Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2012, 4(2):1160-1166

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

HPLC-DAD analysis and antioxidant activity of *Hymenaea martiana* Hayne (Fabaceae)

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ABSTRACT

A qualitative characterization of the main phenolic compounds from ethanol extract of Hymenaea martiana was carried out by high performance liquid chromatography with diode array detection (HPLC-DAD). The total phenolics content of the plant extracts was determined by the Folin-Ciocalteu method. Total flavonoid content also was measured. Antioxidant activities of the extracts were evaluated by using DPPH radical scavenging and β-carotene-linoleic acid bleaching and compared with ascorbic acid, BHA and BHT used as reference compounds. The total phenolic content was of 428.50 ± 5.91 and 705.50 ± 7.22 mg of gallic acid equivalent/g for EtOH and AcOEt extracts, respectively. The total flavonoids content was of 394.90 ± 8.43 and 479.60 ± 10.38 for two extracts, respectively. All extracts exhibited good antioxidant activities. The EtOH extract showed better antioxidant activity than ascorbic acid and BHA using by DPPH method, with a value of IC_{50} of 0.84 ± 0.26 µg/ml. BHT was the most effective antioxidant. The results obtained show that phenolic compounds contribute to the antioxidant activity of the extract. Further studies will be conducted to isolate the chemical constituents responsible for antioxidant activity.

Keywords: *Hymenaea martiana*, Fabaceae, antioxidant activity.

INTRODUCTION

Hymenaea (Fabaceae) is a genus widely distributed from Central to South America, mainly in the Amazon basin [1]. Approximately 25 species from the American continent have been described [2]. Plants from the genus *Hymenaea* are commonly used in Brazilian traditional medicine to treat inflammatory process, bacterial infections, rheumatism and anaemia [3, 4]. The trunk exudes a resin, which is used locally in folk medicine for treatment of wounds, bronchitis and stomach disorder [5]. *Hymenaea* species are known to mainly contain the diterpenoids compounds in the trunk resin and bark extract [6]. Diterpenes of the *enantio*-labdanoic type in the trunk resin and bark extract and *ent*-halimane in the seed pod resin as well as clerodane-type diterpenes were isolated from this genus [7, 8 & 9].

Hymenaea martiana Hayne is a native tree known in the Northeastern region of Brazil as "jatobá". A few examples of biological activities for extracts of this specie have been reported. The previous studies have showed antinociceptive and anti-inflammatory activities related with hydroalcoholic extract obtained from the bark of *H.*

martiana. The exact mechanism that underlies its analgesic and anti-inflammatory profiles remains unclear, but may result from its ability to inhibit the generation of lipoxygenase and/or cyclooxygenase products of the arachidonic acid pathway [10]. Carneiro et al. [11] demonstrated in the bark of *H. martiana*, the presence of glycoside compounds such as astilbin, eucryphin and engelitin, which were capable of antagonising bradykinin responses. This effect may explain the analgesic and anti-inflammatory actions of this plant. Astilbin is a flavonoid initially identified as an active principle present in a crude extract from the bark of this specie which shows antioxidant activity [12]. Astilbin and some related compounds were evaluated for antinociceptive and anti-oedematogenic activities. The results indicated that taxifolin and its tetramethylated derivative exhibited potent and dose-dependent antinociceptive action. Both compounds showed significant anti-oedematogenic effect being more effective than astilbin [13].

In our continuing search of the Brazilian Caatinga medicinal plants to combine biodiversity conservation with drug discovery we demonstrated the antinociceptive effect of the ethanolic extract of *Amburana cearensis* in mice [14] and anti-ulcer activity of ethanolic extract of *Encholirium spectabile* in rodents [15]. Selective spasmolytic effect of a new furanoflavoquinone derivative from diplotropin, a furanoflavonoid isolated from *Lonchocarpus araripensis* also have been demonstrated [16]. There is no previous report on the analysis of the antioxidant activity of *Hymenaea martiana*. The aim of this work was to characterize qualitatively by HPLC-DAD the main phenolic compounds present in the ethanolic extract and to evaluate the antioxidant activity *in vitro* of this plant.

