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Phytochemical study and biological evaluation of *Campsiandra comosa* hexane and methanol extracts

[Estudio fitoquímico y evaluación biológica de los extractos hexánicos y metanólicos de *Campsiandra comosa*]

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Abstract: *Campsiandra comosa* (Fabaceae) is a medicinal Amazonian plant with no previous chemical fractionation. Thus, the objective of the current study was to evaluate extract toxicity on *Artemia salina*, antioxidant activity (DPPH and Fe^{3+}/Phenanthroline), and antibacterial and antiangiogenic activity of *C. comosa*, and to perform a chemical fractionation. Leaf hexane and trunk bark methanolic extracts, after phytochemical prospection, were fractionated. Isolated substances were identified by nuclear magnetic resonance (NMR) (1D and 2D) and mass spectrometry (MS). Chromatographic fractionation allowed the isolation of lupeol, β-sitosterol, 4´,5,7-trihydroxyflavone (apigenin) and quercetin-3,7,3'-trimethylether (pachypodol). Methanolic extracts of branch and trunk barks showed inhibitory activity against *Pseudomonas fluorescens*. Only methanol extracts showed antioxidant activity. Extracts, except those obtained from branch and bark with hexane, showed antiangiogenic activity. Evaluated extracts showed no toxicity against *Artemia salina*. The results show the high biological potential of *Campsiandra comosa* encouraging further fractionation research.

Keywords: *Campsiandra comosa*; Fabaceae; Flavonoids; NMR; Terpenes.

Resumen: *Campsiandra comosa* (Fabaceae) es una planta medicinal amazónica para la que no se han reportado fraccionamientos químicos previos. El objetivo de este trabajo fue evaluar la toxicidad frente a *Artemia salina*, la actividad antioxidante (DPPH y Fe^{3+}/Fenantrolina), la actividad antibacteriana y antiangiogénica de sus extractos y realizar el fraccionamiento químico. Los extractos hexánicos de las hojas y metanólico de las cortezas del tronco fueron fraccionados. El fraccionamiento cromatográfico permitió el aislamiento de lupeol, β-sitosterol, 4',5,7-trihidroxiflavona (apigenina) y quercetina-3,7,3'-trimetiléter (pachipodol), que fueron identificadas mediante análisis de resonancia magnética nuclear (RMN) (1D y 2D) y espectrometría de masas (MS). Los extractos metanólicos de las ramas y cortezas del tronco mostraron inhibición frente a *Pseudomonas fluorescens*. Solo los extractos metanólicos mostraron actividad antioxidante. Los extractos mostraron actividad antiangiogénica, excepto los hexánicos de ramas y cortezas. Los extractos evaluados no mostraron toxicidad frente a *Artemia salina*. Los resultados obtenidos muestran el potencial de *Campsiandra comosa* y estimulan la continuación del fraccionamiento.

Palabras clave: *Campsiandra comosa*; Fabaceae; Flavonoides; RMN; Terpenos

INTRODUCTION

Campsiandra comosa Benth. (Fabaceae, Caesalpinioideae), popularly known in Brazil as *acapurana,* is a medium-sized tree which grows along the margins of watercourses (Silva *et al*., 1988). The species occurs wild in Colombia, Suriname, Venezuela and Brazil, where it is present in the states of Amazonas, Roraima, Amapá and Pará (Souza, 2012). Other species, *Campsiandra laurifolia* Benth, is known by the same popular name, and some interesting activities such as antioxidant activity of the aqueous extract in colitis induced by acetic acid in wistar rats was reported (Souza *et al*., 2021). This similarities in the popular name challenged the present work. Previous work showed that both plant species collected in the Amazon region have an antifungal activity against *Candida albicans, C. glabrata* and *C. parapsilosis* (Rodrigues *et al*., 2014).

Seeds of the species are widely used as food by the local population (Sánchez *et al*., 1987; Padilla *et al*., 2008). In addition, in the Amazon rainforest region, a decoction of *C. comosa* leaves and bark is popularly used against toothache and inflammation, while the peripheral antinociceptive activity of the bark's hydroalcoholic extract has been scientifically validated (Rodrigues *et al.,* 2010). Carrion *et al*. (2013), also demonstrated the activity of dichloromethane and methanol extracts against a strain of *Mycobacterium tuberculosis*. Other previous work have shown their antioxidant potential.

In a chemical study carried out by Hernes & Hedges (2004), *C. comosa*, collected in the Amazon region, showed the presence of tannins in both leaves and wood sample. These appear to be the only published data on the substances that might be present in the species.

Thus, the objectives of the current study were: (i) to evaluate the hexane and methanolic extracts of different parts of *C. comosa* (bark of the trunk, branches, and leaves) on the following activities: antioxidant, antibacterial, antiangiogenic, (ii) to study their toxicity on *Artemia salina*, and (iii) fractionate those extracts that showed to be chemically interesting.

MATERIALS AND METHODS

Plant material

Leaves, bark and branches of *Campsiandra comosa* were collected at Lake Catalão (S 03° 09.396'/ W 059° 55.217'), near the city of Manaus, Amazonas, Brazil, under IBAMA´s license number 16970-1 and SISGEN´s license number AF9CA41. A voucher was deposited in the Herbarium of the National Institute of Amazonian Research – INPA (accession number: 258668). Plant materials were dried in a forced circulation oven below 50°C and ground using a knife mill (Tecnal, model Willye TE- 650, Brazil).

Plant Extraction

Each part of plant material was extracted (1 g of dried plant in: 10 mL of solvent) successively first with hexane, methanol and distilled water. In all cases the extraction was assisted using an ultrasound bath (40 kHz, Unique, Brazil) during 20 min, the solvent separated, and the procedure repeated twice. The hexane and methanolic extracts were concentrated in a rotary evaporator (model 802, Fisatom, Brazil), and the aqueous extract was lyophilized (ALPHA 1-2 LDplus, Christ, Germany). All concentrated extracts were stored in a refrigerator until their phytochemical study.

Extracts were analyzed by thin layer chromatography (TLC) using physical (UV 254 and 365 nm) and chemical (NP/PEG; $Ce(SO_4)_2$; $FeCl_3$ and sulfuric anisaldehyde) revealers. The main group of compounds identified were of flavonoid natures in the methanol and hexane extracts, being these extracts selected for chemical investigation.

Trunk bark methanolic extract fractionation

Bark methanolic extract (10 g) was first dissolved in 500 mL of $MeOH/H_2O$ (1:1) and then extracted three times with dichloromethane (DCM), followed by ethyl acetate (EtOAc) three times.

