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Antioxidant Capacity, Angiotensin I Converting Enzyme (ACE) and Acetylcholinesterase Inhibition by Extracts of the Leaves and Bark of *Hancornia speciosa* Gomes

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ABSTRACT

Purpose: To determine the antioxidant capacity, angiotensin I converting enzyme, and acetylcholinesterase inhibition, by extracts of the leaves and bark of *Hancornia speciosa* Gomes (*H. speciosa*). **Methods:** Antioxidant capacity of methanol extracts of *H. speciosa* was evaluated *in vitro* by the DPPH, FRAP, ORAC and the HPLC-based HX/XO assays. The antihypertension capacities of methanol extracts from *Hancornia speciosa* extracts were evaluated *in vitro*, by their ability to inhibit angiotensin I converting enzyme using analytical reverse-phase high performance liquid chromatography. In addition, thin-layer chromatography was used to screen. **Results:** The methanol extracts (10.0 mg/mL) from the leaves and bark of *H. speciosa* were very effective in the inhibtion of ACE activity (97.63 % ± 2.89 % and 95.07 % ± 3.76 %, with significant IC_{50} values of 79.24 µg/mL and 146.47 µg/mL respectively). In the antioxidant assays, the extracts exhibited low antioxidant capacity in the FRAP and DPPH assays, but were effective in the ORAC assay (ORAC units = 2.70, and 1.74 respectively, compared to Trolox = 1.0). Only the methanol leaf extract exhibited discernable antioxidant capacity in the HPLC-based HX/XO (IC50 = 12.0 ug/uL) assay. Both the hexane and ethanol extracts of bark but only the ethanol extract of leaves inhibited acetylcholinesterase (AChE). The active principle, responsible for acetylcholinesterase inhibition, following purification by column chromatography, and identification by ^{1}H and ^{13}C NMR was determined as lupeol.

INTRODUCTION

Mangabeira is the popular name attributed to *H. speciosa* (Apocynacea), a typical Brazilian species that reproduces readily in sandy soils. Mangabeira is a fructiferous tree, native to many regions and ecosystems in Brazil, ranging from Amapá to Pará, passing through coastal soils known as "tabuleiros" as well as coastal depressions of many northeastern states (especially in Paraíba and Rio Grande do Norte), where it is present in abundance, extending to Espirito Santo and most regions known as the "Brazilian Cerrado". It also grows in neighbouring countries such as Paraguay, Bolivia, Peru and Venezuela [13].

The Apocynaceae are angiosperms which have as a specific characteristic a significant presence of latex in most its species. The latex found in all parts of the mangabeira is adequate for the production of rubber and therapeutic applications. It is used popularly in Brazil to treat arterial hypertension and diabetes, and is said to be efficient in treating inflammation, gastric ulcers and diarrhea, and as a relief for the effects of blunt injuries [17]. Recent research has shown that latex has analgesic and anti-inflammatory proprieties. The latex and the leaves display, according to recent studies, diverse pharmacologic properties and act as antimicrobial, chemopreventive and anticancer agents. This is attributed to compounds such as rutin, proanthocyanidins, bornesitol and quinic acid, among others [7, 22].

Ferreira et al. [8, 9] demonstrated that, constituents of an ethanol extract of mangabeira leaves, significantly inhibit angiotensin I converting enzyme (ACE) which reaffirms its popular use in the control of arterial hypertension.

The fruit, known as mangaba, is highly appreciated in the Northeast of Brazil, due to its pleasant taste and aroma, frequently being consumed raw or in the form of juice or jam, containing a high vitamin C level -139 mg / 100 g [2, 16]. Extracts of the fruit may also be used for the treatment of gastric ulcers, and in the development of cosmetics [10, 24].

Literature surveys reveal several works concerning *H. speciosa*, but mostly they deal with fruit and leaf analyses, whereas latex and bark are understudied.

Considering the pharmacologic potential, already reported for *H. speciosa*, this study aimed: to evaluate the antioxidant capacity, and inhibition of ACE and acetylcholinesterase (AChE) enzymes using the extracts of leaves and bark of this plant.

