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RESEARCH ARTICLE

Antioxidant activity and phenolic acid constituents of two andean *Hypericum* L. species from Colombia

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Abstract

Antioxidant capacity of extracts of different polarity obtained from two *Hypericum* L. species (*H. juniperinum* and *H. mexicanum*) was assessed by means of total polyphenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, ferric reducing antioxidant power (FRAP) assay and oxygen radical absorbance capacity (ORAC) assay. Their phenolic acid composition was also determined by HPLC. The ethyl acetate extract of *H. juniperinum* was the most active in the ABTS, FRAP and TPC assays with 10867.48 µmol TEAC/g, 242.80 mg AAE/g and 491.08 mg GAE/g respectively. On the other hand, the methanol extract obtained from *H. mexicanum* appeared as the most active extract in the DPPH assay (3714.23 µmol TEAC/g). Similarly, the butanol fraction coming from the methanolic extract of *H. mexicanum* showed the highest activity in the ORAC assay (12910.06 µmol TEAC/g). HPLC analysis of the extracts revealed the presence of phenolic acid compounds, such as chlorogenic (50.09 mg/g) and *p*-coumaric acids (63.36 mg/g) in *H. mexicanum* and *p*-coumaric acid (8.45 mg/g) in *H. juniperinum.* A high correlation between antioxidant activity and total polyphenol content was established. Specifically, *H. mexicanum* exhibited the highest ORAC capacity, which may be associated with the high content of chlorogenic and *p*-coumaric acids present in medium to polar extracts. Our results constitute a significant contribution to the study of antioxidant activity and the determination of the phenolic acid profile in both species. The analysed extracts showed promising antioxidant activity that could be useful in the pharmaceutical, cosmetic and food industries.

Keywords

Hypericum juniperinum, *Hypericum mexicanum*, antioxidant activity, phenolic acids, radical scavenging

Introduction

Organisms are continually exposed to reactive oxygen species (ROS); when there is an imbalance between ROS and antioxidant defences in the human body, oxidative stress is produced (1). This phenomenon affects biological macromolecules, causes negative impacts on many cellular functions, and is associated with the pathogenesis of many conditions (2). Antioxidants play a vital role in preventing diseases, as they counteract the deleterious action of ROS (3).

Several research reports have suggested that antioxidants from plants are helpful in mitigating the harmful effects of ROS (4). Plants synthesize a great array of secondary metabolites, such as phenolic compounds (flavonoids, coumarins, lignans etc.), alkaloids and terpenes (5). The crude extracts of plants, rich in phenolic compounds, have been recognized to have medicinal properties and are effective scavengers of oxidants and inhibitors of lipid oxidation (6).

The species belonging to the genus *Hypericum* L. are naturally occurring or have been introduced to all continents, except Antarctica. It represents one of the 100 largest genera of angiosperms in the world. These species are mainly found as herbs, shrubs and sometimes as trees. They are distributed in temperate regions and high tropical mountains, encompassing different habitats (7). The genus *Hypericum* has 469 species reported worldwide. *Hypericum* is native to Europe and Asia and subsequently introduced in the United States (8).

In the catalogue of plants and lichens of Colombia, 54 species belonging to the genus *Hypericum* are described (9). Most of the native species of Central and South American mountain regions are an integral part of the paramo and sub-paramo vegetation types (10). Particularly, *Hypericum mexicanum* L., commonly known as chite or lunaria, is distributed in Colombia in the departments of Antioquia, Boyacá, Cundinamarca and Santander (11). In the rural areas of Bogotá, decoctions obtained from the leaves of the *H. mexicanum* species are used to treat kidney problems and disinfect wounds. In addition, roots mixed with other species of the same genus, such as *H. juniperinum* and *H. myricariifoliu* are consumed to relieve pain (12). On the other hand, the flowers of *H. juniperinum* Kunth. commonly known as *chite* or *guardarocio* are traditionally used to treat coughs and the branches are used to make brooms or fire (13). It occurs in the form of a bush, with leaves arranged in a rosette shape, and is distributed in the paramo along the Colombian eastern mountain range (14).