EXPERIMENTAL SECTION

Plant material

The trunk barks of *Hymenaea martiana* Hayne were collected in Petrolina, State of Pernambuco, Brazil, in November 2009. A voucher specimen (6444) is deposited at the Herbarium Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco.

Extraction

The dried and powdered trunk barks (3000 g) were repeatedly extracted three times during 72 h with 95% EtOH at room temperature. The extractive solution was concentrated under vacuum yielding after distillation of solvent, 90 g of crude ethanol extract (Hm-EtOH). The Hm-EtOH was suspended in a mixture of H_2O:MeOH (7:3) and extracted successively with hexane, $CHCl_3$ and AcOEt in crescent order of polarity to obtain the respective extracts.

Preliminary phytochemical screening

Preliminary phytochemical analysis of the ethanol extract was carried. The presence of alkaloids was tested with Dragendorff's and Mayer's reagents, flavonoids with HCl and Mg powder, phenols with ferric chloride and steroids and terpenoids by Liebermann-Burchard reaction [17].

HPLC-DAD analysis of phenolic compounds

The solvents used in high performance liquid chromatography are of analytic grade from Merck®. A Milli-Q System® (Bedford, MA, USA) was used to purify the water. Analyses of high performance liquid chromatography was performed on a Merck-Hitachi liquid chromatograph LaChrom Elite® equipped with a VRW HITACHI L-2130 pump, a VRW HITACHI L-2300 Diode-Array Detector (DAD), and an auto sampler with a 100 µL loop. The data were acquired and processed using Ezchrom Elite software. The extract was analyzed using a reverse-phase HPLC column: Purospher® STAR RP-18e (250 mm X 4.6 mm i.d., 5 µm) column (Merck). The mobile phase was composed of solvent (A) H_2O/H_3PO_4 0.1% and solvent (B) MeOH. The solvent gradient was composed of A (75-0%) and B (25-100%) for 20 min, then 100% B for 4 min, then again at the initial conditions (75% A and 25% B) for 10 min. A flow rate of 1.0 ml/min was used in a 30 ºC oven, and 20 µL of each sample was injected. The procedure was repeated three times for each sample. Samples and mobile phases were filtered through a 0.22 µm Millipore filter prior to HPLC injection. Spectra data were recorded from to 200 to 400 nm during the entire run.

Total phenolic content

Total phenolic contents were assayed using the Folin-Ciocalteu reagent, it is based on the method reported by Slinkard and Singleton [18], only the volumes have been reduced [19, 20]. An aliquot (40 µl) of a suitable diluted ethanolic extract was added to 3.16 ml of distilled water and 200 µl of the Folin–Ciocalteu reagent, and mix well. The mixture was shaken and allowed to stand for 6 min, before adding 600 µl of sodium carbonate solution, and shake to mix. The solutions were left at 20 °C for 2 hours and the absorbance of each solution was determined at 765 nm against the blank and plot absorbance vs. concentration. Total phenolic contents of the extracts (three replicates per treatment) were expressed as mg gallic acid equivalents per gram (mg GAE/g) through the calibration curve with gallic acid. The calibration curve range was 50–1000 mg/l (R^2 = 0.9993). All samples were performed in triplicates.

Determination of Total Flavonoid Content

Total flavonoid content was determined by using a colorimetric method described previously [21]. Briefly, 0.30 ml of the EtOH and AcOEt extracts or (+)-catechin standard solution were mixed with 1.50 ml of distilled water in a test tube followed by addition of 90 µl of a 5% $NaNO_2$ solution. After 6 min, 180 µl of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before 0.6 ml of 1 M NaOH was added. The mixture was brought to 330 µl with distilled water and mixed well. The absorbance was measured immediately against the blank at 510 nm using a spectrophotometer (QUIMIS, Brazil) in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results were expressed as mg of catechin equivalents per gram of extracts (mg CE/g) through the calibration curve with catechin. The calibration curve range was 50-1000 mg/l.

