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Fatty acid profile and bioactivity from *Annona hypoglauca* seeds oil

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Plants from *Annona* (Annonaceae) genus are present in tropical regions, where they have economic and medicinal potential. Information on the fatty acids profile and bioactivity from seed oil of *Annona* species are incipient. The objective of this work was to investigate *Annona hypoglauca* seeds oil in terms of its yield, composition and biological activity (acetylcholinesterase enzyme inhibition, bactericidal and fungicidal activity). Fatty acids profiles were determined by Gas Chromatography equipped with Flame Ionization Detector. Oil yield reached about 15% and the major constituents detected were ω-9 oleic acid (42.65%) and ω-6 linoleic acid (29.63%). *A. hypoglauca* oil was potent for acetylcholinesterase inhibition (79.55%), and presented high and selective bioactivity against *Candida albicans*.

Key words: *Annona hypoglauca*, ω-9 oleic acid, ω-6 linoleic acid, acetylcholinesterase, *Candida albicans*.

INTRODUCTION

The Annonaceae family consists of about 112 genera and 2,440 species (Couvreur et al., 2011), occurring in tropical regions. Brazil has 33 native genera with about 250 species (Souza and Lorenzi, 2008). Species from *Annona* genus present various activities such as anthelmintic (Ferreira et al., 2013), antioxidant (Julián-

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Abbreviations: BHI, Brain Heart Infusion; **GC-FID,** gas chromatography using flame ionization detector; **AD,** Alzheimer's Disease.

Loaeza et al., 2011), antidiabetic (Florence et al., 2014), anticancer (Wang et al., 2014), antiacetylcholinesterase (Tsai and Lee, 2010), antimicrobial (Jamkhande et al., 2014), among others. Activity has been reported for various parts of the plant such as roots (Jamkhande et al., 2014), stem bark (Dutra et al., 2014), leaves (Matsumoto et al., 2014), fruit pulp (Clerici and Carvalho-Silva, 2011) and seeds (Ribeiro et al., 2014). A huge diversity of substances associated to a range of medicinal and nutritional (Pareek et al., 2011) benefits has also been described.

Annona hypoglauca species has in extracts of its wood antitumor activity against breast adenocarcinoma has been described for *A. hypoglauca* (Suffredini et al., 2007). Other screenings have shown cytotoxic activity against tumor cell lines such as prostate, lung, colon, central nervous system, leukemia, among others (Rinaldi, 2007). Activity of *A. hypoglauca* against the bacterium *Streptococcus mutans* has also been reported (Barnabé et al., 2014). The information on biological activities of *A. hypoglauca* is incipient. This work was addressed to study *A. hypoglauca* seeds oil in terms of its yield, composition and biological activities, which included examination on whether acetylcholinesterase was inhibited by the oil and its possible bactericidal and fungicidal actions.

MATERIALS AND METHODS

Source and processing of *A. hypoglauca* seeds

The plant was identified by Ricardo de Oliveira Perdiz (Studies Center of Amazonian Biodiversity, CENBAM, Brazil) and deposited in UFRR Herbarium (UFRR 3654). The fruits were obtained in Mucajaí city, Roraima, taken to the Environmental Chemistry Laboratory (Research Center, Post-Graduate Course in Science and Technology – NPPGCT-UFRR) and washed. Seeds were removed, washed, and dried at room temperature and then were placed in a drying oven at 40°C with air circulation. Seeds were ground and sieved on a 20 to 40 mesh fabric to obtain a homogeneous granulation (Jorge and Luzia, 2012).

A. hypoglauca oil seeds processing

The oil was obtained by extraction from hexane solvent in a Soxhlet apparatus for 6 h. The solvent was evaporated on rotaevaporator and the oil (10.0328 g) was properly packaged in an amber vial under nitrogen atmosphere and stored in a freezer (Jorge and Luzia, 2012).

Hydrolysis and methylation of lipids

An aliquot (10 mg) of *A. hypoglauca* oil was transferred to a 2 mL cryotube, which contained 100 µL of a mixture made of ethanol (95%) and KOH 1 mol/L (5%). After vortexing for 10 s, esters in the oil were hydrolyzed in a microwave oven (Panasonic Piccolo) at 80 W (power 2) for 5 min. After cooling and neutralization with 400 µL of hydrochloric acid 20%, 20 mg NaCl and 600 µL of ethyl acetate were added. Afterwards, free fatty acids were obtained by using an adapted protocol of the one reported by Christie (adapted from Christie, 1989). Thus, after vortexing for 10 s and rest for 5 min, aliquots (300 µL) of the ethyl acetate layer was taken, placed in microcentrifuge tubes and dried by evaporation. Free fatty acids were methylated using 100 µL of BF_3/methanol (14%) and the reaction mixture was heated for 10 min in a water-bath at 60°C. After dilution with 400 µL methanol, fatty acid methyl esters were analyzed by Gas Chromatography.