Little information is known about the chemical constituents and biological and pharmacological activities of both of the *Hypericum* species, *H. juniperinum* and *H. mexicanum*. Briefly, evidence is the methanolic extract of *H. juniperinum* has antidepressant effects on animal murine models (15). In another study, the acetone extract from a Colombian *H. juniperinum* specimen displayed a high total polyphenolic content (TPC) value and the presence of anthocyanins (16). In the case of *H. mexicanum*, liquid and solid soap formulations of this species have shown antibacterial activity against different strains (11). Likewise, the methanolic extract showed antibacterial activity against *S. aureus*, *E. coli* and *S. epidermidis* and the total extract and the methanolic and chloroform fractions displayed a low minimum inhibitory concentration value in comparison with the *H. perforatum* extract against *S. aureus* (17). Moreover, dimeric acylphloroglucinols isolated from the chloroform fraction of leaves from *H. mexicanum* displayed strong anti-candidal activity (18). Recently, the essential oils (EOs) of these 2 *Hypericum* species were chemically characterized and evaluated against the maize weevil. *H. mexicanum* EO showed a promising fumigant toxicity and repellent action (19).

The antioxidant capacity of a huge spectrum of other *Hypericum* species, mainly from Europe, have been studied. *H. androsaemum, H. ericoides, H. moserianum, H. olympicum* and *H. triquetrifolium* have been evaluated for TPC, flavonoid content and radical scavenging activity (20). Taking into account that *H. juniperinum* and *H. mexicanum* belong to the same genus of *H. perforatum*, the extracts obtained are expected to contain metabolites with considerable antioxidant properties (21). Therefore, the purpose of this study was to investigate the total polyphenolic content, evaluate the antioxidant activity and determine the phenolic acid content of different polarity solvent extracts obtained from these 2 native Colombian species of *Hypericum*.

Materials and Methods

Chemicals and reagents

Milli-Q water (Millipore, Bedford, MA) was used; HPLC grade acetonitrile, methanol, phosphoric acid and formic acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45 µm pore size membrane filter. Stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2´-azo-bis(2-amidinopropane)dihydrochloride (AAPH), fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chlorogenic, caffeic, ferulic, and *p*-coumaric acids, potassium persulfate, Folin-Ciocalteu reagent, gallic acid and ascorbic acid were obtained from Merck® (Germany). All spectrophotometric experiments were performed on a Multiskan Spectrum UV-Vis plate reader (Thermo Scientific®, Finland). The decrease in fluorescence intensity measured in the oxygen radical absorbance capacity (ORAC) assay was performed on a Perkin-Elmer® LS-55 spectrofluorometer (Beaconstield, UK). The chromatographic studies by HPLC were carried out on a Shimadzu® liquid chromatograph from the Prominence UFLC series (Japan).

Plant material

Fresh plant material (leaves, stems, and flowers of both species) was obtained from the vereda Arbolocos belonging to the town of Cuítiva in the department of Boyacá, Colombia, at about 3300 m.a.s.l. *H. juniperinum* Kunth and *H. mexicanum* L. were identified by D.A. Fonseca (Herbario Nacional Colombiano, Universidad Nacional de Colombia). Voucher specimens of *H. juniperinum* Kunth (COL615516) and *H. mexicanum* L. (COL615515) were deposited at the herbarium. The fresh plant material was dried for 48 hr at 40 °C. It was then shredded in a blender (Hamilton Beach, Commercial) for further processing.

Preparation of extracts

The dried material was moistened with 80% methanol. Three percolation extraction procedures were carried out until exhaustion. The extracts obtained were reduced in volume under reduced pressure using a rotary evaporator

at a temperature of 40 °C and 60 rpm. The extract was heated in a water bath at 50 °C until complete dryness. Finally, a portion of the dry extract was taken and redissolved in 80% methanol and a liquid-liquid fractionation was carried out following the methodology described by Kupchan, adapted from (22), to obtain hexane, chloroform, ethyl acetate, butanol and aqueous fractions.

Polyphenolic content and antioxidant activity assays

Folin-Ciocalteu assay

TPC was measured by using the Folin-Ciocalteu colorimetric method, according to the standard methodology (23). The standard was gallic acid, and the absorbance was read at 760 nm. Results were expressed as mg of gallic acid equivalent (GAE)/g of extract.

