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Full Length Research Paper

Phenolic content and antioxidant capacity of four *Cnidoscolus* species (Euphorbiaceae) used as ethnopharmacologicals in Caatinga, Brazil

Tadeu J. S. Peixoto Sobrinho[1]*, Valérium T. N. A. Castro[1], Antonio M. Saraiva[2], Diego M. Almeida[1], Elvis A. Tavares[1] and Elba L. C. Amorim[1]

[1]Laboratório de Produtos Naturais, Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Recife-PE, Brasil.
[2]Laboratório de Análises Microbiológicas, Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Recife-PE, Brasil.

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This study aimed to investigate the phenolic content and antioxidant capacity of four species of *Cnidoscolus* (*C. infestus Pax & K. Hoffm., C. pubescens Pohl, C. quercifolius* Pohl and *C. urens* [L.] Arthur) used as ethnopharmacologicals in the Caatinga. The total phenolic content (TPC), total tannin content (TTC) and total flavonoid content (TFC) were analyzed by spectrophotometric method. The antioxidant activity (AOA) was measured using the qualitative antioxidant assay (QAA), the free-radical scavenging assay (DPPH assay) and the ferrous-ion chelating (FIC) assay. The extract of the leaves of *C. pubescens* had the highest levels of TPC, TFC and TTC. Extracts from the roots of *C. infestus* showed the highest antioxidant activity, while extracts from the roots of *C. pubescens* showed the highest level of chelating activity. The AOA was correlated with TPC, TFC and TTC, but these were not statistical. The parties cited by the population had on average high levels of TPC, TTC, TFC and AOA, while the parties non-cited had high levels of FIC. The results show that the antioxidant activity of plants of the genus *Cnidoscolus*, specifically of the Caatinga, appears to be associated with levels of phenolic compounds and these may explain its popular use.

Key words: 2,2-diphenyl 1-2-picryl-hydrazyl (DPPH), FIC, flavonoids, Folin-Ciocalteu, polyphenols, tannins.

INTRODUCTION

Many ethnopharmacological studies in Brazil have shown that a large number of plant species are used by the local population to treat their diseases; particularly, the family Euphorbiaceae has great medicinal use, as well as the highly cited genus *Cnidoscolus*. This genus, popularly known as "urtiga" or "favela", has 50 to 75 representatives, which are predominantly concentrated in tropical America, almost exclusively in Mexico and Northeastern Brazil (Webster, 1994). The distinct feature of this genus is the presence of stinging trichomes that when stimulated by contact with skin, can cause severe and localized pain (Melo and Sales, 2008).

In Caatinga, the genus is represented by four *Cnidoscolus* medicinal species C. infestus Pax & K. Hoffm., C. pubescens Pohl, C. quercifolius Pohl and *C. urens* [L.] Arthur), which are utilized for a variety of indications, including an anti-inflammatory, an antitumor agent for the genito-urinary system, an antiseptic and to treat kidney infections, dermatological and ophthalmic lesions, bruises, fractures, wounds, warts, dysentery, hemorrhage, appendicitis and rheumatism (Agra et al., 2008; Albuquerque, 2006; Albuquerque et al., 2007; Almeida et al., 2005).

Current studies indicate that many of these folk used, may have high levels of antioxidant activity (Dreifuss et

*Corresponding author. E-mail: tadeu1903@yahoo.com.br. Tel: +55 81 21268511. Fax: +55 81 21268510.

al., 2010; Hevesi et al., 2009). Antioxidants inhibit the formation of damaging reactive oxygen species in the body (Velioglu et al., 1998). Antioxidants can also inhibit the peroxidation of biological molecules by chelating transition metals that generate hydroxyl radicals through the Haber-Weiss and Fenton reactions (Chew et al., 2009). Phenolic compounds, represented mainly by tannins and flavonoids, stand out as the major group of natural antioxidants. They act as efficient scavengers of free radicals and, due to their ability to act as hydrogen donors, they interrupt oxidative chain reactions (Delazar et al., 2006; Higdon and Frei, 2003). A study by Mosquera et al. (2007) evaluated the antioxidant activity of four plant families of Colombian biodiversity (Asteraceae, Euphorbiaceae, Rubiaceae and Solanaceae). The study found that, of the 75 plant extracts that were evaluated, 9 (12%) had IC_{50} values of less than 200 µg/ml and 8 of these species belonged to the family Euphorbiaceae (88.8%).

