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Baccharis spicata (Lam) Baill: Polyphenol screening, determination of their antioxidant activity and their main polyphenolic metabolites

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Abstract
Baccharis spicata is a dioecious plant from South America. The quantitative variation of its polyphenolic compounds was studied in methanolic extracts of its vegetative and reproductive aerial organs. Leaves were found to be the organ with the highest polyphenol concentration. The main compounds have been characterized by thin layer chromatography and high performance liquid chromatography with standard compounds, and studies of structural elucidation were performed by UV-Visible spectrophotometry. The flavonoid rutin and the caffeoylquinic derivatives chlorogenic acid, and 3, 5; 3, 4 and 4, 5 dichlorogenic acids have been found. Studies of antioxidant activity were performed, obtaining a value of IC_{50} of 61.3µg/ml extract. This work sets a precedent in the study of this species and its possible therapeutic use in diseases related to the generation of oxidative stress.

Keywords: *Baccharis Spicata,* polyphenol screening, polyphenolic metabolites

1. Introduction
Baccharis is a genus of North and South America, with more than 500 species belonging to the Asteraceae family.
It is formed by bushes or perennial herbs, often rhizomatous or with gemiferous roots. It has been thoroughly studied from the ethnobotanical and pharmacognostic point of view due to the extensive use of different species throughout its distribution area [1]. In Argentinian folk medicine, from 120 species of this genus, *Baccharis articulata*, *Baccharis trímera,* and *Baccharis crispa* are mainly used as digestive and hepatoprotective in the form of infusions [2].
Its phytochemistry is well studied and a large amount of compounds have been characterized. Within the terpenic derivatives, spathulenol and carquejyl acetate are present in the essential oil, among others; compounds of the neo-clerodane and labdane diterpenic family, as well as, derivatives of oleanolic and ursolic acid, within triterpenes [3].
Flavonoids are mainly represented by methoxylated flavones while flavonoid glycosides are less frequent within the genus [4].
Baccharis spicata is a dioecious bush of 1 to 1.8 meters high, with opposite leaves, of 4 to 8 cm long, striated and erect stems not winged (unlike *B. articulata*, *trimera* and *crispa*), and flower heads of 5 to 6 mm diameter building a false spike in the apex.
It is a native species of the wetlands of the South of Brazil, Paraguay, Uruguay, and center region of Argentina. It is likely to be found in the Paraná Delta, the banks of the River Plate, and fields with little anthropogenic modification [1].
Studies of chemical composition and biological activity previously performed on this species describe compounds of the clerodane diterpene family and their insecticide activity [5], of their composition of essential oils [6], and their antioxidant [7, 8], tripanocide [9], antiviral [10], and cytotoxic activity [11].
Most of these studies have been performed on the whole plant without discrimination of organs or sex. There is no information about the composition of polyphenols of this species.

1.1 Objective: The objective of this study is to determine the dynamics of polyphenols in methanolic extracts of different aerial organs of female and male samples of *Baccharis spicata* and determining the structures of the main polyphenol metabolites and their antioxidant activity.

2. Materials and Methods
2.1 Plant Material
Leaves, stems, and flower heads of female and male samples of *Baccharis spicata* (Lam) Baill collected in La Florida neighborhood, Zárate, Buenos Aires and dried at room temperature.

Reference samples are placed in the herbarium of the Chair of Pharmacobotany, Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

2.2 Extraction of methanolic extracts
Extraction was carried out at room temperature, for 24 h, with 10 mL of methanol 50% (methanol-water, 1:1), over 200 mg dried ground plant material (leaves, stems, and flowers). It was later filtered and the solids were discarded.
In addition, a methanolic extract elaborated with a mix of equal parts of leaves of female and male subjects was performed (MEM).

2.3 Total phenol quantification
The Folin– Ciocalteu method was used [12]. Aliquots (50 µL) of extracts were transferred to test tubes and the volume was taken to 500 µL with deionized water. Then, 250 µL of Folin-Ciocalteu reactive and 1.25 mL of aqueous solution of sodium carbonate 20% were added. After 40 minutes, absorbance was measured at 725 mm. A calibration curve with tannic acid was performed. Total phenol content was expressed as mg tannic acid / g dry material. All measurements were performed in triplicate.

