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Full Length Research Paper

Effect of adding sacha inchi (*Plukenetia volubilis* L.) seeds to a prototype of convenience food draft, on the nutritional composition and sensory acceptance

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The polyunsaturated fatty acids of Sacha Inchi seeds are important in the development of food products with healthy and nourishing properties. The aim of this study was to evaluate the effect of Sacha Inchi (*Plukenetia volubilis L*) on a prototype convenience food. The nutritional composition and sensory acceptance was evaluated using a completely randomized experimental design. Fixed effect-balanced factorial multiple correspondence analyses were used for the different treatments. Four formulations were developed for the prototype type setting (F0, F5, F7.5 and F10) using Sacha Inchi almonds characterized according to their nutritional composition. The fatty acid composition of the total lipids of the seed was determined by gas chromatography. In the food draft type, sensory parameters such as color, odor, flavor and texture in all treatments were evaluated. The results obtained showed a content polyunsaturated fatty acids of 33.74% in the seed. In addition, the acceptance of sensory parameters evaluated by consumers in the draft prototype was greater than 80%. It was observed that lipid content increased up to 3 times and the content of polyunsaturated fatty acids, 4 times in the treatment of F10 with regard to white F0. About 10% almond Sacha Inchi can be effectively incorporated into draft products with suitable timing sensory characteristics and nutritional value, thus, allowing the foods declared under the Colombian law as a high and good source of omega 3.

Key words: Prototype, *Plukenetia volubilis* L, polyunsaturated fatty acids, draft.

INTRODUCTION

Sacha inchi plant is a plant native to the Amazon región, it is known by various names, such as “Gold Inka”, “Inca Inchi” o “Inca peanut”, is a plant of the Euphorbiaceous family and volubilis species is a climbing plant, semi-ligneous, fruits in capsule, with 4 seeds oval and dark brown (Manco 2006). The flour and oil from the seeds are commonly used by the Peruvian natives. The seeds contain approximately, on average, 48% oil and 27% proteins that are rich in essential amino acids (Maurer et al., 2012). The nutritional composition of sacha inchi is

characterized by high levels of essential fatty omega 3 (ω-3) and omega 6 (ω-6),), which have been documented to have effects on the human health by preventing various diseases like arthritis , coronary heart disease , diabetes, hypertension , attention deficit hyperactivity, and inflammatory skin diseases.(Hanssen and Schmitz-Hübsch, 2011) (Gogus and Smith, 2010), according to this context , the use of almonds extracted from the seeds of Sacha inchi has a high potential for nutraceuticals industry (Guillén et al., 2003).

The development, design and innovation of products to help the world population, have spurred the search for alternatives to the production of food with functional properties; hence, the seeds of Sacha inchi *(Plukenetia volubilis* L.) was proposed for use in the oil and cosmetic industries, with little use in the manufacture of food products for human consumption (Guillén et al., 2003), several authors have reported the development of foods with added Sacha inchi as sausage type Frankfurt (Romo, 2015), beverage with cake Sacha inchi (Cárdenas, 2015) and energy bar with addition of seed. (Baéz and Borja, 2013 a). The United Nations Organization for Food and Agriculture (FAO, 2008) has recommended increasing the intake of fatty acids (ω-3) to the general population, and to comply with this recommendation, they enriched a variety of food products such as eggs, yogurt, milk, and spreadable foods (Riediger et al., 2009). Developed countries currently have among other challenges for the populations, the offer to consumers who have little time to prepare their food, through options of healthy processed foods ready for consumption, known as food ready to-eat or convenience (Dutcosky et al., 2006), that are oriented to the trend of snack foods; that are characterized by having a higher energy density and sodium, with high content of carbohydrates and significant sensory characteristics such as taste and texture.

On the basis of the interest of consumers to purchase foods that have a positive impact on human health, in Colombia, they are developing foods from new sources of nutrients with foods considered promising, low production costs and cultivated with few agronomic requirements (Peralta, 2010), This is the case of the seed of Sacha inchi from the biodiversity of Latin American countries, with high nutritional potential (Guillén et al., 2003). The aim of this study is to evaluate the effect of adding Sacha inchi seeds, to a prototype of convenience food draft, on the nutritional composition and sensory acceptance.

MATERIALS AND METHODS

3 kg of seeds were collected of *P. volubilis* L. sedes from the Municipality of Santo Domingo, Antioquia-Colombia, with characteristics of healthy seed grain without broken bark or exposed almond, was used.

Physico-chemical analysis

The following parameters were analyzed: Protein by AOAC No. 954.01, moisture by GTC 1.14, ashes by AOAC No. 923.03, total fat by GTC 6.1. 996.06 total carbohydrates calculated on the basis of its components by difference.

Fatty acid profile of sacha inchi seed

Lipid phase extraction of the Sacha Inchi seed was performed and 0.5 mL of hexane was added to 1.5 mL of the Sacha Inchi extract which was derivatized with 5 mL of boron trifluoride BF3 methylation (Ackman, 1998) in methanol for the trans methylation fatty acids present. The fatty acids were determined using a gas chromatograph (Agilent 7890 B with detector FID, with auto sampler 7963 A), equipped with a split injector relation: 100:1, with a capillary column of silica TRCN-100 (60.0 m x 0.25 mm i.d x 0.20 µm) (brand TEKNOKROMA) and a flame ionization detector; The gases used was Hydrogen (H_2) and air, gas makeup: Nitrogen (N_2). The volume of injection was 1.0 µL. The injector and detector temperatures were 250 and 260°C, respectively. Initial oven temperature of 100°C was gradually increased to 145°C at 8°C/min. Helium was used as carrier gas at a linear flow of about 1.5 ml/min. The retention times and peak areas were processed using OpenLab CDS ChemStation software. For identification of fatty acid retention time and peak areas of the samples with mixture of reference standards, Food Industry FAME mix (37 parts) Mark RESTEK components, diluted in 30 mg/ml of dichloromethane. Fatty acids were quantified through a percentage of the peaks with the total area. All reagents used in the extractions were reagent grade and in the chromatography separation, HPLC grade.

Prototype development of the draft food type

Sacha inchi seeds were hulled for the almond and pre-toasted in a convection oven (brand: UNOX reference BakerTop XBC615), between 80 ± 5°C for 15 min. Then the seeds were finely cut to a particle size between 1.0 and 2.0 mm. The protocol for the development of the prototype food was adapted and standardized in laboratory conditions accordingly (Marpalle et al., 2014). Ingredients such as whole wheat flour, water, brown sugar, instant dry yeast, dietary salt all trademark, obtained from local supermarkets were used.

The ingredients were weighed separately, mixed in a blender (Mixer Spartan reference mark SP500, engine 750W, 110 V / 60Hz, 10vel) for 20 min in a speed of 5 rpm to form a gluten network, and then the Sacha inchi pre-toasted almonds were added at different concentrations and chopped, with respect to whole wheat flour (F5): 5%; (F7.5): 7.5% and (F10) 10%, mixing them for 2 min, except (F0): 0% (white) that had no addition of almonds (Table 1). The mass is left to rest for 40 min and then cut into squares of 2 x 2 cm. It was taken to camera fermentation (brand Javar) with a temperature of 35 ± 5°C and relative humidity of 80% for 1 h to double its size. Subsequently, they were baked at 165°C for 11 min, and finally achieved a draft at 90°C for 30 min (Figure 1). They

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Table 1. Ingredients for treatment of the prototype food.

Ingredients	F0	F5	F7.5	F10
Whole wheat flour (g)	100	100	100	100
Sacha inchi (pieces) (g)	0	5	7.5	10
Brown Sugar (g)	6	6	6	6
Instant dry yeast (g)	1.7	1.7	1.7	1.7
Dietary salt (g)	0.3	0.3	0.3	0.3
Water (ml)	65	65	65	65

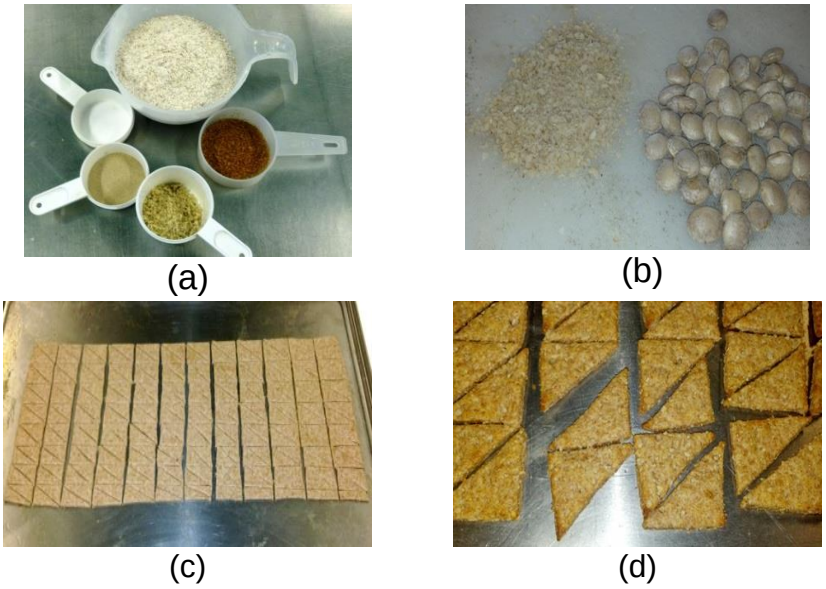

Figure 1. Preparation of food draft prototype. (a) Enlistment (b) Sacha inchi Pieces (c) Rolling and cutting (d) Baked snack.

were cooled on trays for packaging in foil bags, to avoid hygroscopicity and oxidation of fats in the prototype food type setting (Marpalle et al., 2014).

Analysis of the chemical constituent of the prototype food

The following parameters were analyzed: fat by Soxhlet extraction method (NTC 668), moisture by thermo gravimetric method (based on ISO 6496) and protein by Kjeldahl (NTC 4657), the latter worked with a factor of (N × 6.25), the ash by direct incineration method (AOAC 942.05).

Fatty acid profile of the prototype food

They weighed between 1.0 to 2.0 g of the prototype food milled and homogenized. Then hydrolysis of the fat from the seed was conducted with 1,000 g of Celite, 100 mg of BHT and 120 ml of 4N HCl, stirring for 2 min. 2.0 ml Glyceryltriundecanoate of the internal standard (C11: 0) was added to 5.0 mg/ml to start the hydrolysis unit (FOSS reference mark 1047) for 1 h; rinsed and the supernatant was filtered. Subsequently, it was taken to the extraction unit for removal of Soxtec fat (Brand: Reference FOSS 1047) with 50 mL of hexane. The extracted fat was cooled in a desiccator and then the fatty acid profile was analyzed by gas chromatography with flame ionization detector (GC-FID).

