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Full Length Research Paper

Chemical profile, antimicrobial activity, toxicity on *Artemia salina* and anti-acetylcholinesterase enzyme essential oil from *Bauhinia ungulata* L. (Fabaceae) leaves

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Bauhinia ungulata L. species, belongs to the genus _Bauhinia_, popularly known as _pata de vaca_, is among the species of medicinal employ in Brazil, used to treat diabetes. The aim of this study was to characterize the chemical composition, antimicrobial activity, toxicity on _Artemia salina_ and acetylcholinesterase enzyme inhibition by essential oil from _B. ungulata_ L. The chromatographic analysis revealed 18 components, the majority were the β-caryophyllene (15.9%), caryophyllene oxide (9.2%), α-humulene (8.1%), epi-γ-eudesmol (7.5%), α-bisabolol (4.7%), copaene (3.5%), nerolidol (3.3%), α-bisabolol oxide B (2.5%), spathulenol (2.1%). The essential oil showed high toxicity compared to the tests with _Artemia salina_ and inhibited 95.96%±0.62 of the acetylcholinesterase enzyme. The microorganisms show antimicrobial inhibition with _Candida albicans_ (85%), _Bacillus cereus_ (65.5%) and _Staphylococcus aureus_ (66.4%), _Salmonella typhimurium_ (68.7%) and _Citrobacter freundii_ (46.1%). The oil showed great potential when tested in bioassays.

Key words: Volatile oil, cytotoxicity on *microcrustacean*, antifungal, antibacterial, neurodegenerative disease.

INTRODUCTION

Among many medicinal plants, there is the *Bauhinia ungulata* L. species, belonging to the genus *Bauhinia* belonging of Fabaceae family (Caesalpinioideae subfamily) one of the largest of angiosperms, which can be found mainly in tropical areas, with about 250-300 species (Souza and Lorenzi, 2008; Joly, 1993, 1998).

In Brazil this species is popularly known *pata de vaca*, *unha de boi*, *pé de boi*, *escada de macaco*, *unha de jaboti* and *mororó* (Silva and Cechinel, 2002; Lorenzi and Matos, 2002). It is applied in the treatment of diabetes mellitus, cholesterol control as diuretic and expectorant (Correa, 1998). It was also reported the use of *B. ungulata* by Tapebas Indians in *Ceará* state, Brazil for the treatment of diabetes, and this species most frequently studied for its hypoglycemic action (Silva et al., 2002; Pepato et al., 2002; Morais et al., 2005).

Species of this genus have shown molluscicidal activity (Singh et al., 2012), larvicidal activity against *Aedes aegypti* L. (Gois et al., 2011), antiviral against *Arbovirus mayaro* MAYV (Santos et al., 2014), antioxidants (Santos et al., 2014; Port's et al., 2013; Paula et al., 2014), inhibition of the acetylcholinesterase enzyme (Santos et al., 2011), antibacterial action (Cechinel-Filho, 2000, 2009), anti-helminth, in *B. variegata* against *Ferentima posthuma* and *Ascardia galli* (Bairagi et al., 2012) and antitumor activity tested in rats (Rajkapoor et al., 2003).

In addition to the genus activities, aforementioned, there are the major compounds and compounds highlighted, folow. The essential oil from the leaves of another species, *B. acurana*, has as main constituents spathulenol, sesquiterpenes, epi-α-cardinol and caryophyllene oxide (Gois et al., 2011). The major constituents of essential oil from *B. ungulata* are spathulenol and caryophyllene oxide (Gramosa et al., 2009).

Essential oils are a lipophilic moiety of the chemical composition of a plant. Generally, consisting of sesquiterpenes, monoterpenes and phenylpropanoids (Cunha et al., 2004). Aromatic drugs are frequently used to destroy infection causing agents such as bacteria and pathogenic fungi (Costa, 2002). They are also used in industries, food and cosmetics (Bizzo, 2009).

This study aims to characterize the main volatile chemical constituents using gas chromatography and biological activities by essential oil from *B. ungulata* leaves.