2.4 Total hydroxycinnamic acid quantification
It was determined by a modification of the methodology described by Dao and Friedman [13]. Aliquots of 50 µL of each extract were taken to volume (2 mL) with absolute ethanol.
Absorbance was determined at 328 nm. A calibration curve was performed with chlorogenic acid. Values were expressed as mg chlorogenic acid / g dry material. Assays were performed in triplicate.

2.5 Total flavonoid quantification
Aliquots of 0.1 mL of each extract were added to 1.4 mL of deionized water and 0.50 mL of the flavonoid reactive (133 mg aluminium trichloride, 400 mg sodium acetate in 100 mL of solvent constituted by 140 mL methanol, 50 mL water, and 10 mL acetic acid). After 30 min at room temperature, absorbance was measured to 430 nm [14]. A calibration curve was performed with rutin.
Content of flavonoids was expressed as mg rutin / g dry material. All measurements were performed in triplicate.

2.6 Determination of antioxidant power
Total antioxidant activity was studied *in vitro* of a methanolic extract prepared with a mix of equal parts of leaves of female and male subjects by DPPH technique (1,1-Diphenyl-2-picrylhydrazyl) [15]. Briefly, they were taken to a final volume of 1 ml of methanol aliquots of 5, 10, and 20 µl. Then, 2 ml of DPPH reactive to 0.004% was added in methanol and was incubated for 30 minutes. Absorbance was measured at 518 nm and EC_{50} was calculated.

2.7 Polyphenol fingerprinting and compound characterization in thin-layer chromatography
For the MEM, polyphenol fingerprinting was performed by a two-dimensional chromatography using cellulose plates and BAA systems of solvents (butanol: acetic acid: water, 6:1:2) for the first dimension and acetic acid 15% for the second dimension, against rutin. Another chromatography in cellulose plates was also performed, using HCL 1N as mobile phase, against chlorogenic acid.
Chromatograms were observed at UV 254 nm and 365 nm, before and after exposure to ammoniac vapors and revealed with the reactive of natural products (NP 1% in methanol). Rf values of compounds were determined in control drugs and in extracts.

2.8 Hydrolysate: 1 mL of MEM with 5 ml of HCl was submitted to acid hydrolysis for 1 hour. It was filtered and later extracted with 3 fractions of 1 mL each one of ethyl acetate. It was concentrated in rotary evaporator and preparative descendant chromatography was performed in Whatman 3MM paper, using as mobile phase acetic acid 50% and 15%.
Isolated bands were analyzed by TLC of cellulose in the systems Forestal Glacial (acetic acid): chlorhydric acid: water, 30:3:10), TBA (*tert*-Butyl: acetic acid: water, 1:1:1), and acetic acid 60% against control drug of quercetin, and in the HCL 1N system, against control drug of caffeic acid. UV-visible spectroscopic analysis of the isolated compounds was made using standard methodology [16].

2.9 Compound characterization in high performance liquid chromatography
The chromatographic analysis was performed over MEM against control drug rutin, chlorogenic acid, and 3, 4; 4, 5 and 3, 5 dichlorogenic acids, in a HPLC Varian 9050 equipment with detection by diode arrangement at wavelengths of 325 and 255 nm for hydroxycinnamic derivatives and flavonoids respectively, with a Rheodyne loop of 100 µl injection volume. The equipment was controlled by Star Chromatography Workstation software.
Separation was performed in a reversed-phase column Phenomenex Luna C18, (5 µ, 250 x 4.6 mm) with water: acetic acid (98:2) as mobile phase A, and methanol: acetic acid (98:2) as mobile phase B, and a flux of 1.2 ml/minute.
The following gradient was followed: 15% B to 40% B, 30 minutes; 40% B to 75% B, 10 minutes; 75% B to 85% B; 5 minutes.

3. Results
3.1 Quantification of polyphenolic compounds
Results of total phenol, flavonoids, and hydroxycinnamic acids quantification present in methanolic extracts are shown in Figures 1 to 3.
Quantification of these metabolites in the leaves is shown in the MEM is shown in Table 1.

