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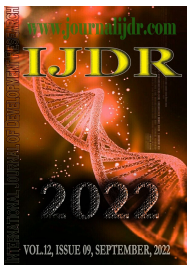

RESEARCH ARTICLE OPEN ACCESS

ISOLATION AND STRUCTURAL ELUCIDATION OF SECONDARY METABOLITES FROM *ALLOPHYLUS SEMIDENTATUS* OF THE EXTRACTS AND THEIR ANTIMICROBIAL, ANTIOXIDANT AND CYTOTOXIC ACTIVITY

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ABSTRACT

Some Sapindaceae plants are used in folk medicine, food and also in the pharmaceutical field. Allophylus semidentatus (Sapindaceae) is a wild plant that is being studied for the first time. In this investigation, the metabolites were extracted by using polar solvent as methanol, hexane, chloroform and ethyl acetate. Chromatographic and spectroscopic techniques were used for the isolation and identification of compounds. Extracts from the branches revealed the presence of steroids/triterpenes, flavonoids, alkaloids and coumarins. Metabolites were isolated by chromatography. The results of ^{1}HNMR and ^{13}CNMR showed the presence of four compounds allantoin, friedelin, campesterol and β-sitosterol. These compounds have been isolated from this plant for the first time. The anticancer, biological and antimicrobial activity of the extracts were also studied. This study highlights the bioactive potential of *Allophylus semidentatus* fractions which can lead to the development of drugs intended for the treatment of cancer.

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INTRODUCTION

Allophylus semidentatus (Miq.) Radlk. belongs to the family Sapindaceae. It is native to Brazil, but not endemic, for its occurrences have been confirmed in the North (Acre, Amazonas, Rondônia), Northeast (Alagoas, Bahia, Ceará), Midwest (Goiás, Mato Grosso), Southeast (Minas Gerais, Rio de Janeiro and São Paulo) and in the South region (Paraná). According to Somner *et al*. (2015), the plant presents phytogeographic domain in Amazonas, Cerrado and Atlantic Forest. Plants of the genus *Allophylus* form the basis of folk medicine in some Asian countries, such as the species *Allophylus serratus*, used in the Indian medical system as an anti-inflammatory and carminative (POONAM *et al*., 2005), and the species *Allophylus Cobbe* L., an herb which grows wild in the mountainous region of Bangladesh. It contains anti-inflammatory activity and is used as an anti-diarrhea agent by health professionals in the east-west region of Bangladesh (TOREQUI, 2012). Due to the lack of information in the technical and scientific literature on the chemical composition and biological activities of the species *Allophylus semidentatus* (Miq.) Radlk., it becomes indispensable to study the anticancer, antioxidant and antimicrobial activity of the extracts, as well as to evaluate their cytotoxicity against cancer cells.

Thus, the present study describes the isolation and structural elucidation of allantoin, friedelin, campesterol and β-sitosterol.

MATERIAL AND METHODS

Plant material: The botanical material collection was carried out in the Capão do Cifloma region at the Botanical Campus of the Federal University of Paraná, Curitiba, Paraná state, Brazil, in July 2018. All the plant material was obtained from three individuals, in a non-sterile phase. Witness material was identified by the taxonomist José Tadeu Weidlich Motta and deposited in the Curitiba Municipal Botanical Museum under registry number MBM 304886. This study was authorized by the National Genetic Heritage Management System (SISGEN) under the AEE0D2F process.

Preparation of extract, fraction and isolates: The plant material of the species *Allophylus semidentatus* (2000g of twigs) was dried, crushed and placed in a Soxhlet equipment with EtOH (6L to 80%, 10 days). This equipment was coupled to a ball condenser (at the top) and a flask (at the bottom) containing 4 glass spheres. The solvent used to perform the extraction was ethanol at 96 °GL. This system was subjected to heating, by means of a blanket.

After the beginning of the reflux, the plant material (branches) was left in extraction for several periods of 6 hours in order to obtain the ethanolic extract. The ethanolic crude extract was concentrated in a roto evaporator until a fluid extract was obtained and did not present any alcohol odor, after which fractions were obtained by a liquid-liquid partition system. Solvents that have different analytical standards and polarities were used in the following order: hexane, chloroform and ethyl acetate. A modified Soxhlet equipment (PI 06011703-7 A2) was used to perform the fractionation. The equipment contained the concentrated crude extract which was connected to a ball condenser and a flask containing glass spheres. The first solvent (hexane) was placed in the flask and refluxed for several hours. This same procedure was repeated for the rest of the solvents. The final extract was called the hydroalcoholic fraction. This procedure was performed for all collected material (branches).