The purpose of this study was to investigate the phenolic content and antioxidant capacity of four species of *Cnidoscolus* referenced in ethnopharmacological surveys performed in the Caatinga, comparing the results of the cited and non-cited parties popularly as ethnopharmacologicals.

MATERIALS AND METHODS

Study area and plant material

Determination of the samples of plant species (*C. infestus*, *C. pubescens*, *C. quercifolius* and *C. urens*) to be collected was based on the results of previous ethnopharmological surveys. For each plant species, parts of the plant traditionally used for folk remedies and parts, which are not traditionally used, were collected. The collections were made in the regions of Caatinga of Northeastern Brazil. In the municipality of Altinho-Pernambuco (08°35'13"S × 36°05'34"W), samples of *C. urens* were collected on July 9, 2008. Samples of *C. infestus* and *C. quercifolius* were collected in the municipality of Soledade-Paraíba (07°04'13"S × 36º20'52"W) on September 19, 2008. Samples of *C. pubescens* were collected in Buíque-Pernambuco (08º37'23"S × 37º09'21"W) on September 27, 2008. The voucher specimens were collected and incorporated into the Herbarium UFP Geraldo Mariz, Department of Botany, Federal University of Pernambuco, numbers 55.098-55.101.

Chemicals and reagents

Anhydrous sodium carbonate, casein, ferrous sulfate heptahydrate, glacial acetic acid, methanol and pyridine were purchased from Vetec (Brazil). Aluminum chloride hexahydrate was purchased from Honeywell (USA). The reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-4′,4″-disulfonic acid disodium salt (Ferrozine) and Folin-Ciocalteu phenol reagent were obtained from Fluka (Switzerland). The standards ascorbic acid, ethylenediaminetetraacetic acid dihydrate (EDTA), rutin and tannic acid were purchased from Vetec (Brazil).

Weights were measured on a Shimadzu analytical balance (AX200) and absorbance readings were performed on a spectrophotometer Shimadzu UV-Vis (UV mini 1240).

Preparation of extracts

The samples of *Cnidoscolus* species were stabilized in an oven for 3 days at 45 ± 5°C, powdered utilizing a Willy mill with vertical knives and standardized by size using sieves, yielding a particle size of 1 mm (16 Mesh). To quantify the phenolic content, the powdered samples (500 mg) were extracted by heating with 50 ml of methanol (80%, v/v) on a hot plate for 30 min, obtaining a final concentration of 10 mg/ml. To evaluate the antioxidant, the pulverized samples were macerated with methanol (80%, v/v) for 48 h and the extracts were obtained following concentration of the filtrates in a rotary evaporator under reduced pressure, obtaining yields between 4.01 and 22.48%. The choice of methanol has been recommended for extraction of phenolic compounds in plant tissues due to its ability to inhibit the oxidation of polyphenols, which may alter the antioxidant activity (Yao et al., 2004).

Determination of the total phenolic content (TPC) and total tannin content (TTC)

The TPC of the extracts was determined by the Folin-Ciocalteu method and the residual phenolic content was determined by the method of precipitation of casein followed by Folin-Ciocalteu, where the TTC is the difference between the levels of the total and residual phenols (Amorim et al., 2008). The TPC was calculated from 1 ml of diluted extract (10 mg/ml, w/v), 5 ml of aqueous solution of Folin-Ciocalteu (10%, v/v), 10 ml of aqueous solution of sodium carbonate (7.5%, w/v) and 84 ml of distilled water. The solution was allowed to stand in the dark for 30 min and the absorbance was measured at 760 nm. To calculate the residual phenolic content, 15 ml of diluted extract (10 mg/ml, w/v) was agitated for 3 h with 1 g of casein, filtered and adjusted to a final volume of 25 ml with distilled water. The residual phenolic content was determined with 5 ml of the filtrate by the Folin-Ciocalteu method. TPC and TTC were expressed as 1 mg of tannic acid per each gram of sample (mg TAE/g). The samples were evaluated with six replicates. The calibration equation of tannic acid was $y = 0.074x + 0.0044$ ($R^2 = 0.9993$).