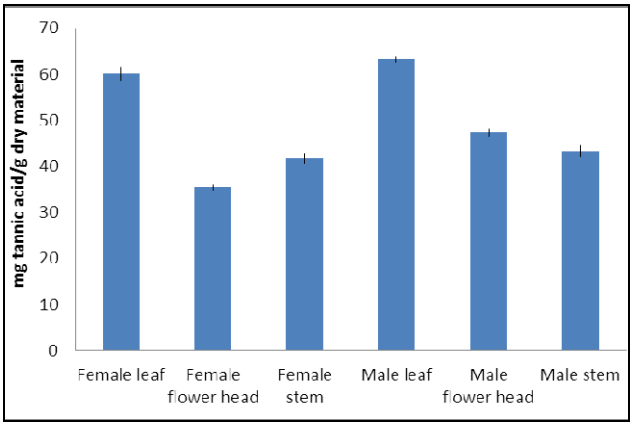

Fig 1: Total phenol content in methanol: water (1: 1) extract

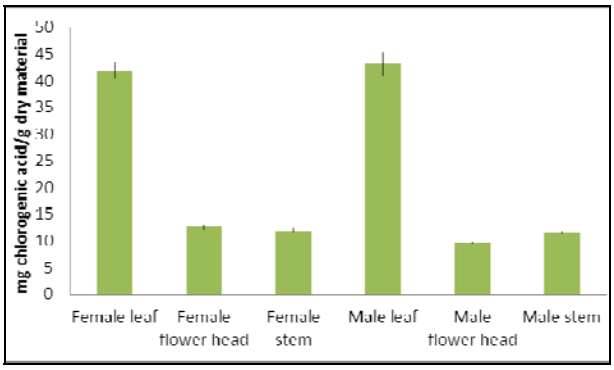

Fig 2: Total hydroxycinnamic acids content in methanol: water (1: 1) extract

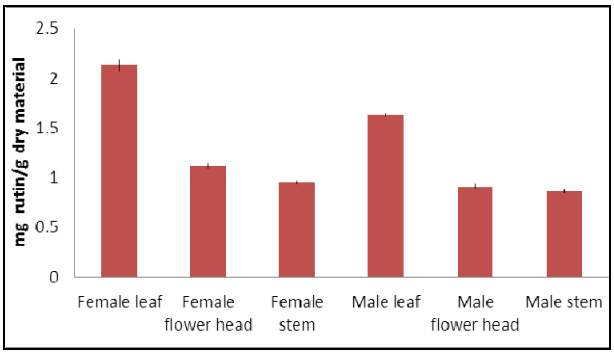

Fig 3: Total flavonoid content in methanol: water (1: 1) extract

Table 1: Content of polyphenolic compounds in methanol: water (1: 1) extract of leaves

	Average	Standard deviation
Total phenols (mg tannic acid/g dry material)	66.33	1.57
Total hydroxycinnamic acids (mg chlorogenic acid/g dry material)	68.66	2.47
Total flavonoids (mg rutin/g dry material)	7.46	0.41

3.2 Determination of antioxidant activity

Results of antioxidant activity of MEM are shown in Figure 4.

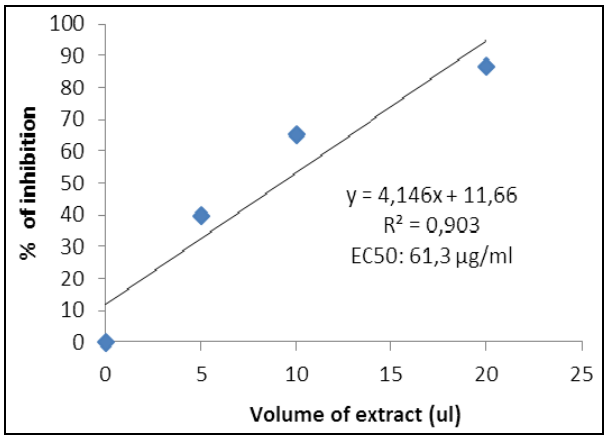

Fig 4: Antioxidant activity of methanol: water (1:1) extract

3.3 Characterization of principal polyphenolic metabolites

Methanolic extract mix (MEM)

In the characterization by thin-layer two-dimensional chromatography, the presence of rutin is observed (Figure 1).