The DCM phase (381 mg) was fractionated using an open column (Ø x h: 3 x 30 cm) filled with Sephadex LH-20 using MeOH (Figure No. 1). Thirty fractions were collected and, after TLC analysis, those most similar were combined. Five of them: 3-4 (187 mg), 10 (44 mg), 11-12 (17 mg), 13-14 (24 mg) and 15-17 (17 mg) were then further fractionated. Fraction 3-4 was re-fractionated on open silica column (18.7 g, Ø x h: 1.3 x 32 cm) using a Hex/EtOAc gradient

(7:3, 6:4, 1:1, 3:7), 100% EtOAc, 1:1 EtOAc/MeOH and 100% MeOH generating 30 subfractions, which were analyzed by TLC. One of them, subfraction 2-6, was re-fractionated on open column of silica (7.5 g, Ø x h: 1.2 x 17.5 cm) using a Hex/DCM gradient (1:1, 4:6, 3:7, 2:8, 1:9), 100% DCM and DCM/MeOH (95:5) obtaining 20 fractions. Subfractions 6 and 12 were analyzed by TLC, then identified by NMR and LC/MS, as substances **1** (3 mg; Rf: 0.2) and **2** (3 mg; Rf: 0.32), respectively.

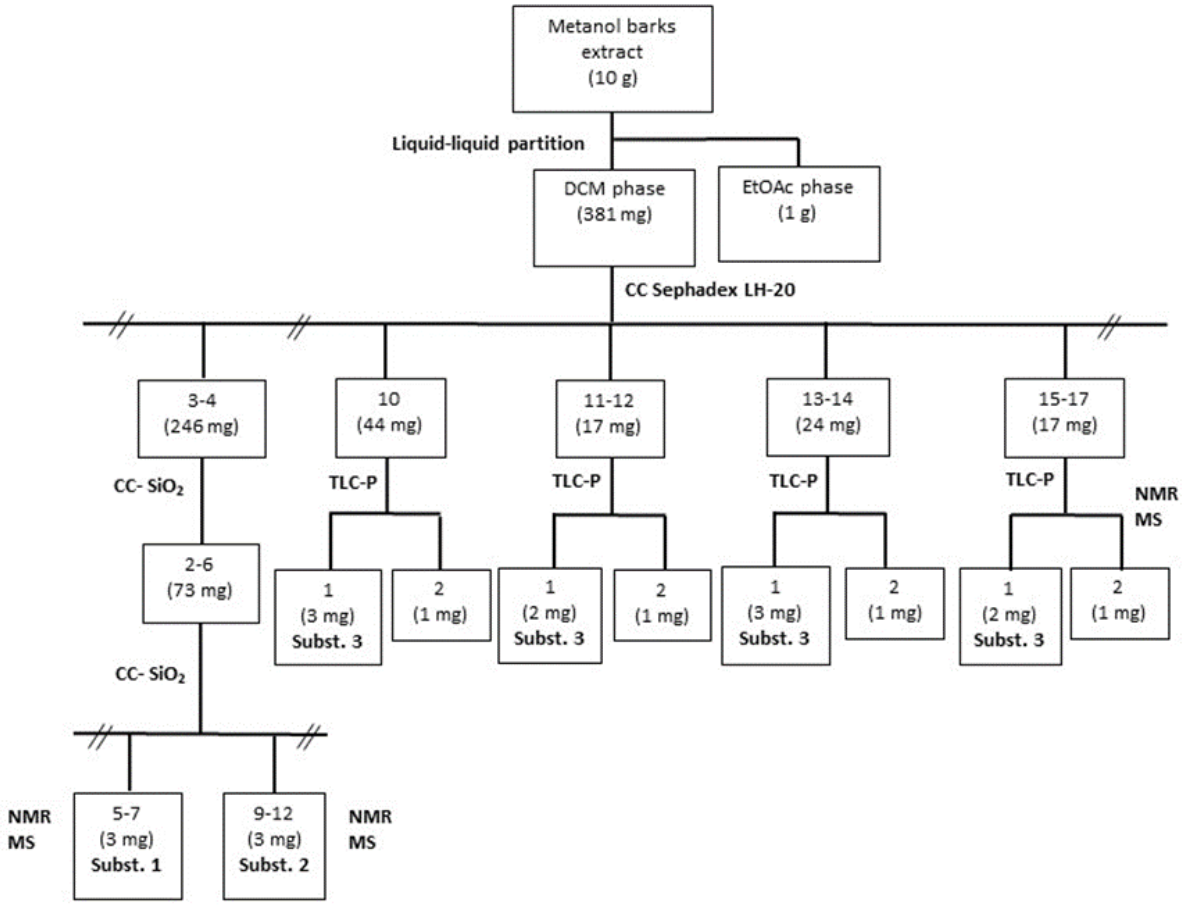

Figure No. 1
Fractionation of *C. comosa* dichloromethane (DCM) phase from metanol barks extract

Subfraction 10 (44 mg) from the first column was purified by preparative TLC (TLC-P), using 20 x 20 cm glass plates coated with silica gel with fluorescence indicator (Macherey-Nagel - 0.2-0, 5 mm) and eluted with DCM/acetone (7:3). This generated subfraction 3, henceforth substance **3** (10 mg; Rf: 0.6). The other 3 subfractions (11-12, 13-14, 15-17) were also purified by TLC-P. All contained the same compound, which was identified as substance **3**.

The EtOAc phase (900 mg) was fractionated in open column (Ø x h: 2,7 cm x 30 cm) of silica gel (100 g), using as eluent a gradient of EtOAc/MeOH (7:3, 6:4, 1:1, 3:7) and MeOH 100%, which yielded 30 subfractions (Figure No. 2). According to TLC analysis, subfractions were mixed, and fraction 7-9 was further fractionated. The fractionation of sample 7-9 (244 mg) in open column (Ø x h: 1.8 x 63 cm) of silica (24 g), eluted with DCM/EtOAc (7:3, 6:4, 1:1, 3:7), EtOAc 100%, EtOAc/acetone (9:1, 8:2, 6:4, 1:1, 9:1) and acetone/MeOH (9:1), yielded 32 fractions. The collected fractions were analyzed by TLC, and subfractions 2-10 combined (named 7-

9_2-10) and identified as substance **3** (8 mg; Rf: 0.5) by ^{1}H and ^{13}C NMR analysis.