Determination of total flavonoid content (TFC)

The TFC of the extracts was estimated by a colorimetric method based on the formation of a flavonoid-aluminum complex (Peixoto Sobrinho et al., 2008). The TFC was calculated using 1 ml of diluted extract (10 mg/ml, w/v), 0.6 ml of glacial acetic acid, 10 ml of pyridine in methanol (20%, v/v), 2.5 ml of aluminum chloride in methanol (5%, w/v) and 10.9 ml of distilled water. The solution was allowed to stand in the dark for 30 min and the absorbance was measured at 420 nm. The results were expressed as 1 mg of rutin per each gram of sample (mg RE/g). Six replicated samples were evaluated. The rutin calibration equation was $y = 0.0251x + 0.0139$ ($R^2 = 0.9994$).

Evaluation of antioxidant activity (AOA)

Qualitative antioxidant assay (QAA)

The QAA was performed using the methodology described by Espin et al. (2000). Aliquots of the extract (20 mg/ml) and the positive controls rutin and ascorbic acid (10.0 mg/ml) were applied to thin layer plates of silica gel. The mobile phase was ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26). After the solvent was completely dry, the plates were sprayed with

methanolic solution of DPPH (40 µg/ml, w/v). The antioxidant activity was qualitatively measured by visually scoring the formation of yellow spots in contrast to the deep violet. The results were expressed as values from 0 to 3, with 0 being the inactive sample and 3 for sample that showed the greatest activity.

Free-radical scavenging activity (DPPH assay)

The DPPH assay was performed in triplicate, based on the method described by Sousa et al. (2007) and modified by Saraiva et al. (2011). Different concentrations of the extract or control (25 to 250 µg/ml), in amount of 0.5 ml, were added to 3 ml of DPPH in methanol (40 µg/ml, w/v). The solution was allowed to stand for 30 min in the dark and then the absorbance was measured at 517 nm. Measurements were compared with a negative control solution of DPPH and were then used as blank concentrations of 0.5 ml of the extract or control with 3 ml of methanol. The final result of the DPPH assay was calculated from a calibration curve obtained for the percentage of radical scavenging activity (Equation 1) versus concentrations of extract or control, and it was expressed as the IC_{50}, that is, the inhibitory concentration of the sample required to reduce the absorbance of the negative control by 50%.

$$\mathrm{RSA}\ (\%) = \frac{\mathrm{ABS}_{\text{negtive control}} - (\mathrm{ABS}_{\text{sample}} - \mathrm{ABS}_{\text{blank}})}{\mathrm{ABS}_{\text{negative control}}} \times 100 \quad (1)$$

Results were also expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg/100 g and were calculated by Equation 2. The IC_{50} of ascorbic acid used for calculation of AEAC was 26.04 ± 1.16 µg/ml.

$$\mathrm{AEAC\ (mg\ AA/100g)} = \frac{\mathrm{IC}_{\text{50 acid ascorbic}}}{\mathrm{IC}_{\text{50 sample}}} \times 100 \quad (2)$$