Oil analysis by GC-FID

Free fatty acids were resolved by Gas Chromatography using HP7820A (Agilent) system equipped with flame ionization detector. An Innowax column (HP) 15 m × 0.25 mm × 0.20 µm was used and the following temperature gradient: 100°C min and 0.7°C/min up to 240°C; injector (1/30 split) to 250 and 260°C detector. Hydrogen was used as carrier gas (3 mL/min) and injection volume was 1 µL. The data acquisition program used was EZChrom Elite Compact (Agilent). The peaks were identified using FAME Mix C14-C22, CRM18917 Supelco fatty acid methyl esters standard.

Biological screening

AChE inhibition assay

Aliquots of a working solution (25 µL) (sample in DMSO 10 mg/mL) were added to microplate wells and positive and negative controls were also prepared. To the first five wells of a column (positive control) 25 µL of an eserine solution prepared at 10 mg/mL (31 mM; 2.7 mM in the whole reaction mixture 275 µL) in Tris/HCl at pH 8.0) was added. Then, 25 µL of acetylthiocholine iodide (ATChI, Sigma A5751) 15 mM; the reaction mixture, 125 µL of 5',5-dithio-bis (2-nitrobenzoate) (DTNB, Sigma D8130) (3 mM) and 50 µL of Tris/HCl (50 mM, pH 8) containing 0.1% (m/v) bovine serum albumin was added to each well. Absorbance was measured at 405 nm every 1 min for 8 times. Then 25 µL (0.226 U/mL) of Electric eel AChE (type VI-S) provided by Sigma (C3389-500UN) in Tris/HCl was added to each well. Absorbance was measured at 405 nm by 10 times (Frank and Gupta, 2005; Ellman et al., 1961).

Filamentous fungi assay

Filamentous fungi used in this test were *Aspergillus flavus* (CCT 4952) and *Fusarium proliferatum* (CML 3287). DMSO was used for sample preparation and the concentration of sample in the assay was 250 mg/mL. Sabouraud broth was used for fungal growth. A spore suspension at a concentration of 5×10^{-5} spores/mL was used after spores counting on a Neubauer chamber. The sample incubation time was 48 h after which absorbance was read at 490 nm on a microtitre plate reader. Data were processed using the Outlier method, Grubbs test with 95% significance level. The percentage of inhibition was calculated by using the formula.

% inhibition = 100-AC-AC × 100AH-AM

AC = absorbance of the sample; AC = absorbance of control sample; AH = absorbance of microorganisms control and AM = absorbance culture medium control.

Antibacterial and antifungal assay

Escherichia coli (ATCC 25922), Salmonella *tiphymurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus sanguinis (*ATCC 49456*)* and *Candida albicans* (ATCC 18804) were used in the assay following the procedures for Minimum Inhibitory

Table 1. Composition and quantification of fatty acids in *A. hypoglauca* seeds oil (percentage in the mixture of fatty acids).

Fatty acids	Retention time (min)	Amount (%)			
		A. hypoglauca	Olive (Carvajal-Zarrabal et al., 2014)	Corn (Carvajal-Zarrabal et al., 2014)	Canola (Carvajal-Zarrabal et al., 2014)
Lauric acid (C12:0)	1.56	0.09	0	0	0
Myristic acid (C14:0)	2.86	0.13	0	0	0
Palmitic acid (C16:0)	4.74	16.35	11.32	7.19	7.50
Palmitoleic acid (C16:1)	4.90	0.55	0.11	0.02	0.20
Stearic acid (C18:0)	6.87	6.65	4.34	4.51	3.30
Oleic acid (ω-9) (C18:1)	7.07	42.65	74.12	32.08	32.0
Linoleic acid (ω-6) (C18:2)	7.56	29.63	7.64	54.26	37.0
α-Linolenic acid (ω-3) (C18:3)	8.20	0.65	0.61	0.10	7.70
Eicosanoic acid (C20:0)	8.98	0.50	0	0	0
Behenic acid (C22:0)	11.04	0.11	0	0	0

