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Potential of *Annona muricata* L. seed oil: phytochemical and nutritional characterization associated with non-toxicity

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SUMMARY: The aim of this study was to evaluate the nutritional quality, phenolic compounds, fatty acid and antioxidant activity *in vitro* as well as a toxicological screening of *A. muricata* seed oil *in vivo*. The chemical composition and quantification of phenolic compounds were determined by the Adolfo Lutz Institute normative. The antioxidant activity was evaluated by DPPH, FRAP and ABTS methods. The oil was extracted by chloroform/methanol and precipitated crude (*AmPtO*) and supernatant oils (*AmSO*) were obtained. The fatty acid profile was evaluated by gas chromatography and total compounds by HPLC-DAD. BALB/C mice received *AmPtO* and *AmSO* (0.5 and 1.0mL·Kg^{-1}) for 14 days. Toxicity parameters were assessed. The major fatty acids in the oil were oleic (39.2%) and linoleic (33%). HPLC-DAD suggested the presence of acetogenins (annonacin: 595 [M-H]$^-$), with a greater presence in *AmPtO*. The *AmPtO* group showed toxicity, which may be related to the acetogenin content in *AmPtO*. The *AmSO* group showed no toxicity and this oil has potential for food or medicinal use.

KEYWORDS*: Acetogenins;* Annona muricata *seeds; Annonacin; Linoleic acid; Oleic acid*

RESUMEN: ***Potencial del aceite de*** **Annona muricata** ***L.: Caracterización nutricional y fitoquímica asociada con no toxicidad.*** El objetivo de este estudio fue evaluar la calidad nutricional, compuestos fenólicos, ácidos grasos, actividad antioxidante in vitro y evaluación toxicológica del aceite de las semillas de la *Annona muricata*. La composición química y los compuestos fenólicos se determinaron de acuerdo con las normas del Instituto Adolfo Lutz y la actividad antioxidante por métodos de DPPH, FRAP y ABTS. El aceite fue extraído con cloroformo/metanol obteniéndose un precipitado crudo (*AmPtO*) y un aceite sobrenadante (*AmSO*). El perfil de ácidos grasos fue evaluado por cromatografía gaseosa y los compuestos fitoquímicos mediante HPLC-DAD. BALB/C recibieron *AmPtO* y *AmSO* (0,5 y 1,0 mL·kg^{-1}) durante 14 días. Se evaluaron los parámetros bioquímicos e histopatológicos. Los ácidos grasos principales fueron oleico (39,2%) y linoleico (33%). HPLC-DAD indicó la presencia de acetogeninas, particularmente anonacina (595 [M-H]$^-$), principalmente en *AMPtO*. *AmPtO* presentó toxicidad y esto puede estar relacionado con las acetogeninas. *AmSO* no presentó toxicidad y tiene potencial para la alimentación o uso medicinal.

PALABRAS CLAVE*: Acetogeninas; Ácido linoleico; Ácido oleico; Anonacina; Semillas de* Annona muricata

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1. INTRODUCTION

Annona muricata L., known as Soursop fruit, is native to America and has recently been aclimatized and established in many continents such as America, Africa and Asia (Nogueira *et al*., 2005). *A. muricata* has a history of popular use over the past few years and has been extensively described in the scientific literature as possessing biological properties such as analgesic and anti-inflammatory (Ishola *et al*., 2014), antidiabetic and antioxidant (Florence *et al.*, 2014). Due to the presence of acetogenins, Annona has anti-tumor and apoptosis-inducing properties (Moghadamtousi *et al.*, 2015).

The soursop fruit and other parts of the plant are considered to be underutilized. One fruit may contain more than 200 seeds, which means the disposal of a large amount of seeds. The main countries which produce soursop are Brazil, Venezuela and Colombia. Bahia, Brazil is considered the world's largest producer of soursop and produces about 8,000 tons of fruit per year. It is used in processing industries in the production of fruit juices and pulps (ADAB, 2015), which results in large amount of seeds that are discarded and non-utilized commercially. The unused structures of the plant, seeds for example, can be a source of herbal products, bio-compounds and pharmaceutical ingredients. However, information on the composition, nutritional value and medicinal uses of the *A. muricata* seeds is still limited. Anuragi *et al*., (2016) report that the graviola seed is rich in oil and can be exploited in the oil industy. Elagbar *et al.,* (2016) identified important fatty acids in the oil of *A. muricata* seeds and emphasized that this oil can be exploited for industrial, cosmetic, and medicinal purposes.