Sensory analysis of the prototype food

Affective method was applied, using a binomial hedonic scale acceptance test (Sancho et al., 2002), evaluating the attributes of color, taste, smell and texture, to analyze the differences between them.

The samples of the prototype food type setting (F0, F5; F7.5; F10) were evaluated by 120 non-regular users of the product of both sexes, in age ranges of 15 to 25; 26 to 35; 36 to 45; 45 to 55, and greater than 55 years, and they were classified into 4 groups chosen at random, each consisting of 30 people who completed the survey type format after consuming the product. The samples were presented in thermo forming packaging (reference AL69P). Statistical analysis entailed applying the multivariate technique called factorial analysis of multiple correspondence (ASM) with additional variable (treatment) using the SPAD version 3.5 program. This analysis allows determining the similarities or dissimilarities between individuals concerning the attributes evaluated in sensory analysis.

Table 2. Nutritional composition of sacha inchi seed *(P. volubilis L).*

Parameter	*Mean ± SD
Humidity (%)	4.7 ± 0.04
Total mineral (%)	2.7 ± 0.04
Total fat (%)	42.3 ± 0.02
Total Protein (%)	25.6 ±1.27
Total carbohydrates (%)	24.5
Total calorie (Kcal / 100 g	481.5

* Dry basis amounts expressed in percentage (%).

Table 3. Fatty acid profile of sacha inchi seed (*P. volubilis* L).

Fatty acid profile	Fatty acids	*Percentage ± De
	Myristic acid	0.02 ± 0.0007
	Palmitic acid	1.85 ± 0.001
Saturated fat	Heptadecanoic acid	0.04 ±0.0003
	Stearic acid	1.26 ±0.002
	Behenic acid	0.01 ±0.0001
	C16: 1 (palmitoleic acid)	0.03 ± 0.001
Monounsaturated fat	C18: 1n9c (oleic acid)	3.4 ± 0.009
	C20: 1n9 (cis-11-eicosenoic acid)	0.17 ±0.001
	C18: 2n6c (linoleic acid)	13.7 ± 0.05
Polyunsaturated fat	C18: 3n3 (a-linolenic acid)	19.9 ± 0.06
	Cis-11,14-eicosadienoico	0.02 ± 0.001

* Dry basis amounts expressed in percentage (%).

Experimental design

There was a design of completely randomized experimental classification of fixed effect, balanced with a single Sacha inchi seed, with four levels of treatments (F0, F5, F7.5, and F10). The prototype draft type food was taken as experimental unit; physico-chemical analysis was performed with fatty acid profile that was analyzed statistically by multivariate analysis of variance MANOVA. The results were generated using SAS University Edition (SAS Institute, 2011), with a confidence level of 95%.

RESULTS

Physicochemical analysis

Physicochemical data seed Sacha inchi is shown in Table 2.

Fatty acid profile of sacha inchi seed

Values were found of polyunsaturated fatty acids such as linolenic acid (n-3) of 19.94% and linoleic acid (n-6) with 13.77% in Sacha Inchi; the values were equally highlighted as a percentage of palmitic fatty acids Saturated with 1.85 ± 0.005. The monounsaturated oleic acid percentage of 3.40 ± 0.009 was obtained (Table 3).

Chemical analysis of the prototype food

The analysis of the prototype chemical properties for the different treatments is shown in Table 4, with values of humidity between 10.3% for F0 and 3.7% for F10; fat showed 0.83% in the treatment of F0 and 4.82 in the treatment of F10. The percentage of protein is found between 15.1% for F0 and 15.8% in F10.

Fatty acid profile of the prototype food

A significant statistical difference ($p<0.05$) (Table 5) between treatments for fatty acids (lauric, palmitic, stearic, palmitoleic, oleic, Cis-11-eicosenoic, linoleic, α-linolenic) was found, with the highest content of the n6 linoleic fatty acid in treating F10. The values obtained from the profile for the treatment of F0 (white) were

Table 4. Compositional analysis food type prototype by treating Sacha inchi.

Parameter	F0	F5	F7.5	F10
Fat (%)	0.83	3.15	3.82	4.82
Protein (%)	15.1	15.8	14.9	15.8
Humidity (%)	10.3	6.0	6.6	3.7
Ash (%)	4.80	4.35	5.20	4.50

Table 5. Acid profile of fatty food type prototype by treating Sacha inchi.

Fatty acid profile	Fatty acids	Treatments			
		F0	F5	F7,5	F10
Saturated fat	C14: 0 (lauric acid)	0.0102 ^{c}	0.015^{b}	0.0193^{a}	0.0091^{d}
	C16: 0 (myristic acid)	0.02^{a}	0.0216^{a}	0.0222^{a}	0.0224^{a}
	C17: 0 (palmitic acid)	0.5358^{d}	0.6051^{c}	0.6686^{b}	0.7281^{a}
	C18: 0 (stearic acid)	0.2044^{d}	0.253^{c}	0.3006^{b}	0.3517^{a}
Monounsaturated fat	C16: 1 (palmitoleic acid)	0.0426^{a}	0.0318^{c}	0.0298^{c}	0.0399^{b}
	C18: 1n9c (oleic acid)	0.3961^{d}	0.5472^{c}	0.6929^{b}	0.8102^{a}
	C20: 1n9 (cis-11-eicosenoic acid)	0.0127^{d}	0.0155^{c}	0.0182^{b}	0.0324^{a}
Polyunsaurated fat	C18: 2n6c (linoleic acid)	1.0611^{d}	1.5899^{c}	2.0425^{b}	2.6315^{a}
	C18: 3n3 (a-linolenic acid)	0.0747^{d}	0.9324^{c}	1.6381^{b}	2.4355^{a}

*Amounts expressed in percentage (%) dry basis. Media in the same row with different letters indicate statistically significant difference ($p< 0.05$).

Table 6. Percentage of acceptance of the prototype food.

Parameter	F0	F5	F7.5	F10
Color	100.0^{a}	96.7^{a}	97.0^{a}	100.0^{a}
Odor	100.0^{a}	90.0^{a}	100.0^{a}	100.0^{a}
Flavor	92.9^{a}	83.3^{a}	94.1^{a}	84.4^{a}
Texture	85.7^{a}	90.0^{a}	91.1^{a}	90.6^{a}

Different superscripts indicate significant statistical difference ($p <0.05$).

0.7704 ± 0.0032; 0.4514 ± 0.0010 and 1.1358 ± 0.0002; while for the treatment of 10, values obtained were 1.1113 ± 0.0013; 0.8825 ± 0.0007 and 5.0670 ± 0.0154for total saturated fatty acids, monounsaturated (MUFA) and polyunsaturated PUFA, respectively. Similarly, an increasing trend of saturated fatty acids was found (MUFA and PUFA) in each of the treatments with regard to white (F0).

Sensory analysis

No statistical difference between treatments was detected by applying multivariate technique MANOVA, to jointly evaluate the sensory variables ($p> 0.05$). 100% of the consumers accepted the color and odor for the F0 and F10. It was also observed that the flavor and texture in general presented percentages of acceptance that ranged between 83.3 and 92.9% for the different treatments (Table 6).

DISCUSSION

The moisture content in the seed of Sacha inchi is within the range of 0 to 13% as reported by (James, 1995), who described this value as suitable for the storage and processing without degradation of triaglycerols by

microorganisms. The protein content of the seed of Sacha Inchi analyzed in this study was similar to that reported by (Gutiérrez et al., 2011) with 24.7% and was slightly lower than that found by (Cisneros et al., 2014) with 27% w/w. These percentages can be related to other oil seeds such as sesame (~25%), peanut (23% w/w) and sunflower (24% w/w) (Bodwell and Hopkins, 1985) to help meet the daily protein requirements for adults (0.66 g/kg per day), as suggested by the World Health Organization (Joint FAO, World Health Organization, 2007).

The lipid content of the Sacha inchi are consistent with those reported by other authors (Bondioli et al., 2006) and (Follegatti-Romero et al., 2009) that found the characteristic values of oil seeds to be between 30 and 54% (McKevith, 2005). Notably, the Sacha inchi analyzed in this work has high lipid content as found with regard to chia seeds (26.7 to 35%), safflower (27.5%) and soybeans (16.5%) (Chirinos et al., 2013).The values of Polyunsaturated Fatty acids (PUFA), linolenic (n-3) and linoleic acid (n-6) found in this study are lower than those reported by other authors (Gutiérrez et al., 2011) and (Garmendia et al., 2011). The differences can be attributed to weather conditions and physiological maturity of the fruit (Ruiz et al., 2013). According to Gebauer et al.(2006), in relation to the recommendations of ω-3 and source food with benefits for cardiovascular health, about 2 g/day are suggested; which implies that the seed itself may be a food source of ω-3 cardio-protective benefits. Considering the effect of the addition of Sacha inchi in treatments, these increased to 3 times the total lipid content and about 4 times the content of PUFA in the treatment of F10 with regard to white (F0). Similarly, there was a decrease in the percentage of moisture in the prototype food draft type, which can reduce microbial growth as an indicator of the useful shelf life of the product.

In relation to the data obtained in the fatty acid profile of the prototype, the food draft showed significant differences ($p<0.05$) in the values of ω-3 between treatments F0: 0.07%; F5: 0.9%; F7.5: 1.6% and F10: 2.4%; and for ω-6 and there were significant differences ($p<0.05$) between treatments F0: 1.0%; F5: 1.6%; F7.5: 2% and F10: 2.6%. Baéz Pazmiño et al. (2013 b), developed an energy bar with addition of Sacha inchi seed and other lipid materials reporting values of ω-3 (6.2%) and ω-6 (3.1%) (46), which when compared with the values of ω-3 and ω-6 were lower compared to those found in this study. However, prototype of the drafted food developed from the treatment of F5 can be considered high and a good source of omega 3, according to resolution 333 of 2011 requirements for nutrition labeling of the Ministry of Social Protection in Colombia (Resolución 333 de 2011). Addition of Sacha inchi seed does not significantly change the acceptance of consumers who evaluated the prototype of the drafted food type; even by permitting the addition of this oilseed with high nutritional value without altering the sensory characteristics by up to 10% food. Similarly, Marpalle et al. (2014), developed functional bread with added flaxseed flour, where they found acceptance in the sensory parameters evaluated with 10% added of wheat flour flaxseed.