DPPH Free Radical Scavenging Assay

The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay [22, 23]. Sample stock solution (1.0 mg/ml) of Hm-EtOH was diluted to final concentrations of 243, 81, 27, 9, 3 and 1 µg/ml, in ethanol. One ml of a 50 µg/ml DPPH ethanol solution was added to 2.5 mL of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control – absorbance of the sample)/ absorbance of the control] x 100. Ethanol (1.0 ml) plus plant extracts solutions (2.5 ml) were used as a blank. DPPH solution (1.0 ml) plus ethanol (2.5 ml) was used as a negative control. The positive controls (ascorbic acid, BHA and BHT) were those using the standard solutions. Assays were carried out in triplicate. The IC_{50} values were calculated by linear regression using by GraphPad Prism 5.0 program.

β-Carotene Bleaching Test

The β-carotene bleaching method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion [24]. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants. β-carotene (2 mg) was dissolved in 10 ml chloroform and to 2 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40 ^oC and 100 ml of distilled water was added, then the emulsion was vigorously shaken during two minutes. Reference compounds (ascorbic acid, BHA and BHT) and sample extracts were prepared in ethanol. The emulsion (3.0 ml) was added to a tube containing 0.12 ml of solutions 1 mg/ml of reference compounds and sample extracts. The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50 ^oC for 120 min, when the absorbance was measured again. Ascorbic acid, BHA and BHT were used as positive control. In the negative control, the extracts were substituted with an equal volume of ethanol. The antioxidant activity (%) was evaluated in terms of the bleaching of the β-carotene using the following formula: % Antioxidant activity = $[1 - (A_0 – A_t) / (A_0^0 – A_t^0)] \times 100$; where A_0 is the initial absorbance and A_t is the final absorbance measured for the test sample, A_0^0 is the initial absorbance and A_t^0 is the final absorbance measured for the negative control (blank). The results are expressed as percentage of antioxidant activity (% AA). Tests were carried out in triplicate.

Statistical analysis

All determinations were conducted in triplicates and the data are expressed as mean ± SD. Values were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. The many pharmacological effects of phenolic compounds are linked to their ability to act as strong antioxidants [25]. These compounds are considered as secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wounding, and UV radiation, among others. These compounds occur ubiquitously in plants and are a very diversified group of phytochemicals derived from phenylalanine and tyrosine [26] and may be classified into different groups as a function of the number of phenol rings that they contain and based on the structural elements that bind these rings to one another [27]. Distinctions are thus made between the phenolic acids (e.g. hydroxybenzoic and hydroxycinnamic acids), stilbenes, coumarins, tannins, lignans, lignins and flavonoids.

High performance liquid chromatography with diode array detection (HPLC-DAD) constitutes a crucial, reliable technique for the characterization of phenolic compounds due to its versatility, precision and relatively low cost [28]. Most frequently, reversed-phase (RP) C_{18} columns, a binary solvent system containing acidified water and a polar organic solvent (acetonitrile or methanol) and UV-Vis diode array detection are used an so far constitute a crucial and reliable tool in the routine analysis of plant phenolic compounds [26]. In fact, the spectra from different phenolic classes (hydroxycinnamic and hydroxybenzoic acids as well as flavonoids) allow the identification of phenolic structures present in the samples [27].

Preliminary phytochemical analysis demonstrated that Hm-EtOH contain phenols, flavonoids, steroids and terpenoids. Phenolic profiles at 320 nm for the Hm-EtOH evaluated are presented in Fig. 1. Fig. 2 shows the UV λ_{max} values of peaks eluted in Hm-EtOH extract. The chromatogram shows the presence of four peaks with retention times between 12 and 15 min. Based on their UV-Vis spectral data and their retention time, the compounds have UV band characteristic for phenolic compounds, possibly cinnamic acid, flavan-3-ol or flavanone derivatives. These compounds are under investigation.