The characterization of the functional quality of *A. muricata* seeds may favor their use in the human diet, especially as a functional food. Such products are commonly known to contain bioactive compounds with properties that prevent degenerative diseases and other morbidities. Typically, anti-inflammatory and antioxidant properties exerted by medicinal plants are attributed different fatty acids, phenolic compounds (Dimitrios, 2006), alkaloids or other bioactive compounds, which are present in plant extract and oil. However, natural compounds can have toxic effects and interfere in the vegetable's consumption.

Given the above, the aim of this study was to evaluate the nutritional quality of *A. muricata* seeds and its oil through the chemical composition, quantification of phenolic compounds, antioxidant activity *in vitro* and toxicological evaluation *in vivo*.

2. MATERIALS AND METHODS

2.1. Sample preparation

The seeds were kindly provided in two batches (sample 1 and 2), by a of fruit pulp industry located in Ilhéus, Southern Bahia, Brazil. The seeds were identified and registered in the Herbarium of the State University of Bahia - Paulo Afonso HUNEB-Collection (n° 28720).

The seeds were separated for grinding manually, subjected to crushing, and stored at -20 °C until analysis of composition. Before crushing, the seeds used for oil extraction were previously lyophilized to facilitate crushing and better extraction of ethereal components,

2.2. Oil Extraction

Oil extraction was performed according to Blig-Dyer (1959) using methanol and chloroform as solvents. The decanted phase in the extraction process was collected and concentrated in a rota-evaporator equipment at 30 rpm under vacuum (695 mmHg) at 50-55 °C for 45 minutes, and was then subjected to a final drying by means of direct contact with nitrogen gas to enable the volatilization of residual solvent and the precipitation of the crude oil *(AmPtO)*. The oil was centrifuged at 2300g/5min (4 °C) to separate precipitated lipids and for obtaining the supernatant oil *(AmSO)*. The patent for this extraction process was deposited at the Intellectual Property Institute (INPI) (BR102016029177-1).

2.3. Centesimal composition

Centesimal composition was performed on fresh samples, in triplicate, and the determination of lipids, proteins, dietary fiber, moisture and ash was performed according to the analytical method of the Adolfo Lutz Institute (2008). The quantification of carbohydrates was performed by percentage differences in comparison to other components.

2.4. Quantification of phenolic compounds

The Folin and Ciocalteu assay was used for the quantification of total phenolic compounds using gallic acid as a standard in lyophilized seeds (Instituto Adolfo Lutz, 2008). Aqueous and methanolic extracts in the sample were analyzed in a concentration of 0.5 $mg{\cdot}mL^{-1}$ in a spectrophotometer VIS/UV at 760 nm. A standard curve was plotted using gallic acid (0 to 8 $mg{\cdot}mL^{-1}$). The results were expressed as milligram of tannic acid (ETA) and gallic acid (EGA) equivalents per gram of the dry weight sample.

2.5. *In vitro* antioxidant activity

An *AmSO* extract was made by adding the oil (0.5g) to a methanol solution (50%), and after one hour of incubation at room temperature (28±1 ºC), acetonic solution (70%) was added, followed by distilled water to a final volume of 100 mL (Rufino *et al.*, 2007). *DPPH* (2,2-diphenyl-1-picrylhydrazyl) *free radical scavenging assay* was made as per

Roesler *et al.* (2007) to obtain the final result, which is expressed in IC_{50} (g sample/g DPPH). The *ABTS* [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] *method* was used with standard Trolox and UV/VIS at 734nm (Rufino *et al.*, 2006). A *ferric reducing antioxidant potential (FRAP) assay* was made using the standard ferrous sulphate and spectrophotometer VIS/UV at 595nm (Rufino *et al.*, 2006).