Conclusion

The Sacha inchi is a food containing polyunsaturated fats and a good source of protein. Incorporating Sacha inchi seeds in a new prototype of food such as a snack could be considered as a novel and nutritious ingredient. Levels from 5% Sacha inchi seed are allowed for declaring the foodstuff as a high and good source of ω-3. The sensory evaluation revealed an acceptance of the prototype food developed, which added up to 10% of Sacha inchi seeds, which can be a potential health benefit due to the content of ω-3, to which they have been described a protective effect against cardiovascular diseases.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Ackman R (1998). Remarks on official methods employing boron trifluoride in the preparation of methyl esters of the fatty acids of fish oils. J. Am. Oil Chem. Soc. 75(4):541-545.

Baéz PLL, Borja AAK (2013a). Elaboración de una barra energética a base de Sacha inchi (Plukenetia volubilis) como fuente de omega 3 y 6.

Baéz PLL, Borja AAK (2013b). Elaboración de una barra energética a base de Sacha Inchi (Plukenetia volubilis) como fuente de omega 3 y 6 ingenieria de Alimentos, Universidad San Francisco de Quito, Quito. Retrieved from http://repositorio.usfq.edu.ec/handle/23000/2380

Bodwell C, Hopkins D (1985). Nutritional characteristics of oilseed proteins. New protein foods 5.

Bondioli P, Della BL, Rettke P (2006). Alpha linolenic acid rich oils. Composition of *Plukenetia volubilis* (Sacha Inchi) oil from Peru. Rivista Italiana Delle Sostanze Grasse 83(3):120-123.

Cárdenas PVS (2015). Elaboración y caracterización de una bebida a partir de la semilla de sacha inchi (*Plukenetia volubilis* L.). Ingeniería Química, Universidad Central del Ecuador, Quito.

Chirinos R, Zuloeta G, Pedreschi R, Mignolet E, Larondelle Y, Campos D (2013). Sacha inchi (*Plukenetia volubilis*): a seed source of polyunsaturated fatty acids, tocopherols, phytosterols, phenolic compounds and antioxidant capacity. Food chem. 141(3):1732-1739.

Cisneros FH, Paredes D, Arana A, Cisneros-Zevallos L (2014).

Chemical composition, oxidative stability and antioxidant capacity of oil extracted from roasted seeds of Sacha-inchi (*Plukenetia volubilis* L.). J. Agric. Food Chem. 62(22):5191-5197.
Dutcosky SD, Grossmann MVE, Silva RSS, Welsch AK (2006). Combined sensory optimization of a prebiotic cereal product using multicomponent mixture experiments. Food Chem. 98(4):630-638.
FAO (2008). Grasas y ácidos grasos en nutricion humana, Consulta de expertos, Ginegra, 10-14 de Noviembre de 2008 (Vol. 91): FAO y Fundación Iberoamericana de Nutrición (FINUT).
Follegatti-Romero LA, Piantino CR, Grimaldi R, Cabral FA (2009). Supercritical CO 2 extraction of omega-3 rich oil from Sacha inchi (*Plukenetia volubilis* L.) seeds. J. Supercrit. Fluids 49(3):323-329.
Garmendia F, Pando R, Ronceros G (2011). Efecto del aceite de sacha inchi (*plukenetia volúbilis* l) sobre el perfil lipídico en pacientes con hiperlipoproteinemia. Rev. Perú. Med. Exp. Salud Publica *28*(4):628-632.
Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM (2006). n-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. Am. J. clin. Nutr. 83(6):S1526-1S535.
Gogus U, Smith C (2010). n-3 Omega fatty acids: a review of current knowledge. Int. J. Food Sci. Technol. 45(3):417-436.
Guillén MD, Ruiz A, Cabo N, Chirinos R, Pascual G (2003). Characterization of sacha inchi (*Plukenetia volubilis* L.) oil by FTIR spectroscopy and 1H NMR. Comparison with linseed oil. J. Am. Oil Chem. Soc. 80(8):755-762.
Gutiérrez LF, Rosada LM, Jiménez Á (2011). Chemical composition of Sacha Inchi (*Plukenetia volubilis* L.) seeds and characteristics of their lipid fraction. Grasas y aceites 62(1):76-83.
Hanssen H, Schmitz-Hübsch M (2011). Sacha inchi (*Plukenetia volubilis* L.) nut oil and its therapeutic and nutritional uses. Nuts and Seeds in Health and Disease Prevention. Academic Press, San Diego, pp. 991-994.
James CS (1995). Determination of the fat content of dairy products by the Gerber Method. Analytical Chemistry of Food. Blackie Academic and Professionals, an imprint of Chapman and Hall, Glasgow, UK, pp. 93-95.
Joint FAO, World Health Organization (2007). Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation.
Manco CEI (2006). Cultivo del Sacha Inchi : Programa Desarrollo Rural Sostenible-PDRS.
Marpalle P, Sonawane SK, Arya SS (2014). Effect of flaxseed flour addition on physicochemical and sensory properties of functional bread. LWT - Food Sci. Technol. 58(2):614-619.
Maurer NE, Hatta-Sakoda B, Pascual-Chagman G, Rodriguez-Saona LE (2012). Characterization and authentication of a novel vegetable source of omega-3 fatty acids, sacha inchi (*Plukenetia volubilis* L.) oil. Food Chem. 134(2):1173-1180.
McKevith B (2005). Nutritional aspects of oilseeds. Nutr. Bull. 30(1):13-26.
Peralta E (2010). Producción y distribución de semilla de buena calidad con pequeños agricultores de granos andinos: Chocho, Quinua, Amaranto. Quito: Programa Nacional de Leguminosas y Granos Andinos.Estación Experimental Santa Catalina. INIAP.
Resolución 333 de 2011 (2011). Por la cual se establece el reglamento técnico sobre los requisitos de rotulado o etiquetado nutricional que deben cumplir los alimentos envasados para consumo humano. Available at: http://www.icbf.gov.co/cargues/avance/docs/resolucion_minproteccion_0333_2011.htm
Riediger ND, Othman RA, Suh M, Moghadasian MH (2009). A systemic review of the roles of n-3 fatty acids in health and disease. J. Am. Diet. Assoc. 109(4):668-679.
Romo DVA (2015). Evaluación del proceso de obtención de salchicha tipo frankfurt con adición de diferentes niveles de torta de plukenetia volubilis (sacha inchi) y dos tipos de proteína de origen vegetal.
Ruiz C, Díaz C, Anaya J, Rojas R (2013). Análisis proximal, antinutrientes, perfil de ácidos grasos y de aminoácidos de semillas y tortas de 2 especies de Sacha inchi (Plukenetia volubilis y Plukenetia huayllabambana). Rev. Soc. Quím. Perú 79(1):29-36.
Sancho J, Bota E, De Castro J, Puig VE (2002). Introducción al análisis sensorial de los alimentos.
SAS Institute (2011). The SAS system for Windows (Version Release 9.2 SAS Inst., Cary, NC.).

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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:

$$\text{Mortality Probability (\%)} = \frac{r}{n} x100$$

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:

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

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

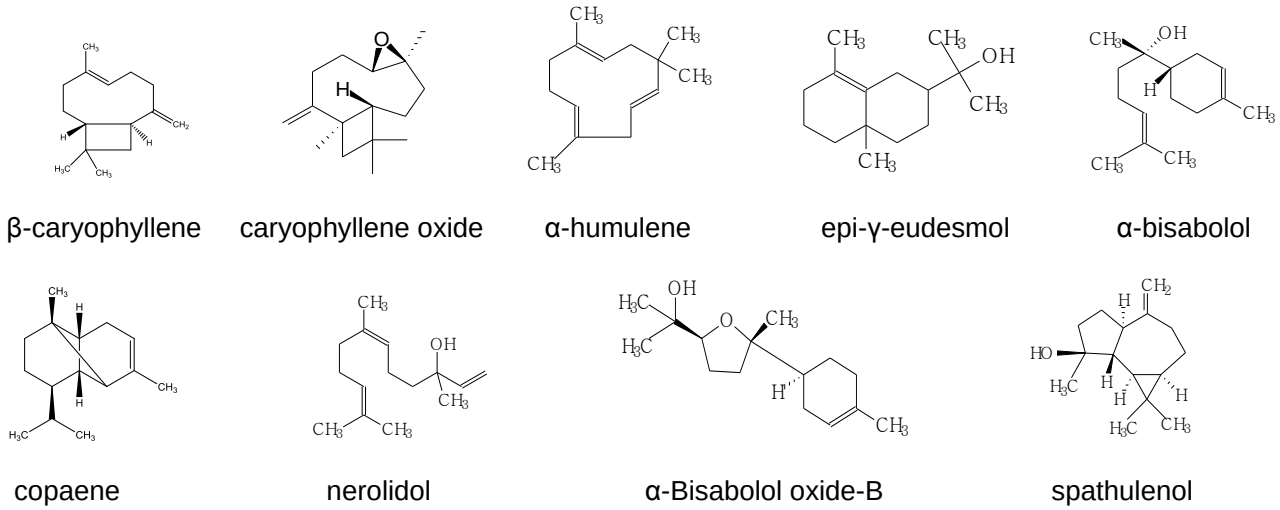

Figure 1. Chemical structures of the major constituents from essential oil from leaves of *B. ungulata* L.

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)