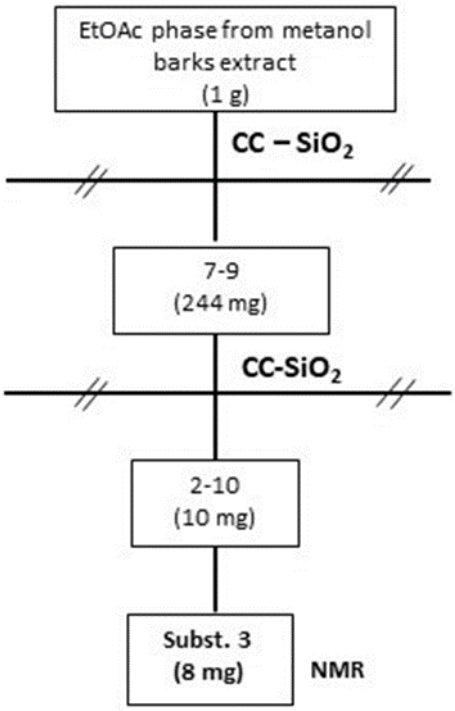

Figure No. 2
Fractionation of *C. comosa* ethyl acetate (EtOAc) phase metanol barks extract

Leaves hexane extract fractionation

The hexane extract was also analyzed by TLC, using light UV 254 and 365 nm as physical developers and NP/PEG, $Ce(SO_4)_2$, $FeCl_3$ and sulfuric anisaldehyde as chemical developers. Additionally, terpenes and flavonoids were detected when TLC plates were sprayed with $FeCl_3$ and NP/PEG.

Fractionation of the leaves hexane extract (6 g) was carried out in an open column (Ø x h: 3 cm x 30 cm) of silica, with a mobile phase Hex/EtOAc (8:2, 7:3, 6:4, 1:1), 100% EtOAc and EtOAc/MeOH (1:1), from which 39 subfractions were obtained (Figure No. 3). These subfractions were analyzed byTLC using physical and chemical developers and showed, when developed with UV 365 nm and NP/PEG, intense yellow and blue fluorescence, for subfractions 19-21 which were collected and refractionated.

Fractionation of sample 19-21 (100 mg) was carried out in open column (Ø x h: 23 cm x 1 cm) with silica (10 g) using $CHCl_3$/MeOH (98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9; 90:10) as mobile phase and 100% MeOH and yielding 24 subfractions. Subfractions 2-3, when analyzed by TLC, showed yellowish spots in anisaldehyde, dark in $FeCl_3$ and with very intense yellow and blue spots, when developed in NP/PEG, and observed under 365 nm UV light. Accordingly, they were mixed and re-fractionated.

Sample 19-21_2-3 (12 mg) was fractionated on open column (Ø x h: 8 cm x 1 cm) of silica (1.2 g), using the $CHCl_3$/MeOH system (98:2, 96:4, 90:10, 1:1) to 100% MeOH, yielding 13 subfractions. The collected subfractions were again evaluated by TLC and developed in UV light 254 and 365 nm, sulfuric anisaldehyde, $FeCl_3$ and NP/PEG. Fraction 1 (7 mg) indicating the presence of flavonoids in TLC, but still in mixture. Accordingly, fraction 1 was selected for further re-fractionation.

The fractionation of fraction 19-21_2-3_1 was performed on an open column (Ø x h: 7 cm x 1 cm) of silica (0.7 g) using a single system

($CHCl_3$/MeOH 99:1), yielding 3 fractions. The separation of this column was monitored with 365 nm UV light, and the substance of interest was collected as fraction 1. Then, it was analyzed by ^1H NMR and identified as substance **4** (2.5 mg; Rf: 0.2).

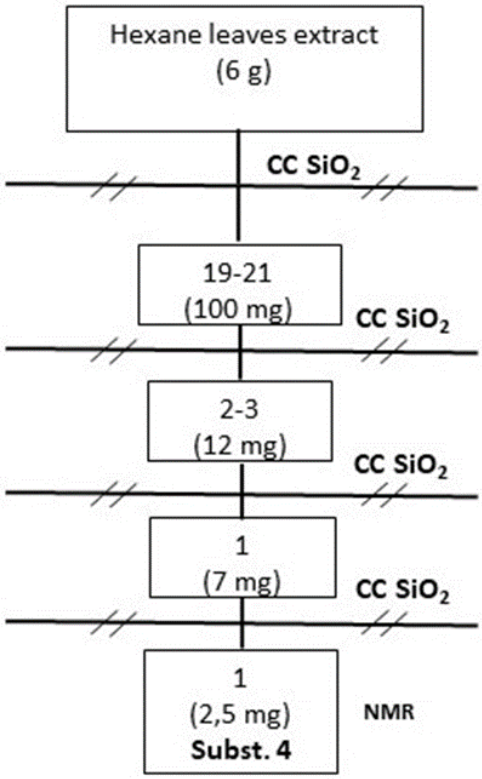

Figure No. 3
Fractionation of *C. comosa* hexane leaves extract

Structure identification

To determine the chemical structure, the fractions were dissolved in $CDCl_3$ (Cambridge Isotope Laboratories) and analyzed by Nuclear Magnetic Resonance (NMR) (Fourier 300, Bruker spectrometer), operating at 300 for ^1H and 75 MHz for ^{13}C respectively. Chemical NMR shifts were expressed in ppm, using TMS as internal reference. NMR data was processed using the TopSpin software (Bruker). The substances exact masses were obtained using a MicroTOF-Q II (Bruker) mass spectrometer using APCI and ESI sources in positive ionization mode. LC-DAD-MS analyzes were obtained by Prominence UFLC chromatography (Shimadzu), equipped with a LC-20AT binary pump, SPDM-20A diode array detector (DAD) and SIL-20A automatic injector. The analyzes were performed on a Shim-Pac XR-ODS C18 column (2.0 mm x 50.0 mm id), using an elution gradient with water (0.1% HCOOH)/acetonitrile (0.1% HCOOH) of 100/0% until 4 min, 100/80% up to 10 min, 80% at 10-15 min, 80-100% up to 17 min and 100% at 17-20 min. Flow rate was 0.4 mL/min with a Split of 0.06 mL (MS)/0.34 mL (desc), UV detection was set at 190-400 nm and pressure at 2800 psi. Spectrometer settings were: APCI source voltage, 4.5 kV (positive mode) and 2.6 kV (negative mode); capillary temperature 250°C; full-scan mode (100-900 Da).