2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity

The cationic radical $ABTS^{\bullet+}$ was generated through an oxidation reaction of ABTS with potassium persulfate. The ability of the samples to trap the ABTS radical was evaluated by means of the decrease in the absorbance after 30 min of reaction at a wavelength of 732 nm (24). Results were expressed as µmol of Trolox equivalent antioxidant capacity (TEAC)/g of extract by constructing a standard curve using Trolox as an antioxidant.

DPPH radical scavenging activity

The ability of the samples to trap the DPPH radical was evaluated by means of the decrease in the absorbance after 30 min of reaction at a wavelength of 517 nm, following the method of Cao *et al*. with some modifications (25). For each sample studied, the percentage of radical inhibition was calculated, and the results are expressed as µmol of TEAC/g of extract by constructing a standard curve using Trolox as an antioxidant.

Ferric reducing antioxidant power (FRAP) assay

The test was carried out in an acetic acid-sodium acetate buffer (pH 3.4) containing TPTZ and $FeCl_3$. 900 µl of this solution, 50 µl of sample and 50 µl of distilled water were used. After 60 min of reaction time, the absorbance was determined at a wavelength of 593 nm. For each sample, the reading of the absorbance of the blank without chromophore was taken into account. The reference curve was constructed using ascorbic acid as the primary standard. The activities of the samples were expressed as mg of ascorbic acid equivalent (AAE)/g of extract (26).

ORAC assay

The ORAC assay was determined by the following methodology. In a quartz cell, 3 ml were prepared from the following solution: 21 µl of a 10 µM solution of fluorescein, 2899 µl of 75 mM phosphate buffer (pH 7.4), 50 µl of 600 mM AAPH, and 30 µl of extract (27). Fluorescence was recorded on a Perkin-Elmer LS-55 spectrofluorometer with a thermostatted multicell. The ORAC value (µmol TEAC/g of extract) was calculated by a calibration curve using different concentrations of Trolox and the differences in areas under the fluorescein decay curve between the blank and the sample, which was compared against the Trolox curve, according to Equation 1:

$$ORAC = \frac{(AUC - AUC^0)}{(AUC_{trolox} - AUC^0)} f[Trolox]$$

(Eqn. 1)

Where AUC is the area under the curve of the samples, AUC^0 is the area under the curve for the control, AUC_{Trolox} is the area under the curve for the Trolox and *f* is the dilution factor of the extracts.

Determination of phenolic acids by HPLC

The extracts were filtered (pore size 0.45 µm) and dilutions were made using Milli-Q water. The phenolic compounds were eluted at the following conditions: mobile phase acetonitrile/acidified water (40/60 v/v); flow of 1 ml/min, 25 °C and isocratic conditions. The UV-Vis spectrum ranged from 200 to 600 nm for all peaks; the identification and quantification of the compounds was done with calibration curves for each of the phenolic acids (chlorogenic, caffeic, ferulic and *p*-coumaric acid). The results were expressed as mg of phenolic acid/g of extract.

Statistical analysis

All experiments were carried out in triplicate. Regressions were calculated with a significance level of 95% ($P < 0.05$) using the Statgraphics Plus version 5.0 program (Statistical Graphics Corp., Rockville, MD). Correlation coefficients (Pearson's correlation coefficient, *r*) were determined using Excel software (Microsoft, 2010 version).

Results

In this study, *in vitro* antioxidant activities and determination of the main phenolic acids were established in different polarity solvent extracts obtained from two *Hypericum* species.

Phenolic content and antioxidant activity assays

Folin-Ciocalteau assay is not a specific reagent for polyphenolic compounds, since other compounds can reduce it. Despite that, the assay is reproducible, easy to implement and accessible, since the reagent is commercially available (28). The TPC and antioxidant activity results for *H. mexicanum* and *H. juniperinum* extracts are presented in Table 1.

The TPC values for the butanol and ethyl acetate fractions of *H. mexicanum* and the methanolic and butanolic fractions of *H. juniperinum* were very similar and oscillated between 211.87 and 269.84 mg GAE/g of extract; however, the ethyl acetate extract of *H. juniperinum* showed the highest value for TPC in this assay (491.08 mg GAE/g of extract). The aqueous extract of both species showed the lowest TPC values, 1.70 for *H. juniperinum* and 44.53 mg GAE/g of extract for *H. mexicanum*.