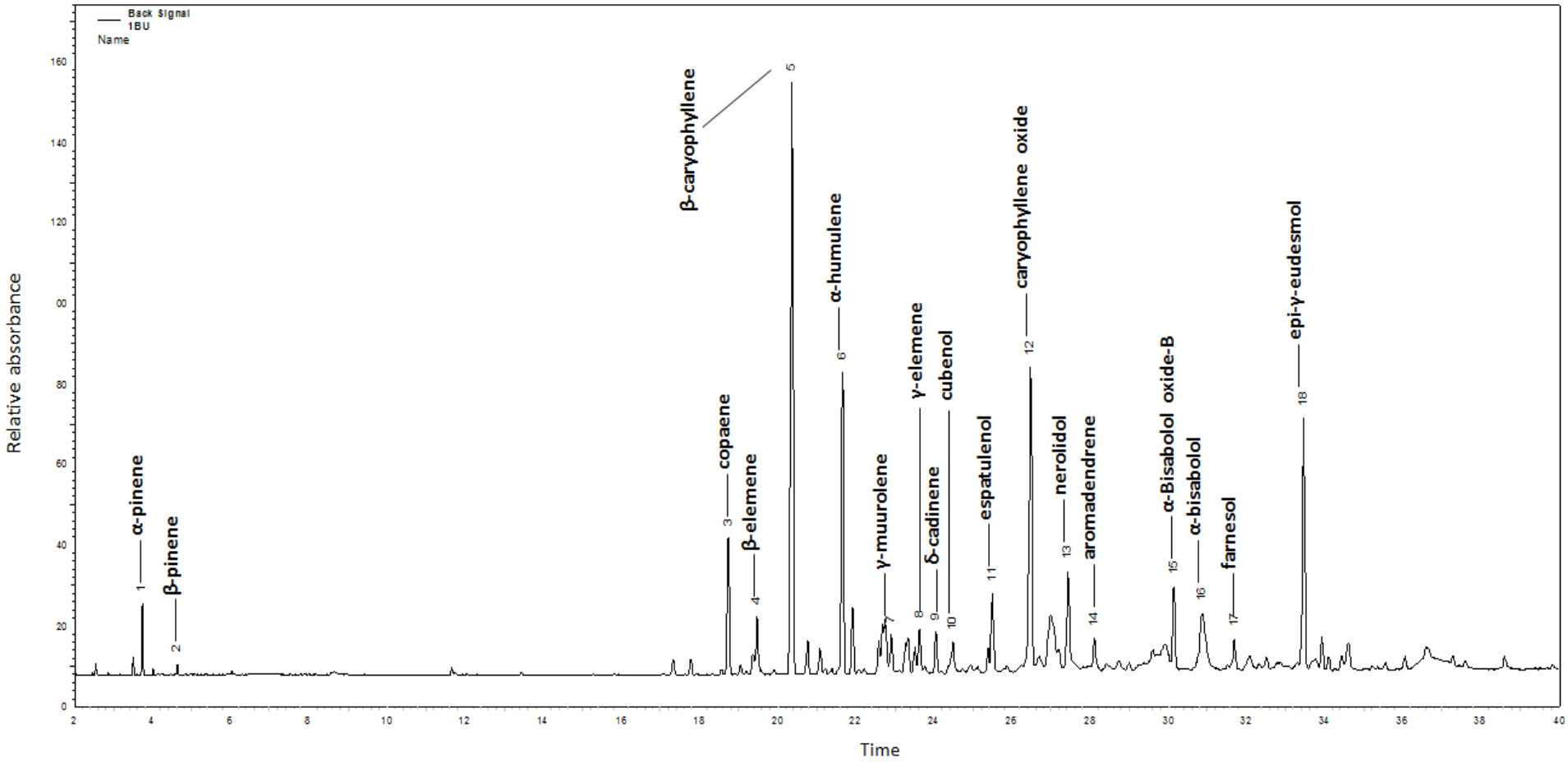

Figure 2. Chromatogram of total ions essential oil from leaves of *B. ungulata* L.

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

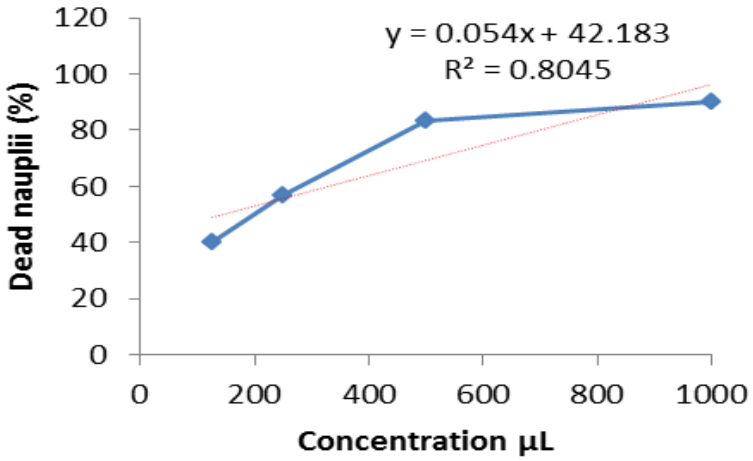

Figure 3. Curve ahead activity of *A. salina* for essential oil from leaves of *B. ungulata* L.

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).

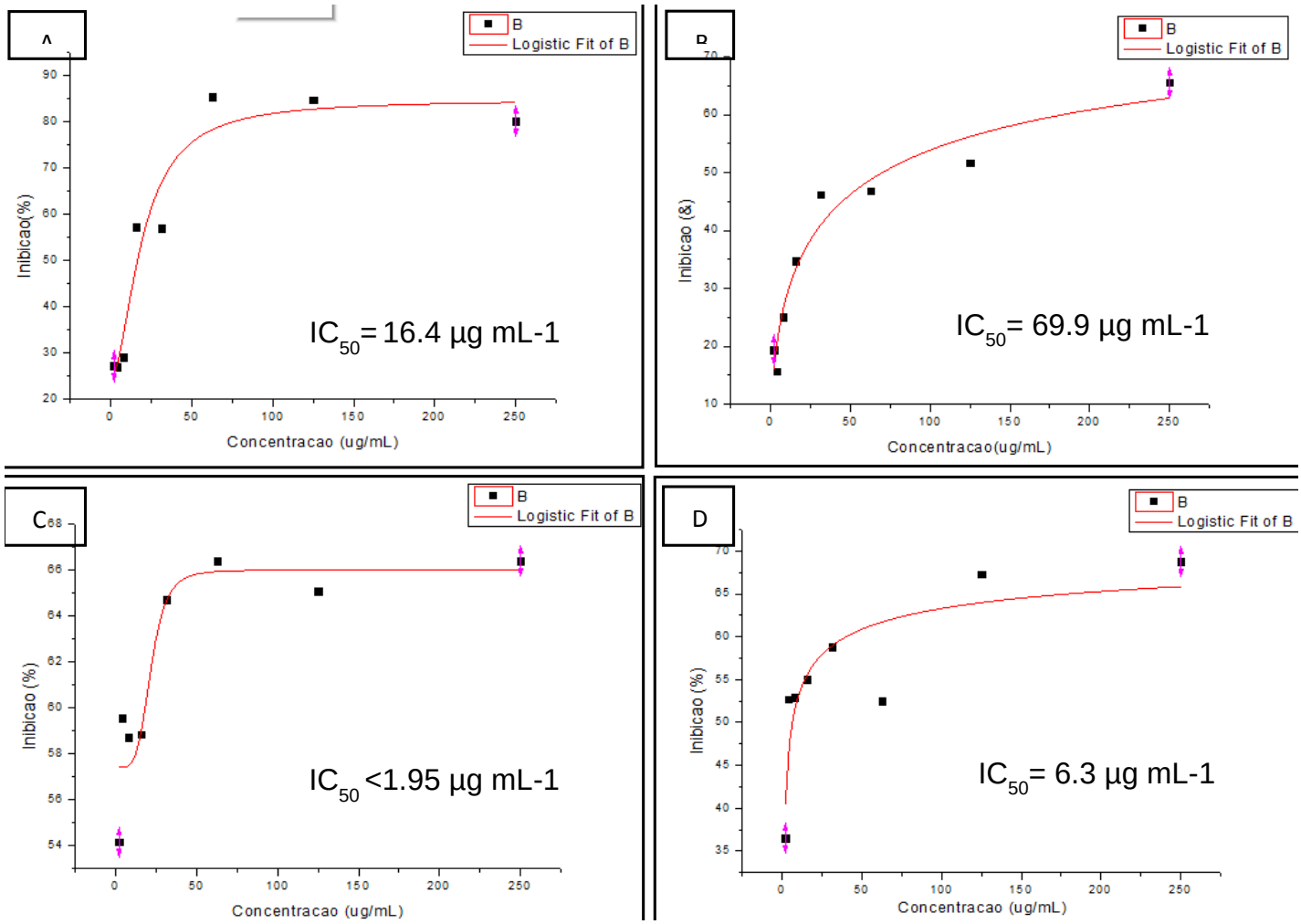

Figure 4. Activity curve for essential oil from leaves of *B. ungulata* (L) front of the A) *Candida albicans*; B) *Bacillus cereus*; C) *Staphylococcus aureus*; D*) Salmonella typhimurium.*

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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REFERENCES

Alcantara JM, Yamaguchi KKL, Silva JRA, Veiga Junior VF (2010). Chemical composition and biological activity of essential oils from the