Qualitative phytochemical analysis: The fractions of *Allophylus semidentatus* was screened for the presence of phytochemicals constituents such as saponins (Frothing test), tannins (NaOH test), coumarins (alkaline KOH test), alkaloids (Dragendorff test), flavonoids (Magnesium test) and steroids (Liberman-Bouchard test) as well as triterpenes for qualitative analysis.

NMR Spectrum of ethyl acetate fraction: NMR spectrum of isolated *Allophylus semidentatus* obtained from the ethyl acetate fraction (2 mg/ml of D_2O) was recorded by using BRUKER DPX 200 spectrometer, Nuclear Magnetic Resonance equipment, Bruker DPX 200, operating at 4.7 Tesla and observing the ^{1}H and ^{13}C nuclei at 200.13 and 50.62 MHz, respectively, in D_2O solution. The experiments were run at room temperature in which the solvent (HOD) peak did not interfere with any peaks. After dissolution, 1 ml of the *Allophylus semidentatus* solution was transferred to 5 mm NMR tube. The sample tube was inserted in the magnetic field and allowed to reach thermal equilibrium for 10 min before performing the experiment.

Bacterial strains: Four human clinical bacterial strains were selected for the present study, namely *Escherichia coli, Staphylococcus aureus, Pseudomonas aeurginosa* and *Candida albicans*. They were all obtained from the Microbiology Laboratory at the Department of Pharmacy in Ponta Grossa State University, Paraná, Brazil.

Inoculum preparation: Nutrient broth was prepared in test tubes and autoclaved at 15 lbs pressure for 15 min. All bacterial strains were separately inoculated in the sterilized nutrient broth and incubated at 37 °C for 24 h.

Antibacterial activity: The antimicrobial activity of the ethyl acetate fraction of *Allophylus semidentatus* was assessed by the determination of the Minimum Inhibitory Concentration (MIC) by the broth microdilution technique (CLSI, 2015). The concentration range varied from 125 µg/mL to 4000 µg/mL. The final volumes of the test tubes: 200 µL: 100 µL of the extract or diluted fraction + 100 µL of the microbial suspension.

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH): The method was performed as described by (MENSOR *et al*. 2001). The methanolic solution of DPPH was prepared at the time of use at a concentration of 0.03mmol/mL. Ascorbic acid samples and master solutions were also prepared at a concentration of 1mg/mL. From this concentration, different dilutions were carried out in such a way that each curve had a minimum of 5 points and included the IC_{50} concentration (necessary concentration to reduce the initial concentration of DPPH by 50%). The reaction was carried out in a microplate with a flat bottom of 96 wells, placing 142µL of each diluted solution with the addition of 58 µL of DPPH solution. For each sample and master, a blank was prepared containing 142µL of the solution of each concentration and 58µL of methanol. A control was also prepared containing 142µL of methanol and 58µL of DPPH solution. After 30 minutes of reaction under the protection of light, the reading of the absorbances of the samples was performed in triplicate in a spectrophotometer with a wavelength of 518nm, corresponding to the maximum absorption of the radical in the study. The percentage of antioxidant activity (AA%) was calculated as a percentage from the following equation:

AA% = [(Abs. of sample – Abs. of blank) x 100] / (Abs. of control – Abs. of blank)

Evaluation of toxicity against Artemia salina: Eggs from the crustacean of the species Artemia salina were acquired and the test was prepared according to the methodology cited by Meyer, et al. (1982). Crustacean eggs were hatched in saline water, prepared with 14.31g of sea salt and dissolved in 400mL of purified water. For this amount, 200mg of crustacean eggs were added. The acidity of the water was regulated to a pH between 8.0 and 9.0 to avoid the death of the crustaceans. The hatching temperature was controlled between 27 and 30°C and the saline solution was constantly stirred and aerated for a period of 48 hours. During the whole process, lighting (20W) was maintained on the hatching container. Subsequently, solutions were prepared with the extracts and fractions at concentrations of 1000µg/mL, 750µg/mL, 500µg/mL, 250µg/mL and 100µg/mL, in triplicate, 10 crustaceans were placed in each jar in order to observe the live crustaceans. The flasks with the solutions of different concentrations were placed in an oven for the elimination of the solvent (methanol), including the flasks that were used as control, which only contains the solvent used for the dissolution of the samples. After evaporation of the solvent, 10 crustaceans were added to each bottle in an approximate volume of 2.5 mL of saline solution using a glass dropper. After 24 hours, it was observed how many of the crustaceans died in the presence of each of the concentrations analyzed. For the determination of the LC50, the statistical program IBM SPSS Statistics 21 (Probit) was used with a confidence interval of 95%. The fractions will be considered active when the LC50 is less than 1000µg/mL (MEYER et al., 1982). The analysis of variance was evaluated by the ANOVA test and the statistical difference was by the results obtained by the Tukey test, being for the values of $p<0.05$, they were considered significant.