MATERIALS AND METHODS

Reagents

Angiotensin I converting enzyme (ACE) from rabbit lung, captopril, trifluoroacetic acid (TFA), HCl, hippuric acid, *N*-hippuryl-L-histidyl-L-leucine (HHL), sodium chloride and Trizma base, were obtained from Sigma-Aldrich Chemie (Deisenhofen, Gemany); acetonitrile from Fluka/Riedel de Haen (Seelze, Germany); acetic acid, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid, hypoxanthine, ethanol, methanol, xanthine, and xanthine oxidase were purchased from Merck (Darmstadt, Germany); K_2HPO_4 and KH_2PO_4 were purchased from Serva (Heidelberg, Germany); ascorbic acid, acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE), 5, 5- dithiobis [2-nitrobenzoic acid] (DTNB), DPPH (2,2-diphenyl-1-picrylhydrazyl), $FeCl_3.6H_2O$, salicylic acid, and Trolox from Sigma-Aldrich Chemie (Steinheim, Germany); 2,4,6,-tripyridyl-*s*-triazine complex (TPTZ) from Riedel de Haen (Seelze, Germany) while 3,3′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals (Neuss, Germany). All solutions were made up in doubly distilled water, DMSO or methanol.

Plant material

The plant material was collected in April 2014, in the district Lagoa do Mato in the city de São José de Mipibú - RN locations of Brazil, and identified by the Department of Biology, Federal University of Ceará (UFC). Voucher specimens (N°55710), of the collected plant are deposited in the Herbarium Prisco Bezerra, UFC, and identification was carried out by Sarah Sued Gomes.

Extraction procedure

The leaves and bark extracts were obtained by two different methods namely Soxhlet extraction and steeping.

Soxhlet apparatus

The leaves and bark samples, were dried at 37°C to constant weight, and ground to a fine homogeneous powder prior to extraction. Plant powders (10 g) were extracted with *n*-hexane in a Soxhlet apparatus (3 h) to remove lipid. After drying, the powders were subsequently extracted with methanol (3 x 3 h) and the solvent removed by rotary evaporation under reduced pressure at 37°C in vacuo.

Steeping (cold extraction)

The leaves and bark samples were dried to constant weight. Dried plant powders (1.56 kg from leaves and 2.223 Kg from bark) were placed in two separate bottles and submitted to cold extraction with *n*-hexane (3 x 9 L) and then with ethanol (3 x 9 L). Following filtration, the extracts were combined and organic solvent was removed by rotary evaporation at 35°C in vacuo.

Chromatographic fractionation

The *n*-hexane extract (10.006 g) of *H. speciosa* from bark was subjected to column chromatography (40 x 6 cm) on silica gel (200 g) using gradient elution with n-hexane, dichloromethane, ethyl dichloromethane, ethyl acetate and methanol in order of increasing polarity. 146 fractions were collected and grouped, after checking the fractions by thin layer chromatography (TLC). Fractions containing identical compounds were combined, giving 35 combined fractions.

Angiotensin I converting enzyme assay

The activity of ACE, was determined using the substrate HHL (5mM - 2.15 mg/mL) dissolved in 50 mM Tris-HCl (pH 8.3) containing 0.3 M NaCl. The buffer was used to dilute enzyme and substrate. Each sample (25 µL dissolved in methanol) was added to the substrate solution (100 µL) and incubated at 37°C for 10 minutes. The reaction was initiated by addition of ACE solution (10 µL, 200 mU/mL) in 50 mM Tris-HCl, pH 8.3 containing 0.3 M NaCl, and the mixture was incubated at 37°C for 30 minutes with continuous agitation at 450 rpm. The reaction was stopped by addition of 1 M HCl (100 µL). Reaction products were analysed by analytical reverse-phase HPLC. The antihypertension agent Captopril was used as positive control, at a concentration of 2 ng/mL and either methanol or hexane was used as negative controls. The IC_{50} value was defined as the concentration required to inhibit 50 % of ACE activity under the assay conditions, and was determined by regression analyses of ACE inhibition (%) versus the log of the inhibitor concentration [12].