In HCL 1N system, the presence of chlorogenic acid is observed (Figure 2).

The analysis by high performance liquid chromatography confirms the results above mentioned, determining the presence of rutin (tr: 34.97 minutes), chlorogenic acid (tr: 12.529 seconds), and isomers of dichlorogenic acid (3,4; 4,5; 3,5 dicaffeoylquinic acids) (tr: 35.767; 32.008, and 32.773 minutes) (Figures 5 to 7).

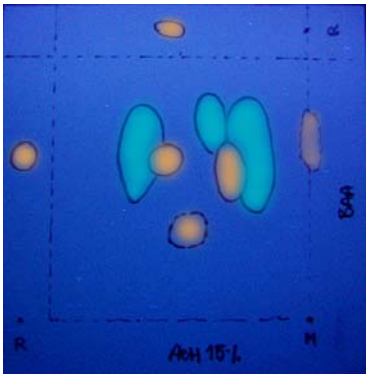

Fig 5: Characterization of rutin byTLC-2D

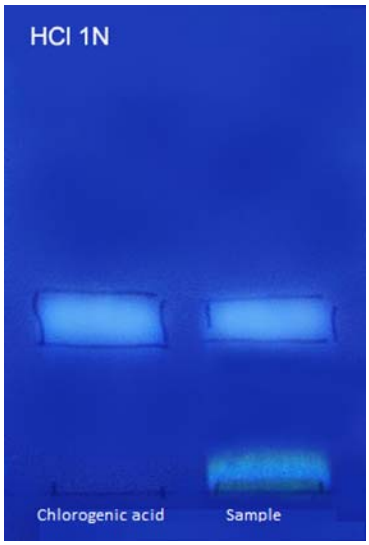

Fig 6: Characterization of chlorogenic acid by TLC

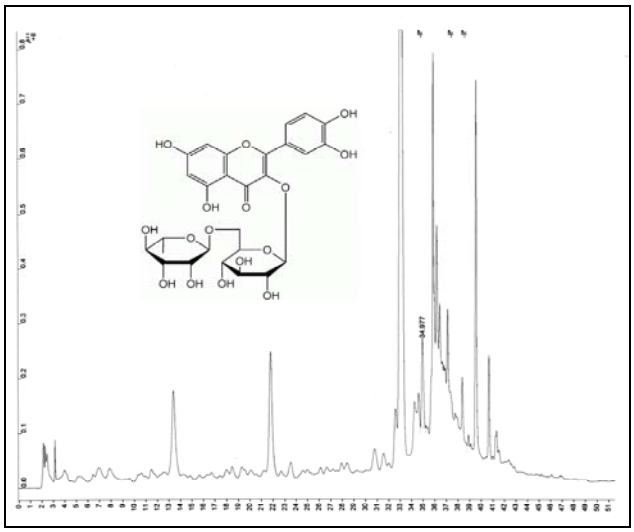

Fig 7: Characterization of rutin by HPLC

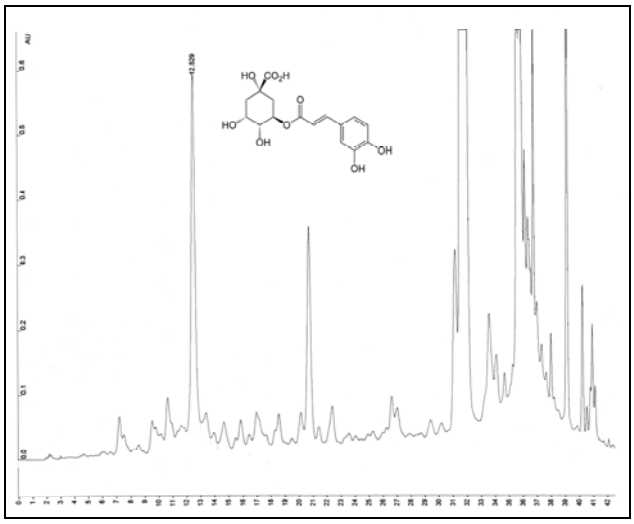

Fig 8: Characterization of chlorogenic acid by HPLC

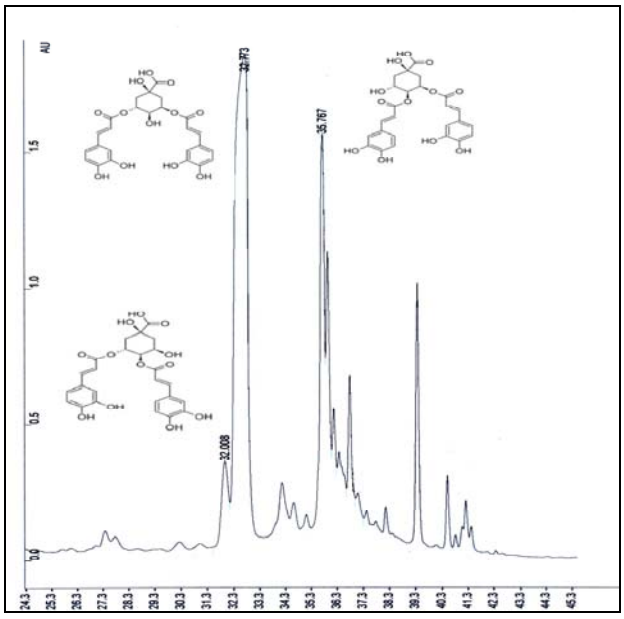

Fig 9: Characterization of isomers of dichlorogenic acid by HPLC

3.4 *Characterization of polyphenolic compounds in the hydrolysate*

An aglycone was isolated by paper descendant chromatography, whose chromatographic (Figures 3, 4, and 5) and spectroscopic characteristics (Figures 8, 9, and 10; Table 2) match with those of the flavonoid