Biological Assays

Artemia salina toxicity assay

Artemia salina toxicity assay of *C. comosa* extracts were conducted following the methodology of Meyer *et al*. (1982) and Martins *et al*. (2014), at different concentrations (1000, 500,

250, 125 and 62.5 µg/mL) in 5% dimethyl sulfoxide (DMSO). Samples were evaluated in triplicate, using 5% DMSO and potassium dichromate as negative and positive controls respectively. Extract toxicity was determined via the CL_{50}, where extracts are considered inactive with CL_{50} > 1.0 mg/mL, moderately active with CL_{50} between 100 and 900 µg/mL, and strongly active with CL_{50} below 100 µg/mL (Anderson *et al*., 1991). Statistical analysis was performed using the GraphPad Prism 6 program.

Antioxidant activity

Antioxidant potential of *C. comosa* extracts was evaluated using two methods 2,2-diphenyl-1-picryl-hydrazil (DPPH), and Fe^{3+}/phenanthroline method (Martins *et al*., 2014). A calibration curve was prepared using ascorbic acid at different concentrations. After checking curve linearity (R: 0.98), extract antioxidant activity was evaluated.

For the DPPH assay: 990 µL of DPPH and 10 µL were added to each extract (at concentrations of 0.5 mg/mL).

For the assay with Fe^{3+}/Phenantroline: 980 µL of phenanthroline followed by 10 µL of Fe^{3+} were added to each extract (at concentrations of 0.5 mg/mL).

Measurements were performed with a spectrophotometer (FENTOM-Cirrus 80ST) at 517 nm (DPPH) after 30 minutes of reaction, and 508 nm (Fe^{3+}/Phenanthroline) after 1 hour of reaction. Variation in extract absorbance was compared to the values of the ascorbic acid standard for quantitative evaluation of extract antioxidant potential. All extracts were evaluated in triplicate, with extract replaced by 10 µL of methanol as a negative control.

Antibacterial activity

The antibacterial test was performed using the microdilution method in 96-well-microplates, determined on spectrophotometer at 625 nm (Model Multiskan Go, Thermo Scientific), according to the Clinical and Laboratory Standards Institute methodology (CLSI, 2015), with adaptations. The strains used [*Aeromonas hydrophila* (IOC/FDA 110-36), *Citrobacter freundii* (ATCC 8090), *Edwardsiella tarda* (ATCC 15947), *Escherichia coli* (ATCC 11775), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 10145), *Pseudomonas fluorescens* (ATCC 13525), *Serratia marcescens* (ATCC 13880), *Salmonella enteritidis* (ATCC 6951), *Yersinia enterocolitica* (ATCC 9610), *Staphylococcus aureus* (ATCC 12600)]were provided by the Reference Microorganisms Laboratory of the National Institute for Quality Control in Health, of the Oswaldo Cruz Foundation, Rio de Janeiro. The inhibitory activity of each extract was obtained through the difference in optical densities between two readings: after and before bacterial growth in contact with the extract. The antibiotic oxytetracycline at 125 µg/mL was used as a positive control and the culture medium itself, with 5% DMSO, as a negative control.

Antiangiogenic activity

For this test, a chorioallantoic membrane (CAM) assay was performed on fertilized *Gallus domesticus* eggs following Nguyen *et al*. (1994), with adaptations.

Extracts were dissolved in ethanol (1 mg/mL) and analyzed at 100, 500 and 1000 µg/mL, in triplicate. The same volume of ethanol was also used as negative control for each test. To measure antiangiogenic activity, a photographic camera was used to record each egg in triplicate. The images obtained were used to count the blood vessels that intercepted the disc and vessels present in an area of 0.9 cm^2, with results expressed as percentage of vessels ± standard deviation from the mean.

RESULTS AND DISCUSSION

Isolation and identification of substances

Phytochemical study of the methanolic extract of bark from *C. comosa* trunks allowed the isolation of 3 components: *β*-sitosterol (**1**), lupeol (**2**) and 4',5,7-trihydroxyflavone (apigenin) (**3**) isolated from the DCM phase (**3**) was also identified in the EtOAc phase; and quercetin-3,7,4'-trimethylether (pachypodol) (**4**) was isolated from the leaves hexane extract, which were identified by NMR and mass analysis (Figure No. 4).

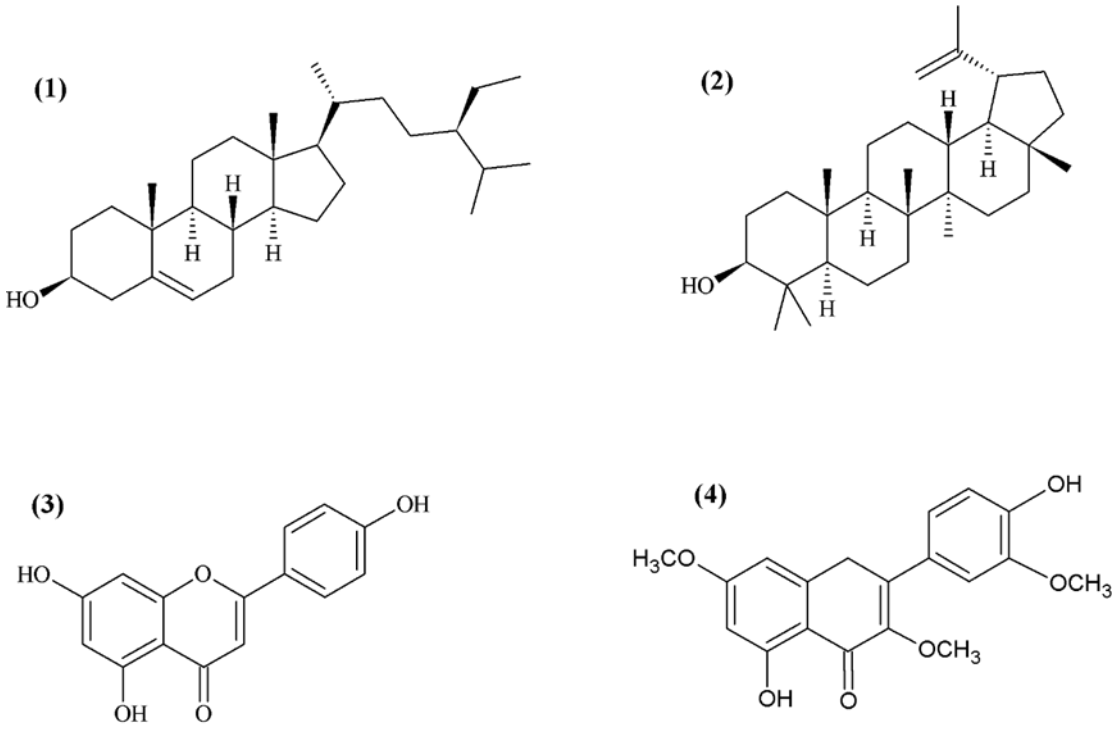

Figure No. 4
Chemical structures of *β*-sitosterol (1), lupeol (2), apigenin (3) and quercetin-3,7,4'-trimethylether (4)