MATERIALS AND METHODS

Plant material and essential oil extraction

The leaves of *B. ungulata* were collected along the *Água Boa* River, near the BR 174, at 11 Km in *Boa Vista*, *Roraima*, Brazil, in January 2016 (dry season). The plant material was identified by José Ferreira Ramos (National Institute for Research in the Amazon, INPA), and a voucher specimen (272558) was deposited at the INPA Herbarium.

Fresh leaves (600 g) were cut into smaller pieces with scissors and putting in a Clevenger apparatus to obtain the essential oil by hydrodistillation. Water droplets were removed from the essential oil by anhydrous sodium sulfate and stored at -20°C before analysis (Rubiolo et al., 2010; Sefidkon, 2002).

GC/FID analysis

The essential oil was analyzed on a HP 7820A Gas Chromatograph (GC) equipped with a flame ionization detector (FID) using a capillary column (HP5 30 m × 0.32 mm × 0.25 µ, Agilent). Column temperature: 50°C (0 min) at 3°C min^{-1} up to 230°C. Gun: 250°C Split (1:30). FID Detector: 250°C. Carrier gas: hydrogen at 3 mL min^{-1}. Vol injection: 1 µL mL^{-1}. Essential oil was diluted at 1% in chloroform. Data acquisition software used was Compact EZChrom Elite (Agilent). The quantitative analysis was accomplished using standard areas from the chromatograms obtained by GC-FID.

Gas chromatography/mass spectrometry analysis

A GCMS-QP2010 ULTRA (Shimadzu) was used. Column: Rxi-1MS, 30 m × 0.25 mm × 0.25µ (Restek). Column Temperature: 50°C (3 min), 3°C min^{-1} to 250°C. Injector: 250°C Split (1:10), GC-MS interface at 250°C. MS detector (electron impact at 70 eV) temperature was 250°C. Carrier gas: helium at 1.5 mL min^{-1}. Vol injection: 1 µL. Essential oil was diluted at 0.1% in chloroform. Data acquisition software used was GC-MS Solution (Shimadzu) together with NIST11 library. Identification of peaks was made by comparison of the mass spectra obtained by GC-MS spectra with the NIST11 library and also by comparing the Kovats indices calculated by GC-FID and literature data.

Determination of toxicity on *Artemia salina*

The essential oil was solubilized in Tween 20 (1%) and saline supplemented with water to give concentrations (1000, 500, 250 e 125 µL mL^{-1}). They were transferred to tubes (3 mL) and added 10 organisms (nauplii *Artemia salina*). The tests were performed in triplicate for each concentration. Saline without extract was used as negative control also in triplicate and was subjected to the same experimental procedure. This system was incubated at room temperature for 24 h, with aeration and other tubes kept under illumination. After 24 h, the number of dead and live larvae in each tube was counted. Thereafter, the probability of mortality was calculated according to the formula:

$$\text{Mortality Probability (\%)} = \frac{r}{n} \text{x} 100$$

Where: r = number of dead nauplii; n = total number of *A. salina* in each tube.

It was given the lethal concentration 50% LC_{50}, using the statistical program Microsoft Excel 2010 (Meyer et al., 1982; Mclaughlin et al., 1993).

Acetylcholinesterase (AChE) inhibition assay

Aliquots of a working solution (25 µL) (sample in Tween/DMSO/30%) was added to microplate wells, positive and negative controls were also prepared. To the first five wells of

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a column (positive control), 25 µL of an eserine solution prepared at 10 mg mL^{-1} (in Tris/HCl at pH 8.0) was added. Thereafter, 25 µL of acetylthiocholine iodide (ATChI, Sigma A5751); the reaction mixture, 125 µL of 5'.5-dithio-bis (2-nitrobenzoate) (DTNB, Sigma D8130) and 50 µL of Tris/HCl (pH 8) containing 0.1% (m/v) bovine serum albumin were added to each well. Absorbance was measured at 405 nm every 1 min for 8 times. Then 25 µL (0.226 U mL^{-1}) of Electric eel AChE (type VI-S) provided by Sigma (C3389-500UN) in Tris/HCl were added to each well. Absorbance was measured at 405 nm by 9 times (Frank and Gupta, 2005; Ellman et al., 1961). Percentual inhibition was calculated using the formula:

$$\% \text{ inhibition} = 100 - \frac{(PCA)}{(PSA)} X\, 100$$

Where PCA = (absorbance of the sample with enzyme - absorbance of the sample without enzyme); PSA = (absorbance of negative control with enzyme - absorbance of negative control without enzyme).