DPPH is a purple, stable radical with a maximum absorption in the visible spectrum ranging from 515 to 528 nm. When the radical traps an electron or a radical species, it reduces the intensity of the purple colour until it turns yellow. This assay has been extensively used for determining the antioxidant activity of diverse plant natural ex-

Table 1. Total polyphenolic content (TPC) and antioxidant activity results for *H. mexicanum* and *Hypericum juniperinum* extracts. Values are expressed as: TPC: mg GAE/g of extract, ABTS: µmol TEAC/g of extract; DPPH: µmol TEAC/g of extract; FRAP: mg AAE/g of extract and ORAC: µmol TEAC/g of extract

Plant	Extract	TPC	ABTS	DPPH	FRAP	ORAC
Hypericum mexicanum	Methanol crude extract	82.09±2.9	3082.19±7.3	3714.23±282.3	31.15±3.1	7619.61±576.5
	Aqueous	44.53±1.5	1065.43±416.9	409.11±5.8	13.84±0.6	1866.45±171.0
	Butanol	235.34±23.8	8500.86±732.9	1187.20±34.8	70.09±3.6	12910.06±877.7
	Ethyl acetate	268.78±6.0	7557.33±387.3	1513.76±43.9	80.97±5.1	5447.49±535.6
	Chloroform	133.44±9.0	4498.31±237.4	1326.48±68.3	77.25±7.7	5447.15±337.6
	n-hexane	86.91±2.4	3172.11±159.1	1072.40±79.2	11.59±0.7	2164.08±256.8
Hypericum juniperinum	Methanol crude extract	211.87±4.5	2115.32±106.3	1528.41±2.0	99.21±4.1	159.17±220.7
	Aqueous	1.70±0.06	19.20±1.1	6.94±0.2	0.51±0.05	14.53±1.8
	Butanol	269.84±17.6	4672.20±260.6	1615.76±46.3	97.32±4.1	3990.45±252.7
	Ethyl acetate	491.08±33.3	10867.48±992.6	3196.87±184.0	242.80±14.3	12204.40±487.2
	Chloroform	157.62±6.0	3590.72±349.2	896.59±44.2	39.93±2.2	2754.12±60.6
	n-hexane	102.72±2.8	2995.60±34.4	548.87±23.6	19.13±0.1	1384.91±45.2

tracts, including those from fruits and vegetables because it allows analysis of a high number of samples in a short period of time and is sensitive enough to detect antioxidant compounds at low concentrations (28). In the DPPH assay, the values ranged from 6.94 to 3,714.23 µmol TEAC/g of extract. The aqueous fractions showed lower values in both species, 409.11 µmol TEAC/g of extract for *H. mexicanum* and 6.94 µmol TEAC/g of extract corresponding to *H. juniperinum*. The methanol extract of *H. mexicanum* had the highest radical scavenger effect (3,714.23 µmol TEAC/g of extract), followed closely by the ethyl acetate extract of *H. juniperinum* (3,196.87 µmol TEAC/g of extract).

The ABTS scavenging assay is applicable for both hydrophilic and lipophilic compounds because the $ABTS^{\bullet+}$ radical cation is soluble in water and methanol, and it is not affected by ionic strength, therefore it can be carried out in different media to determine the antioxidant activity (28). Antioxidant activity is measured when the blue-green $ABTS^{\bullet+}$ radical cation that is formed gradually loses its colour. In this assay, the ethyl acetate and butanol extracts from both species displayed the highest antioxidant activity. The ethyl acetate extract of *H. juniperinum* exhibited the highest scavenging capacity of the $ABTS^{\bullet+}$ radical cation with a value of 10,867.48 µmol TEAC/g of extract followed by the butanol extract (4,672.20 µmol TEAC/g of extract). For *H. mexicanum*, the most active sample was the butanol extract with 8,500.86 µmol TEAC/g of extract followed by the ethyl acetate extract (7,557.33 µmol TEAC/g of extract). The less active samples on this assay were the aqueous fractions (19.20 µmol TEAC/g of extract for *H. juniperinum* and 1,065.43 µmol TEAC/g of extract for *H. mexicanum*).