The ferrous-ion chelating (FIC) assay

The FIC assay was performed in triplicate as described by Chew et al. (2009). Different dilutions of the extract (1000 to 7000 µg/ml) or control (10 to 100 µg/ml), in amount of 1 ml, were mixed with 1 ml of $FeSO_4$ in methanol (0.1 mM, v/v), followed by 1 ml of ferrozine in methanol (0.25 mM, v/v). The solution was allowed to stand for 10 min in the dark and the absorbance was measured at 562 nm. Measurements were compared with a negative control consisting of 1 ml of methanol (75%, v/v), 1 ml of FeSO4 in methanol (0.1 mM, w/v) and 1 ml of ferrozine in methanol (0.25 mM, w/v). The extract or EDTA was used to dilute the samples with 2 ml of methanol (blank). The capacity of the sample to chelate ferrous ions was calculated from a calibration curve obtained for the percentage of chelating activity (Equation 3) versus concentrations of extract or control, and this capacity was expressed as the IC_{50}, that is, the sample concentration required to reduce absorbance of the negative control by 50%.

$$CA\ (\%) = \frac{\mathrm{ABS}_{\text{negative control}} - (\mathrm{ABS}_{\text{sample}} - \mathrm{ABS}_{\text{blank}})}{\mathrm{BSA}_{\text{negative control}}} \times 100$$

Statistical analysis

The Kolmogorov-Smirnov test confirmed the normality of the obtained data. Data were expressed as means ± standard deviation and were analyzed using ANOVA (one-way followed by a multiple comparisons Tukey test). A Pearson correlation test was used to compare the phenolic content with the inhibitory concentrations of the samples. $p < 0.05$ value was considered as significant difference. The GraphPad Prism 5 software was used to perform statistical analysis and graphs.

RESULTS AND DISCUSSION

Phenolic content

Overall, there was a wide variation between the levels of TPC in the extracts analyzed, with the highest levels in the leaves of *C. pubescens*, while the aerial parts of *C. infestus* and the bark of *C. quercifolius* contained the lowest levels. Leaf extracts of *C. pubescens* had the largest TTC (about 40% of total phenols), while the aerial parts of *C. infestus* and roots of *C. quercifolius* did not contain detectable tannins. The roots of *C. pubescens* did not contain detectable flavonoids; however, their leaves exhibited the highest content of flavonoids. The Pearson correlation test showed a significant relationship between TPC × TTC and TPC × TFC (r = 0.9200 and r = 0.7903, respectively); however, TTC and TFC did not correlate statistically. The results of the phenolic content are as shown in Table 1.

Our results show that the phenolic content of *Cnidoscolus* samples are far above those found by Kuti and Konuru (2004) for *Cnidoscolus aconitifolius* (Mill.) IM Johnst and *Cnidoscolus chayamansa* McVaugh, which had measured TPCs of 2.91 ± 0.02 and 1.22 ± 0.02 mg/g, respectively. Another study of leaf samples from *C. aconitifolius,* where the evaluated samples were fresh and treated post-harvest, showed TPC ranging from 7.0 ± 1.0 to 12.0 ± 2.0 mg/g (Oboh, 2005). Previously, Ogundade et al. (2009) analyzed a set of vegetables consumed in Nigeria and they confirmed that *C. aconitifolius* has a TPC of 29.4 mg/g.

These results indicate that the popular use of these species may be associated with the phenolic content of the plant parties cited in the ethnopharmacological surveys. For example, the results from the extracts of the leaves of *C. pubescens* that are compared with samples not listed in ethnopharmacological studies suggest that choice should be linked to its high phenolic content (Albuquerque et al., 2007; Almeida et al., 2005). In recent years, research on plants of Caatinga that are traditionally used to treat various diseases has identified promising pharmacological activities. These activities have been generally attributed to the presence of phenolic substances because several species that have medicinal uses also contain high levels of these compounds (Almeida et al., 2005; Monteiro et al., 2006). For example, a study by Araújo et al. (2008), in a rural community, observed that there is a strong association between the content of tannins and the healing and anti-inflammatory activities effects popularly attributed to the plants. However, when the levels of flavonoids of the plants in folk remedies and randomly selected plants were

Table 1. Total phenolic content (TPC), total tannins content (TTC) and total flavonoids content (TFC) of the samples of four species of the genus *Cnidoscolus* used as ethnopharmacologicals in the Caatinga, Northeastern Brazil.