Antibacterial and yeast assay

Two Gram-negative: *Salmonella typhimurium* (ATCC 13311) e *Citobacter Freundii* (ATCC8090), two bacterium Gram-positive: *Staphylococcus aureus* (ATCC 25923), and *Bacillus cereus* (ATCC 11778) and one fungus (yeast) *Candida albicans* (ATCC 18804) yeast were used in the assay. Concentrations assayed were 250, 125, 62.5, 31.25, 15.6, 3.9 and 1.95 µg mL^{-1} (Zacchino and Gupta, 2007). Samples were weighed and dissolved in DMSO to 500 mg mL^{-1}. 124 µL of this solution was added to a flask containing 2976 µL of BHI (Brain Heart Infusion) broth (working solution) for bacterium and 2976 µL of Sabouraud for yeasts. A pre-inoculum was prepared in which the bacteria and the yeast, stored under refrigeration, were transferred with a platinum loop to test tubes containing 3 mL of freshly made BHI broth. The tubes were incubated at 37°C for 24 h. Then, the pre-inoculum (500 µL) was transferred to tubes containing 4.5 mL of sterile distilled water. The tubes were homogenized and the concentration adjusted to 0.5 of McFarland turbidity standard (108 CFU mL^{-1}), thereby obtaining the inocula used in the bioassays.

Assays were performed in 96-microwell plates in triplicate. 100 µL of BHI broth was added to each well. In the first well, 200 µL of working solution were also added. The solution was homogenized and 100 µL transferred to the next well and so on until the last well, from where 100 µL was discarded. Then, 100 µL of microorganism inocula were added to wells. Eight different concentrations of each sample were tested. A positive control devoid of the working solution allowed us to examine microorganism growth. A negative control, which lacked the inoculum made it possible to discount the color coming from the working solution. A control plate containing 100 µL of BHI culture medium and 100 µL of sterile distilled water were added to the experiment as a control of BHI broth sterility.

Another control was also prepared, containing the standard antibiotics Ampicillin (antibacterial), miconazole and nystatin (antifungals) to observe the activity of these antibiotics over the microorganisms. Microorganism growth was measured in ELISA plate reader (492 nm) immediately after ending the experiment (0 h). They were incubated at 37°C and read again after 24 h of experiments, ending the test. Results were calculated as percentual inhibition using the formula:

$$\% \text{ inhibition} = 100 - AC1 - AC2 \times 100AH - AM$$

AC1 = absorbance of the sample; AC2 = absorbance of control sample; AH = absorbance of microorganisms in the control control and AM = absorbance of culture medium control.

RESULTS AND DISCUSSION

The yield of essential oils of *B. ungulata* in this study was 0.066%, higher than that obtained by Gramosa et al. (2009) who in their study developed this species in the Northeast, which was 0.007%. The yields of essential oils obtained from the genus *Bauhinia* varies between *bauhinia rufa* with 0.37% (Silva and Camara, 2014) and *B. acuruana* with 0.01% (Gois et al., 2011).

Table 1 shows the chemical components identified by chromatographic analysis GC-FID and GC-MS with their Kovats index and mass and Figure 1 shows chemical structures of the major constituents identified in the essential oil in *B. ungulata*.

The nine major components identified in this study are β-caryophyllene (15.9%), caryophyllene oxide (9.2%), α-humulene (8.1%), epi-γ-eudesmol (7.5%), α-bisabolol (4.7%), copaene (3.5%), nerolidol (3.3%), α-bisabolol oxide B (2.5%), spathulenol (2.1%). The structures are shown in Figure 1.