Concentration (MIC) described below. Concentrations assayed were 500, 250, 125, 62.5, 31.25, 15.6, and 3.9 µg/mL (Zacchino and Gupta, 2007). Samples were weighed and dissolved in DMSO to 50 mg/mL. Forty µL of this solution was added to a flask containing 960 µL of BHI (Brain Heart Infusion) broth (working solution). 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 18 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 (10^8 CFU/mL), thereby obtaining the inocula used in the bioassays. Assays were performed in 96-microwell plates in duplicate. One hundred µL of BHI broth was added to each well. In the first well 100 µL of working solution was 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 was 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 permitted us 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. 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 AND DISCUSSION

Fatty acids profile and quantification by gas chromatography using flame ionization detector (GC-FID)

Extraction of *A. hypoglauca* seeds provided 15.05% yield in oil. This oil was subjected to hydrolysis and methylation before being analyzed by GC-FID. Table 1 shows the identified fatty acids (%) from seed oil of *A. hypoglauca*. The relative abundance of fatty acids in seeds of other oil-making plants is also shown. The ω-9 in *A. hypoglauca* is higher than the corn oil and canola and ω-6 higher than the olive oil and ω-3 was similar to the concentration in olive oil and superior to the corn oil, canola was superior. These unsaturated fatty acids are important to human health, because they act on reducing blood lipid, cholesterol, but the human body does not produce such acid (Lopez-Huertas, 2010; Simopoulos, 2006).

Bioactivity of *A. hypoglauca*

Inhibition of acetylcholinesterase

But fatty acids can go beyond reducing blood lipids and reduce cardiovascular problems. The fatty acids present in vegetable oils or fish fats may have anti-inflammatory activities (Calder, 2005; Calder, 1998; Zhao et al., 2005), mainly against neuroinflammation. The neuroinflammation can lead to brain disorders such as Alzheimer's disease (AD) and Parkinson's disease, but there is a relationship between the use of polyunsaturated fatty acids present in oils or fats at low risk of developing these disorders (Bazinet and Layé, 2014). Thus, seek for new therapeutic agents from natural products such as extracts, essential oils and fixed oils with potential for inhibition of acetylcholinesterase (AChE) to treat people with AD is a modern challenge (Mukherjee et al., 2007). In this way, in testing the capacity of AChE inhibition, *A. hypoglauca* seed oil showed to be able to inhibit 79.55% of AChE activity. According to Vinutha et al. (2007) inhibitions values higher than 50% indicate potent inhibition; agents providing values below 30% are considered weak inhibitors and those leading to 30 to 50% inhibition are considered moderate inhibitors. The best known and prevailing neurodegenerative diseases are Parkinson and Alzheimer (AD). Symptoms of the latter include a regression of various physiological functions, causing difficulties in language, memory, emotional

Table 2. Bioactivity of oil from *A. hypoglauca* seeds against fungi and bacteria.

% Oil inhibition against yeast			
Concentration (µg mL^{-1})	***C. albicans***	**Miconazole (%)**	**Nystatin (%)**
500	91.08	92.33	93.30
250	90.61	91.05	90.77
125	91.01	90.71	90.14
62.5	90.02	90.55	90.18
31.25	91.09	91.15	90.92
15.625	94.57	91.61	91.49
9.375	91.45	91.52	91.26
3.90625	91.27	91.28	91.42

% Oil Inhibition against filamentous fungi		
Concentration (µg mL^{-1})	***A. flavus***	***F. proliferatum***
250	11.69%	3.69%

% Oil inhibition against bacteria gram(-)				
Concentration (µg mL^{-1})	***E. coli***	**Ampicillin (%)**	***S.* Typhimurium**	**Ampicillin (%)**
500	30.867	100.0	77.637	100.0
250	21.169	100.0	7.234	100.0
125	17.737	100.0	24.939	100.0
62.5	15.499	100.0	59.829	100.0
31.25	16.792	96.270	57.241	100.0
15.625	18.781	95.375	17.277	100.0
9.375	22.910	94.529	56.930	100.0
3.90625	26.789	79.559	63.763	100.0

% Oil inhibition against bacteria gram(+)				
Concentration (µg mL^{-1})	***S. aureus***	**Ampicillin (%)**	***S. sanguinis***	**Ampicillin (%)**
500	24.902	100.0	17.658	100.0
250	28.937	100.0	14.117	3.223
125	23.917	100.0	19.110	2678
62.5	20.177	100.0	14.389	0.000
31.25	12.106	100.0	7.490	0.000
15.625	8.268	86.713	18.021	0.000
9.375	13.780	34.744	20.835	0.000
3.90625	5.807	14.862	25.465	0.000

or personality behavior, and cognitive abilities (Singh et al., 2013). Since the number of people afflicted of AD increases exponentially, it is estimated that in 2050 about 115 million people might be affected by AD around the world (WHO, 2012).