2.6. Fatty acid profile

Fatty acids profile was determined by the capillary column gas chromatographic method applied to the oil methyl esters. The amount of total fatty acids (sum of free and bound fatty acids) in the oil was obtained by transesterification into the corresponding methyl esters (fatty acid methyl esters (FAME)), through saponification with NaOH (0.5N) in methanol, followed by methylation with a solution of boron trifluoride (BF_3) catalyst (12% in methanol). The FAME were extracted with iso-octane and stored in an inert atmosphere (N_2) in the freezer at −18 °C. The FAME separation was performed on a gas chromatograph (Varian® 3800) equipped with a flame ionization detector and a fused silica capillary column Elite-WAX (30m×0.32 mm×0.25 mm). The analysis parameters were: injector temperature of 250 °C and detector temperature of 280 °C. The following thermal program was used: 150 °C for 16 min, then increased by 2 °C·min^{-1} up to 180 °C, held for 25 min, followed by an increase of 5 °C·min^{-1} up to 210 °C, held for 25 min. Helium was used as carrier gas at 1.3 mL·min^{-1}. Nitrogen gas was used as make up gas (30 mL·min^{-1}); the flow of hydrogen gas and air were provided at 30 and at 300 mL·min^{-1}, respectively. The injections were performed in duplicate for each extraction in a volume of 1 µL.

The FAME were identified by comparing retention times with known mixed standards (189–19-1AMP FAME Mix C4–C24; Sigma-Aldrich®). The quantification of fatty acids, expressed in milligrams per gram of lipids, was performed by adding internal standard (C23:0 Sigma®, USA) and calcuated using equation 1. Reported yields were averaged from duplicate extractions (Nascimento *et al.*, 2003):

$$Concentration\ (mg \cdot g^{-1}) = (A_x \times W_{is} \times CF_x) / = (A_{is} \times W_s \times CF_s) \times 1000 \quad (1)$$

Where: A_x Area of methyl ester fatty acid peek in the chromatogram of the sample.

W_{is} Weight (in milligrams) of internal standard added to the sample.

CF_s Conversion factor of fatty acid methyl ester to fatty acid.

A_{is} Area of internal standard methyl ester of fatty acid peek in the chromatogram of the sample.

W_s Sample weight (in milligrams).

CF_x Correction factor response of each fatty acid methyl ester ionization detector, relative to 23:0

2.7. Physiochemical screening of crude oil

The *AmPtO* sample was separated as precipitate (solid residue in low temperature) and *AmSO* (supernatant oil). *AmPtO* (50 mg) was dissolved in methanol and acetonitrile (1:1) and extracted in solid phase extraction (SPE). A Sep-Pak silica cartridge (Waters) was sequentially conditioned with 5 mL of hexane and 5 mL dichloromethane to prevent the cartridge from drying. *AmPtO* was passed through the cartridge and eluted with 10 mL of dichloromethane, ethyl acetate and methanol. The eluates were dried under reduced pressure in a rotatory evaporator at 40 °C to yield 14.0 mg dichloromethane, 13.5 mg ethyl acetate and 8.0 mg of methanol fractions. After evaporating to dryness by rotary evaporator, the residues were dissolved in methanol and acetonitrile, filtered through a 0.45-µm nylon syringe filter (Whatman) and injected into the HPLC system. The standard stock and working solutions were stored at 4 °C.

All reagents used were of analytical grade. HPLC-grade methanol and acetonitrile were purchased from Tedia and Merck, respectively. The deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA).

The thin layer chromatographic profiles were made with *AmSO, AmPtO* and their fractions obtained by SPE extraction according to Grzybowski *et al.,* (2012). Chromatoplates were made with hexane: chloroform: nitroethane: ethyl acetate: acetone: methanol: acetonitrile: water (12:2:4:4:1:2:1.6:0.1, v/v) as mobile phase. The spray for acetogenin constituents was specifically Kedde´s reagent (alkalinized 3,5-dinitrobenzoic acid).