The FRAP assay consists of the electron transfer and the power to reduce iron to its intensely blue-coloured ferrous ion. The FRAP mechanism is through a single electron transfer (SET), so it is not valid to compare this method with those where a radical scavenging mechanism is involved (29). Similar to the previous test, the most active sample in the FRAP assay was the ethyl acetate fraction of *H. juniperinum* with 242.80 mg AAE/g of extract followed by the methanol (99.21 mg AAE/g of extract) and butanol (97.32 mg AAE/g of extract) fractions. Again, the fraction with the lowest antioxidant activity was the aqueous fraction from *H. juniperinum.* Furthermore, the FRAP values for *H. mexicanum* ranged from 11.59 to 80.97 mg AAE/g of extract, corresponding to the hexane and the ethyl acetate fraction respectively.

In the ORAC assay, the $ROO^{\bullet}$ reacts with a fluorescent probe to produce a non-fluorescent species, which can be quantitated by fluorescence decay; as the product formed decreases, the radical scavenging capacity seems to be higher (30). The two species had similar values on the ORAC test in each solvent. The butanol extract of *H. mexicanum* and the ethyl acetate extract of *H. juniperinum* possessed the highest values of antioxidant activity, 12,910.06 and 12,204.40 µmol TEAC/g of extract, respectively. Similar to the previous antioxidant tests, the less active samples were the aqueous fractions of both plants, showing ORAC values of 14.53 and 1,866.45 µmol TEAC/g of extract for *H. juniperinum* and *H. mexicanum* respectively.

Antioxidant and chemical studies of these species are limited; however, some comparisons are possible. The results obtained in the TPC assay agree with the data reported earlier (16), who described high polyphenol content (100.37 mg GAE/g plant dry material) in *H. juniperinum*. Moreover, in studies with different *Hypericum* species, the ethyl acetate extract showed the highest content of phenolic compounds, while the hexane and chloroform fractions were relatively poor in this kind of compounds (20). Studies on other *Hypericum* species showed that less polar solvents than water tend to perform better scavenging activity on DPPH and ABTS assays. The ethyl acetate fraction of *Hypericum hyssopifolium* also showed the highest value for the DPPH test. Also, flavonoids like quercetin and glycosyl quercetin derivatives were isolated from these fractions (31). Additionally, a study with *Hypericum ascyron* extracts showed higher ABTS scavenging capacity in the ethyl acetate extract than in the methanolic one, but in the FRAP assay, the reducing power was better for the

methanol extract (483.32 µmol Trolox/g sample). Nevertheless, quercetin-3-*O*-β-D-galactoside, quercetin-3-*O*-β-D-glucoside and kaempferol were isolated from the ethyl acetate extract and provided better results on these assays (32), providing evidence of the relation between the presence of those metabolites and their antioxidant power. The results obtained for the ORAC test in both species were similar to those reported for other *Hypericum* species, where the highest ORAC values of the crude methanol extract were for *H. caprifoliatum* (820 µmol Trolox/g of extract) and *H. carinatum* (347 µmol Trolox/g of extract) and the lowest was for *H. polyanthemun* (240 µmol Trolox/g of extract) (33).

Relationships between antioxidant assays and TPC results have been described for other species. The ethanol extract of *Hypericum lysimachioides, H. triquetifolium* and *H. scabroides* showed high scavenging ability in the DPPH assay and a TPC of 307, 267 and 333 mg GAE/g sample respectively (34, 35). Those values are higher than the value of the methanol extract from *H. mexicanum* (82.02 mg GAE/g sample) but similar to the ethyl acetate fraction (268.78 mg GAE/g sample) of the same species. In comparison with *H. juniperinum* values, the ethyl acetate extract had better results for TPC (491.08 mg GAE/g sample). This accounts for the different behaviour of the extracts in each solvent due to the extract composition.

According to our results, there is a direct correlation between the concentration of phenolic compounds and the antioxidant potential. The TPC of the *H. juniperinum* extracts showed high correlations with the antioxidant activity values. Pearson's correlation coefficient (r) for DPPH, ABTS, FRAP and ORAC were 1.00, 0.98, 0.98 and 0.97 respectively. High correlations between these variables were observed with determination coefficients (r^2) greater than 95%. In *H. mexicanum* extracts, the correlations between TPC and antioxidant activity were lower than those obtained in *H. juniperinum*, with Pearson's correlation coefficients of 0.97, 0.83, 079 and 0.72 for ABTS, FRAP, DPPH and ORAC respectively. It is important to note that only ABTS had a high correlation with TPC (r^2 = 94%).