quercetin. Original spectrum is characterized by the presence of maximums at 256 nm, 296 nm (sh), and 368 nm. With the addition of sodium methoxide, a bathochromic of 78 nm of the band I with decomposition is evidenced, corresponding to a structure sensitive to alkali, from a flavonoid with 3 and 4´free hydroxyls. Also, the appearance of a peak of 330 nm reveals a hydroxyl in position 7. With the addition of $AlCl_3$, a bathochromic shift is observed, which partially reverts before HCl addition, confirming the presence of a dihydroxyl group in ring B. These spectroscopic features match with the ones reported for quercetin.

Furthermore, the presence of caffeic acid was characterized, coming from the hydrolysis of the chlorogenic acids and its derivatives, by thin-layer chromatography with a control drug of this substance (Figure 6).

Fig 10: Characterization of quercetin by TLC (Mobile phase butanol: acetic acid: water 6:1:2)

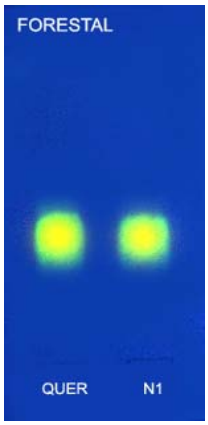

Fig 11: Characterization of quercetin by TLC (Mobile phase: Forestal)

Fig 12: Characterization of quercetin by TLC (Mobile phase: acetic acid 60%)