Figure 2 shows the chromatographic profile for *B. ungulata* oil. 18 components corresponding to 65.5% of essential oil composition were identified. Among the compounds identified, nine are majority corresponding to 61.1%.

In *Boa Vista*, *Roraima*, Brazil compared to studies Gramosa et al. (2009), the composition varies mainly in the major constituents. Secondly, according to Oliveira et al. (1998), plant species, in general, may present variations in relation to the yield and chemical composition of essential oils according to the part of the plant studied as well as their interactions with the environment, climate, micro-organisms and also genetic factors.

Gramosa et al. (2009) identified 13 compounds, which represent 95.9% of the essential oil content, the majority were spathulenol (47.7%), caryophyllene oxide (18.3%), humulene epoxide II (5.2%), β-caryophyllene (4.2%), α-humulene (3.5%) and α-copaene (2.9%).

Duó-Bartolomeu et al. (2014) identified: germacrene-D, biciclogermacrene, β-elemene, trans-cariofilene, α-humulene, espatulenol, trans-nerolidol, β-ionone, e β-elemene.

It is observed so spathulenol presented itself as the main constituents for the essential oil of *B. ungulata* in studies of Gramosa et al., (2009), with a percentage of 47.7. However in the present study this compound presents a lesser amount (2.1%).

According to Figueiredo et al. (2008) and Simões et al. (2004), depending on the part of the plant, type of collection, climatic conditions and extraction methods, essential oils, may suffer variations in their income.

In this study it was found that the highest percentage constituent was the β-caryophyllene (15.9%), a fact equivalent to the study of Neto (2006), where this substance was also the majority with 25.65%.

Oils with high concentrations of β-caryophyllene

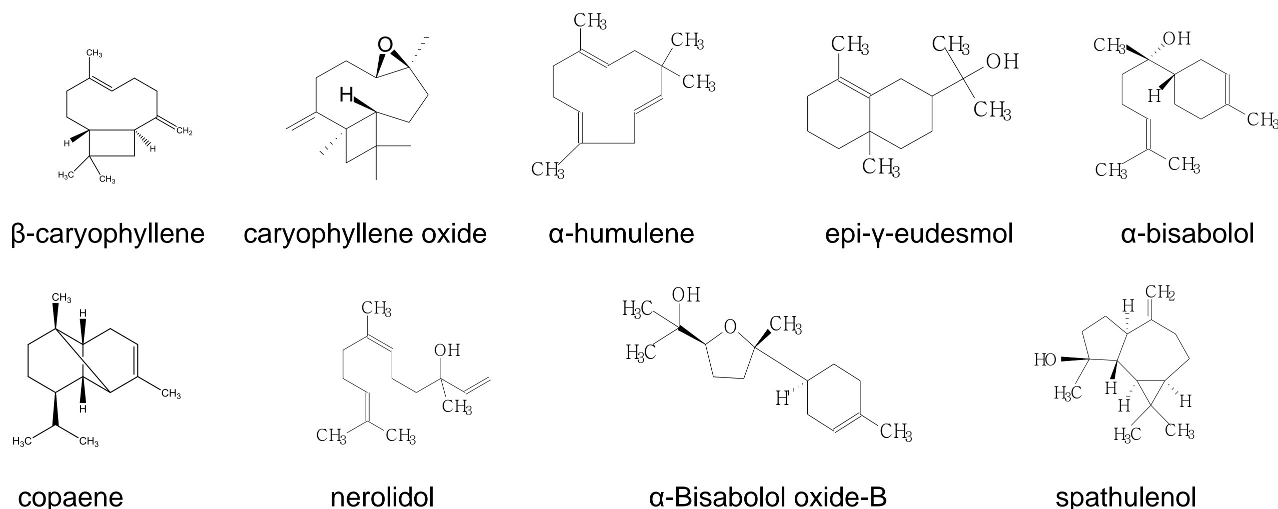

Figure 1. Chemical structures of the major constituents from essential oil from leaves of *B. ungulata* L.