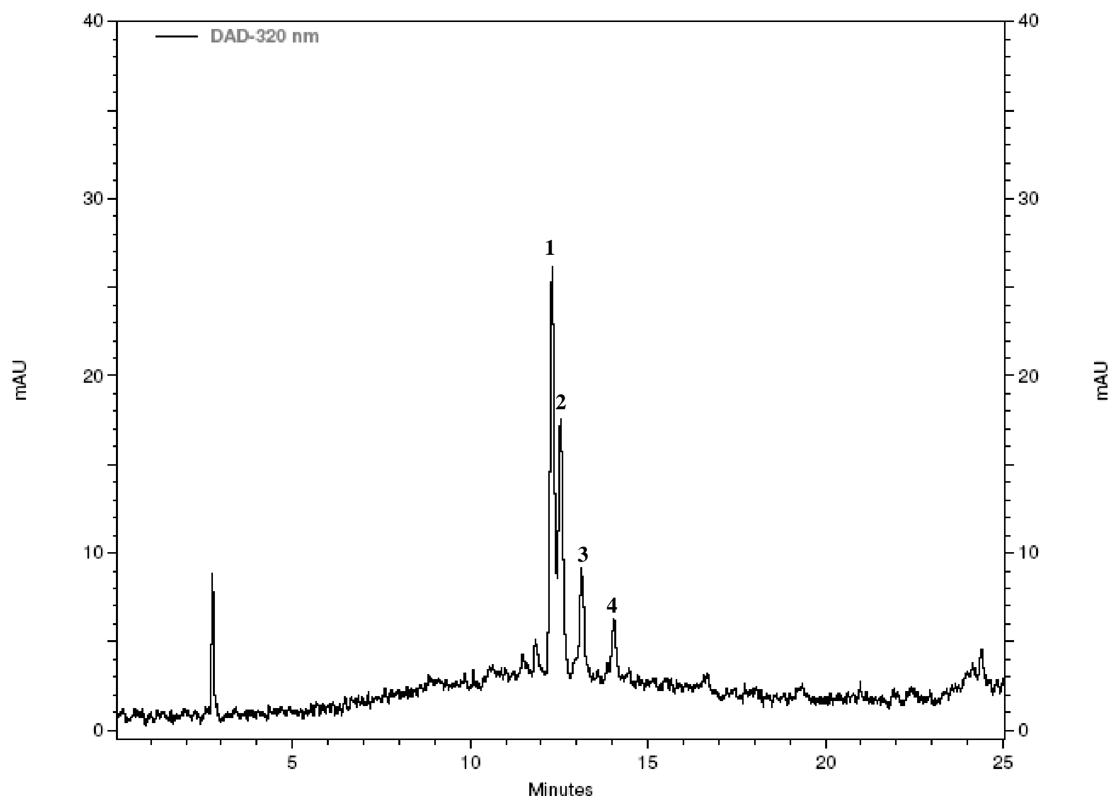

Fig. No 1 HPLC-DAD phenolic profiles for the Hm-EtOH recorded at 320 nm.

Thus, Figures 1 and 2 show the chromatographic and spectral characteristics of ethanol extract of *Hymenaea martiana* by HPLC-DAD, respectively. The possible classes of phenolic compounds were identified by comparing their retention time and UV-Vis spectral data to known previously injected standards as well as by comparison with values of literature.

Table 1 summarizes the results from the quantitative determination of phenolic and flavonoids as well as the effect of extracts from *Hymenaea martiana*, ascorbic acid, BHA and BHT on the DPPH free radical scavenging and β-carotene-linoleic acid bleaching test.

The total phenolic contents of the extracts were determined by Folin-Ciocalteu method as gallic acid equivalents in milligrams per gram (mg GAE/g) while total flavonoid contents were calculated as catechin equivalents in milligrams per gram (mg CE/g). Among the four extracts, ethyl acetate extract (AcOEt) was containing highest (705.50 ± 7.22) amount of phenolic compounds followed by crude ethanol extract (428.50 ± 5.91). The most popular assay for determination of total phenols is by the use of Folin-Ciocalteu reagent. This reagent consists of a mixture of phosphomolybdic and phoshotungstic acids, in which the molybdenum and tungsten are in the 6^+ state. On reduction with certain reducing agents, the so-called molybdenum blue and tungsten blue are formed, in which the mean oxidation state of the metals is between 5 and 6. The degree of the color change is proportional to the antioxidant concentrations. It is known that Folin-Ciocalteu reagent reacts not only with phenols but also with a variety of other types of compounds. It is obvious that the total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quantity or quality of the phenolic constituents in the extracts. In addition, there may be some interference rising from other chemical components present in the extract, such as sugars or ascorbic acid [29]. The Folin-Ciocalteu reagent measures a sample's reducing capacity, but this is not reflected in the name "total phenolic assay". Numerous publications applied the total phenols assay often found excellent linear correlations between the total phenolic profiles and the antioxidant activity [30]. For the total