^{1}H-NMR analysis of fraction 6 (Figure No. 5) allowed the identification of substance **1** as β-sitosterol based on the following data: 0.69 ppm (s, 3H, H-18), 0.81 ppm (d, J = 6,9 Hz, 3H, H-26), 0.83 ppm (d, J = 6,6 Hz, 3H, H-27), 0.85 ppm (s, 3H, H-29), 0.93 ppm (s, 3H, H-21), 1.01 ppm (s, 3H, H-19), 3.53 ppm (m, 1H, H-3), 5.35 ppm (d, J = 5,2 Hz, 1H, H-6). MS spectra showed *m/z* of 397 $[414 + H\text{-}H_2O]^+$ (positive mode) corresponding to molecular formula $C_{29}H_{50}O$. NMR data were compared to Lozano *et al*. (2020), and confirmed.

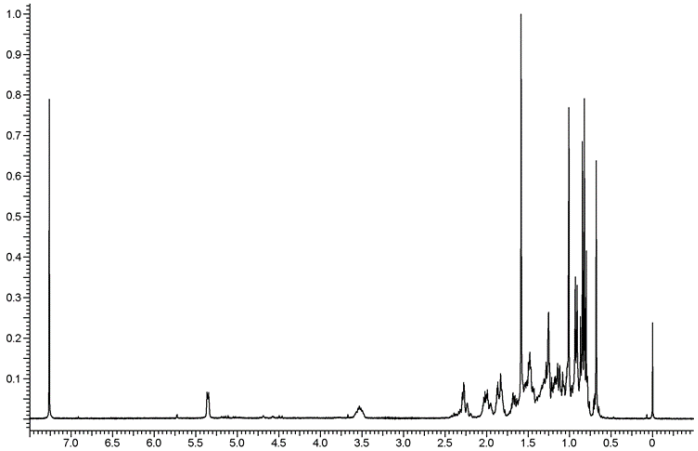

Figure No. 5
1H-RMN spectra of β-sitosterol, in CDCl3, 300 MHz

^{1}H-NMR analysis of fraction 12 (Figure No. 6) allowed the identification of substance **2** as lupeol based on the following data: 0.76 ppm (s, 3H, H-24), 0.79 ppm (s, 3H, H-28), 0.83 ppm (s, 3H, H-25), 0.94 ppm (s, 3H, H-27), 0.97 ppm (s, 3H, H-23), 1.03 ppm (s, 3H, H -26), 1.68 ppm (s, 3H, H-30), 1.92 ppm (m, 2H, H-2), 2.38 ppm (dt, J = 10,8; 5,7 Hz, 1H, H-19), 3.19 ppm (m, 1H, H-3), 4.57 ppm (dd, J = 2,2; 1,2 Hz, 1H, H-29b), 4.69 ppm (d, J = 2,2 Hz), 1H, H-29a). These data were compared with Cursino *et al*. (2009) and confirmed. The mass spectra supported the expected mass: *m/z* 409 [426 + H-H_2O]$^+$ (positive mode), corresponding to the molecular formula $C_{30}H_{50}O$.

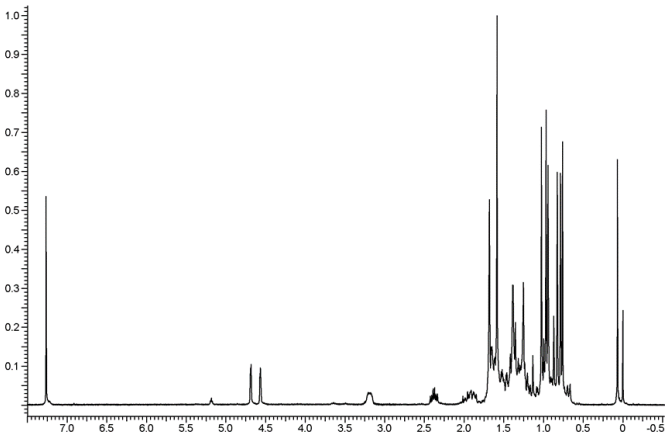

Figure No. 6
1H-RMN spectra of lupeol, in CDCl3, 300 MHz

NMR analysis of the subfraction (2-10) (Figure No. 7) allowed compound **3** was identified as 4',5,7-trihydroxyflavone (apigenin), based on ^{1}H NMR and HSQC spectra showed two hydrogens at 7.90 ppm (d, 1H, J = 8.7 Hz) and 6.91 ppm (d, 1H, J = 8.7 Hz) that correlated with the carbons at 128.39 ppm and 116.4 ppm, respectively, suggesting relationships with the hydrogens of a flavonoid B ring. A singlet at 6.75 ppm linked to a carbon at 103.2 ppm was assigned to hydrogen and carbon, located in position 3 of a flavone C ring. Two other doublets at 6.46 ppm (J = 2 Hz) and 6.17 ppm (J =2 Hz) were linked, respectively, with carbons at 94.4 ppm and 99.3 ppm, suggesting that they were related to hydrogens and carbons at positions 6 and 8 of the flavonoid A ring. The data obtained by HMBC analysis revealed all the couplings between hydrogens and carbons. In addition, correlations of the hydrogens at 6.91 ppm (H-2 ', H-6'), 7.90 ppm (H-3', H-5') with the carbon at 161.65 ppm (C-4) confirmed the position of the ring B hydroxyl. The data obtained were compared to Fathiazad *et al*. (2006). The mass spectrum generated the ions *m/z* 269 (negative mode) and *m/z* 271 (positive mode), which corresponds to the expected molecular formula $C_{15}H_{10}O_5$.