The presence of phenolic compounds had been previously described for both *H. mexicanum* and *H. juniperinum* and for other *Hypericum* species. Preliminary phytochemical studies with *H. mexicanum* and *H. juniperinum* have reported the presence of terpenes/steroids, phenolics, flavonoids, quinones, tannins, saponins and coumarins (15, 17). In addition, Mejía-Agudelo *et al*. isolated the flavonoid quercetin and chlorogenic acid methyl ester (5-*O*-caffeoylquinic acid methyl ester) from an ethyl acetate fraction of *H. juniperinum* (36). Furthermore, the presence of quercitrin and rutin was also established in the ethyl acetate fraction of *H. juniperinum* by means of HPLC (15). More recently, a complete characterization of the phenolic profile from stems, roots and leaves of *H. mexicanum* has been described (18).

Concerning our study, in general, the most active antioxidant extracts, in both plants, were the ethyl acetate and butanol. Polyphenolic compounds may have a wide range of polarities, thus both organic and aqueous extracts showed a significant antiradical and antioxidant activity. The fact that most of the assays presented the best results with the ethyl acetate fraction indicates that the responsible compounds for the antioxidant activity correspond to medium to high polarity metabolites, such as flavonols (i.e., quercetin and kaempferol), which show a high ability to scavenge radicals (37). Indeed, the composition of the phenolics in *Hypericum* species are mainly quercetin and kaempferol derivatives (38). This is important since these kinds of compounds are well known for their good antioxidant potential (39). Their antioxidant capacity depends on different structural features, such as the hydroxyl and carbonyl groups arrangement around the molecule, which mainly determines the metal-chelating potential, the presence of hydrogen or electron-donating substituents in order to reduce free radical and the structural ability of the flavonoid to delocalized unpaired electrons, leading to the formation of a phenoxyl radical (40). For flavonols, such as quercetin and kaempferol, it is widely suggested that the C3 free hydroxyl group is responsible for the high oxidation inhibition; as well as the pattern of substitution of the other phenolic hydroxyls in the A and B rings contributes to the activity (41). Furthermore, the butanol extract was the second most active in the tests, suggesting the presence of more polar compounds, such as quercetin and kaempferol glycosyl derivatives (38). Likewise, the most polar extract (aqueous) was the least active in most of the assays, which might be because this extract could present a low content of reactive antioxidants, such as glycosyl flavonoids like rutin, hyperoside, isoquercitrin and quercitrin, which are recognized compounds in *H. perforatum*, rutin and quercitrin in *H. juniperinum* and isorhamnetin-3-*O*-glucoside and quercetin-3-*O*-glucoside in *H. mexicanum* (15, 18, 39).

Although the mechanism of action of phenolic compounds is not completely elucidated yet, for definition, the role of the hydroxyl group attached to the aromatic ring is to interrupt the radical chain reaction. Due to the structural substitution pattern of glycosyl compounds, antioxidant and antiradical potential might decrease when compared to the aglycone due to the masking of phenolic groups (38). There are 2 antioxidant mechanisms proposed, the hydrogen atom transfer (HAT) and SET. ABTS and DPPH are considered mixed methods (SET- and HAT-based), while the FRAP test is based on the SET reaction, which is pH dependent and the ORAC test is based on the HAT mechanism (28). The mechanism is relevant when it is associated with the antioxidant capacity detection methods. Most of the assays were done at non-physiological pH values; therefore, it is advisable to perform assays that occur through different mechanisms of action. In the same line, more studies to estimate the antioxidant action under *in vivo* models are necessary.

Phenolic acids detection and quantification

Phenolic acids can be found in almost all plants, and they have become important because of their possible protective action against diseases where oxidative damage is present (42). Phenolic acids can be classified as hydroxybenzoic or hydroxycinnamic; the latter exhibits a great antioxidant activity due to the conjugation effects of

the carbonyl with the double bond. Hence, in this study, only hydroxycinnamic acids (chlorogenic, caffeic, *p*-coumaric and ferulic acid) were quantified (Table 2). As an example of the HPLC analysis, the chromatographic profiles of the *Hypericum* methanolic extracts are shown in Fig. 1.