HPLC was performed in a Shimadzu Prominence LC-20AT equipped with a (SPD-M20) diode array detector (HPLC-DAD) (Shimadzu Corp. Kyoto, Japan). The samples were injected into a Rheodyne 7125i injector with a 50 µL loop. The column heater was set at 30 °C. The chromatographic separation was performed with a Luna Phenomenex C-18 column (250 mm x 4.6 mm x 5 µm, Phenomenex). The compounds were separated using a mobile phase consisting of water (A) and acetonitrile:methanol (1:1, B). The separation gradient was: 0-20 min 70-100% B, 20-40 min 100%, 45 min stop at a flow rate of 1.0 mL·min^{-1}. A 50 µL sample was injected and the detection of compounds was performed using light with a wavelength of 215 nm. The infrared absorption spectra were recorded in KBr pellets using a Varian 640 FT-IR spectrophotometer with a PIKE ATR accessory operating in the 4000-400 cm^{-1} range. Liquid chromatograph Electrospray ion source mass spectrometer (LC-ESI-MS) was obtained in negative electrospray mode using an Esquire 3000 Plus instrument (Bruker). Thin-layer chromatography plates were run using 60 F_{254} silica gel (Macherey-Nagel).

2.8. *In vivo* toxicity evaluation

2.8.1. Animals and ethical considerations

BALB/C mice of 8-10 weeks old were used in this study. The animals were housed at 22 °C and received water and food *ad libitum* until the time of the experiment. Animal procedures were performed in accordance with the recommendations of the Ethics Committee of the Institute of Health- Federal University of Bahia Sciences (CEUA/ICS 029/2012).

2.8.2. Experimental protocols

The experimental protocol aimed to verify the toxicity of repeated doses (ANVISA, 2010) of 14 days-exposure daily to *AmPtO* and *AmSO* doses was 0.5 $mL \cdot Kg^{-1}$ animal weight and 1.0 $mL \cdot Kg^{-1}$ animal weight, respectively. The oil samples were diluted in saline and emulsified using DMSO (dimethylsulfoxide, 0.5%, Sigma). The animals were divided into three groups, each group with 6 animals. On day 14 (D14), the animals were euthanized (150 $mg \cdot Kg^{-1}$) thiopental associated with 10 $mg \cdot Kg^{-1}$ lidocaine) and histopathological and biochemical samples were taken for the analysis of toxicity.

AmPtO protocol groups were defined as follows: the *CtrlAmPtO* group (control): received saline orally (200 µL of saline + DMSO 0.5%); the *AmPtO0.5* group received crude oil orally at 0.5 $mL \cdot kg^{-1}$ (oil diluted in 200 µL of saline + DMSO 0.5%); the *AmPtO1.0* group received crude oil orally at 1.0 mL/kg (oil diluted in 200 µL of saline + DMSO 0.5%).

The *AmSO* protocol was followed with the same oil doses (0.5 $mL \cdot kg^{-1}$ and 1.0 $mL \cdot kg^{-1}$) and the groups were defined as *CtrlAmSO* group; *AmSO0.5* group; *AmSO1.0* group. *AmSO* was dissolved in saline followed by filtration (0.2 µm). The filtration process allowed separating the precipitate in the crude oil of the supernatant liquid fraction (*AmSO*). The waste precipitates were retained in the filter, thus, a quantity of 40% of AmSO weight was added to correct the average percentage weight loss during filtration.

2.8.3. Histopathological, physical, biochemical and haematological analysis

The animals were weighed on D1 and D14 of the protocol. On D14, before euthanasia, three independent observers trained in animal experiments evaluated the overall appearance of the animals, according to the score of toxicity signs: (0) normal appearance and coat; (1) discrete change in coat and discrete cachexia; (2) coat change and cachexia (Lerco *et al.*, 2003). Liver and spleen were collected for weight evaluation. The ratio of liver weight/body weight of the animal was calculated to identify liver relative weight (Ritter *et al.*, 2012).

Liver and kidney samples were paraffin embedded twice after fixing with 10% formalin using a microtome; the paraffin blocks were cut into serial 3 µM sections. In the staining step, the slides were immersed in hematoxylin-eosin (HE). Histopathological changes were evaluated by two different pathologists, blindly.

