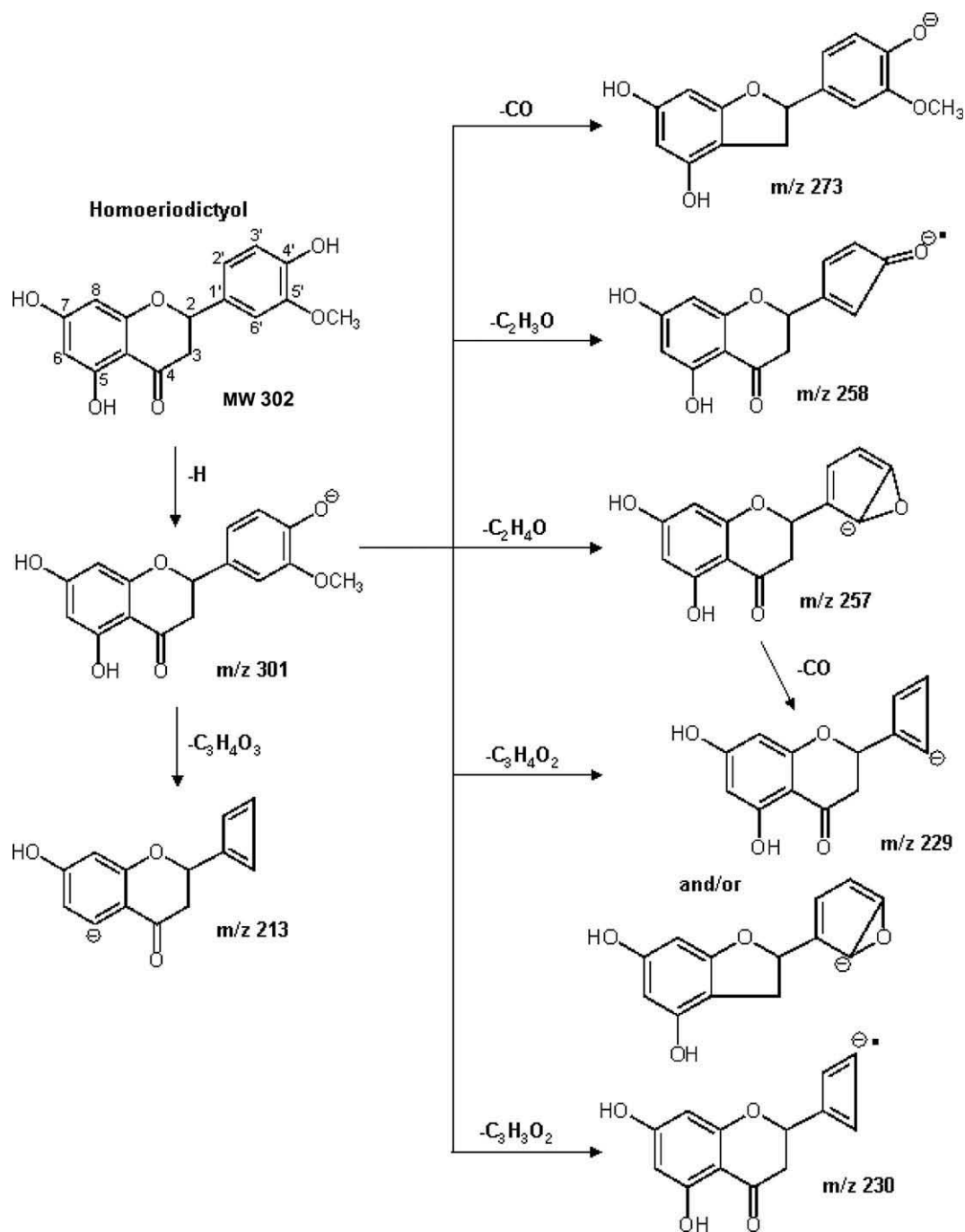
effects, by scavenging reactive oxygen and chlorine species, thus protecting against gastric and colonic cancer.[17] Moreover, some biological effects of flavonoids, that only require small concentrations, have also been linked to modulatory actions in the cell, by influencing the cellular processes of signal transduction mediated by oxidants, which further modulate the inflammatory response.[18]

Identification of the individual constituents of the extracts is therefore of main importance, since it will enable to further determine the bioactive compounds, which are responsible for their health effects, and the underlying biological mechanisms of action. Nevertheless it should be stressed that phenolic compounds undergo chemical modifications in vivo which may change some of their biological effects including their antioxidant properties.