NMR spectra analysis of subfraction 1 (Figure No. 8), obtained from the hexane extract of leaves, allowed the substance to be identified as 3,7,3'-trimethoxy-quercetin (pachypodol) (**4**). Its spot revealed by NP-PEG on TLC indicates characteristics of a flavonoid. The ^{1}H NMR spectra of this substance showed signs in the region of aromatic hydrogens at 7.71ppm (d, J = 2.0 Hz), 7.68 ppm (dd, J = 8.4; 2.0 Hz) and 7.05 ppm (d, J = 8.4 Hz). These signals are characteristic of an aromatic ring with an ABX system, where the hydrogens H-5' and H-6' are coupling and generating a doublet with *J* = 8.4 Hz (*orto*), as well as H-2' and H- 6', both *d* with *J* = 2.0 Hz, indicating *meta* coupling of a flavonoid B ring. Two signals were also observed at 6.45 ppm (d, J = 2.2 Hz) and 6.36 ppm (d, J = 2.2 Hz), both doublets, with a low coupling constant value, showing that they are separated by four bonds, characteristic of hydrogens H-8 and H-6 respectively, of a flavonoid A ring. No singlets were observed at 7.9 ppm and 8.5 ppm, ruling it

out as an isoflavonoid. Likewise, no singlet between 6.3 ppm and 6.7 ppm, nor signs at 5 ppm and 3 ppm, were observed, which would be hydrogens in position 2 and 3 in a flavonoid C ring, making clear the existence of substitutions at these positions.The last three signs observed were singles at 3.99 ppm, 3.88 ppm and 3.86 ppm, characteristic of methoxyls linked to substituents on a flavonoid skeleton. A signal at 12.65 ppm showed the presence of a quelated hydroxyl in position C-5 of the flavonoid A ring, linked to the oxygen of carbonyl C-4 of C ring of the flavonoid. One final sign in the form of singlet was observed at 6.01 ppm. This signal could not be distinguished with the hydrogen spectrum alone, requiring two-dimensional analysis. HSQC correlation analysis and HMBC allowed to distinguish the methoxy positions. The data were compared to Ali *et al*. (2008) and confirmed.

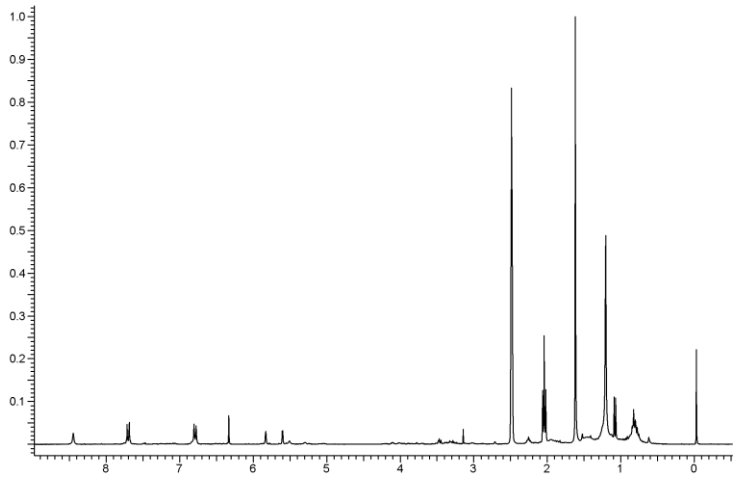

Figure No. 7
^{1}H-RMN spectra of 4',5,7-trihydroxyflavone (apigenin), in DMSO-d_6, 300 MHz

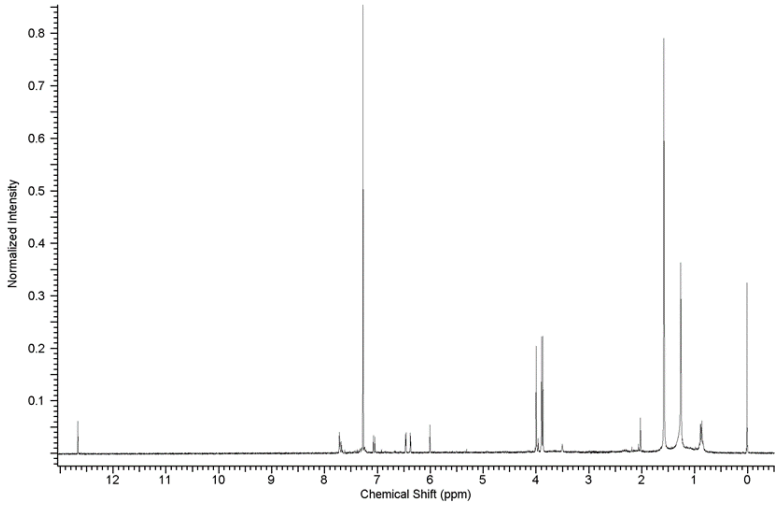

Figure No. 8
^{1}H-RMN spectra of quercetin-3,7,4'-trimethylether (pachypodol), in $CDCl_3$, 300 MHz

Toxicity to Artemia salina

C. comosa toxicity against *Artemia salina* showed a lethal concentration of 50% of the individuals (LC_{50}) higher than 1000 µg/mL, indicating that none of evaluated extracts was toxic. Thus, the result of this test showed that *C. comosa* extracts

are non-toxic, which can be correlated mainlywith of flavonoids, whose presence already demonstrated in the chemical analysis above. Most flavonoids are classified as safe for humans to eat and possess low toxicity being also active for different biological properties (Tapas *et al*., 2008; Akroum *et al*., 2010).

Antioxidant activity

Results from antioxidant test were interpreted as showing very active or active and moderate activity, when compared to ascorbic acid (AA) via the standard calibration curve. Branch and bark methanolic extracts showed moderate and active activity in the DPPH assay, respectively (Table No. 1). For the Fe^{3+}/Phenanthroline assay, leaves and branch methanolic extracts showed moderate activity, while the bark methanolic extract was active (Table No. 2). Other extracts showed no activity, regardless of the method used.

Table No. 1
Absorbance values for antioxidant activity of *C. comosa* extracts by the DPPH method. Data are expressed as ascorbic acid standard equivalent

Extracts		Method using DPPH▪						
		Media Values			Standard Deviation			Activity
		$[\Delta ABS_{517}]$	$[AA]_{eq}$	Equiv.	$\lvert\Delta ABS_{517}\rvert$	$[AA]_{eq}$	Equiv.	
	Leaves	0,006	-0,015	-102,877	0,004	0,034	418,845	NA
Hexane	Branches	0,001	-0,058	-95,818	0,002	0,020	40,583	NA
	Barks	0,004	-0,038	-179,379	0,003	0,027	102,768	NA
	Leaves	0,123	0,977	5,117	0,002	0,017	0,089	NA
MeOH	Branches	0,209	1,709	2,933	0,012	0,103	0,171	MO
	Barks	0,349	2,897	1,727	0,009	0,072	0,043	AT

Legend: NA: not active; MO: moderate active; AT: active.