Blood glucose levels, total cholesterol, urea and creatinine were performed in semi-automatic Bioplus 2000 equipment using commercial kits (Doles) according to the manufacturer. The quantitation of leukocytes was performed by manual counting, with dilution in Turk liquid (200µL liquid Turk + 20 µL of blood with Ethylenedeamine Tetra Acetic Acid (EDTA)).

2.9. Statistical analysis

One-way analysis (ANOVA) and Tukey Test correction to post-test (for data with normal distribution) and Kruskal Wallis and Dunn as post-test (for data without normal distribution) were used to determine statistical significance among groups using GraphPad V5 (GraphPad Software Inc., San Diego, CA, USA) software. The results were considered significant when $p < 0.05$.

3. RESULTS

3.1. Centesimal composition, phenolic compounds and *in vitro* antioxidant activity

The results of centesimal composition (wt %) of *A. muricata* seeds *in natura* were: moisture 34.7±0.02; ash 1.3±0.01; proteins 9.85±0.31; lipids 18.3±0.28; fibers 24.7±0.52 and carbohydrates 11.15. The polyphenols of aqueous extracts in the *A. muricata* lyophilized seeds showed a variation of 310-320mg%, which is 3.2±0.02 mg TAE/g and 3.1 ±0.02 mg GAE/g (tannic acid and gallic acid equivalents, respectively). Methanol extracts showed turbidity, making them impossible to read in the spectrophotometer.

The *In vitro* antioxidant capacity, AmSO showed 370133.0 $\mu g \cdot mL^{-1}$ DPPH (DPPH assay); 40.2 µmol trolox/g (ABTS assay) and 50.7 µmol $Fe_2SO_4 \cdot g^{-1}$ (FRAP assay). Regarding the DPPH assay, the lowest value of the sample, able to reduce DPPH by 50% (IC_{50}), had better antioxidant activity. The assessment of the ABTS and FRAP methods in a better antioxidant activity was based on the higher amount of equivalent in trolox or ferrous sulfate per gram of sample, respectively. Overall, in comparison with standard gallic acid (1.30 $\mu g \cdot mL^{-1}$ DPPH; 18.917.0 µmol trolox/g; 41946.7 µmol $Fe_2SO_4 \cdot g^{-1}$), AmSO had low antioxidant activity.

3.2. Fatty acid profile

A. muricata seed oil had a total of 75% unsaturated fatty acids (Table 1). The fatty acid profile of the two oil samples analyzed, each in duplicate, were similar. Considering the percentage of unsaturated fatty acids, 40% was monounsaturated, represented by 39.2% oleic acid (n-9). The second highest detected percentage (33%) of fatty acid was linoleic (n-6). The oil contained a low content of α-linolenic acid (n-3). Regarding the saturated content (24.5%), the most representative fatty acid was palmitic (19.3%).

3.3. Phytochemical screening

Figure 1-A shows the chromatographic profile in chromatoplate of the oil, precipitate and the fractions obtained by SPE extraction. This clearly revealed predominant rose-wine coloration spots (positive Kedde's reaction) on the acetogenin family members (Figure 1-A). The absorption in the UV spectrum at 215 nm was observed in HPLC-DAD analyses (Figure 1-B and 1-C). The presence of acetogenins was confirmed by the LC-ESI-MS analysis, which detected several peaks with molecular weights (*m*/*z* values), which are typical of these compounds. In all the samples, the most abundant peaks corresponded to the molecular weight corresponding to annonacin ($C_{35}H_{64}O_7$; mol. wt. 595 $[M\text{-}H]^-$). Fractionation of the extract (precipitate residue) by SPE allowed the observation of the major acetogenin (Figure 1-B and 1-C), which was confirmed to be annonacin (*m*/*z* 595, $[M\text{-}H]^-$, mainly in the methanol fraction (Figure 1-D).

3.4. *In vivo* toxicity

3.4.1. Survival

In the *AmPtO* protocol, the *AmPtO1.0* group showed an important reduction in survival in the early days of the Protocol and reached a percentage lower than 50%. In the *AmSO* protocol, all the animals showed 100% survival.