Table 1. Chemical composition (%) of essential oils from fresh leaves of *B. ungulata* L. leaves.

Peak	Compounds	Kovats Index	Area	%
1	α-pinene	972	228798	0.8
2	β-pinene	996	53858	0.2
3	copaene	1370	999332	3.5
4	β-elemene	1389	502121	1.8
5	β-caryophyllene	1413	4531553	15.9
6	α-humulene	1447	2302563	8.1
7	γ-muurolene	1480	305438	1.1
8	γ-elemene	1499	363997	1.3
9	δ-cadinene	1510	302924	1.1
10	cubenol	1522	388178	1.4
11	spathulenol	1549	611896	2.1
12	caryophyllene oxide	1575	2611601	9.2
13	nerolidol	1600	941423	3.3
14	aromadendrene	1618	287445	1.0
15	α-Bisabolol oxide-B	1672	711066	2.5
16	α-bisabolol	1691	1333254	4.7
17	farnesol	1713	327718	1.2
18	epi-γ-eudesmol	1760	2122981	7.5
	Others		9548036	33.5
	Identified		18926146	66.5
	Total		28474182	100.0

showed good correlation with the free radical scavenging by dpph, and acetylcholinesterase inhibition (Alcântara et al., 2010); i*n vivo* tests reduced cell death in neurodesgenerativas diseases such as Parkinson's and Alzheimer (Ferreira, 2014).

The α-bisabolol (4.7%) was not identified in other regions for the species *B. ungulate,* indicating that it may have been the formation of a new chemotype for this species in *Boa Vista*, RR.

The α-bisabolol compound has correlation with activity against acetylcholinesterase. According to Nurulain et al. (2015), the compound binds directly to the receptor a7-nAChRs, leading to inhibition of acetylcholine.

It also has anti-inflammatory action (Kim et al., 2011)