Quantitative HPLC determination of hippuric acid

For the analysis of hippuric acid from HHL by analytical HPLC, a C18, reverse-phase (5 µm), column (250 × 4 mm I.D.; Latek, Eppelheim, Germany) was used, and the mobile phase consisted of 0.05 % TFA in deionized water (solvent A) and 0.05 % TFA in acetonitrile (solvent B) with the following gradient: 95 % A to 60 % B in 10 min, maintained for 2 min.,

followed by a return to 5 % B in one min. and equilibration for 5 minutes. Detection was by UV absorbance at 228 nm, with a flow-rate of 1.0 mL/min. Instrument control, and data handling were performed with the HP Chemstation software on a PC. In order to determine the IC_{50} of the extracts, data were adjusted to a non-linear regression.

DPPH assay

The free radical scavenging capacity of the substances was determined by using the DPPH radical discoloration method [23]. The extracts were diluted in methanol at concentrations between 0.25 and 1.0 mg/mL. Twenty microliters of the different concentrations was placed in 96 well plates in duplicate. The reaction was initiated by adding 180 µL of DPPH solution (20 µg/mL in methanol). The absorbance was read at 515 nm over 45 min with an Universal Micro plate reader Elx 800 (Bio-Tek Instruments Winooski, VT), Steady-state levels were reached within the first 10 minutes versus a control (methanol). The concentration of DPPH radical was calculated from a standard curve of DPPH between 1.0 and 100 µg/mL measured simultaneously.

For comparison, the IC_{50} values for each extract (concentration of each extract where 50% of the DPPH radical is scavenged) were calculated with the DPPH values at 10 minutes, for different concentrations using the Table curve program (Jandel Scientific, Chicago, IL).

Ferric reducing ability of plasma (FRAP) assay

The ferric reducing ability of plasma (FRAP) assay measures the ability of antioxidant to reduce $[Fe^{3+}\text{- (TPTZ)}]^{3+}$ to blue colored ferrous complex $[Fe^{2+}\text{- (TPTZ)}]^{2+}$. The dilution of the different substances was identical to that described for the DPPH assay. Ten microliters of substance was incubated with 30 µL of water and 300 µL of FRAP reagent, consisting of 25 mL of acetate buffer (300 mM sodium acetate buffer, pH 3.6), 2.5 mL of TPTZ (10 mM TPTZ in 40 mM HCl), and 2.5 mL of $FeCl_3$ solution (20 mM $FeCl_3.6H_2O$ in water) at 37°C. Ten µL of the extracts, plus distilled water (30 µL) at 37°C were added to a 96 well-plate, and 300 µL of FRAP reagent was added to initiate the reaction. All reagents were freshly prepared and warmed to 37°C before measurement. A calibration curve of ferrous sulfate (0.01 - 1.0 mM), was measured simultaneously and the results are expressed in mM Fe^{2+}/L.

The absorbance was read at 595 nm. The reaction of all extracts reached a steady-state level after 5 minutes. A linear regression curve was generated at the 5 min. reaction time-point for different concentrations of extracts with the Microcal Origin 5.0 program. Using these

regression curves, the EC_1 values were calculated, as the concentrations of antioxidant (µM) giving an absorbance equivalent to a 1 mM Fe(II) solution according to [20].

Oxygen radical absorbance capacity (ORAC) assay

3,3′-Azo-bis-(2-amidinopropane) dihydrochloride (AAPH) (Wako Chemicals) was used as a peroxyl radical generator, and fluorescein (0.21µM in ORAC buffer) was used as a redox-sensitive fluorescent indicator [11].