Evaluation of hemolytic activity: For the determination of the percentages of hemolysis, it was carried out according to the method of Banerjee et al. (2008), adapted. 5mL of commercial sheep blood, previously homogenized with slight agitation, were transferred to a centrifugation test tube for a period of 5 minutes at 3000rpm with 3mL of cold PBS solution (10°C). It was necessary to perform 7 washes with PBS solution, where the supernatant was discarded. In the last wash, the supernatant was colorless. At this stage, a 2% dilution was made with the erythrocytes that were at the bottom of the test tube, PBS solution was used as a diluent. To carry out the hemolysis test, Eppendorf with 100% hemolysis (positive control) were prepared, using a 1% triton solution, basal hemolysis (blank), control with the solvent and the samples. In carrying out the positive control, 200µL of drinking water was pipetted into 200µL of 2% erythrocyte solution. While for the blank, 200µL of PBS solution was pipetted into 200µL of 2% erythrocyte solution. In the solvent control, 20µL of ethanol plus 180µL of PBS solution were added, in 200µL of 2% erythrocyte solution. For sample preparation, solutions of 1000µg/mL, 500µg/mL, 200µg/mL and 100µg/mL were prepared in 200µL of 2% erythrocyte solution. For a better dissolution of the samples, 10% ethanol of the total volume was added to each of the samples. Both the samples, the controls and the blank were performed in triplicate.

Subsequently, the Eppendorf were homogenized with a slight manual agitation. After this, they were incubated for a period of three hours in an oven with controlled temperature at 37°C. At the end of the incubation, the Eppendorf were centrifuged at 3000 rpm for 5 minutes. The absorbance reading was performed in a 96-well Elisa microplate, from which 200µL of the supernatant of the samples were pipetted. The reading was performed in a Multiscan FC microplate spectrophotometer from the company Themo Scientific®, at a wavelength of 540nm.

Cytotoxicity assay: The cytotoxicity of the extract and fractions was tested on cell lines and the cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide method (SHAYNE *et al*. 1999). Confluent cultures in 96-well plate were exposed to different concentrations of the extract and fractions (10 -1000 µg/ml) with three wells for each concentration. Then 10 µl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 hours of incubation at 37°C, the supernatant was removed, and 200 ml of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm.

Anticancer activity _ MTT assay: The anticancer activity of the extract and fractions against H295R cell lines by using MTT assay were carried out (SHAYNE *et al*. 1999). Cells were seeded (3 x 10^4/well) inside 96-well plates in 100 mL of growth medium (MEM) containing 10% FCS mixture in each well incubated at 37°C in a 5% CO_2 incubator. After 24 hours of monolayer cell cultivation, the medium was removed and replaced by a 100 mL of varying concentrations (10-1000 µg/mL) of the fetal bovine serum in MEM medium containing 2% FCS in respective wells. Control cells were maintained in MEM medium containing 2% FCS incubated at 37°C in a 5% CO_2. After 72 hours of incubation, 20 mL of MTT (5 mg/mL) in PBS solution/well were added and incubated at the above said condition for 4 hours, and then were observed for the crystal formation. The medium was replaced by 100 mL of DMSO solution in each well and the Optical Density (OD) of each well was measured by using an Elisa reader at 620 nm. All experiments were carried out in triplicate and the data in the form of mean ± SD were presented.

Statistic analysis: The analysis of variance was evaluated by the ANOVA test and the statistical difference was by the results obtained by the Tukey test, being for the values of $p<0.05$, they were considered significant.

RESULTS AND DISCUSSION

Phytochemical analysis: The phytochemical constituents of *Allophylus semidentatus* was qualitatively estimated and the results were displayed in Table 1. In addition, study Steroids and Triterpenes were observed at high level, Flavonoids and Coumarins were documented at medium level, and the Tannins, Saponins and alkaloids were absent.