Bioassays with filamentous fungi

A. hypoglauca oil showed low inhibition of fungi viability (*A. flavus*, 11.69%; and *F. proliferatum*, 3.69%, Table 2). These unsatisfactory results discarded the use of the oil as a means to control these filamentous fungi. *A. flavus* and *F. proliferatum* are phytopathogenic fungi that generate large economic damage. For *A. flavus*, for example, various types of grains contaminated with the mycotoxin aflatoxin lead to asperlogilosis in humans with severe effects on the respiratory tract (Hedayati et al., 2007). In turn, *F. proliferatum* can produce rot in some crops, such as corn on the cob; more recently, it has been reported that the fungus causes rot of soybean root (Chang et al., 2015).

Bioassay with *C. albicans*

When testing the capacity of *A. hypoglauca* seeds oil against *C. albicans* outstanding results were obtained

(Table 2). In some concentrations, oil from seeds of *A. hypoglauca* was superior to the inhibition promoted by the standard utilized. It is worth noting that inhibition caused by the oil was greater than 94% in the concentration of 15.625 mg/L, while at the same concentration miconazole and nystatin only reached 92% inhibition (Table 2). *C. albicans* is an opportunistic pathogen host in humans. The patient may even need to be hospitalized because of *C. albicans* infection called candidemia. People with low immunity, the elderly, patients with cancer, diabetes, surgery, among, others, have increased risk of fungal infections (Giolo and Svidzinski, 2010).

Bioassays with bacteria

The antibacterial activity test using the *A. hypoglauca* seed oil against *E. coli*, S. *tiphymurium*, *S. aureus*, *S. sanguinis* and *C. albicans* showed notable inhibition, except for *E. coli* which was poorly inhibited: 31% with 500 mg L^{-1} oil and 27% with 3.90625 mg L^{-1}. The antibacterial activity against *S. typhimurium* was greater. Using 500 mg L^{-1} of seed oil the inhibition extent reached 78% and at the lowest concentration tested (3.90625 mg L^{-1}) the inhibition level was rather high (64%). *E. coli* and *S. typhimurium* are Gram(-) bacteria that may cause diarrhea, intense fever and even death. They are transmitted principally by fecal-oral contamination, very common in countries without basic sanitation, as well as the improper handling of food (Moura et al., 2012; Butler, 2011). The activity of the oil against *S. aureus* was not satisfactory, since less than 30% inhibition was observed at all concentrations used. Similarly, *S. sanguinis* inhibition was below 40% at all concentrations. The low inhibition of *A. hypoglauca* seed oil towards some bacterial strains and high inhibition against *C. albicans* is indicative of a selective action mechanism, which deserves further investigation. *S. aureus* and *S. sanguinis* are Gram(+) bacteria which also cause grave health problems for humans, for instance skin infections and pneumonia (*S. aureus*), periodontal disease and severe endocarditis (*S. sanguinis*) (Evans et al., 2014; Sung et al., 2008). The WHO (World Health Organization) (2014) pointed out that there is global concern about the indiscriminate use of antibiotics, because this makes it that fungi and bacteria develop resistance to current drugs. Thus, it is necessary to look for new drugs that meet this need.

Conclusion

The oil from *A. hypoglauca* seed can be considered as an alternative source of vegetable oil. It can be used as raw material in the pharmaceutical and food industries, as it is constituted by essential fatty acids, where ω-9 and ω-6 unsaturated fatty acids occur in high proportion. Of note was the potent inhibition that the oil exerted on AChE, about 80% of the activity was suppressed. Although the bioactivity against filamentous fungi (*A. flavus* and *F. proliferatum*) was low, it was excellent for inhibiting the yeast *C. albicans*, exceeding 90%. At some concentrations, the activity of the oil was superior to that displayed by clinically used standards. The inhibition level did not reach 50% against *E. coli* and was above 77% for S. *tiphymurium*. The inhibition of Gram-positive bacteria, *S. aureus* and *S. sanguinis* was unsatisfactory. The excellent bioactivity of the oil against *C. albicans* may be related to selectivity and this is the most outstanding action detected for this oil.

Conflict of interests

The author(s) did not declare any conflict of interest.

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