4. Experimental

4.1. Reagents

Sodium carbonate, Folin–Ciocalteau 2 N reagent, hydrogen peroxide, sodium phosphate monobasic monohydrate (99%) and so-
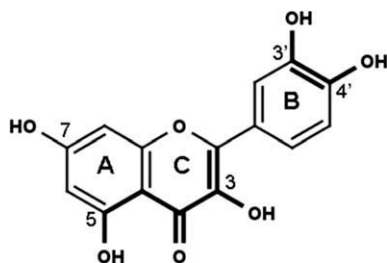
Scheme 1. Proposed fragmentation pathway typified for *m/z* 301 ion (homoeriodictyol deprotonated molecule fragment ion) resulting from hydrogen abstraction of the OH group attached to $C_{4'}$. To note that the hydrogen abstraction can also occur at C_5 and C_7, resulting in similar ion structures.

dium phosphate dibasic (>99%), potassium phosphate monobasic (99.5%), potassium phosphate dibasic (99%), sulfuric acid (95–97%) and EDTA (99%) were supplied from Merck (Darmstadt, Germany). Catechin, quercetin (98%), horseradish peroxidase enzyme (HRP), 2,2′-aziono-bis (3-ethylbenzthiazoline sulfonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH; 95%) and DTNB were obtained from Sigma Chemical Co. (St. Louis, MO, USA). L-Ascorbic acid, aluminum chloride, sodium borohydride (95%), sodium nitrite, sodium hydroxide, formic acid, sodium hypochlorite (6–14% chloroactive), acetic acid and methanol were from Riedel-de Haën (Seelze/GmbH, Germany). Gallocatechin, catechin, chlorogenic acid, epicatechin, vanillin, daidzin, eriocitrin, orientin, luteolin-3′,7-di-o-glucoside, taxifolin, vitexin, narirutin, luteolin-7-o-

glucoside, naringin, prunin, rutin, apigetrin, astragalin, myricetin, fisetin, eryodictiol, morin, daidzein, linarin, fortunellin, quercetin, naringenin, luteolin, genistein, kaempferol, tamarixetin, isoliquirtigenin, apigenin, isorhamnetin, chrysoeriol, diosmetin, 6-hydroxyflavanone, formononetin, 6-hydroxyflavone, prunetin, chrysin, biochanin A, acacetin and genistein-4′,7-dimethylether flavonoid standards were all from Extrasynthese (France). All reagents were of the highest quality available and were used as supplied.

4.2. Plant extracts

Aqueous ethanol (50:50) extracts from *G. robertianum* and *U. tomentosa* were purchased from Homeopathic pharmacy of Santa

Figure 6. Structural features responsible for the radical scavenging effect of flavonoids. Three structural features are marked in bold. These features are: the catechol group (3′,4′-OH) in the B-ring, the C2–C3 double bond in the C-ring, that enables the conjugation of the B-ring to the 4-oxo group, and the 3- and 5-OH groups together with the 4-oxo group.

Justa (Lisbon, Portugal), and Homeopathic pharmacy in Copacabana (Rio de Janeiro, Brazil), respectively.

4.3. Determination of phenolic content

The amount of phenolic groups in the ethanol extracts of the plants under study was measured using the Folin–Ciocalteu reagent, as described by Sharma et al.[33] with small modifications. The Folin–Ciocalteau reagent oxidizes phenolic groups, in alkaline solutions, giving origin to a blue chromophore that absorbs at 700 nm.[34] To express the phenolic group content in catechin equivalents, a standard curve with catechin was established. One tenth (0.1 mL) of Folin–Ciocalteu reagent (a mixture of phosphomolibdenium and phosphotungstenium acids) and 0.2 mL of 10% Na_2CO_3 were added to aliquots of 1 mL of either the extracts (in appropriate dilutions) or the standard catechin in various concentrations. The mixtures were then placed in a water bath (90–100 °C) for 1 min. After boiling, the mixtures were cooled and the phenolic concentrations were measured spectrophotometrically at 700 nm in a Unicam UV/Visible spectrometer UV2, controlled by the Vision 32 software.