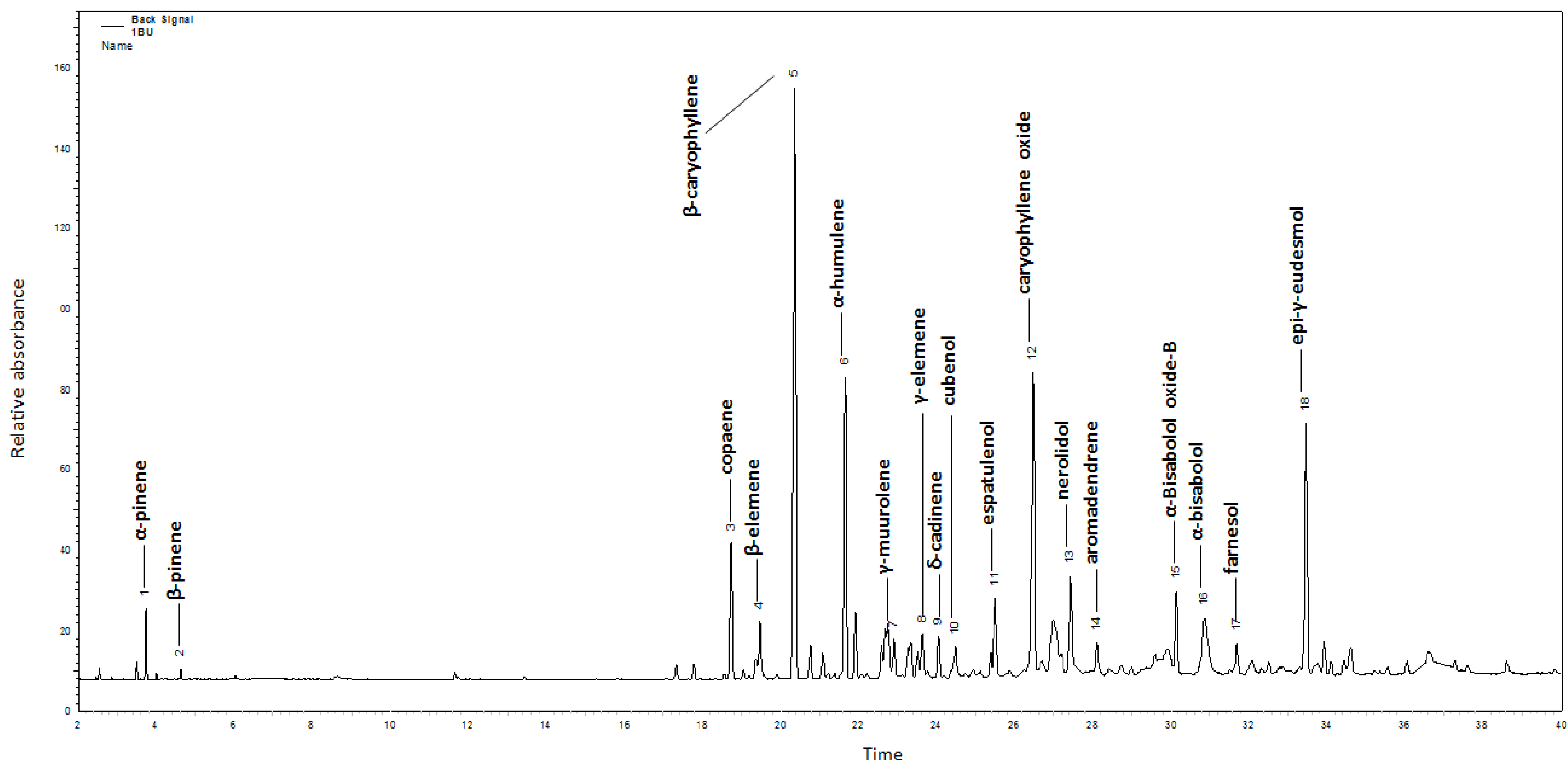

Figure 2. Chromatogram of total ions essential oil from leaves of *B. ungulata* L.

and biological activity against bacteria and fungi, as well as, *Aedes aegypt, S. aureus, Pseudomonas aeruginosa, Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum* (Vila et al., 2010). The Figure 3 shows the bioactivity of essential oil from *B. ungulata* leaves for *A. salina*.

Interpretations of the results of toxicity were carried out taking into account the above literature, which can be classified as highly toxic LC_{50} values between 0-500 µg mL^{-1}; moderate toxicity between 500-1000 µg mL^{-1} and low toxicity or nontoxic values above 1000 µg mL^{-1} (Meyer et al., 1982; Lopes et al., 2002; Rodriguez et al., 2004).

Through the straight equation formula Y = A + BX, we can calculate the CL_{50}. Considering Y = 50, A = 42.183 e B = 0.054, is the value of X is equal to 144.75 µg mL^{-1}. It can be considered that the essential oil of *B. ungulata* has high toxicity, based on the LC_{50} value found to be less

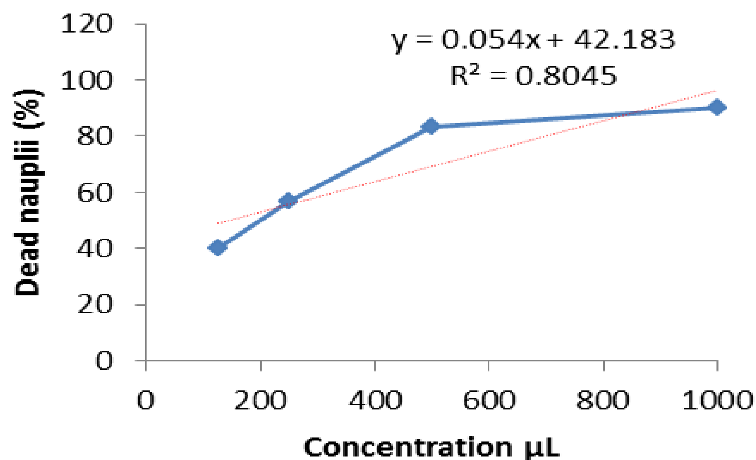

Figure 3. Curve ahead activity of *A. salina* for essential oil from leaves of *B. ungulata* L.