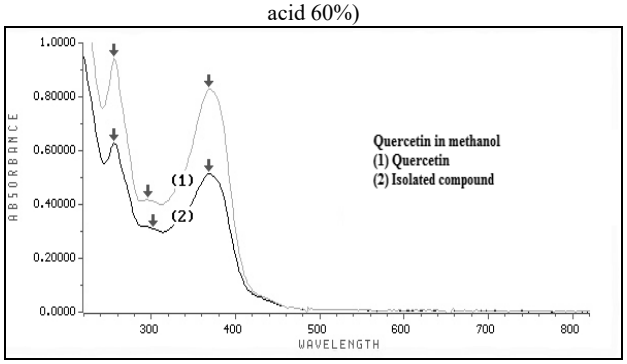

Fig 13: Spectra of isolated aglycone and quercetin in methanol

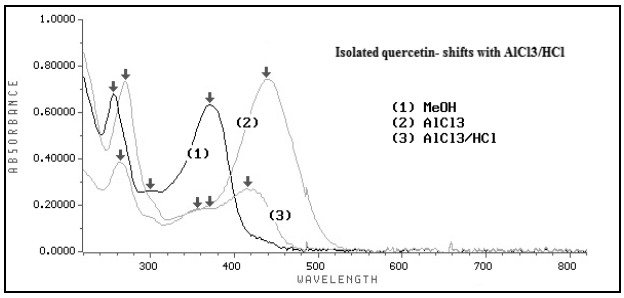

Fig 14: Spectral shifts of isolated aglycone with $AlCl_3$ and HCl

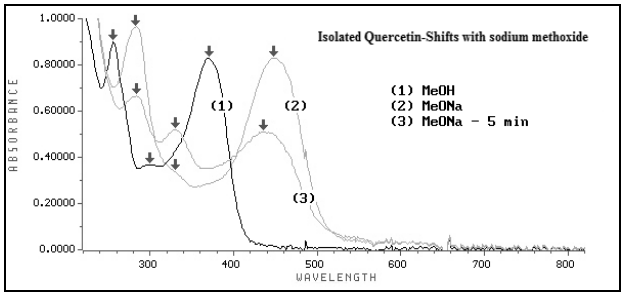

Fig 15: Spectral shifts of isolated compound with sodium methoxide

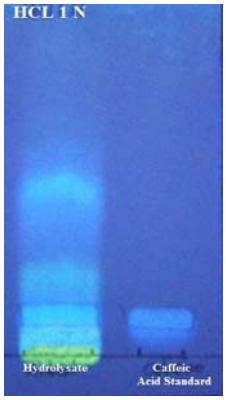

Fig 16: Characterization of caffeic acid by TLC

Table 2: Spectral shifts with reagents of isolated compound and comparison with quercetin standard

Samples	Signals (nm)		
Quercetin standard in MeOH	256	304sh	370
Isolated quercetin in MeOH	256	296sh	368
Isolated quercetin/Sodium methoxide	282	330	448
Isolated quercetin/AlCl3	268	358sh	434
Isolated quercetin/AlCl3/HCl	264	370sh	416

5. Discussion

It should be stressed that this study evaluates polyphenol dynamics in the flowering stage, since it analyses female and male samples.

According to results shown in Figures 1, 2, and 3, the methanolic extracts made from leaves presented a higher concentration with regard to the stem and flower heads for all the analyzed metabolites.

There are no significant differences associated with sex in the total phenol and hydroxycinnamic acid content for the analyzed organs.

As far as the analysis of flavonoids, they showed a significant difference associated to sex when female and male flower heads are compared, with a higher concentration of these metabolites in the female materials. There was not a significant difference in the flavonoid content for leaves and stems between both sexes.

Regarding the identity of the main compounds, the flavonoid rutin was detected in the MEM, and within the caffeoylquinic derivatives, chlorogenic acid, and 3, 4; 4, 5, and 3, 5 dichlorogenic acids were found. Determination of quercetin and caffeic acid in the hydrolysate confirms the presence of rutin and chlorogenic acid in the MEM.

Rutin is a flavonoid of widespread distribution in the plant realm, although it is not common in *Baccharis* genus and has been only documented in *B. gaudichaudiana, B. thesioides,* and *B. trimera*. It is interesting to notice that, according to bibliography, the main chemotaxonomic characteristic of this genus is the presence of free methoxylated flavonoid aglycones [4], so the presence of rutin constitutes an important differential when it comes to make the identification of the species by chemical methods.

On the other hand, the presence of the mentioned metabolites, allowed obtaining an EC_{50} value for the determination of the antioxidant activity (DPPH) of 61.3 µg of dry material / ml, being considered a promising value of the mentioned activity.