Purification of metabolites through column chromatography: The crude ethanol extract was concentrated and partitioned with hexane, chloroform and ethyl acetate, then the solvent was evaporated to obtain the fraction powder. The hexane, chloroform and ethyl acetate extracts were further subjected to silica gel CC eluting with n-hexane/ ethyl acetate (4:6-0:1 v/v) and then with ethyl acetate /MeOH (9.5:0.5-0 :1 v/v) to give three fractions (Fr.1-3). Fr. 1 was carried out on CC silica gel eluted with n-hexane/ethyl acetate (4:6-0:1 v/v) to obtain 1 (18.6 mg). Fr. 2 and Fr. 3 were carried out on CC silica gel eluted with ethyl acetate /MeOH (9.5:0.5−0:1 v/v) to obtain 2 and 3 (12.8 and 1.46mg, respectively).

NMR Spectrum of fractions (1-3): The ^{1}HNMR spectrum (Fr.1) (200 MHz, d6 DMSO) (Fig. 1) of the isolated beige powder showed signals with chemical shifts (ppm) in various regions of the spectrum. Three signals could be observed and corresponds to the integration of a proton, two signals in the form of a singlet with shifts of 10.58 and 8.08ppm, and a doublet with a shift of 6.92ppm (J=8.01Hz). In the central region of the spectrum, a singlet with a shift of 5.82ppm could be observed, which corresponds to two protons. Lastly, we could see a doublet with a shift of 5.25ppm (J=8.01Hz), which corresponds to a proton. This last displacement was coupled with the proton signal with 6.92ppm, that being the only correlation between the neighboring protons of the spectrum. In the ^{13}CNMR spectrum (Fr.1) (50 MHz), (DMSO d6) (Fig. 2) four signals could be observed, three of them with chemical shifts at (173.80, 157.40 and 156.83ppm) which correspond to carbons of amide-type carboxylic groups and another displacement at 62.44ppm.

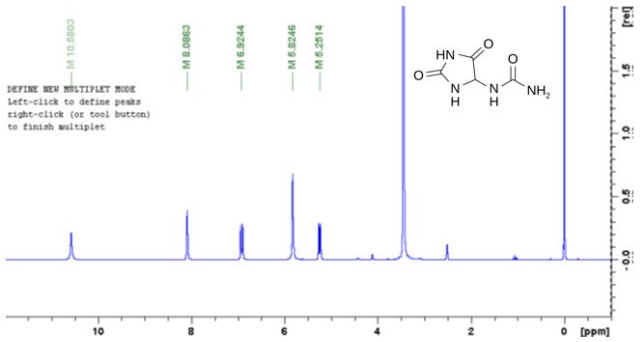


Figure 1. Structural conformation Allantoin through 1HNMR analysis

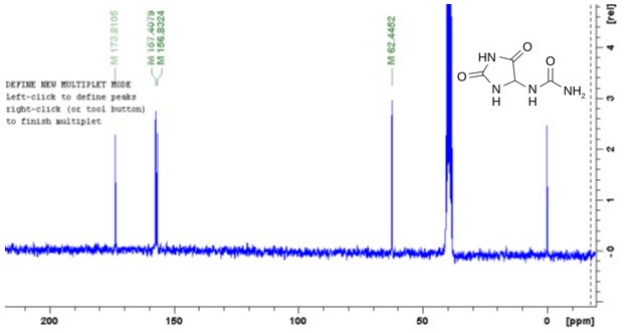


Figure 2. Structural conformation Allantoin through ^{13}CNMR analysis

Two of the three signals correspond to carbon bonded to electronegative atoms that donate electronic density by resonance effect that causes shielding of the carbon nucleus, such as nitrogen. Based on the ^{1}H and ^{13}C NMR spectral data, by comparing them with those found in the literature (FERREIRA, 2000), the isolated compound (Fr.1) was identified as 2,5-dioxo-4-imidazolidinyl urea, which is commonly known as Allantoin. The ^{1}HNMR spectrum (Fr.2) (200 MHz, $CDCl_3$) (Fig. 3) of the isolated needle-shaped crystals showed signals with chemical shifts (ppm) in various regions of the spectrum, in which seven singlets between δ 0.75 and δ 1.37 (δ 0.75, δ 0.92, δ 0.98, δ 1.03, δ 1.21, δ 1.28 and δ 1.37) indicated the presence of methyl groups in the molecule at carbons C-24, C-25, C-29, C-30, C-26, C-27 and C-28, respectively, suggesting that it may be a triterpene. The signal at δ 0.89 showed the presence of a doublet attributed to the methyl hydrogens located at position 23.