Aliquots of 20 µL, of a 10 mM stock solution of Trolox in DMSO were stored at -20°C and melted, and diluted with ORAC buffer, to a final concentration of 20 µM immediately before use. The ORAC buffer, contained 75 mM potassium hydrogen phosphate/potassium dihydrogen phosphate at pH 7.4. Freshly prepared sample solutions (1.0 mM), in methanol or DMSO, were diluted in ORAC buffer, to a final concentration of 20 µM. Ten microliters of the sample, buffer, or Trolox solution together with 170 µL of fluorescein solution were placed in quadruplate in a 96 well plate and incubated at 37 °C for 10 min. The exterior wells of the plates were not used for experimental determinations. The reaction was initiated by addition of 20 µL of APPH solution (103.5 mg/2 mL buffer) and freshly mixed on ice immediately before use.

The decline of fluorescence was measured at 37°C every 2 min. until completion at 122 min. using a Cytoflour 4000 fluorescent microplate reader, excitation wavelength Ex 530/25 nm, and emission wavelength Em 585/30 nm (Perspeptive Biosystems, MN). The net area under the curve (AUC) of the standards and samples was calculated. The final ORAC values were calculated using the regression equation between the Trolox concentration and the net AUC and are expressed as relative ORAC units, where 1 ORAC unit equals the inhibition of the declining fluorescence produced by 1 µM Trolox.

Hypoxanthine-xanthine oxidase HPLC-based antioxidant assay

The hypoxanthine/xanthine oxidase HPLC-based assay, conducted according to the methods described in [18] and [19], was used to assess the antioxidant capacities of the raw methanol extracts of leaves and bark. Stock solutions of 10 mg/mL were assessed. For the hypoxanthine/xanthine oxidase assay, the relevant sample concentration range (0-1000 µg) in methanol, was added to 2 mL plastic microfuge tubes in duplicate, and the solvent was removed under a stream of nitrogen.

The dried residues were suspended in phosphate buffer (1.0 mL), and 5.0 µL of a 1:5 dilution of xanthine oxidase in $(NH_4)_2SO_4$ (3.2 M) were added to initiate the reaction. The tubes were incubated at 37°C until reaction completion at 3 hours. After incubation, 20 µL of the reaction mixtures were analyzed by analytical HPLC.

HPLC Analysis of dihydroxyphenols in the HPLC-based antioxidant assay

This was conducted as described by Owen et al. [17]. Briefly the UV detector was set at 325 and 278 nm for the detection of the products produced by ROS attack on salicylic acid (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) and hypoxanthine (uric acid), respectively. The amounts of dihydroxyphenol (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) and uric acid produced were determined from the calibration standard curves generated for authentic standards at the relevant wavelength.

Instrument control and data handling were performed with the HP Chemstation software on a PC.

Thin layer chromatography (TLC) with bioassay detection for AChE inhibition

The TLC method of Ellman et al. [5] as modified by Rhee et al. [21] was used to determine inhibition of acetylcholinesterase. The ethanol and hexane extracts of leaves and bark of *H. speciosa* were dissolved in methanol, to a concentration of 10 mg/mL. Then 2.5 µL of each sample was spotted on the baseline of silica gel TLC plates (0.25 mm silica gel plate F254; Macherey-Nagel, Germany), with eserine as reference standard. The TLC plates, were sprayed with DTNB/ATCI reagent (1 mM DTNB and 1 mM ATCI in buffer A) until the silica was saturated with the solvent. The plates were allowed to dry for 3–5 minutes and then resprayed with 3 U/ml of AChE enzyme. A yellow background appeared, with white spots (halos) indicating inhibition of AChE becoming visible after 10 minutes. These were observed and recorded within 15 minutes, because they disappeared after 20–30 minutes.

RESULTS AND DISCUSSION

Inhibition of Angiotensin I-converting enzyme

The inhibition of ACE activity of plant extracts of *H. speciosa* was evaluated using a HPLC assay. The assay system was calibrated with captopril, a positive control which showed ACE inhibitory activity with an IC_{50} value of 6.85 mM.

The methanol extracts of the leaves and bark of *H. speciosa* show extensive inhibition of ACE activity (97.63% ± 2.89% and 95.07% ± 3.76% at a concentration of 10 mg/mL) with significant IC_{50} values of 79.24 µg/mL and 146.47 µg/mL respectively (Table 1).

Table 1. Inhibition of Angiotensin I-converting enzyme of methanol extracts from the leaves and bark of *Hancornia speciosa* Gomes.