Table 2. Results of inhibition of acetylcholinesterase and their respective standard deviations.

Sample	% Inhibition
B. ungulata	95.96 ± 0.62
Eserine	91.93 ± 1.30
Galantamine	94.36 ± 1.14

Table 3. Antimicrobial activity of the essential oil from *B. ungulata* leaves

Microorganisms	% inhibition of growth (µg mL^{-1})							
	250	125	62.5	31.25	15.62	7.81	3.90	1.95
C. albicans	80.13	84.76	85.44	56.95	57.29	29.02	26.90	27.18
B. cereus	65.55	51.67	46.82	46.25	34.74	25.02	15.72	19.41
S. aureus	66.41	65.08	66.41	64.71	58.84	58.69	59.56	54.16
S. typhimurium	68.78	67.32	52.48	58.83	55.03	52.96	52.73	36.51
C. freudii	46.16	45.76	41.66	41.41	22.31	24.64	12.19	15.69

than 500 µg mL^{-1}.

According to Amarante et al. (2011), the plant extracts and derivatives that have a high toxicity against *A. salina* are high potential indications for biological activities. This finding reinforces the importance of the method as it is very useful to use this bioassay, when you want to develop biological studies. The results of the inhibition tests for acetylcholinesterase essential oil *B. ungulata* are as shown in Table 2.

The oil showed a good inhibitory potential, corresponding to 95.96%, and can see that the standards used for Eserine and galantamine showed inhibition of 91.93 and 94.36%, respectively, which were lower than the inhibition of essential oil. Savalev et al. (2003) observed synergistic interactions among the components (1.8-cineole, camphor, α-pinene, β-pinene, borneol, caryophyllene oxide, linalool and bornyl acetate) which come from the species *Salvia lavandulaefolia*. Among those components mentioned above was found the essential oil of *B. ungulata* in the presence of α-pinene, β-pinene and caryophyllene oxide; certainly there was synergism of compounds present in the oil of the species studied by high inhibition of the enzyme, highlighting the chemical components β-caryophyllene and α-bisabolol, which has a close relationship with neurodegenerative diseases and reported by Nurulain et al. (2015), Ferreira (2014), Alcantara et al. (2010) and Santos et al. (2015).

Another biological activity of essential oil of *B. ungulata* leaves deserves attention as well as the bioactivity against the pathogenic microorganisms (Table 3).