3.4.2. Physical, biochemical and haematological analysis

Table 2 shows the biochemical and physical parameters of *in vivo* experiments. The *AmPtO1.0* group showed a statistical difference in blood glucose level compared to the *CtrlAmPtO* group and *AmPtO0.5* group ($p < 0.001$ and $p < 0.01$, respectively). The *AmPtO1.0* group also showed weight loss (variable average -1.3g) compared to the other experimental groups. The *AmPtO0.5* group was characterized by moderate signs of toxicity (score 1) and the *AmPtO1.0* group showed more intense toxicity signs (score 2). In the *AmSO* protocol, liver weight was inversely proportional to oil doses ($p < 0.01$ and $p < 0.001$, respectively for *AmSO0.5* and *AmSO1.0*) in comparison to the control group (*CtrlAmSO*) and was evidenced by liver relative weight (ratio of 0.027) in relation to body weight.

TABLE 1. Fatty acid profiles in *Annona muricata* L. seed oils

		Sample 1		Sample 2		Sample 1 and 2	
Fatty acids	**Name**	**mgFA/g fat**	**mgFA%**	**mgFA/g fat**	**mgFA%**	**Average (mg FA%)**	***Standard deviation***
C16:0	*Palmitic*	128.72	17.78	150.80	20.83	19.31	*2.16*
C16:1n7	*Palmitoleic*	11.29	1.56	11.72	1.62	1.59	*0.04*
C18:0	*Steararic*	29.76	4.11	36.20	5.00	4.56	*0.63*
C18:1n9*c*	*Oleic*	283.87	39.22	283.65	39.19	39.21	*0.02*
C18:2n6*c*	*Linoleic*	250.53	34.61	227.00	31.36	32.99	*2.30*
C18:3n3	*α-Linolenic*	9.21	1.27	8.70	1.20	1.24	*0.05*
C20:0	*Arachidonic*	3.26	0.45	3.91	0.54	0.50	*0.06*
C20:1n9	*Gadoleic*	nd	nd	0.95	0.13	0.07	*0.09*
C22:0	*Behenic*	1.09	0.15	n.d.	n.d.	0.08	*0.11*
C24:0	*Lignoceric*	1.19	0.17	0.91	0.13	0.15	*0.03*
Σ Saturated		164.02	22.49	191.83	26.50	24.50	*2.84*
Σ Unsaturated		554.9	76.66	532.01	73.50	75.08	*2.23*
Σ Monounsaturated		295.19	40.78	296.32	40.94	40.86	*0.11*
Σ Polyunsaturated		259.74	35.88	235.7	32.56	34.22	*2.35*