leaves and stems of *Rhodostemono* daphne *parvifolia Madriñán* (Lauraceae). Acta Amaz. 40(3):567-572.
Amarante CB (2011). Phytochemical study biomonitored by toxicity tests front of *Artemia salina* and antiplasmodial activity aninga stem (Montrichardia linifera). Acta Amaz. 41(3):431-434.
Bairagi SM, Abhijeet AA, K*n*imase P (2012). *In vitro* Anthelmintic Activity of *Bauhinia variegata* Bark (Leguminoseae*).*
Bizzo RH (2009). Essential oils in Brazil: General Aspects, development and research. Química Nova. 3(32):588-594.
Cechinel-Filho V (2000). Major Advances and Prospects on Natural Products Area Assets: developed studies in Niqfar/Univale. Química Nova 5:680-685.
Cechinel-Filho V (2009). Natural and Synthetic Products with Therapeutic Potential: 15 years Studies at the core of Chemical investigations - Pharmacist at Niqfar/Univale. Rev. Fitos. 4(2):6-23.
Costa FA (2002). Pharmacognosy**,** Lisboa: Fundação calouste gulbenkian. pp. 1-1031.
Cunha AP (2004). Medicinal plants and plant products with ethics, Lisboa: Fundação calouste gulbenkian. pp. 9-310.
Duó-Bartolomeu AC, Fiori GML, Bastos DV, França SC, Pereira MAS, Taleb-Contini SH (2014). Compostos voláteis de Bauhinia forficata L.. 2014. (Apresentação de Trabalho/Simpósio).
Ferreira DAS (2014). The protective effect evaluation of β-caryophyllene in cellular models of neurodegenerative diseases. College science pharmaceutical in Ribeirão Preto. Doctoral thesis pp. 1-71.
Figueiredo AC, Barroso JG, Pedro LG, Scheffer JC (2008). Factors Affecting Secondary Metabolite Production in Plants: VolatileComponents and Essential Oil. A Review. Flavour Fragr. J. 23:213-226.
Gois RWS, Sousa LM, Lemos TLG, Arriaga AMC, Andrade-Neto M, Santiago GMP, Ferreira YS Alves, PB, Jesus HCR (2011). Chemical Composition and Larvicidal Effects of Essential oil from *Bauhinia acuruana* (Moric.) against *Aedes aegypti*. J. Essent. Oil Res. 23:59-62.
Gramosa NV, Freitas JVB, Neto MND (2009). Volatile components of the essential oil from *Bauhinia ungulata* L. J. Essent. Oil Res. 21(6):495-496.
Joly AB (1993). Botany: Introduction to plant taxonomy.11ª. Ed., Nacional: São Paulo.
Joly AB (1998). Botany: introduction to vegetal taxonomy. 12ª. Ed., Nacional editor: São Paulo.
Kim S, Jung E, Kim JH, Park YH, Lee J, Park D (2011). Inhibitory effects of (-) α- bisabolol on LPS- induced inflamatory responde in RAW264 macrophages. Food Chem. Toxicol. 49(10):2580-2585.
Lopes WB (2002). Development of an alternative method to the use of laboratory animals for toxicity evaluation of plant extracts. Rev. Eletrôn Horizonte Científico. 1:1-11.
Lorenzi H, Matos FJA (2002). Medicinal plants in Brazil: native and exotic grown*,* Nova Odessa editor: São Paulo.
Mclaughlin JL, Chang CJ, Smith DL (1993). Simple bench-top bioassays (BS & PD) for discovery of plant antitumor compounds-review of recent progress in human medicinal agents from plants. Nova York: Kinghorn & Balandrini. pp. 112-137.
Meyer BN, Ferrigni NR, Putnam LB, Jacobsen DE, Nichols and McLaughlin JL (1982). A convenient general bioassay for active plant constituents. Planta Méd. 45:31-34.
Morais SM, Dantas JDP, Silva ARA, Magalhães EF (2005). Medicinal plants used by the Tapebas Indians of Ceara. Bras. J. Pharmacogn. 15(2):169-177.
Neto MM (2006). Contribution to the knowledge of chemical plant in northeastern Brazil. Dissertation (Master in Chemistry) – Federal University of Ceará. Fortaleza. pp. 1-170.
Nurulain S, Prytkova T, Sultan AM, Ievglvskyi O, Lokke D, Yang KHS, Petroianu G, Howarth FC, Kabbani N, Oz M (2015). Inhibitory actions of bisabolol on α 7- nicotinec acetylcholine receptors. Rev. Neurosci. 306:91-99.
Oliveira F, Akisue G, Akisue KM (1998). Pharmacognosy. São Paulo: Atheneu editor. 1-412.
Paula SC, Canteli VCD, Hirota BCK, Campos R, Oliveira VB, Kalegari M, Silva CB, Silva GM, Miguel OG, Miguel MD (2014). Antioxidant potential of *Bauhinia Ungulata.* Rev. Ciênc. Farm. Básic. Apl. 35(2):217-222.
Pepato MT, Keller EH, Baviera AM, Kettelhut IC, Vendramini RC, Brunetti IL (2002). Anti-diabetic activity of *Bauhinia forticata* decoction in Streptozotocin-diabetic rats. J. Ethopharmacol. 81:191-197.
Port's S P, Chisté RC, Godoy HT, Prado MA (2013). The phenolic compounds and the antioxidant potential of infusion of herbs from the Brazilian Amazonian region. Food Res. Int*.* 53:875-881.
Rajkapoor B, Jayakak B, Murugesh N (2003). Antitumour Activity of *Bauhinia variegata* on Dalto's Asiatic lymphoma. J. Ethnopharmacol. 89:107-109.
Rodriguez AG, Texeira JRA, Salles FG, Vital JP, Peixoto DS. (2009). *Artemia salina* bioassay for detection of toxins in foods. Estudos 36(5):795-808.
Rubiolo P, Sgorbini B, Liberto E, Cordero C, Bicchi C (2010). Essential oils and volatiles: sample preparation and analysis. A review. Flavour Fragr. J. 25:282-290.
Santos AE, Kuster RM, Yamamoto KA, Salles TS, Campos R, Meneses MDF, Soares MR, Ferreira D (2014). Quercetin and quercetin 3-glicosides from *Bauhinia longifolia* (Bong) steud. Show anti-Mayaro virus activity. Parasit. Vectors 7:130.
Santos RC, Melo Filho AA, Chagas EA, Takahashi JA, Ferraz VP, Montero IF, Ribeiro PRE, Melo ACGR, Holanda LC (2015). Chemical composition, antimicrobial and antiacetylcholinesterase activities of essential oil from *Lantana camara* (Verbenaceae) flowers. J. Med. Plants Res. 9(35):922-928.
Santos KM, Gonçalves OS, Paiva MJN, Lacerda GA (2011). Acetylcholinesterase Inhibition from Startin extracts of *Bauhinia variegata L, Bauhinia Var. Candida* (Aiton) Buch – ham and *Bauhinia ungulata L.* Rev. Soc. Bras. Med. Trop. 44(6):741-783.
Santos MP, Limab ES, Moraesc MO, Costac PM, Meirac AS, Pessoac CÓ, Valented LMM, Juniora VFVN (2014). Profile of flavonoids and evaluation of the antioxidant potential and of cytotoxic *Bauhinia purpurea* (Fabaceae) the Amazon Region. Química Nova 37(1):89-94.
Savalev S, Okello E, Perry NSL, Wilkins RM, Perry EK (2003). Synergistic and antagonistic interactions of anticholinesterase terpenoides in *Salvia lavandulaefolia* essential oil. Pharmacol. Biochem. Behav. 75:661-668.
Sefidkon F (2002). Essential oil of *Lantana camara* L. occurring in Iran. Flavour Fragr. J. 17:78-80.
Silva FRMB, Szpoganicz B, Pizzolatti MG, Willrich MAV, Sousa E (2002). Acute effect of *Bauhinia forticata* on serum glucose levels in normal and alloxan- induced diabetic rats. J. Ethnopharmacol. 83:33-37.
Silva KLC, Camara CAG (2014). Chemical composition and evaluation of acaricide potential of the essential oil from the leaves of bauhinia rufa species Steud against the mite Rajad the *tetranichus urticae koch*. in: 54º comgresso brasileiro de quimica cbq, natal. Resumo, Natal:cbq. pp. 1-54.
Silva LK, Cechinel-Filho V (2000). Plants Bauhinia Gender: Chemical Composition and Potential Pharmacological. Química Nova 3:449-454.
Simões CMO, Spitzer V (2004). Volatele oils. In: Simões CMO, Schenkel EP, Gosmann, G, Mello JCP, Mentz LA, Petrovick PR (Org.). Farmacognosia: da planta ao medicamento. 5ª ed. Porto Alegre: Editora UFRGS/ Florianópolis: Editora UFSC.
Singh KL, Singh DK, Singh VK (2012). Characterization of the molluscicidal activity *Bauhinia Variegata* and *mimusops clengi* plant extracts against the fasciola vector *Limnaea acuminate*. Rev. Inst. Med. Trop. 53(3):135-140.
Souza VC, Lorenzi H (2008). Systematic Botany: Illustrated Guide to the identification of the families of native and exotic Phanerogams in Brazil, based on APG II. 2ª. ed.; Instituto Plantarum: Nova Odessa, São Paulo.
Vila R, Santana AI, Rosés RP, Valderrama A, Castelli MV, Mendonça S, Zacchio S, Gupta MP, Canigueral S (2010). Composition and biológical activity of essential oil from leaves of *Plinia cerrocampanensis* a new souce of α- bisabolol. Bioresouce Technol. 101:2510-2514.

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Antifungal and cytotoxicity activities of *Anacardium othonianum* extract

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The antifungal activities of the crude ethanolic extract of *Anacardium othonianum* (Anacardiaceae) leaves (EE) and the *n*-hexane (HF), EtOAc (EF), *n*-BuOH (BF) and hydromethanolic (HMF) fractions were assayed against *Candida albicans* (ATCC 64548) and *Trichophyton rubrum* (Tr1). Additionally, the cytotoxicities were also evaluated against normal human lung fibroblasts (GM07492A). The EE yielded minimum inhibitory concentration (MIC) values of 78.12 and 312.50 µg/mL for *C. albicans* and *T. rubrum*, respectively, and no cytotoxicity was observed. The EF and BF fractions exhibited enhanced antifungal activities when compared with the MIC values obtained for the EE fraction, and no cytotoxicity was observed for either fraction. Thus, the EF fraction, which displayed the higher antifungal activity, was purified, leading to the isolation of the following compounds: amentoflavone (1), gallic acid (2), protocatechuic acid (3), and ethyl 3,4,5-trimethoxybenzoate (4). HPLC analysis confirmed the presence of compounds 1-4 and 1-3 in the EF and BF fractions, respectively, in different proportions. The results suggest that the antifungal activities of the EE, EF and BF fractions may be attributed mainly to the actions of 1, 2 and 3.

Key words: *Anacardium othonianum* extract, antifungal and cytotoxicity activities, HPLC analysis.

INTRODUCTION

Anacardiaceae consists of approximately 76 genera and 600 species, which are divided into five tribes:

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Anacardieae, Dobineae, Rhoeae, Semecarpeae and Spondiadeae (Vogl et al., 1995). The genus *Anacardium* (Anacardieae) consists of 10 species typically found in tropical climates (Mitchell and Mori, 1987). *Anacardium othonianum* Rizz., which is known in Brazil as “caju-de-árvore-do-cerrado”, “cajuzinho”, and “cajuí”, is native to the Brazilian savannah. This species is regionally important and has widespread acceptance as a food product (Bessa et al., 2013). The Brazilian traditional medicine has allusions to the employment of *A. othonianum*, which has mainly been used in the treatment of inflammation, diabetes, pain and respiratory diseases, such as coughing and the flu (Vieira et al., 2006). Chemical examinations of the *Anacardium* species have revealed the major presence of tannins, phenolic acids, sterols, flavonoids, biflavonoids, phenolic lipids and saponins (Konan and Bacchi, 2007; Luiz-Ferreira et al., 2010; Dinda et al., 1987; Correia et al., 2006; Arya et al., 1989).

Dermatomycoses are common dermatological conditions of the skin, hair and nails caused by superficial fungal infections, affecting more than 20-25% of the people in the world, mainly in tropical and subtropical regions. These diseases alter the individuals’ quality of life and are considered a public health issue (Havlickova et al., 2008; Simonnet et al., 2011). The etiologic agents of dermatomycoses comprise dermatophytes, yeasts and non-dermatophytic filamentous fungi. Epidemiological studies have verified that most patients with superficial fungal infections had dermatophytes or yeast depending on the geographic location (Silva et al., 2014; Kemna and Elewski, 1996). The treatment available for dermatomycoses includes both topical and systemic antifungal drugs, such as the allylamines (terbinafine), triazoles (fluconazole, itraconazole and voriconazole), imidazoles (ketoconazole) and griseofulvin (Meis and Verweij, 2001). The resistance to the available therapeutic agents, for example, in the case of terbinafine, has led to the need for new drugs (Mukherjee et al., 2003). Plant crude extracts have demonstrated antifungal activity in many studies (Kuete et al., 2009; Mbaveng et al., 2008).

Antifungal effects have not been reported for *A. othonianum*. Therefore, herein, the authors described the antifungal activities of the crude ethanolic extract (EE); the *n*-hexane (HF), EtOAc (EF), *n*-BuOH (BF), and hydromethanolic (HMF) fractions; and isolated compounds of interest against the following microorganisms: *Candida albicans* and *Trichophyton rubrun*. Additionally, the cytotoxicities of these compounds were also evaluated.