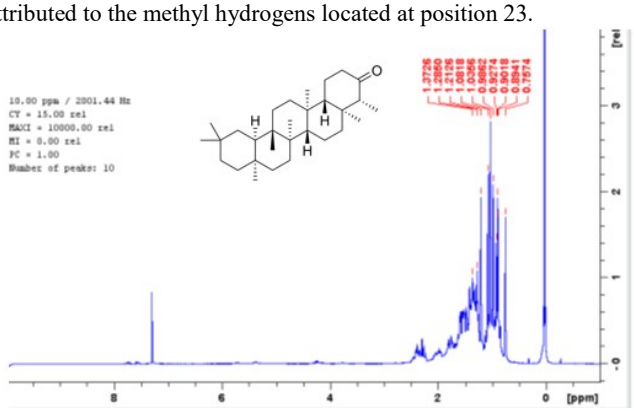


Figure 3. Structural conformation Friedelin through ^{1}HNMR analysis

In the ^{13}CNMR spectrum (Fr.2) (50MHz), CDCl3) (Fig. 4) several signals corresponding to carbons could be observed, which presented signals below 60 δ, characteristic of carbons of the sp^3 type. The signal at δ 213.21 was attributed to the C-3 carbonyl group corresponding to the keto group. On the other side, the signal at δ 6.81 indicated the presence of a C-23 methyl group, which is quite protected, attributed to the γ-gauche effect promoted by the carbonyl.

The spectral data of the substance (Fr.2) compared to those identified in the literature (ALMEIDA *et al.*, 2011; ARAGÃO *et al.*, 1990), suggest that the chemical structure of the isolated substance was triterpene pentacyclic 3-oxo-friedelan, which is commonly known as Friedelin.

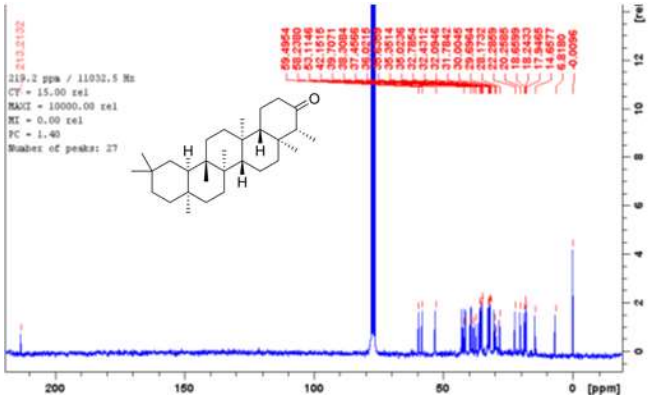


Figure 4. Structural conformation Friedelin through ^{13}CNMR analysis

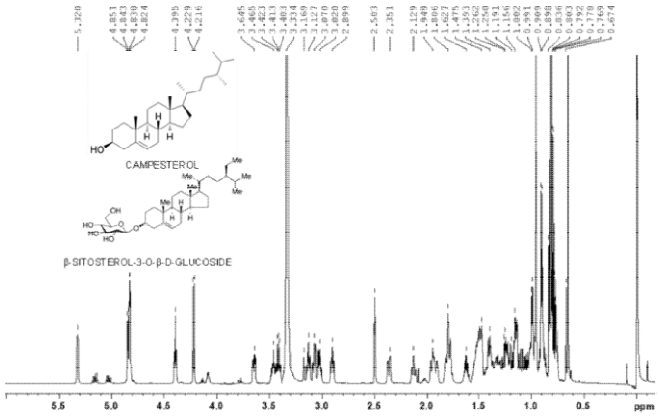


Figure 5. Structural conformation β-sitosterol-3-o-β-D-glucoside and campesterol through ^{1}NNMR analysis