4.4. Determination of flavonoids

The total flavonolic content in the ethanol extracts of the plants under study was determined by a colorimetric method, as described by Gunes et al.[35] with small modifications. This method is based on the formation of a red aluminum complex where the flavonoid acts as a bidental ligand. Aliquots of 2 mL of water were added to 0.2 mL of standard, or diluted extract, where it applies, together with 0.2 mL of $NaNO_2$ (5%, w/v). The mixtures were stirred, left at room temperature for 5 min and then 0.2 mL of $AlCl_3 \cdot 6H_2O$ (10%, w/v) was added. After 6 min stirring, 1.4 mL of NaOH 1 M and 2 mL of water were added. Subsequently, the mixtures were measured spectrophotometrically at 318 nm.

4.5. Estimation of antioxidant activity

4.5.1. ABTS assay

This assay is based on the formation of the radical cation $ABTS^{\cdot+}$ from ABTS, in the presence of both H_2O_2 and a peroxidase. The $ABTS^{\cdot+}$ has a characteristic absorption band at 730 nm.[26] The addition of different volumes of the extracts (corresponding to different phenol group contents) results in the disappearance of the radical, estimated by a decrease in absorbance. A calibration curve for the antioxidant L-ascorbic acid was established, correlating the decreases of absorbance with L-ascorbic acid concentrations. The antioxidant capacity of the plant extracts was then expressed as μmol of L-ascorbic acid equiv/μmol of phenol. The reaction mixture contained 2 mM ABTS, 28.3 μM hydrogen peroxide and 9.3 mM horseradish peroxidase (HRP) in 50 mM sodium phosphate buffer (pH

7.4) in a total volume of 3 mL. The reaction was monitored spectrophotometrically at 730 nm until a stable absorbance, due to $ABTS^{\cdot+}$ formation, was obtained. Different amounts of either L-ascorbic acid or plant extracts were then added, and the decreases in absorbance were determined. The slope of the curve of L-ascorbic acid equivalents against phenol concentrations of the extracts, gives the antioxidant activity of the plant extract, expressed in L-ascorbic acid equivalents (μmol of L-ascorbic acid/μmol of phenol).

4.5.2. DPPH· assay

The DPPH radical is quite stable and has a high absorption at 517 nm. Upon reaction with an antioxidant, the absorbance at this wavelength decreases, reflecting the ability of the molecules under study to reduce the radical, enabling to evaluate their antioxidant activity.[25] The final mixture contained 100 μM DPPH· dissolved in ethanol and different volumes of the extracts diluted in 50% (v/v) ethanol/water mixtures (corresponding to different phenol group contents). The decrease in absorbance at 517 nm, caused by the addition of the phenolic compounds, was followed for 10 min. The DPPH· scavenged, after 10 min, for each phenolic concentration, was determined by using an extinction coefficient of 8317 M^{-1} cm^{-1}. This coefficient was obtained from a calibration plot (ranging 0–100 μM) established using standard DPPH· solutions. The slope of the plots of DPPH· scavenged against phenolic concentrations, gives the moles of DPPH· scavenged/mol of phenol.