FA: fatty acids. nd: not detected

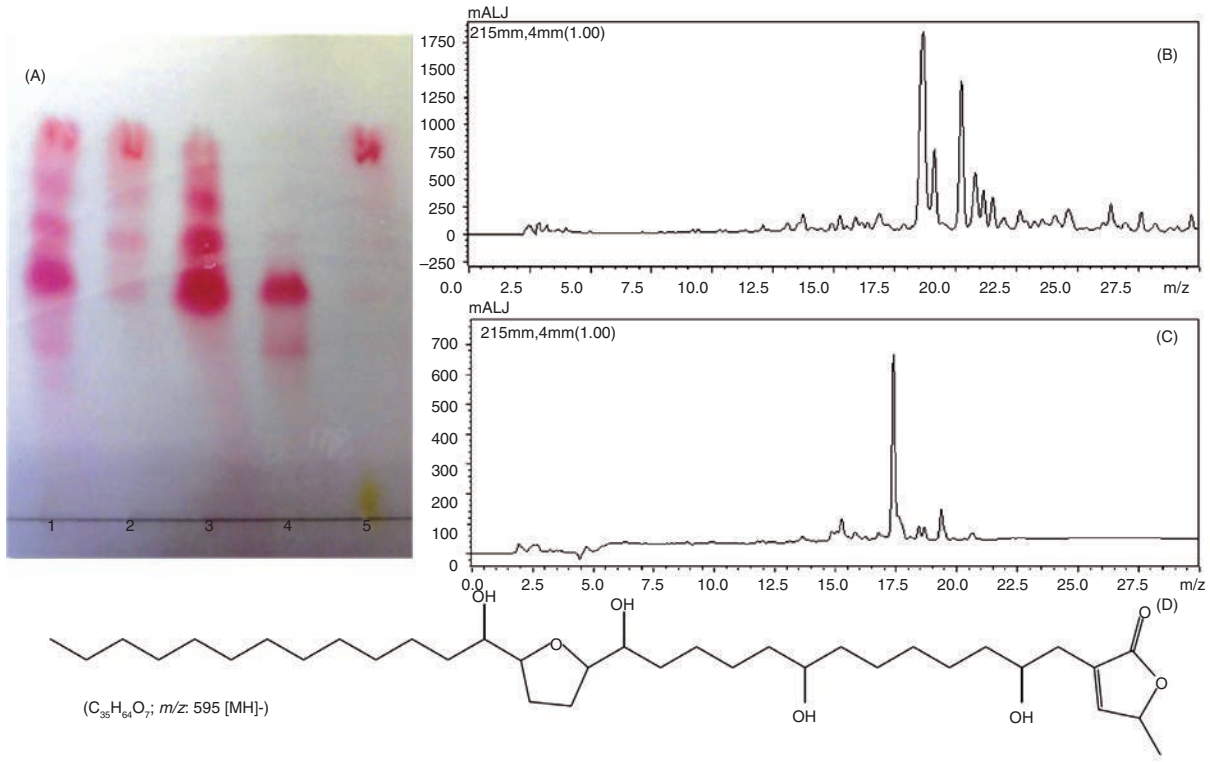

FIGURE 1. A) Thin layer chromatograph of extract and fractions from *A. muricata* seed oil. Spray with Kedde´s reagent. Numbers 1: precipitate residue, 2: dichloromethane fraction; 3: ethyl acetate fraction; 4: methanol fraction and 5;oil extract. B and C) HPLC-DAD chromatograms (215 nm) of precipitate residue (B) and methanol fraction (C) from *A. muricata* seeds oil. (D) Annonacin molecular structure.

3.4.3. Histopathological analysis

Figures 2 (A, B, C) and 3 (A, B, C) show the impact of toxicity in the liver tissue. In the *AmPtO* protocol, the *CtrlAmPtO* group showed preserved morphology with isomorphic hepatocytes arranged in cords, directed to the centrilobular hepatic veins and hepatic sinusoid, with thin walls with the frequent presence of Kupffer´s cell. Portal spaces were regularly distributed and the typical polygonal morphology of hepatocytes and evident cell nucleus were observed. The hepatocytes of the *AmPtO0.5* group and *AmPtO1.0* group showed focal and minimal histological changes. The *AmPtO0.5* and *AmPtO1.0* groups showed discrete hepatic steatosis (Figure 2-B and 2-C, black circle). The liver section of the *AmPtO0.5* group showed some hemorrhagic areas with necrosis (Figure 2-B, red arrow). Histopathological changes were not identified in the case of the *AmSO protocol*.

Figures 2 (D, E, F) and 3 (D, E, F) show the kidney histopathological analysis. The *CtrlAmPtO* group showed normal characteristics of the renal parenchyma. Magnification at 100x revealed an eosinophilic cortex with the abundance of proximal convoluted tubules along with well distributed and uniform renal corpuscles. Magnification at 400x allowed for the observation of glomeruli with Bowman´s capsule and capillary tube supported by delicate mesangium, discarding features of glomerulonephritis (Figure 2-D). The abundant proximal convoluted tubules had preserved cubic epithelium and strongly eosinophilic cytoplasm which differed from the distal convoluted tubules. The *AmPtO0.5* group had similar characteristics, however, the glomeruli showed discrete atrophy and restricted Bowman´s space (Figure 2-E, white arrow), and the presence of capillary hyperemia in the medullary area between the collecting tubules. The *AmPtO1.0* group had some tubules with more eosinophilic cytoplasm, the presence of precipitates, more basophilic cell nucleus associated with karyolysis and coagulative necrosis (Figure 2-F, asterisk). In the *AmSO* protocol (Figure 3) similar morphological characteristics in the cortical area, glomeruli with proportional size, the presence of a capsule and Bowmn´s space, simple cubic and upright tubular epithelium were observed. In the medullary zone, the tubules' normal collectors between the various blood capillaries, with low columnar cubic epithelium with visible lumen were also observed.