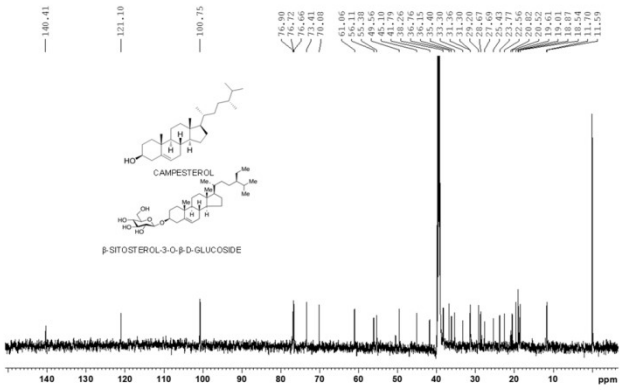


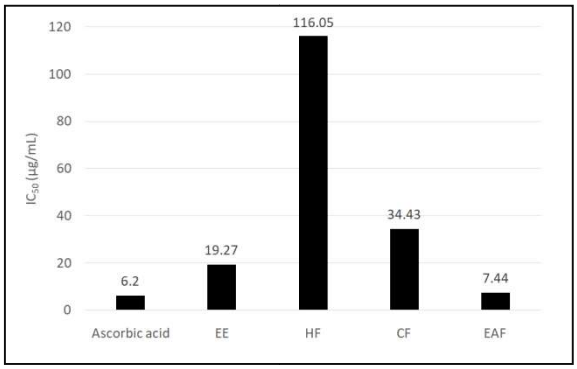
Figure 6. Structural conformation β-sitosterol-3-o-β-D-glucoside and campesterol through ^{13}CNMR analysis

The ^{1}HNMR spectrum (Fr.3) (200 MHz, $CDCl_3$) (Fig. 5) showed a chemical shift in the range of 0.89-0.99 ppm, suggesting the presence of methyl protons (12H, H-21, 26, 27, 29). A singlet observed at 0.67 ppm was attributed to methyl (H-18) protons. A proton bound to the olefinic bond was observed at 5.32 ppm. The signals observed at 0.83 and 0.89 ppm were attributed to methyl protons of the isopropenyl fraction. The methyl proton (H-21) was observed at 0.99 ppm with coupling constant J = 6.5 Hz. The glucose proton was observed at 2.89-3.64 ppm as a multiplet. The proton of the -CH glycoside group was observed at 4.21 ppm.

Proton H-3 was observed at 2.90 ppm. The hydroxyl protons of the sugar fraction (β-sitosterol-3-o-β-d-glycoside) showed resonance at 3.46-3.64 ppm (J = 4.5 Hz) and were assigned to C-2', C-3'C-4' and C-6' respectively(ANDRADE, 2003; PESHIN, 2017). In the ^{13}CNMR spectrum (Fr.2) (50MHz), $CDCl_3$) (Fig. 6) of the compound 35 carbon signals were indicated, in which 6 were for the sugar portion and 29 were assigned to the sugar portionaglycone. The carbon signals of the sugar fraction observed at 61.06 (C-6'), 70.08 (C-4'), 73.41 (C-2'), 76.66 (C-5'), 76 .72 (C-3') and 100.75 (C-1') ppm were quite consistent with those of the glucose portion. The aglycone portion signals were observed at 140.41 (C-5), 121.10 (C-6), 76.90 (C-3), 56.11 (C-14), 55.38 (C- 17), 49.56 (C-9), 45.10 (C-24), 41.79 (C-13), 39.28 (C-4), 38.26 (C-12), 36, 76 (C-1), 36.15 (C-10), 35.40 (C-20), 33.30 (C-22), 31.36 (C-7), 31.30 (C-8), 29.20 (C-2), 28.67 (C-25), 27.69 (C-16), 25.43 (C-23), 23.77 (C-15), 22.56 (C-28), 20.82 (C-11), 19.61 (C-26), 19.01 (C-19), 18.87 (C-27), 18.54 (C-21) , 11.70 (C-29) and 11.70 (C-18) ppm. These data confirmed that the compound is a mixture of β-sitosterol-3-o-β-D-glucoside and campesterol.

The chemical shift of 19.61 and 18.87 ppm was attributed to two methyl groups. The signals observed in the fields 11.70, 19.01 and 18.54 ppm were attributed to the methyl group angular moiety linked at C-18, C-19 and C-21. The signals at 140.41, 36.15 and 41.79 ppm were assigned to the quaternary carbon at point C-5, C-10 and C-13. The signals observed at 31.30, 49.56 and 56.11 ppm were attributed to protons at C-8, C-9 and C-14, respectively. The chemical change at 36.76, 29.20, 38.26, 31.36, 49.56, 20.52, 38.26, 56.11, 23.77, 27.69, and 55.38ppm were attributed to the cyclohexyl and cyclopentyl of rings A, B, C and D. The cyclic hinge carbon linked to the side chain was observed at 35.40 ppm. The anomeric carbon 100.73 (C-1') and proton (H-1') at 4.21 with 76.90 ppm, respectively, confirmed the glycemic moiety at position 3.