4.5.3. HOCl scavenging assay

This assay, based on the HOCl-mediated oxidation of thionitrobenzoic acid (TNB) to dithionitrobenzoic acid (DTNB) was performed as described by Ching et al.[36] and Fernandes et al.[37] with minor modifications. The TNB oxidation, followed by the absorbance decrease at 412 nm, can be prevented when a HOCl scavenger compound is present in the reaction mixture. HOCl was prepared, before use, by adjusting the pH of a 2 mM NaOCl solution to pH 6.2 with 0.5 M H_2SO_4; the HOCl concentration was determined spectrophotometrically using $\varepsilon_{235nm} = 100$ M^{-1} cm^{-1}. HOCl was diluted appropriately for each assay. TNB was prepared by incubating 1 mM DTNB in 50 mM potassium phosphate buffer pH 6.6 (supplemented with 5 mM EDTA) with 20 mM $NaBH_4$ for 30 min at 37 °C. TNB concentration was determined spectrophotometrically using $\varepsilon_{412nm} = 13,600$ M^{-1} cm^{-1}. For each assay, DTNB was diluted with the buffer. The assays were performed at room temperature and the reaction mixtures (3 mL final volume) contained equimolar parts of TNB, HOCl (40 μM), and plant extracts, in appropriate concentrations. The absorbance was measured at 412 nm, 5 min after the addition of HOCl. The amount of TNB unchanged (not oxidized) after incubation, is calculated and expressed as percentage of the TNB initial concentration value.

4.6. Analysis of non-volatiles

For chemical characterization of the non volatile fraction of the extracts, solid phase extraction followed by high performance liquid chromatography with diode array detection (SPE/LC-DAD) was firstly used for purification/separation purposes. The SPE assays were performed on a manifold (Supelco, Visiprep SPE™) with a vacuum pump (KnF Laboport, Neuberger) using Oasis HLB cartridges (6 mL, 200 mg; Waters). The cartridges were conditioned twice with 6 mL of methanol followed by 6 mL of ultra-pure water acidified with formic acid (pH 2.5) and slowly aspirated (−0.2 bar). After loading the samples (−0.4 bar), that is, 1 mL of each plant extract plus 2 mL ultra-pure water (pH 2.5), the column was washed twice with 2 mL of ultra-pure water (pH 2.5) followed by vacuum drying for 15 min. Subsequently, the elution took place with three times of 2 mL of methanol, followed by evaporation under a gentle stream of purified nitrogen to a final volume of 1.5 mL. The vials were then

closed with a seal using a hand crimper and, after agitation by vortex (Velp Scientifica, Zx³), were placed into the automatic liquid sampler tray for LC-DAD analysis. For blank assays the same procedure as above was performed using pure water (pH 2.5) instead of plant extracts. LC-DAD analyses were carried out on an Agilent 1100 Series LC system by the LC3D ChemStation software (Agilent Technologies, Waldbronn, Germany), using a Symmetry Shield C8 column, 150 mm × 3.9 mm, 5 μm particle size with a guard column (20 × 3.9 mm) (Waters, USA). The mobile phase consisted on a mixture of methanol (solvent A) and ultra-pure water with 0.1 M of formic acid (pH 2.5) (solvent B). The samples were analyzed using a 50 min linear gradient for which the content of solvent A was progressively increased from 30% to 80% with a flow rate of 0.5 mL min⁻¹. The injection volume was 20 μL with a draw speed of 200 μL min⁻¹. The detector was set at 260, 280, 330, 370 and 500 nm. Aiming at optimization of the separation conditions and further purification, the standard addition of forty four flavonoids diluted in methanol was performed by spiking the samples with pure standards and by comparing the retention parameters and the UV/vis spectral data. In each extract, whenever the presence of a particular flavonoid or phenolic compound was detected/suspected, the corresponding peak was isolated into a different vial for subsequent confirmation and/or identification. The compounds identification/characterization was carried out on a LCQ Duo quadrupole ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) equipped with an ESI source. The flow rate of the electrospray solutions to the ion source was 5 μL min⁻¹. The capillary was heated at 200 °C and maintained at a voltage of 4–5 kV. The sheath gas flow rate was optimized. The pressure measured with the convection gauge during ESI experiments was normally 0.97 Torr and the base pressure in the ion trap with helium added was typically 1.31 × 10⁻⁵ Torr. MS data were acquired in the positive or negative ionization mode, where adequate. Full-scan mass spectra were recorded in the range m/z 100–1000 Da, using a scan time of 50 ms, and three micro-scans were summed. MS/MS experiments were performed for unequivocal identification. The collision energy was increased until both precursor and product ions could be observed. To acquire and process data, XCalibur™ software was used.

Acknowledgments

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.01.045.

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