Extracts of *H. speciosa*	IC_{50} (µg/mL)
Captopril (positive control)	6.85nm
EMLS	79.24
EMBS	146.47

EMLS= Methanol extract from leaves by Soxhlet extraction
EMBS= Methanol extract from bark by Soxhlet extraction

ACE inhibition capacity in *H. speciosa* leaf extracts was previously reported by Ferreira et al., [7, 8] assigning the flavonoid rutin as the main inhibitor. Ethanol extracts of the leaves of *H. speciosa*, also promote dramatically reduced vasorelaxant effects in rat aorta, and mesenteric arteries, through endothelium production of nitric oxide (NO) via phosphatidyl-inositol 3-kinase (PI3K) activation [7, 8]. Vasodilators are clinically used to treat hypertension indicating their potential as antihypertension agents [14].

Our data supports and consolidates that of Ferreira et al. [7, 8] with confirmation of ACE inhibition by leaf extracts, and furthermore showing that bark extracts are also very active in this respect. This is the first report of ACE inhibition capacity by bark extracts of *H. speciosa*. These results support the use of *H. speciosa* extracts in traditional Brazilian medicine as antihypertension agents.

Antioxidant capacity of methanol extracts from the leaves and bark of *Hancornia speciosa* Gomes

The antioxidant capacity of the methanol extracts of leaves and bark of *H. speciosa* was determined by monitoring the production of hydroxylated benzoic acids (DHBA) due to attack of ROS, on salicylic acid in the HX/XO assay, as well as by DPPH, FRAP and ORAC assays. The IC_{50} values are given in Table 2.

Table 2. Antioxidant capacity of methanol extracts from the leaves and bark of *Hancornia speciosa* Gomes

SAMPLES	Assay			
	DPPH (mg/mL) (IC_{50})	FRAP (mg/mL) (EC)	ORAC (Units)	HX/XO (ug/uL) (IC_{50})
EMLS	285.49	387.4	2.70	12
EMBS	264.81	647.89	1.74	*
Trolox	66.00	62.44	1.00	2.32

EMLS= Methanol extract from leaves by Soxhlet extraction

EMBS= Methanol extract from bark by Soxhlet extraction

***** No significant activity at the concentrations tested.

The methanol extracts, obtained by Soxhlet extraction of leaves and bark of *H. speciosa*, were used to evaluate the antioxidant capacity in the HX/XO, DPPH, FRAP, and ORAC assays, and shows that in comparison to Trolox (synthetic form of vitamin E) the extracts were only positive in the ORAC assay (ORAC units = 2.70, and 1.74 respectively, Trolox = 1.0). This represents the first report of antioxidant capacity of *H. speciosa* bark and leaf extracts in the literature.

Thin layer chromatography (TLC) with bioassay detection of AChE inhibition

The formation of a white halo around the TLC "spots" is indicative of AChE enzyme inhibition. The size of the halos were measured (mm), and compared with the positive standard eserine. The ethanol extracts of the leaves and bark, and the hexane extract of the bark, gave positive results with regard to AChE inhibition, whereas the hexane extract of the leaves did not (Table 3). Among the different extracts from *H. speciosa*, the hexane extract of the bark, represented the most promising potential for inhibition of AChE. For this reason we selected this extract (EHC) for fractionation and purification of the active principle.

Evaluation of AChE inhibition of the fractions, obtained by column chromatography on silica gel of EHC, showed that fractions 136-143 and 144-146 were the most active. These fractions were pooled, and after removal of solvent, gave a white powder (25 mg) which was subjected to ^{1}H and ^{13}C NMR analysis, and identified as the triterpene lupeol.