flavonoid content, the highest value was observed in AcOEt extract (479.60 ± 10.38) while the crude ethanol extract (EtOH) presented 394.90 ± 8.43 mg CE/g. The total flavonoid content of the hexane and chloroform ($CHCl_3$) extracts were note determined. The complexation of phenolics with Al(III) has been used for the development of spectrophotometric methods for determination of total caffeic acid, total flavonoids and total tannins. The modification of the $AlCl_3$ assay proposed by Zhishen et al. [31] included the reaction of phenolic extract with sodium nitrate followed by the formation of flavonoid–aluminum complex. The absorbance of the solution is then read at 510 nm. Simple phenolics have absorption maxima between 220 and 280 nm [26].

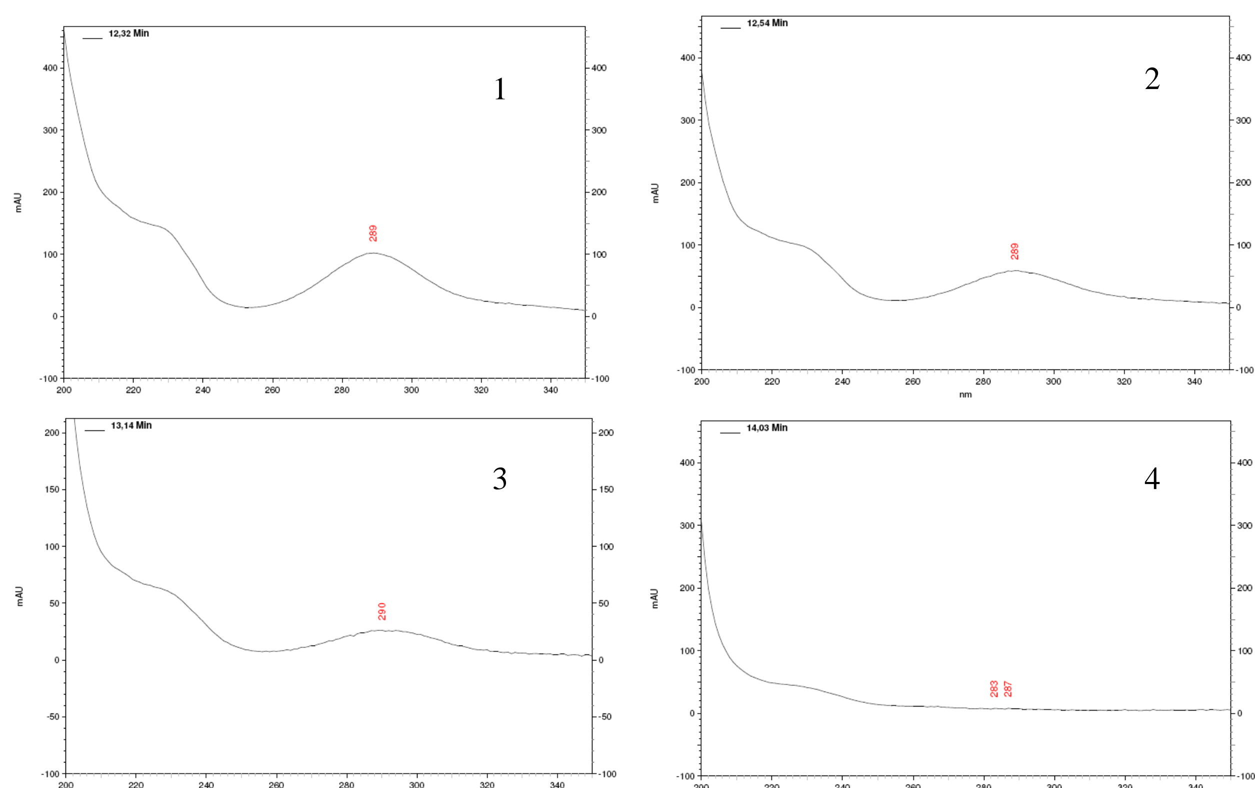

Fig. No 2. UV spectra of the peaks shown in the HPLC chromatogram of Hm-EtOH

In the present study, the antioxidant ability of the *H. martiana* extracts was investigated through some *in vitro* models such as radical scavenging activity using, 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and β-carotene-linoleate model system. Antioxidant activity on method of DPPH was expressed as IC_{50} which is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. Lower IC_{50} value indicated higher antioxidant activity. In β-carotene-linoleate model system the antioxidant activity was expressed as percentage of antioxidant activity (%AA).

The DPPH reactivity is one popular method for screening of the free radical-scavenging ability of compounds that has been extensively used for screening antioxidants from fruit and vegetables juice or extracts. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. The degree of color change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound or extract under test [32]. The data showed that the all extracts exhibited good free radical scavenging activity. The EtOH extract showed better antioxidant activity than ascorbic acid and BHA using by DPPH method, with a value of IC_{50} of 0.84 ± 0.26 μg/ml. In addition, AcOEt (IC_{50} 2.56 ± 0.72 μg/ml) and hexane (IC_{50} 3.40 ± 2.20 μg/ml) extracts were more effective than ascorbic acid as antioxidant. BHT was the most effective antioxidant, with a value of IC_{50} of 0.70 ± 0.24 μg/ml. It appears that the extracts of *H. martiana* have compounds with a strong hydrogen-donating capacity and can efficiently scavenge DPPH radicals. The presence of phenolic compounds in the extract shown to be essential for scavenger properties [33].