Antibacterial activity: The antibacterial activity of branches fractions was tested against four clinically isolated bacterial strains (Table 2). According to the classification used by Santos et al (2004), the ethyl acetate fraction of the branches showed significant activity against the bacterium S. *aureus* (125 µg. mL^{-1}), while the hexane and chloroform fractions showed moderate activity (250 µg. mL^{-1}) for the same bacterium. In relation to *P. aeruginosa*, the crude extract and the chloroform fraction showed weak activity (500 µg. mL^{-1}). The crude extract and the hexane, chloroform and ethyl acetate fractions showed moderate activity against the bacterium *C. albicans* (250 µg.mL^{-}1). These results may be related to the presence of triterpenes, steroids and flavonoids observed in the qualitative phytochemical analyses, as well as the metabolites isolated from the plant, which have proven antimicrobial activity in several studies (SOUZA *et al.*, 2014). The antimicrobial mechanism of triterpenes is not yet fully elucidated, but it is probably linked to the disruption of the plasma membrane, which causes the cell death of the microorganism (SALEEM *et al*. 2010).



EE: Ethanolic extract; HF: Hexanolic fraction; CF: Chloroform fraction; EAF: Ethyl acetate fraction

Graphic 1. DPPH radical scavenging activity of extracts and fractions of *Allophylus semidentatus*

DPPH radical scavenging activity: For the evaluation of the results of this analysis, the percentages of inhibition of the DPPH were calculated and, by linear regression, it was possible to calculate the

Table 1. Phytochemical composition of *Allophylus semidentatus* fractions

Name of the sample	Saponins	Tannins	Coumarins	Alkaloids	Flavonoids	Steroids Triterpenes
Hexanolic fraction	--	--	+	--	--	--
Chloroform fraction	--	--	+	++	+	++
Ethyl acetate fraction	--	--	++	--	++	+++

--: Absence; +: Low level; ++: Moderate level; +++: High level.

Table 2 Antibacterial activity of *Allophylus semidentatus* fractions

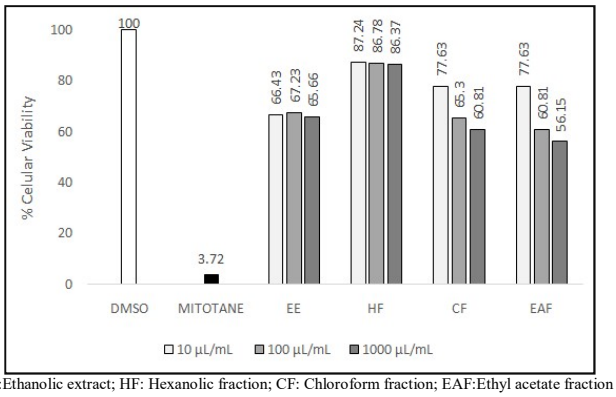
Microorganism	CIM (mg/mL)			
	EE	HF	CF	EAF
S. aureus ATCC 25923	500	250	250	125
E. coli ATCC 25922	100	100	1000	1000
P. aeruginosa ATCC 27853	500	500	1000	1000
C. albicans ATCC 40175	250	250	250	250

EE:Ethanolic extract; HF: Hexanolic fraction; CF: Chloroform fraction; EAF:Ethyl acetate fraction

Table 3. Toxicity test with *Artemia salina* with different concentrations of fractions of the branches of *Allophylus semidentatus*