MATERIALS AND METHODS

General

^{1}H- and ^{13}C-NMR spectra were recorded in CD_3OD on Bruker 400 and 500 NMR spectrometers using tetramethylsilane (TMS) as an internal standard. A positive-ion mode HRESIMS spectrum was obtained on a Bruker Daltonics HRMS ultrOTOF-Q-ESI-TOF instrument using electrospray ionization. Analytical and preparative HPLC analyses were performed on Shimadzu LC-20AD and LC-6AD systems, with a degasser DGU-20A5, an SPD-M20A series PDA detector or an SPD-20A series UV-VIS detector, a CBM-20A communication bus module, and a Reodyne manual injector or SIL-20A autosampler. A SHIMADZU Shim-pack ODS (particle diameter = 5 µm, 250 × 4.60 mm, and 250 × 20 mm) column equipped with a pre-column were used in the HPLC separations. The MeOH employed was of HPLC grade (J. T. Baker), and ultrapure water was obtained from Millipore Direct-Q UV3 system. Sample preparation was accomplished using silica gel 90 reverse-phase ODS (Fluka, 230-400 mesh).

Plant

The leaves of *A. othonianum* Rizz. were obtained from the Brazilian Cerrado in the Goiás State in the city of Montes Claros de Goiás (17°48′15.9" S and 50°54′19.5" W), in September 2012. A voucher specimen (HJ3793) was deposited in the Herbarium Jataiense Germano Guarim Neto of the Universidade Federal de Goiás, Brazil (Herbarium HJ).

Extraction and isolation

The powdered air-dried leaves of *A. othonianum* (1.17 kg) were extracted with ethanol, and the solvent was eliminated under reduced pressure producing 30.8 g of the crude extract. The crude extract (EE, 24 g) was then resuspended in MeOH:H_2O (2:8, v/v) and partitioned with *n*-hexane, EtOAc and *n*-BuOH. The solvent of the obtained phases was removed using a rotary evaporator, yielded 0.2, 1.9, 12.4, and 1.2 g, respectively. The EtOAc fraction (1.9 g) was purified by solid phase extraction with a silica gel 90 reverse-phase ODS chromatographic column using MeOH:H_2O (30:70 v/v, 50:50 v/v) and 100% MeOH as eluents, yielded three fractions. Fraction 3 (1.2 g) yielded compound 1. Fraction 1 was dissolved in MeOH:H_2O (35:65, v/v) and subjected to preparative RP-HPLC purification using MeOH:H_2O:Acetic acid (58:41.9:0.1, v/v/v), UV detection at 254 nm, and a 9.0 mL/min flow rate, which yielded seven fractions. Fractions 3, 4 and 6 gave compounds 2 (140 mg, retention time (t_R)= 3.54 min), 3 (260 mg, t_R= 7.75 min) and 4 (200 mg, t_R= 14.45 min), respectively.

Amentoflavone (1)

^{1}H-NMR (400 MHz, CD_3OD) δ: 7.83 (d, *J*=2.3, 1H, H-2'), 7.79 (dd, *J*=2.3 and 8.6, 1H, H-6'), 7.41 (d, *J*=8.8, 2H, H-2''' and H-6'''), 7.01 (d, *J*=8.6, 1H, H-5'), 6.61 (d, *J*= 8.8, 2H, H-3''' and H-5'''), 6.50 (s, 1H, H-3''), 6.48 (s, 1H, H-3), 6.29 (d, *J*=2.0,1H, H-8), 6,26 (s, 1H, H-6''), and 6.07 (d, *J*=2.0, 1H, H-6).^{13}C-NMR (100 MHz, CD_3OD) δ: 184.2 (C-4), 183.8 (C-4''), 165.9 (C-2), 165.0 (C-7, C-2'' and C-7''), 162.0 (C-5 and C-5''), 161.0 (C-4'''), 159.4 (C-4'), 159.0 (C-9), 156.5 (C-9''), 132.8 (C-2'), 129.3 (C-2''' and C-6'''), 128.9 (C-6'), 123.0 (C-1'), 123.1 (C-1'''), 121.0 (C-3'), 117.4 (C-5'), 116.8 (C-3''' and C-5'''), 105.0 (C-10, C-8'' and C-10''), 104.0 (C-3''), 103.4 (C-3)**,** 100.1 (C-6''), 100.0 (C-6), and 95.1 (C-8). HR-ES-MS *m/z* 539.0936 (calculated for $C_{30}H_{18}O_{10}$ $[M+H]^+$, 539.0978).

Gallic acid (2)

^{1}H-NMR (500 MHz, CD_3OD) δ: 6.95 (s, H-2 e H-6, 2H). ^{13}C-NMR

(100 MHz, CD_3OD) δ: 170.4 (C-7), 146.4 (C-3 and C-5), 139.6 (C-4), 121.9 (C-1) and 110.3 (C-2 and C-6).

Protocatechuic acid (3)

^{1}H-NMR (500 MHz, CD_3OD) δ: 7.45 (d, *J*= 2.0, 1H, H-2), 7.44 (dd, *J* = 8.0 and 2.0 Hz, 1H, H-6) and 6.81 (d, *J*= 8.0 Hz, 1H, H-5). ^{13}C-NMR (100 MHz, CD_3OD) δ: 170.5 (C-7), 151.6 (C-4), 146.1 (C-3), 123.9 (C-6), 123.4 (C-1), 117.8 (C-2), 115.8 (C-5).

Ethyl 3,4,5-trimethoxybenzoate (4)

^{1}H-NMR (500 MHz, CD_3OD) δ: 6.93 (2H, s, H-2 and H-6), 4.16 (q, *J*= 7.1 Hz, 2H), 3.38 and 3.10 (OCH_3), 1.24 (t, *J*= 7.1 Hz, 3H). ^{13}C-NMR (100 MHz, CD_3OD) δ: 168.6 (C-7), 146.5 (C-3 and C-5), 139.7 (C-4), 121.7 (C-1), 110.0 (C-2 and C-6), 61.7 (C-1'), 50.0, 50.0 and 49.9 (OCH_3), 14.6 (C-2').

Chromatographic studies

Chromatographic separations of the samples were carried out on a Phenomenex Gemini C18 (Octadecylsilane) (5 µm, 250 x 4.60 mm) column with a pre-column. For the experiments, the elution conditions were as follows: methanol/water (0.1% acetic acid) gradient from 5 to 100% methanol for 30 min, then elution with 100% methanol for 10 min, oven at 40°C, and flow = 1.0 mL/min. The analysis also includes 3 min to regress to the initial conditions and 15 min of equilibration. The chromatogram wavelength was set at 254 nm, and UV data were recorded between 190 and 500 nm. The 1-mg samples (EE, EF, BF and 1-4) were weighed and dissolved in 1 mL of methanol. The sample solutions were filtered and then transferred to a vial.

Antifungal assay

The following microorganisms were employed for evaluation of the antifungal activity: *Candida albicans* (ATCC 64548) and *Trichophyton rubrum* (Tr1, earlier identified by Profa. Dra. Ana Marisa Fusco Almeida). The *T. rubrum* strain was preserved and cultured in Petri dishes having Sabouraud agar. It was incubated and subcultured for 7 days at 28°C. The strain was suspended in 0.85% saline solution, counted in a Neubauer chamber and final concentration of 5.0 x 10^3 CFU/mL was achieved by dilution in RPMI medium (Ghannoum et al., 2006). The strain *C. albicans* was cultivated in RPMI-1640. The inoculum was adjusted to 0.5 McFarland scale to yield a cell concentration of 1×10^6-5×10^6 yeast/mL (CLSI, 2008a). The extracts, fractions and compounds 1-4 were resuspended in dimethyl sulfoxide (DMSO) to concentrations of 4 mg/mL and subsequently diluted in RPMI medium with L-glutamine (pH 7.2) with 0.165 mol/L morpholine propane sulfonic acid (MOPS) complemented with 2% glucose. Amphotericin B (Sigma Chemical Co. Saint-Louis, USA) was prepared in DMSO and tested in the concentrations of 0.00625 to 32 µg/mL. Minimum inhibitory concentration (MIC) values for each sample were obtained in triplicate using document M-38 A2 adapted to 96-well microplates for *T. rubrum* (CLSI, 2008b). Each well contained 100 µL of a twofold serially diluted sample and 100 µL of RPMI medium, with 100 µL being transferred to the next well, sequentially. Then, 100 µL of the *T. rubrum* inoculum was added to obtain a final sample volume of 200 µL with concentrations ranging from 1250 to 2.44 µg/mL. For amphotericin B, 100 µL of the twofold sample dilution was added to 100 µL of the inoculum. The microplates were incubated in an orbital shaker at 120 rpm and 28°C for 7 days. The MIC was determined as the minimum concentration of the tested sample for which no growth was visualized, and then 30 µL of a 0.01% resazurin aqueous solution (Sigma-Aldrich) was added to determine the microorganism viability. The minimum inhibitory concentration (MIC) values against *C. albicans* for each sample were determined in triplicate using the reference method M-27 A3 (CLSI, 2008a) with modifications. Each well contained 100 µL of a twofold serially diluted sample and 100 µL of RPMI medium, with 100 µL being transferred to the next well, consecutively. Then, 100 µL of the *C. albicans* inoculum was added to obtain a final sample volume of 200 µL with concentrations ranging from 1250 to 2.44 µg/mL. For amphotericin B, 100 µL of the twofold drug dilution was added with 100 µL of the microorganism. The orbital shaker at 120 rpm and 35°C for 2 days was used to incubate the microplates. Then, the viability of *C. albicans* was determined by the addition of a 2.0 % 2,3,5-triphenyltetrazolium chloride aqueous solution (Sigma-Aldrich). The MIC was characterized as the smallest concentration of the tested sample for which no development was observed.

Cytotoxicity assay

The cytotoxicity was measured using the *in vitro* Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics) according to manufacturer´s instructions, using a normal human lung fibroblasts (GM07492A), and as previously described (Alvarenga et al., 2015).

Statistical analysis

Inhibitory concentration at 50% cell growth inhibition (IC_{50}) was obtained with the GraphPad Prism 5 software. One-way ANOVA was used for the comparison of means ($p < 0.05$).

RESULTS AND DISCUSSION

The effects of the crude ethanolic extract from the leaves of *A. othonianum* (EE) on the growth of the selected fungi are shown in Table 1. The lowest MIC value was obtained for *C. albicans* (MIC= 78.12 µg/mL), and the EE showed no cytotoxicity. Once, Suffness and Pezzuto (1990), extracts that present IC_{50} values lower than 30 µg/mL indicated cytotoxic action. In addition, the EE displayed a MIC of 312.5 µg/mL against *T. rubrum*.