Species (Voucher number)	Part used	Popular usage*	TPC (mg TAE/g)	TTC (mg TAE/g)	TFC (mg RE/g)
C. infestus Pax & K. Hoffm. (55.101)	Aerial parts	Non-cited	4.02 ± 0.15[a]	ND	3.36 ± 0.15[a]
	Roots	Cited	16.31 ± 0.19[b]	5.99 ± 0.14[a]	1.75 ± 0.15[b]
C. pubescens Pohl (55.100)	Leaves	Cited	23.00 ± 0.10[c]	8.72 ± 0.16[b]	39.37 ± 0.57[c]
	Roots	Non-cited	8.96 ± 0.12[d]	3.70 ± 0.20[c]	ND
C. quercifolius Pohl (55.099)	Barks	Cited	3.58 ± 0.16[a]	ND	0.60 ± 0.05[b]
	Leaves	Non-cited	18.32 ± 0.60[e]	4.10 ± 0.24[c]	26.51 ± 1.78[d]
C. urens (L.) Arthur (55.098)	Aerial parts	Non-cited	12.10 ± 0.48[f]	5.42 ± 0.79[a]	0.89 ± 0.06[b]
	Roots	Cited	7.21 ± 0.28[g]	2.10 ± 0.31[d]	4.86 ± 0.28[e]

Values are mean ± standard deviation. Values followed by the same letter in column are not statistically different (n = 6, $p < 0.05$); TAE = tannic acid equivalent; RE = rutin equivalent. ND = not detected. *Cited or non-cited as folk remedy in ethnopharmacologic surveys.

Table 2. Free-radical scavenging (DPPH assay) and ferrous-ion chelating (FIC) activity of samples from different parts of four species of the *Cnidoscolus* identified in ethnopharmacological surveys in the Caatinga, Northeastern Brazil.

Standard/Species	Part used	Yield (%)	Antioxidant activity (AOA)			
			Free-radical scavenging (DPPH assay)			FIC assay
			QAA	IC_{50} (µg/ml)	AEAC (mg AA/100g)	(IC_{50} µg/ml)
Rutin	-	-	-	22.96 ± 1.99[a]	114.16 ± 9.84[a]	-
EDTA	-	-	-	-	-	15.26 ± 0.58[a]
C. infestus Pax & K. Hoffm.	Aerial parts	7.73	0	1741.68 ± 53.06[b]	1.50 ± 0.05[d]	239.42 ± 39.25[b]
	Roots	9.08	3	122.87 ± 5.37[c]	21.23 ± 0.94[c]	398.04 ± 19.24[c]
C. pubescens Pohl	Leaves	14.23	3	179.15 ± 4.03[d]	14.54 ± 0.33[d]	256.25 ± 13.28[b]
	Roots	4.55	2	152.92 ± 3.49[e]	17.04 ± 0.40[e]	90.29 ± 7.12[d]
C. quercifolius Pohl	Barks	9.30	0	1867.24 ± 53.66[f]	1.40 ± 0.04[b]	299.37 ± 14.60[e]
	Leaves	22.48	2	426.66 ± 42.66[g]	6.15 ± 0.59[f]	361.73 ± 14.58[c]
C. urens (L.) Arthur	Aerial parts	10.19	1	1361.41 ± 76.06[h]	1.92 ± 0.11[b]	177.69 ± 11.84[f]
	Roots	4.01	1	423.07 ± 34.43[g]	6.19 ± 0.50[f]	278.68 ± 35.88[be]

Values are expressed as mean ± standard deviation. Values followed by the same letter in column are not statistically different (n = 6, $p < 0.05$); QAA = Qualitative antioxidant assay; 0 = inactive; 1 = low active; 2 = moderately active; 3 = very active; IC_{50} = inhibitory concentration; AEAC = ascorbic acid equivalent antioxidant capacity; FIC = ferrous-ion chelating activity.

compared, no significant differences were observed. This result suggests that the healing and anti-inflammatory indications of medicinal plants in the community are not related to flavonoids.