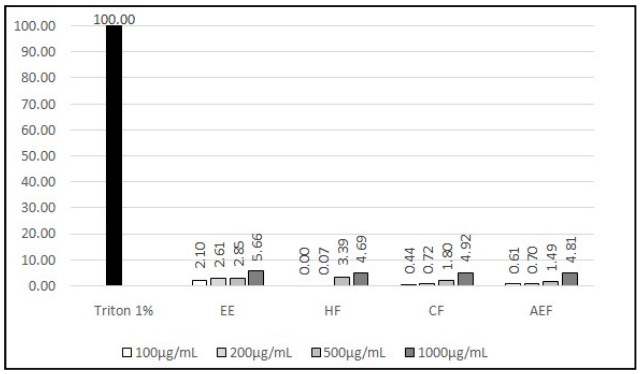
Lethality (µg/mL)						LC_{50} (µg/mL)	IC 95% (µg/mL)
Concentration	100	200	500	750	1000	>1000	
EE	0	1	4	6	12	722.87	------
HF	2	3	7	16	21	888.73	562,80-1024,60
CF	0	1	2	12	19	>1000	753,65-1156,85
EAF	0	0	1	3	13	>1000	------
SQ	-	-	-	-	-	142.27	122,48-186,91

EE: Ethanolic extract; HF: Hexanolic fraction; CF: Chloroform fraction; EAF: Ethyl acetate fraction; SQ: Quinidine sulfate



EE:Ethanolic extract; HF: Hexanolic fraction; CF: Chloroform fraction; EAF:Ethyl acetate fraction

Graphic 2. Inhibition of growth of H295R cells against extracts of the species *Allophylus semidentadus*



EE:Ethanolic extract; HF: Hexanolic fraction; CF: Chloroform fraction; EAF:Ethyl acetate fraction

Graphic 3. Percentage of hemolysis of the crude extract and fractions of the branches of *Allophylus semidentatus*

IC_{50}, which was the concentration of the sample that reduced the 50% of the initial concentration of the DPPH. The samples were compared with the pattern of ascorbic acid (Graph. 1)and showed the different IC_{50} values of the crude extract and fractions of *Allophylus semidentatus* obtained by the reduction of the DPPH radical. The crude extract and the fractions were compared to the standard of ascorbic acid, where the fraction with values closer to the IC_{50} of the ascorbic acid was the fraction of ethyl acetate from the branches, with 7.44µg/mL. This result agrees with the results of the phytochemical study, which is a positive reaction to the flavonoid determination method. As we already know, these are the metabolites responsible for the antioxidant action.

Evaluation of toxicity against Artemia salina: For a sample to be cataloged or characterized as toxic against microcrustaceans, it is necessary that the samples present an LD_{50} value of less than 1000 µg/mL according to Meyer et al. (1982). According to this, only the fractions that were slightly toxic were the hexanolic fraction and the chlorophomic fraction. Table 3 shows the relationship between the number of dead crustaceans and the LC_{50} calculated from the statistical program IBM SPSS Statistics 21 (Probit), for the branch fractions of *Allophylus semidentatus*

Evaluation of hemolytic activity: The fractions presented a percentage of hemolysis less than 5%, from which we could say that they did not present significant hemolytic activity.

These percentages are in relation to the positive control used (1% newt). Graph 2 shows the results.

Anticancer activity _ MTT assay: The (Graph. 3) shows the proliferation of H295R cells in the presence of the tested extracts and fractions, quantifying the amount of MTT reduced by a colorimetric method (EISENBRAND, 2002). At concentrations of 10 µL/mL and 100 µL/mL, growth inhibition around 70% was observed, except for the hexane fraction of the branches. These results were observed at 24 hours of incubation. The highest cytotoxicity inhibition rates were 56.15% and 60.81% at a concentration of 1000 µL/mL for the ethyl acetate and chloroform fractions of the branches, respectively. The presence of steroids and triterpenes in the chloroform fraction and ethyl acetate of the branches, as suggested by phytochemical analysis, may have an impact on the cell viability of several tumor cells (PATOCKA, 2003). Numerous steroids have been described regarding antitumor activity. It is believed that this activity is linked to aromatase inhibition and these inhibitors block the conversion of androgen to estrogen, which can induce the production of tumor cells (INÁCIO, 2012). Triterpenes seem to act by inhibiting DNA enzymes, which causes cellular apoptosis (VECHIA *et al.,* 2009).

Conclusion

The efforts undertaken in the present study clearly revealed the presence of biologically active compounds of secondary metabolites produced by the plant *Allophylus semidentatus*.

In the literature, it is reported that the isolated compounds show promising antibacterial, antioxidant and anticancer properties. No research studies have been reported on the medicinal properties of the plant *Allophylus semidentatus*. Thus, the present study was useful to know some of the secondary metabolites produced, as well as to carry out *in vivo* studies of the fractions obtained from *Allophylus semidentatus*.

Declaration of Competing Interest: The authors have no conflicts of interest to declare.

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