Table No. 2
Absorbance values of antioxidant activity by the Fe^{3+}/phenanthroline method of *C. comosa* extracts. Data are expressed as standard ascorbic acid equivalent

Extracts		Method using Fe^{3+}/phenanthroline						
		Media Values			Standard Deviation			Activity
		$[Fe^{2+}]$	[AA]eq	Equiv.	$[Fe^{2+}]$	[AA]eq	Equiv.	
	Leaves	0,0032	0,075	73,14	0,020	0,031	24,139	NA
Hexane	Branches	0,000	0,123	41,84	0,016	0,024	9,254	NA
	Barks	0,010	0,138	38,16	0,028	0,042	9,902	NA
	Leaves	1,051	1,707	2,93	0,024	0.036	0,061	MO
MeOH	Branches	0,976	1,593	3,15	0,071	0,108	0,209	MO
	Barks	1,715	2,707	1,85	0,032	0,049	0,033	AT

Legend: NA: not active; MO: moderate active; AT: active

Results obtained showed antioxidant activity mainly in bark-derived methanolic extracts, possibly dueto the presence of flavonoids.

The effectiveness of substances to act as antioxidant is characteristic of hydroxyl groups present in molecules with an aromatic ring, such as flavonoids and coumarins (Olszowy *et al*., 2019). Thus, the *C. comosa* methanol extract antioxidant activity is confirmed by the presence of phenolic substances, revealed as dark spots by $FeCl_3$ and intense yellow inflorescence by NP/PEG, as well as by the isolation of the flavonoids apigenin and quercetin-3,7,4'-trimethylether, in which the substance apigenin, already has shown to have antioxidant activity (Shahidi & Ambigaipalan, 2015; Salehi *et al*., 2019). It is also possible that other substances not yet identified may contribute to the antioxidant action of *C. comosa* extracts.

Antibacterial activity

Antibacterial test results were expressed as a percentage of inhibition of the samples, compared to a standard antibiotic (oxytetracycline). Samples were evaluated at a concentration of 1000 µg/mL, and inhibition in microorganismal growth calculated according to the spectrophotometric reading. According to the data obtained, and interpretation of the scale in Holetz *et al*. (2002), *C. comosa* extracts showed weak antibacterial activity.

The highest percentages of inhibition were achieved by methanolic extracts from branches and bark, with 69% and 52% inhibition, respectively for *Pseudomonas fluorescens* (Table No. 3). No inhibition was observed from the leaves methanol extract.

The analysed *C. comosa* extracts showed a maximum inhibition of 69% for the gram-negative strain at a concentration of 1000 µg/mL.

Table No. 3
Inhibition percentages, obtained by extracts of *C. comosa* against gram-negative bacteria strains

Extracts		Microorganisms				
		P. fluorescens	*S. marcescens*	*S. enterica*	*A. hidrophyla*	*K. pne*
Hexane	Leaves	-	7%	26%	-	
	Branches	-	27%	-	45%	
	Barks	-	11%	-	-	2
MeOH	Branches	69%	-	-	-	
	Barks	52%	-	-	-	

Antiangiogenic activity

Tested *C. comosa* extracts showed antiangiogenic activity (Figure No. 9S), with the leaves methanolic extractbeing active in concentrations of 100, 500 and 1000 µg/mL, producing 15, 75 and 85% of blood vessel inhibition formation, respectively. Lower antiangiogenic activity was observed for branches methanolic extracts 15% of inhibition vessels at concentrations of 100 and 500 µg/mL and 60% at 1000 µg/mL). Barks methanolic extract was not active at a concentration of 100 µg/mL but showed 10% and 30% of inhibition at concentrations of 500 and 1000 µg/mL, respectively (Figure No. 10).

The leaves hexane extract showed high antiangiogenic activity, with inhibition at concentrations of 100 and 500 µg/mL, decreasing 80% and 100% of new blood vessels respectively.

However, the concentration of 500 µg/mL was considered high, while at a concentration of 1000 µg/mL, the extract was toxic as it killed the embrio. The hexane, branch and bark extracts were toxic at a concentration of 100 µg/mL (Figure No. 11).

Various natural substances, such as phenolics and terpenoids, have significant antiangiogenic effects, with different action mechanisms being considered as promising options for the development of strategies or drugs aimed at pathological angiogenesis (Lewandowska *et al*., 2014; Ribeiro *et al*., 2018). Among the substances isolated from *C. comosa* described in this research, apigenin and lupeol are considered to have important, but differing, pharmacological properties, among them capacity as potent angiogenesis inhibitors (Fang *et al*., 2006; Siddique & Saleem, 2011; Soares *et al*., 2017).