Antioxidant activity

The results of the QAA analysis of the extracts showed that several *Cnidoscolus* samples contained antioxidant activity. Samples of roots from *C. infestus* and leaves from *C. pubescens* showed the highest level of antioxidant activity, whereas samples of aerial parts from *C. infestus* and bark from *C. quercifolius* did not have detectable antioxidant activity (Table 2).

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant or free radical terminators (Cao et al., 1997), because it inhibit directly the formation of damaging reactive oxygen species at body (Velioglu et al., 1998). The DPPH assay evaluates the ability of the extract to donate hydrogen or to scavenge free radicals (Almey et al., 2010).

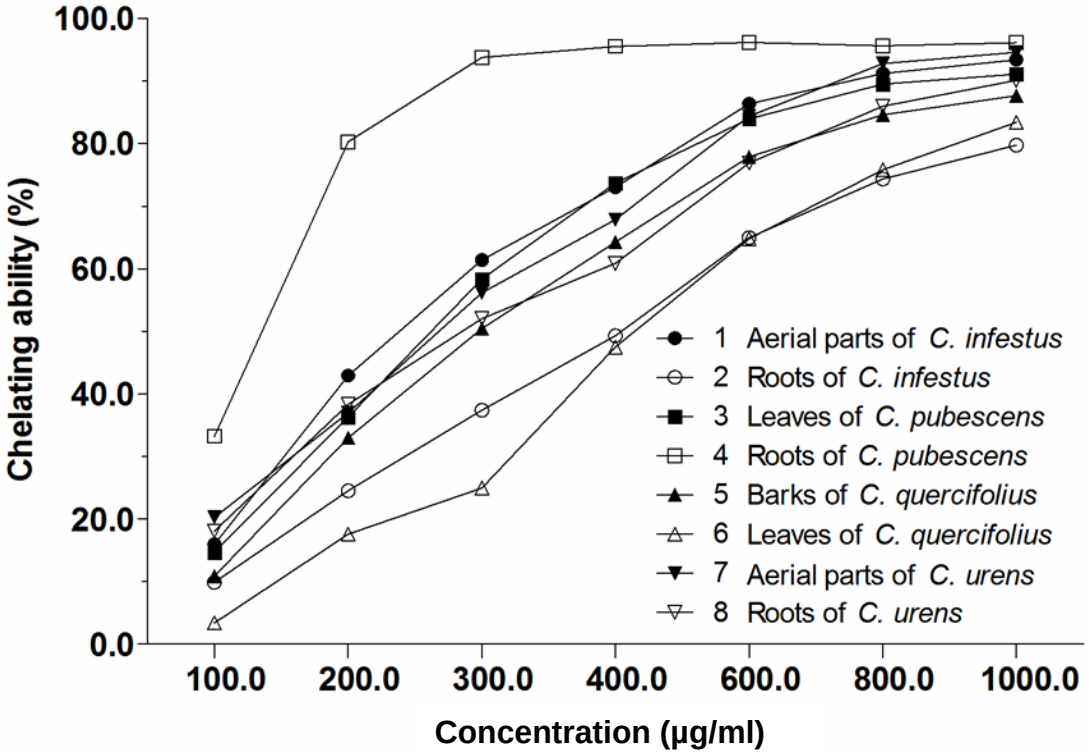

Figure 1. Results of the ferrous-ion chelating (FIC) assay of different parts of four species of *Cnidoscolus* from Northeastern Brazil.

Differences were observed in the radical scavenging activity of the species evaluated (Table 2). According to Melo et al. (2010), the results of the antioxidant activity can be classified based on the performance of the crude extract as compared to the control used: 1) good activity (IC_{50} < 68.88 µg/ml, up to three times the inhibitory concentration of the control); 2) moderate activity (68.88 µg/ml < IC_{50} < 160.72 µg/ml, between three and seven times the inhibitory concentration of the control); 3) low activity (IC_{50} > 160.72 µg/ml, exceeding seven times the inhibitory concentration of the control). Using this classification, the extract from the roots of *C. infestus* and *C. pubescens* showed moderate free-radical scavenging activity, while the other plant species showed low antioxidant activity.