Concerning the chlorogenic acid concentration, *H. mexicanum* values ranged from 1.53 to 50.09 mg/g. The aqueous fraction had the highest concentration (50.09 mg/g) followed by the butanol extract (14.40 mg/g). No chlorogenic acid concentration was detected in the *n*-hexane fraction for *H. mexicanum*. On the other hand, *H. juniperinum* presented significantly lower chlorogenic acid concentrations ranging from 0.85 to 2.47 mg/g, with no detection in the chloroform extract. Neither *H. mexicanum* extracts nor *H. juniperinum* extracts reported the presence of caffeic acid. *p*-coumaric acid concentration ranged from 1.48 to 63.36 mg/g in *H. mexicanum*, with the aqueous (63.36 mg/g) and butanol (44.06 mg/g) extracts having the highest concentration, similar to the behaviour for chlorogenic acid determination. In the *n*-hexane extract of *H. mexicanum*, *p*-coumaric acid was not detected. For *H. juniperinum*, only the methanol and butanol extracts presented *p*-coumaric acid with lower values than *H. mexicanum*, ranging from 4.82 to 8.45 mg/g. Finally, ferulic acid was not detected in any of the *H. juniperinum* extracts. In *H. mexicanum*, it was only detected in the ethyl acetate (6.92 mg/g), methanol (1.71 mg/g) and chloroform (1.14 mg/g) fractions.

To the best of our knowledge, this is the first time that hydroxycinnamic acids are determined and quantified for *H. juniperinum*. For *H. mexicanum*, it was reported that the presence of caffeic and cinnamic acids in leaves but not in stems and roots. They also did not detect chlorogenic acid in any of the organs analysed (18). In comparison with our results, we did not detect caffeic acid, but we could determine the presence of *p*-coumaric, chlorogenic and ferulic acids in aerial parts of *H. mexicanum*. Other species of *Hypericum* have also confirmed the presence of phenolic acids. In *H. humifusum*, among other phenols, caffeic (0.056 mg/g sample) and chlorogenic acids (0.064 mg/g sample) were identified (43). Those acids were also quantified in the aqueous extracts of *H. perforatum*, *H. androsaemum*, *H. undulatum* and *H. foliosum*, obtaining chlorogenic acid values of 4.34, 34.18, 6.45 and 29.89 respectively (44). Additionally, *p*-coumaric acid was found in *H. monoatum* (10 mg/kg dried plant material) (45).

The phenolic acids in *H. juniperinum* and *H. mexicanum* can also contribute to the antioxidant activity of the extracts, including the 5-*O*-caffeoylquinic acid methyl ester, already isolated from *H. juniperinum*. The hydrogen donating capacity of phenolic acids depends on the pres-

Table 2. Phenolic acids occurring in the different solvent polarity fractions obtained from *H. mexicanum* and *H. juniperinum*. Concentrations are expressed as mg acid/g of extract

Plant	Extract	Chlorogenic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid
Hypericum mexicanum	Aqueous	50.09 ± 4.51	n.d.	63.36 ± 5.41	n.d.
	Methanol	1.53 ± 0.04	n.d.	2.56 ± 0.08	1.71 ± 0.07
	Butanol	14.40 ± 1.12	n.d.	44.06 ± 3.21	n.d.
	Ethyl acetate	9.64 ± 0.78	n.d.	17.93 ± 0.12	6.92 ± 0.32
	Chloroform	2.39 ± 0.12	n.d.	1.48 ±0.08	1.14 ± 0.07
	n-Hexane	n.d.	n.d.	n.d.	n.d.
Hypericum juniperinum	Aqueous	1.83 ± 0.02	n.d.	n.d.	n.d.
	Methanol	2.47 ± 0.14	n.d.	4.82 ± 0.28	n.d.
	Butanol	1.18 ± 0.09	n.d.	8.45 ± 0.61	n.d.
	Ethyl acetate	0.85 ± 0.02	n.d.	n.d.	n.d.
	Chloroform	n.d.	n.d.	n.d.	n.d.
	n-Hexane	1.26 ± 0.07	n.d.	5.09 ± 0.40	n.d.