Isolated metabolites in this study are common within the plant realm and have an interesting theoretical framework when establishing possible biological activities for this plant. Chlorogenic acid is the ester of quinic acid with caffeic acid. It has widespread distribution among vascular plants and it has been studied intensively, having found evidence of its antioxidant activity [17] and chelating activity of Fe^{2+} [18], which explains its action in different models of animal pathologies.

At the hepatic level, its protective activity against damage caused by paraquat [19], ischemia/reperfusion [20], carbon tetrachloride [21], and bacterial lipopolysaccharides [22] has been investigated.

Moreover, its cytotoxic activity in tumor cells has been studied. It is an inductor of apoptosis by caspase-dependent pathway and mitochondria-dependent in U937 human leukemic cells [23]. It is also an inductor of cytotoxicity in tumor oral cells, by prooxidant mechanisms in high doses [24], with evidence found of its mutagenicity in CHO cells in presence of manganese [25] and its prooxidant activity and ability of DNA rupture of plasmids before a Cu^{++} [26]. This mechanism is antagonistic with the evidence that indicates the chlorogenic acid as antioxidant *in vivo* and *in vitro* abovementioned, and with the protective activity of DNA rupture in plasmids induced by monochloramine [27]. In addition, it is an inhibitor of the Matrix metallopeptidase 9, involved in the metastatic dissemination by hydrolysis of the extracellular matrix [28].

Dichlorogenic acids (isomers 3,4; 4,5; and 3,5 dicaffeoylquinic) are also antioxidant [29], and their analgesic [30] and hypertensive activity [31] have been documented, as well as the anti-hepatotoxic activity of isomer 3,4 dicaffeoylquinic acid [32]. Likewise, a strong inhibiting activity of the integrase of HIV-1 virus [33, 34] has been found, inhibiting replication, from these compounds, which has led to structure-activity studies of derivatives obtained by pharmacomodulation [35]. It has also been demonstrated that isomer 3,5 caffeoylquinic prevents damage caused by lipopolysaccharide in endothelial cells [36].

Inhibition of nuclear translocation of the transcription factor NF κB in macrophages [37] has been documented, as well as induction of apoptosis by activation of caspases 3 and 8 in human colon cancer cells [38].

Rutin has been widely studied for its pharmacological activity against different models of animal disease and associated molecular mechanisms. Regarding the hepatic function, protective action against damage caused by carbon tetrachloride [39] and paracetamol [40] has been proved.

As far as the cardiovascular system, vasorelaxant and hypotension activity [41] have been proved, as well as, antiplatelet [42], and cardio protective in models of myocardial infarction induced in healthy rats treated with streptozotocin [43].

Its antioxidant and Fe $^{2+}$ chelating activity are well known [44], which would explain, to a large extent, the activities before mentioned. In addition, the superoxide dismutase activity simile of the complexes of this flavonoid with transition metals has been described [45].

Protective activity against oxidative stress induced by overload of plasmatic iron has been documented [46], in diabetes-induced rats with streptozotocin [47] and with cyclophosphamide [48].

Genotoxic activity at high concentrations has also been shown [49], but just as the chlorogenic acid, it is protective against DNA damage. This activity was demonstrated with models of induced damage by benzo (a) pyrene in hepatic cells [50].

It also has a nephroprotective activity, studied in models of kidney damage induced by ischemia/reperfusion [51] and cisplatin exposure [52], and gastroprotective activity was proven in models of ethanol-induced damage, aspirin, acetic acid, pylorus ligation, and restriction in low temperatures [53]. This activity has also been documented for damage induced by chloridric acid /absolute ethanol. In this regard, it also presents antimicrobial activity against *Helicobacter pylori* and stimulating of gastric mucus secretion [54].

6. Conclusions

B. spicata is an understudied species and the presence of metabolites with proven pharmacological activity makes it necessary to continue deepening in its phytochemistry and possible therapeutic use. This article is the first one that reports the presence of rutin, chlorogenic acid, and 3,4; 4,5; and 3,5 dichlorogenic acids, for this species, apart from describing the quantitative composition of polyphenols in the different plant organs.

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