Among the four fractions, including the *n*-hexane (HF), EtOAc (EF), *n*-BuOH (BF), and hydromethanolic (HMF) fractions, achieved by liquid-liquid partitioning of the EE, the EtOAc fraction (EF) displayed the highest antifungal activity, with MIC values of 4.88 and 39.06 µg/mL against *C. albicans* and *T. rubrum*, respectively, and no cytotoxicity was observed for this fraction.

According to these results, the EF fraction exhibits promising activity. The *n*-BuOH fraction (BF) also exhibited potential antifungal activity against *C. albicans* and *T. rubrum* (MIC= 19.53 and 39.06 µg/mL, respectively) and did not significantly inhibit the growth of the normal cell line.

When compared with the EE, the *n*-hexane fraction (HF) provided inferior antifungal activity against *C. albicans* (MIC > 1250 µg/mL). However, HF displayed the same MIC value against *T. rubrum* (MIC= 312.5 µg/mL), but HF was considered cytotoxic when compared with the

Table 1. Minimum inhibitory concentration (MIC) values and cytotoxic activities obtained for the crude ethanolic extract of *A. othonianum*, fractions and isolated compounds.

Samples	MIC [µg/ml]		Cell line GM07492A[a]	
	C. albicans	*T. rubrum TR1*	IC_{50}[b] [µg/ml]	IC_{50}[b] [µM]
EE	78.12	312.50	> 400	-
HF	> 1250	312.50	235.3[c]	-
EF	4.88	39.06	> 400	-
BF	19.53	39.06	> 400	-
HMF	78.12	156.25	> 400	-
1	19.53	312.50	-	> 400
2	312.50	9.76	-	> 400
3	> 1250	39.06	-	> 400
4	> 1250	625.00	-	> 400
Amphotericin B	0.25	0.25	-	n.t.

[a]GM07492A (human lung fibroblasts); [b]IC_{50} 50% cytotoxic concentration values for the cell line after 24 h of treatment. Significantly different from the negative control ($P < 0.05$).

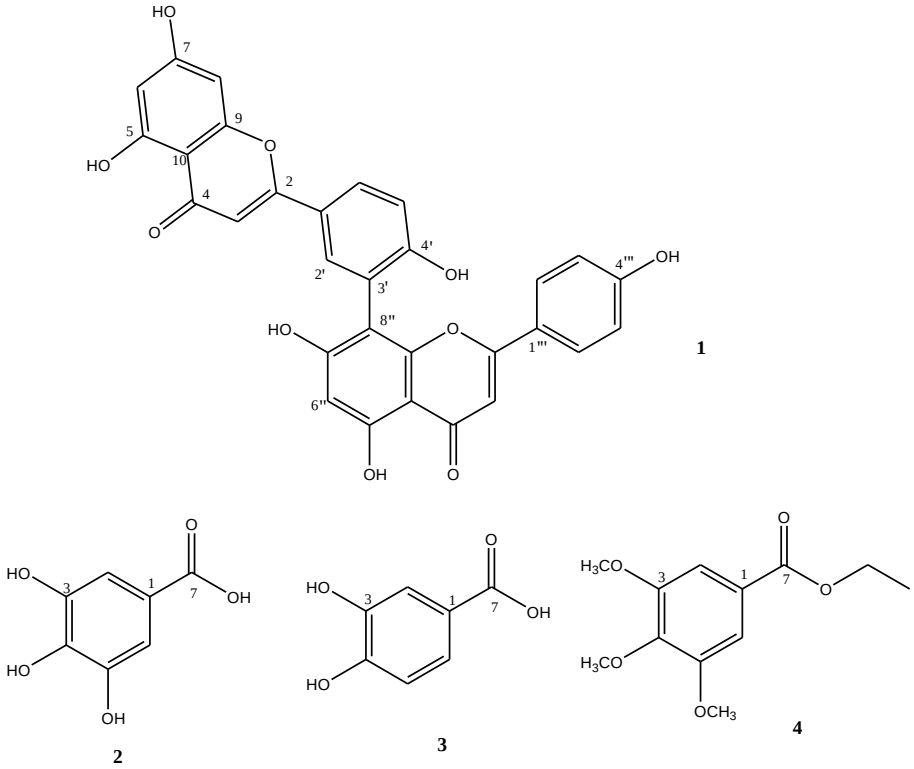

Figure 1. Chemical structures of the isolated compounds.

other fractions and the crude extract, with an IC_{50} value of 235.3 µg/mL. Although, this IC_{50} compared with the reference (Suffness and Pezzuto, 1990) did not indicate promising cytotoxic activity.

The hydromethanolic fraction (HMF), when compared with the EE, displayed an equal MIC value against *C. albicans*. Meanwhile, the result obtained for the HMF against *T. rubrum* was superior (MIC= 156.25 µg/mL) to that of EE, and no cytotoxic effects were observed for HMF. Thus, the HF and HMF fractions are not promising antifungal agents against the strains tested in this work when compared with the EF and BF fractions.

The EF fraction was purified by chromatography, yielding four compounds, namely, amentoflavone (1), gallic acid (2), protocatechuic acid (3) and ethyl 3,4,5-trimethoxybenzoate (4) (Figure 1), as established by

Table 2. Area (%) of the isolated compounds 1-4 in the crude ethanolic extract (EE) of *A. othonianum*, the EtOAc fraction (EF) and the *n*-BuOH fraction (BF).

Samples	1 (%)	2 (%)	3 (%)	4 (%)
EE	18.9 (7.8)[a]	2.45 (1.0)[a]	3.87 (1.6)[a]	2.44 (1.0)[a]
EF	24.8 (14.9)[a]	1.67 (1.0)[a]	4.48 (2.68)[a]	2.56 (1.5)[a]
BF	17.4 (6.6)[a]	2.64 (1.0)[a]	2.76 (1.0)[a]	n.d.[b]

[a]Percent relative to the lower area (%); [b]not detected.

NMR spectra and comparisons with earlier recorded data (Markham et al., 1997; Gottlieb et al., 1991; Hur et al., 2003; Wu and Darcel, 2009). To the best of the authors' knowledge, this is the first report on compounds 1-4 in *A. othonianum*.

Regarding the antifungal activities of the compounds, only 1 displayed significant inhibitory effects against *C. albicans*, with a MIC value of 19.53 µg/mL. Compounds 2 and 3 presented significant inhibitory effects against *T. rubrum TR1* (MIC values of 9.76 and 39.06 µg/mL, respectively). Again, no cytotoxicity was observed for the isolated compounds (IC_{50} > 400 µM). However, the isolated compounds displayed weaker antifungal activities when compared with amphotericin B, which was used as a positive control (Table 1). Additionally, compounds 1-4 have previously been shown to possess antifungal activities (Mbaveng et al., 2008; Leal et al., 2009; Kuete et al., 2009; Soares et al., 2014). Flavonoids have been found to be active against a wide range of microorganisms; their mechanism of action is possibly due to their capacity to complex with proteins and with bacterial cell walls (Cowan, 1999).

The structures of gallic acid (2) and ethyl 3,4,5-trimethoxybenzoate (4) were quite related, differing in the occurrence of three free hydroxyl substituents and an acidic moiety in 4 versus 3 methoxy groups and an ethyl acetate group in 4. The presence of three methoxyl groups in the latter compound might cause a decrease in the activity, suggesting that the three free OH groups could play a role in the antifungal activity. This finding is in accordance with the data obtained by Leal et al. (2009). Additionally, the mechanism of action of phenolic compounds comprises enzyme inhibition (Cowan, 1999).

Protocatechuic acid (3) and gallic acid (2) also showed related chemical structures, diverging only in the occurrence of two hydroxyl moieties in 3, which decreased the activity. Once more, these results suggested that the presence of the three free OH groups appears to be necessary for antifungal activity.

Regarding the chemical composition of the bioactive extract (EE) and fractions (EF and BF) (Figure 2a-c), an HPLC-DAD analysis was performed using a C18 column. The chromatograms were recorded using a methanol/water (0.1% acetic acid) gradient from 5 to 100% methanol over 30 min, then elution with 100% methanol for 10 min. These conditions were similarly employed to evaluate the presence of compounds 1-4 in the samples, based on an evaluation of retention times (λ = 254 nm) and UV spectra from a DAD detector of the compounds previously isolated from EF (Figure 2d).

Compounds 1-4 were recognized in different proportions (Table 2) in the ethanol extract from the leaves of *A. othonianum* (EE) and the EtOAc fraction (EF) (Figure 2a and b). Additionally, compound 4 was not detected in the *n*-BuOH fraction (BF) (Figure 2c). Conversely, the amentoflavone (1) was always present at a higher concentration relative to 2, 3 and 4. Based on the data obtained from the chromatogram and MIC values of EF, BF and compounds 1-3, when compounds 1-3 were combined in the fractions, the antifungal activity was improved, at least in the case of *C. albicans*.

Thus, the data obtained suggest that the antifungal activity of *A. othonianum* may be mainly attributed to the effects of amentoflavone (1), gallic acid (2) and protocatechuic acid (3). Frequently plant extracts showed better activity than the isolated compounds, this situation can be explained by synergy effects (Wagner and Ulrich-Merzenich, 2009). Although, in this study the isolated compounds displayed increased antifungal activity, when compared with the crude extract (EE).

Conclusions

To the best of the authors' knowledge, the antifungal activity of *A. othonianum* and the occurrence of compounds 1-4 are reported for the first time in this work. Though, the antifungal activities of compounds 1-4 have been documented previously. In conclusion, the antimicrobial activity of the crude extract of the leaves of *A. othonianum* may be due to the occurrence of compounds 1-3. The evaluated extract, together with fractions EtOAc (EF) and *n*-BuOH (BF) and compounds 1-3, could be beneficial for the research of new antifungal medications. Nevertheless, the toxicological and pharmacological studies of the analyzed samples will check this proposition.

Conflict of Interests

The authors have not declared any conflict of interests.