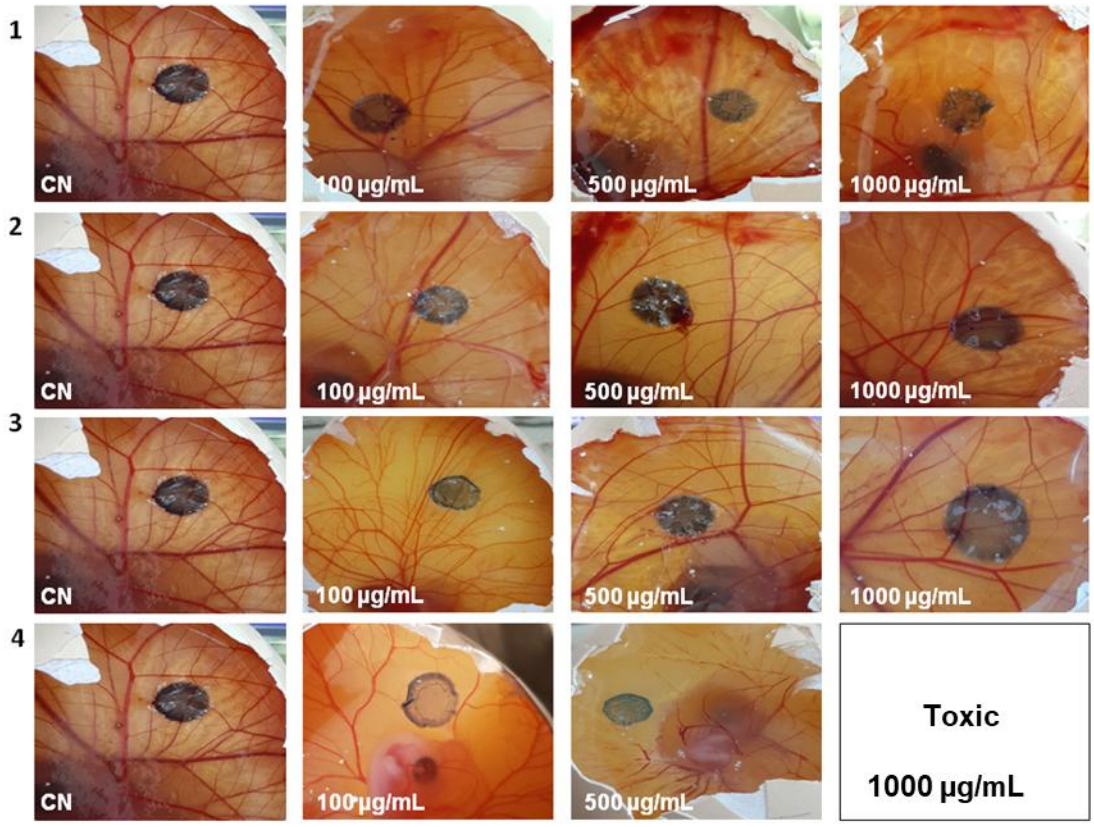

Figure No. 9
Photographs of the *in vivo* test of the antiangiogenic activity of *C. comosa* extracts. (1) leaves methanol extract; (2) branches methanol extract; (3) barks methanol extract; (4) leaves hexane extract

Apigenin has inhibitory effects on hypoxia-induced vascular endothelial growth factor mRNA (VEGF), against the human endothelial umbilical artery (HUVEC), human prostate cancer cells (PC-3, DU 145, LNCaP), of the colon (HCT-8), breast (MCF-7), and pancreas, among others (Osada *et al*., 2004; Fang *et al*., 2006; Melstron *et al*., 2011).

Lupeol also has antiangiogenic potential, confirmed in *in vivo* and *in vitro* assays. This terpene substance is effective against human melanoma cells (SK-MEL-2), lung carcinoma (A549), and murine melanoma (B16-F10) (You *et al*., 2003). In an *in vivo* assay by the HET-CAM method, lupeol inhibited blood vessels by 100 µg/mL (39.41%) and 250 µg/mL (44.12%), which was more active than the current antiangiogenic drug bevazicumab (Avastin®) at a concentration of 250 µg/mL with 24.62% inhibition of the vessels. Lupeol can inhibit the formation of new blood vessels and the deregulation of angiogenic genes such as MMP-2 and 9, VEGFa, fkt-1 and HIF-1a that are associated with the ability to form tumors (Vijay-Avin *et al*., 2014).

Thus, *C. comosa* could be an important focus for the development of antiangiogenic agents, but complementary tests are needed to determine their action mechanism.

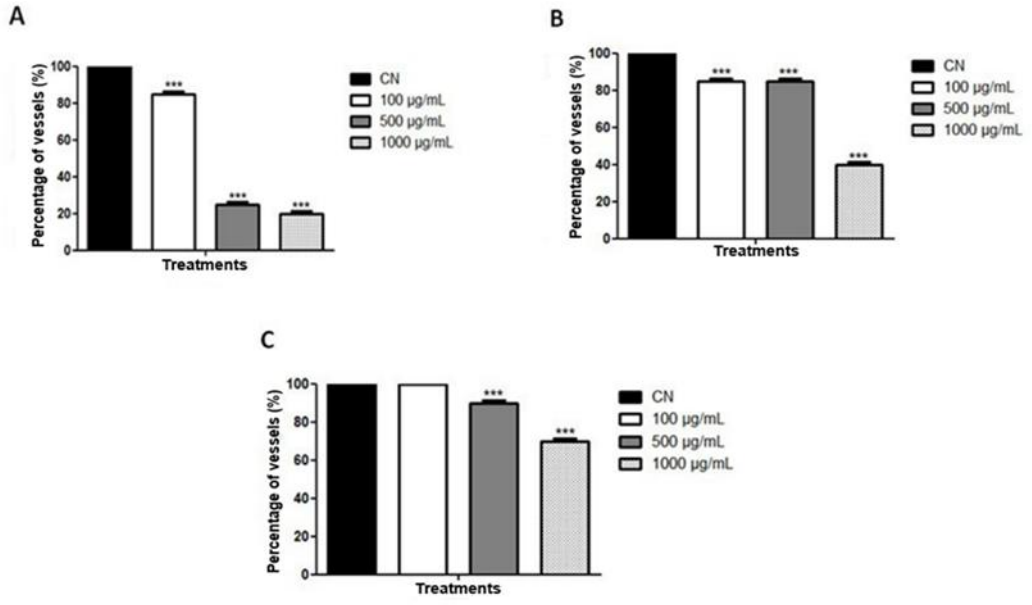

Figure No. 10
Antiangiogenic activity of *C. comosa* methanol extracts: (A) leaves; (B) branches and (C) barks. Results were expressed as mean ± standard deviation, n=3. (***) represents a statistically significant difference (p<0.001) in relation to the negative control (CN)

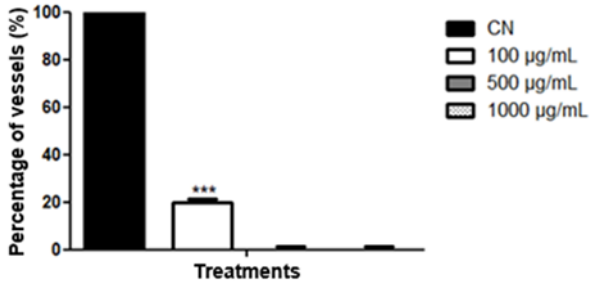

Figure No. 11
Antiangiogenic activity of *C. comosa* leaves hexane extract. The results were expressed as mean ± standard deviation, n=3. (***) represents a statistically significant difference (p<0.001) in relation to the negative control (CN)

CONCLUSION

This is the first report in the literature on the isolation and identification of substances from *Campsiandra comosa*, in addition to the studies of toxicity, antioxidant, antibacterial and antiangiogenic potential of their extracts. The current study revealed that there are differences in the action of the extracts, either by plant part used and/or by type of extract. The isolated compounds, in turn, showed a relationship with the properties identified in the extracts, for example, apigenin that correlates its potential with antioxidant activity and lupeol with antiangiogenicactivity.

The biological and chemical properties found in the *C. comosa* extracts should, therefore, encourage further phytochemical studies.

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