The results of the QAA and the free-radical scavenging assay (DPPH assay) were highly correlated (r = -0.8927), suggesting that qualitative analysis can be used to preliminarily evaluate the free-radical activity of plant tissues, serving as a predictor for the discovery of new antioxidant compounds. The Pearson correlation test showed a negative relationship between the inhibitory concentrations (IC_{50}) of DPPH × TPC (r = -0.6776), TTC (r = -0.6779) and TFC (r = -0.4112). These results suggest, in part, that the higher the phenolic content of *Cnidoscolus* samples, the higher the antioxidant activity.

Differently from the activity performed by DPPH (primary antioxidant), the FIC assay is considered secondary antioxidant (Chan et al., 2007), because it act in the inhibition of the peroxidation of biological molecules by chelating transition metals that generate hydroxyl radicals through the Haber-Weiss and Fenton reactions, preventing the formation of reactive oxygen species (Chew et al., 2009).

The Figure 1 shows that the chelating capacity of the extracts increased proportionally to the concentration; however, for the extract of the roots of *C. pubescens*, the chelating capacity was statistically stabilized when the concentration reached 300 µg/ml ($p < 0.0001$). The highest chelating activity was found for the extract of the roots of *C. pubescens* (Table 2), while the extract from the roots of *C. infestus* and the leaves of *C. quercifolius* showed low chelating activity. The Pearson correlation test did not reveal any relationship between FIC assay and the phenolic content and free-radical scavenging activity.

Much research has shown a significant relationship between the phenolic content in plant extracts and antioxidant activity. Nazaruk (2008) observed a strong relationship between the methanol extracts (R^2 = 0.9523) and ethyl acetate fractions (R^2 = 0.8048) of leaves and flowers of five species of *Cirsium* (*Asteraceae*) from Northeastern Poland. In addition, a separate study showed a strong relationship between the total antioxidant capacity and the TPC and TFC of the ethyl acetate fractions extracted from six species of *Ficus*

(*Moraceae*). The butanol fractions showed a strong association between TPC and TFC, suggesting that these groups are responsible for the antioxidant capacity of the samples (Saleh and Hameed, 2009).

Some authors have proposed that the antioxidant activity of the flavonoids is due to the presence of an aromatic hydroxyl group. For example, the flavonols rutin and quercetin have higher antioxidant activities than ascorbic acid, which is considered a powerful reducer (Soares et al., 2005). However, a study by Malenčić et al. (2008) with acetone extracts of twenty samples from the hybrid plant *Glycine max* (L.) Mer. (*Fabaceae*) showed no correlation between the antioxidant activity and the TFC; however, a correlation was observed for polyphenols (r = 0.6696), tannins (r = 0.7465) and proanthocyanidins (r = 0.6538).

Al-Duais et al. (2009) examined TPC using different antioxidant methodologies (DPPH, ORAC, TEAC and FRAP) and showed that phenolic compounds of *Cyphostemma digitatum* (Forssk.) Desc. (*Vitaceae*) are not responsible for the antioxidant capacity of the species. Other authors also found that the presence of phenolic compounds may be related to the chelating activity. For example, Rumbaoa et al. (2009) assessed the five varieties of *Ipomoea batatas* (L.) Lam (*Convolvulaceae*) and observed a correlation between TPC and FIC (r = -0.800).

Conclusion

Our results show that the species identified in the ethnopharmacological surveys conducted specifically in Caatinga can serve as a guide to search for compounds with antioxidant activity. The levels of polyphenolic compounds in these species may explain, in part, the popular use of these plants, because the plants popularly used as folk remedies had a higher level of antioxidant activity.

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