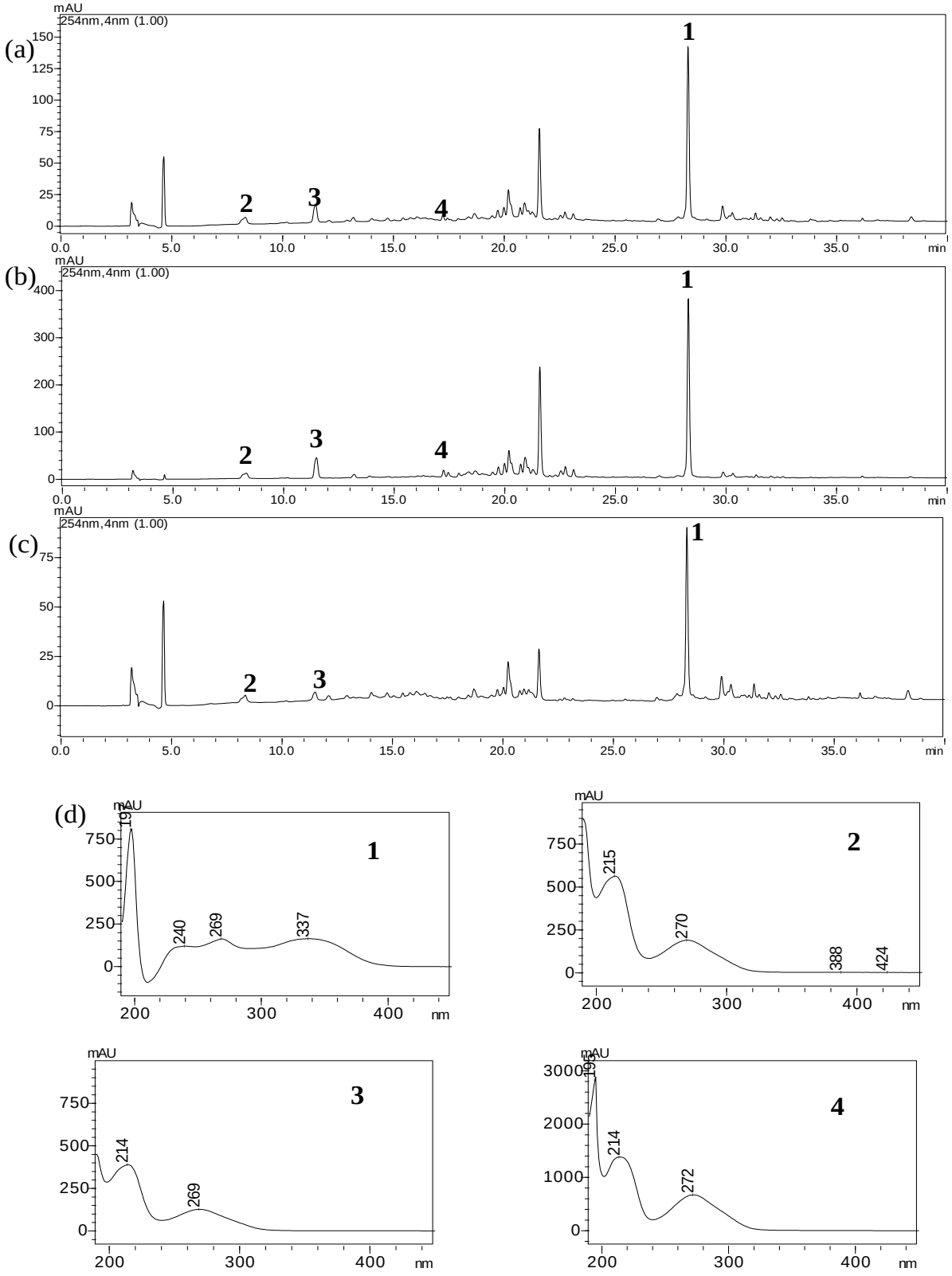

Figure 2. HPLC-DAD chromatogram of (a) the crude ethanol extract from *A. othonianum* leaves, (b) the EtOAc fraction and (c) the *n*-BuOH fraction and (d) UV-DAD spectra of compounds 1-4. Chromatographic conditions: methanol/water (0.1% acetic acid) linear gradient from 5 to 100% methanol over 30 min and 100% methanol for 10 min, including 3 min to return to the initial condition and 15 min of equilibration, oven at 40°C, and detection at λ = 254 nm. The flow-rate was 1.0 mL/min.

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REFERENCES

Alvarenga TA, Bertanha CS, de Oliveira PF, Tavares DC, Gimenez VM, Silva ML, Cunha WR, Januário AH, Pauletti PM (2015). Lipoxygenase inhibitory activity of *Cuspidaria pulchra* and isolated compounds. Nat. Prod. Res. 29(11):1083-1086.

Arya R, Babu V, Ilyas M, Nassim KT (1989). Phytochemical examination of the leaves of *Anacardium occidentale*. J. Ind. Chem. Soc. 66(1):67-68.

Bessa NGFde, Borges JCM, Beserra FP, Carvalho RHA, Pereira MAB, Fagundes R, Campos SL, Ribeiro LU, Quirino MS, Chagas Júnior, AF, Alves A (2013). Prospecção fitoquímica preliminar de plantas nativas do cerrado de uso popular medicinal pela comunidade rural do assentamento vale verde – Tocantins. Rev. Bras. Pl. Med. 15(4):692-707.

Clinical Laboratory Standards Institute CLSI (2008a). Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne.

Clinical Laboratory Standards Institute CLSI. (2008b). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne.

Correia SJ, David JP, David JM (2006). Metabólitos secundários de espécies de Anacardiaceae. Quím. Nova 29(6):1287-1300.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12(4):564-82.

Dinda B, Chatterjee J, Banerjee J (1987). Sterols from *Anacardium occidentale*. J. Indian Chem. Soc. 64(10):647-648.

Ghannoum MA, Arthington-Skaggs B, Chaturvedi V, Espinel-Ingroff A, Pfaller MA, Rennie R, Rinaldi MG, Walsh TJ (2006). Interlaboratory study of quality control isolates for a broth microdilution method (Modified CLSI M38-A) for testing susceptibilities of dermatophytes to antifungals. J. Clin. Microbiol. 44(12):4353-4356.

Gottlieb HE, Kumar S, Sahai M, Ray AB (1991). Ethyl brevifolin carboxylate from *Flueggea microcarpa*. Phytochemistry 30(7):2435-2438.

Havlickova B, Czaika VA, Friedrich M (2008). Epidemiological trends in skin mycoses worldwide. Mycoses 51(Suppl. 4):2-15.

Hur JM, Park JG, Yang KH, Park JC, Park JR, Chun SS, Choi JS, Choi JW (2003). Effect of methanol extracts of *Zanthoxylum piperitum* leaves and of its compound, protocatechuic acid, on hepatic drug metabolizing enzymes and lipid peroxidation in rats. Biosci. Biotechnol. Biochem. 67(5):945-950.

Kemna ME, Elewski BE (1996). A U.S. epidemiologic survey of superficial fungal diseases. J. Am. Acad. Dermatol. 35(4):539-542.

Konan NA, Bacchi EM (2007). Antiulcerogenic effect and acute toxicity of a hydroethanolic extracts from the cashew (*Anacardium occidentale* L.) leaves. J. Ethnopharmacol. 112(2):237-242.

Kuete V, Nana F, Ngameni B, Mbaveng AT, Keumedjio F, Ngadjui BT (2009). Antimicrobial activity of the crude extract, fractions and compounds from stem bark of *Ficusovata* (Moraceae).J. Ethnopharmacol.124(3):556-561.

Leal PC, Mascarello A, Derita M, Zuljan F, Nunes RJ, Zacchino S, Yunes RA (2009). Relation between lipophilicity of alkyl gallates and antifungal activity against yeasts and filamentous fungi. Bioorg. Med. Chem. Lett. 19(6):1793-1796.

Luiz-Ferreira A, Almeida AC, Cola M, Barbastefano V, Almeida AB, Batista LM, Farias-Silva E, Pellizzon CH, Hiruma-Lima CA, Santos LC, Vilegas W, Brito AR (2010). Mechanisms of the gastric antiulcerogenic activity of *Anacardium humile* St. Hilon ethanol-induced acute gastric mucosal injury in rats. Molecules 15(10):7153-7166.

Markham KR, Sheppard C, Geiger H (1997). ^{13}C NMR studies of some naturally occurring amentoflavone and hinokiflavone biflavonoids. Phytochemistry 26(12):3335-3337.

Mbaveng AT, Ngameni B, Kuete V, Simo IK, Ambassa P, Roy R, Bezabih M, Etoa FX, Ngadjui BT, Abegaz BM, Meyer JJ, Lall N, Beng VP (2008). Antimicrobial activity of the crude extracts and five flavonoids from the twigs of *Dorstenia barteri* (Moraceae). J. Ethnopharmacol. 116(3):483-489.

Meis JFGM, Verweij PE (2001). Current management of fungal infections. Drugs. 61(Suppl. I):13-25.

Mitchell JD, Mori SA (1987). The cashew and its relatives (*Anacardium*: Anacardiaceae). Mem. N. Y. Bot. Gard. 42(4):1-76.

Mukherjee PK, Leidich SD, Isham N, Leitner I, Ryder NS, Ghannoum MA (2003). Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob. Agents Chemother. 47(1):82-86.

Silva LB, de Oliveira DB, da Silva BV, de Souza RA, da Silva PR, Ferreira-Paim K, Andrade-Silva LE, Silva-Vergara ML, Andrade AA (2014). Identification and antifungal susceptibility of fungi isolated from dermatomycoses. J. Eur. Acad. Dermatol. Venereol. 28(5):633-640.

Simonnet C, Berger F, Gantier JC (2011). Epidemiology of superficial fungal diseasesin French Guiana: a three-year retrospective analysis. Med. Mycol. 49(6):608-611.

Soares LA, Gullo FP, Sardi JdeC, Pitangui NdeS, Costa-Orlandi CB, Sangalli-Leite F, Scorzoni L, Regasini LO, Petrônio MS, Souza PF, Silva DH, Mendes-Giannini MJ, Fusco-Almeida AM (2014). Anti-trichophyton activity of protocatechuates and their synergism with fluconazole. Evid. Based Complement. Alternat. Med. (2014):957860.

Suffness M, Pezzuto JM (1990). Assays related to cancer drug discovery, in: Hostettmann K (Ed.). Methods in Plant Biochemistry: Assays for Bioactivity. Academic Press, London. pp. 71-133.

Vieira RF, Costa TSA, da Silva DB, Ferreira FR, Sano SM (2006). Frutas nativas da região Centro-Oeste. 2nd ed.; Embrapa Recursos Genéticos e Biotecnologia, Brasília.

Vogl O, Qin M, Mitchell JD (1995). Oriental lacquers. 7. Botany and chemistry of Japanese lacquer and the beauty of the final art objects. Cell. Chem. Technol. 29(3):273-286.

Wagner H, Ulrich-Merzenich G (2009). Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine 16(2-3):97-110.

Wu X-F, Darcel C (2009). Iron-catalyzed one-pot oxidative esterification of aldehydes. Eur. J. Org. Chem. 8:1144-1147.

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