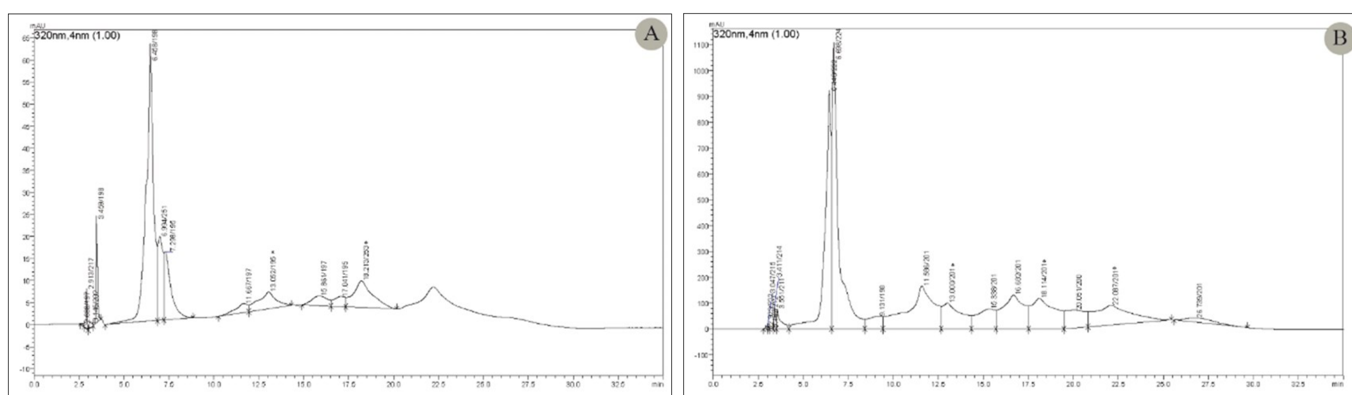

Fig. 1. HPLC profile of methanolic extract of *H. juniperinum* (**A**) and *H. mexicanum* (**B**). Phenolic acids retention time (min): Chlorogenic acid (13.2), *p*-coumaric acid (18.2) and ferulic acid (22.1).

ence of OH substituents in ortho- and para- positions and the unsaturated bonds. These characteristics also determine the capacity of these compounds to donate electrons, similar to flavonoids. Several studies have been conducted to determine the antioxidant capacity of these compounds, suggesting that the more OH substituents at the aromatic ring are present, the more antioxidant capacity they have (46). Therefore, since caffeic acid was not detected, we could suggest that chlorogenic acid would be more active as an antioxidant in the methanolic extract of *H. juniperinum*. Additionally, the amount of ferulic acid in the ethyl acetate fraction of *H. mexicanum* and *p*-coumaric acid in the butanol fraction of *H. juniperinum* might have contributed to the good results in the antioxidant activity.

Conclusion

The information available about the biological activities of extracts and fractions obtained from both *Hypericum* L. species is scarce or in some cases absent. There are some preliminary studies with these species, but none of them have focused on the study of antioxidant activity and the profile of organic acids. Our results provide new insights into the antioxidant activities of both *H. mexicanum* and *H. juniperinum* species. For the first time, results of the antioxidant activity of hydrophilic and hydrophobic extracts of both species are reported; the antioxidant tests used allowed us to discriminate the oxidative mechanisms by transfer of hydrogen (ORAC) and by electron transfer (FRAP). Furthermore, phenolic acids were determined and quantified for these Colombian Andean species; interestingly, this is the first time the presence of phenolic acids is reported in *H. juniperinum* and in addition, some phenolic acids that had not been previously identified in *H. mexicanum* were found. Chemical constituents of these extracts can produce health benefits and possible applications in the pharmaceutical, cosmetic and food industries. Nevertheless, a more complete chemical characterization would be necessary. In addition, further investigation on the mechanism of action and safety is crucial for the evaluation of their potential as prophylactic agents.

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Authors contributions

ACP and AFA performed the experiments. BR, JHG and JCML analyzed and interpreted the data. MR and JCML conceived and designed the experiments. JHG and JCML drafted the manuscript. All authors revised and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: The *Ministerio de Ambiente y Desarrollo Sostenible* granted permission to collect samples and perform this research (Contrato de Acceso a Recurso Genético y sus Productos Derivados No 121, Otrosí No 12).

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