Table 3. Inhibition of acetylcholinesterase by ethanol and *n*-hexane extracts of leaves and bark of *Hancornia speciosa* Gomes

Samples	Result	Size of halo (mm)
Positive standard (Eserine)	Positive	9
EHC	Positive	13
EHF	Negative	**
EEC	Positive	8
EEF	Positive	9
EHC – hexane	Negative	**
EHC – dichloromethane	Negative	**
EHC – ethyl acetate	Positive	12
EHC – methanol	Positive	11
EHC – (1/3)	Negative	**
EHC – (74/88)	Negative	**
EHC – (90/98)	Positive	6
EHC – (104/106)	Positive	7
EHC – (112-118)	Negative	**
EHC – (120/126)	Negative	**
EHC – (128-132)	Negative	**
EHC – (136/143)	Positive	16
EHC – (144/146)	Positive	15

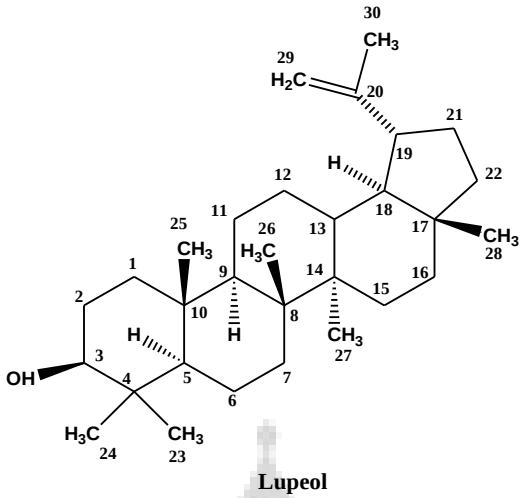
****** Not active.

EHC – Hexane extract from bark; **EHF** – Hexane extract from leaves; **EEC** – Ethanol extract from bark; **EEF** – Ethanol extract from leaves; **EHC** – Hexane extract from bark (fractions).

Chromatographic Fractionation

The analysis of the fractions (EHC-136-143), EHC (144-146) resulted in 25 mg of a solid powder. Nuclear Magnetic Resonance analysis ^{1}H and ^{13}C NMR: ^{13}C NMR (125Mz; CD_3Cl_3), **Lupeol:** δ_C 38.14 (**1C**); 27.34 (**2C**); 77.47 (**3C**); 38.46 (**4C**)**;** 5**C** (55.48); 18.38 (**6C**)**;** 34.30 (**7C**); 40.95 (**8C**); 50.44 (**9C**); 37.19 (**10C**); 21.07 (**11C**); 25.19 (**12C**); 37.92 (**13C**); 42.95 (**14C**); 27.55 (**15C**); 35.68 (**16C**); 43.11 (**17C**); 48.39 (**18C**); 48.11 (**19C**); 151.03 (**20C**); 29.76 (**21C**); 40.12 (**22C**); 28.14 (**23C**); 14.64 (**24C**); 16.10 (**25C**); 16.29 (**26C**); 14.64 (**27C**);

18.13 (**28C**); 109.52 (**29C**); 19.43 (**30C**). ^{1}H NMR (500Mz; CD_3Cl_3) δ_H: 0.77-1.69 (each 3H, s, Me × 7), 3.20 (1H, *dd*, *J* = 5.4, 10.6 Hz, **H-3**), 4.57 (1H, s, **H-29a**), 4.69 (1H, s, **H-29b**). In comparison with the literature [13], it was proposed that it is Lupeol (**Figure 1**).



Lupeol

Figure 1: Structure of the triterpene lupeol

CONCLUSION

Methanol extracts from the leaves and bark of *H. speciosa* were very effective in the inhibition of ACE activity (97.63 % ± 2.89 % and 95.07 % ± 3.76 %, with significant IC_{50} values of 79.24 µg/mL and 146.47 µg/mL respectively). In the antioxidant assays, the extracts exhibited low antioxidant capacity in the FRAP and DPPH and HX/XO assays, but were effective in the ORAC assay (ORAC units = 2.70, and 1.74 respectively, compared to Trolox = 1.0). Both the hexane and ethanol extracts of bark, but only the ethanol extract of leaves, inhibited acetylcholinesterase (AChE). The active principle, responsible for acetylcholinesterase inhibition, following purification by column chromatography, and identification by ^{1}H and ^{13}C NMR was identified as lupeol.

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