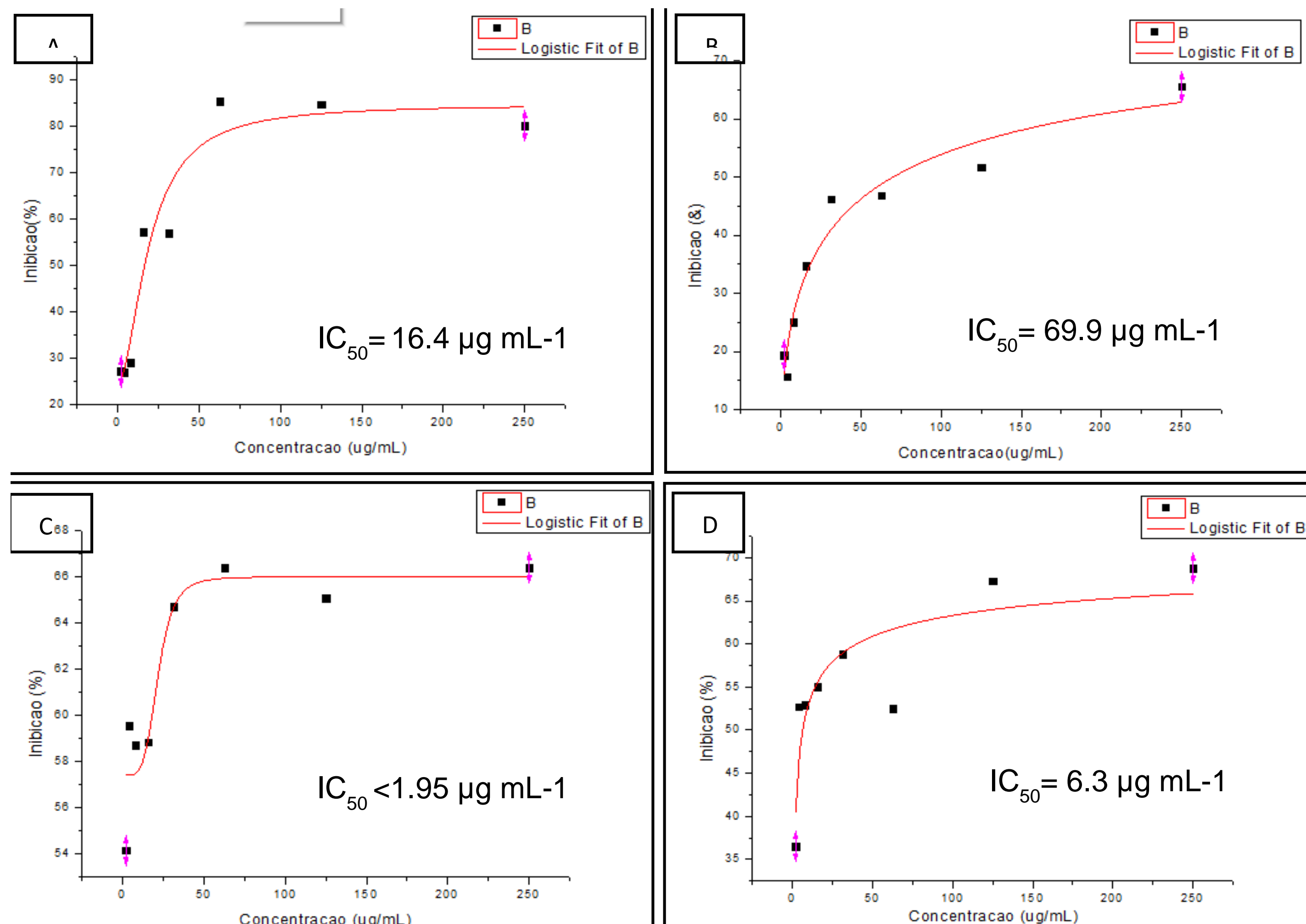

Figure 4. Activity curve for essential oil from leaves of *B. ungulata* (L) front of the A) *Candida albicans*; B) *Bacillus cereus*; C) *Staphylococcus aureus*; D*) Salmonella typhimurium.*

Graphs were plotted with the aid of software origin 8.0, as can be seen in Figure 4 and using equation Y = A2 + (A1-A2) / (1 + (x/x0) ^p) was calculated from IC_{50}.

By analyzing the percentages of inhibition and IC_{50}, it can be seen that there has been satisfactory inhibition of four of the tested microorganisms, whereas inhibition was greater than 50%. Checking greater emphasis on the *S. aureus* which was inhibited in all eight concentrations tested and showed a IC_{50} < 1.95 µg mL^{-1} as well as *C. albicans* to inhibit the microorganism concentration 15.62 µg mL^{-1} of essential oil, this value was found to be in agreement with the IC_{50} (16.4 µg mL^{-1}). It is noteworthy that the three highest concentrations were those that had greater inhibition ranging from 80-85%.

The Figure 4 shows graphs of the minimum inhibitory concentrations of the essential oil to the front microorganisms studied. The bacterium *C. freundii* showed an inhibition of 46.16%, thus less than 50%, not showing satisfactory results.

Species of this genus *Bauhinia* action also demonstrated inhibition of acetylcholinesterase (Santos et al., 2011), antibacterial action (Cechinel-Filho, 2000, 2009), anthelmintic (Bairagi et al., 2012) and antitumor activity (tested in rats) (Rajkapoor et al., 2003).

Conflict of Interests

The authors have not declared any conflict of interests.

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