The antioxidant activity of extracts was also evaluated by the β-carotene/linoleate bleaching method. This method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid

oxidation in an emulsion. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The rate of the β-carotene bleaching can be slowed down in the presence of antioxidants [34]. This method is one of the antioxidant assays suitable for plant extracts. The addition of EtOH, AcOEt extracts and BHA and BHT prevented the bleaching of β-carotene to different degrees. There was no statistically significant difference between the antioxidant activities presented by EtOH extract and synthetic antioxidants BHA and BHT.

Table No 1. Total phenolics (TP), total flavonoids (TF) and antioxidant activity of extracts from *Hymenaea martiana*

	TP (mg GAE/g)	TF (mg CE/g)	DPPH (IC_{50}, μg/ml)	β-carotene bleaching (% AA)
EtOH	428.50 ± 5.91	394.90 ± 8.43	0.84 ± 0.26	81.35 ± 17.20
Hexane	68.46 ± 6.17	---	3.40 ± 2.20	9.39 ± 1.14
$CHCl_3$	40.13 ± 2.17	---	75.70 ± 8.20	33.86 ± 2.64
AcOEt	705.50 ± 7.22	479.60 ± 10.38	2.56 ± 0.72	65.34 ± 15.69
Ascorbic acid	---	---	5.83 ± 0.28	0.79 ± 2.21
BHA	---	---	1.67 ± 0.30	80.93 ± 3.45
BHT	---	---	0.70 ± 0.24	86.77 ± 1.14

The IC_{50} values were obtained by interpolation from linear regression analysis with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. Values are given as mean ± SD (n=3).

CONCLUSION

This study showed that the ethanol extract of *H. martiana* and the ethyl acetate extract obtained by partition contain substantial amount of phenolics which are responsible for its marked antioxidant activity as assayed through *in vitro* models. Several reports have conclusively shown close relationship between total phenolic content and antioxidative activity of the fruits and vegetables [35]. Nowadays, the interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products to replace synthetic antioxidants which are being restricted due to their carcinogenicity. *H. martiana* could be a good source of antioxidant phenolics. Further research will be completed to reach the isolation and identification of main phenolic constituents of the extracts.

Acknowledgements
The authors are grateful to Brazilian agencies CNPq and FACEPE for financial support.

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