TABLE 2. Biochemical and physical parameters *in vivo* toxicity experimental protocols

	AmPtO protocol			***AmSO protocol***		
	CtrlAmPtO	***AmPtO0.5***	***AmPtO1.0***	***CtrlAmSO***	***AmPtO0.5***	***AmPtO1.0***
Biochemical parameters						
Blood glucose (mg/dL)	**74.8** ± 33.52	**85.4** ± 22.09 **	**246.6** ± 114.16 ###	**127.4** ± 51.20	**198.0** ± 10.56	**207.2** ± 34.94
Total Cholesterol (mg/dL)	**112.4** ± 29.87	**149.0** ± 17.50	**126.2** ± 32.39	**103.4** ± 55.34	**160.6** ± 37.31	**174.25** ± 20.63
Creatinine (mg/dL)	**0.37** ± 0.06	**0.34** ± 0.08	**0.37** ± 0.10	**0.40** ± 0.13	**0.31** ± 0.05	**0.24** ± 0.07
Urea (mg/dL)	**28.8** ± 12.39	**28.6** ± 3.20	**25.8** ± 5.26	**31.8** ± 6.87	**29.4** ± 0.35	**30.4** ± 10.31
Total Leucocyte	**1930.0** ± 721.63	**3900.0** ± 885.3	**2790.0** ± 1346.9	**1840.0** ± 641.68	**1550.0** ± 583.1	**2250.0** ± 623.5
Physical parameters						
Liver weight (g)	**0.852** ± 0.20	**0.860** ± 0.23	**1.052** ± 0.18	**1.00** ± 0.09	**0.810** ± 0.09 ##	**0.686** ± 0.02 ###
Spleen weight (g)	**0.077**± 0.01	**0.066** ± 0.01	**0.079** ± 0.03	**0.087**± 0.02	**0.087** ± 0.01	**0.079** ± 0.01
Animal body weight (g)	D[0]: 23.2 ± 2.1	D[0]: 23.3 ± 1.2	D[0]: 23.4 ± 1.3	D[0]: 23.8 ± 1.3	D[0]: 23.8 ± 1.0	D[0]: 25.0 ± 1.2ç
	D[13]: 24.5 ± 1.7	D[13]: 24.2 ± 1.7	D[13]: 22.1 ± 2.8	D[13]: 24.5 ± 0.8	D[13]: 24.4 ± 1.1	D[13]: 25.6 ± 1.1
	Variation (g):**+1.3**	Variation (g):**+0.9**	Variation (g):**-1.3**	Variation (g):**+0.8**	Variation (g):**+0.6**	Variation (g):**+0.6**
Liver relative weight	0.035	0.036	0.048	0.041	0.33	0.027
Toxicity signs	**0**	**1**	**2**	**0**	**0**	**0**

Mean ± standard deviation values. (g) grams. Data from a total of 5 animals per group. Score toxicity signs: (0) coat and normal appearance; (1) discrete change in coat and discrete cachexia; (2) altered coat and cachexia. AmPtO- crude oil seeds *Annona muricata* L. AmPtO- supernatant oil seeds *Annona muricata* L. *AmPtO* Protocol: *CtrlAmPtO*- animals receiving saline + DMSO 0.5%, orally. *AmPtO0.5*- animals receiving *AmPtO* dose of 0.5 mL/Kg body weight + 0.5% DMSO, orally. AmPtO1.0- animals receiving *AmPtO* dose of 1.0 mL/Kg body weight + 0.5% DMSO, orally. (###) p <0.001, compared to *CtrlAmPtO* group. (**) p <0.01, compared to *AmPtO1.0* group. *AmSO* Protocol: CtrlAmSO- animals receiving saline + 0.5% DMSO, orally. *AmSO0.5*- animals receiving *AmSO* dose of 0.5 mL/Kg body weight + 0.5% DMSO, orally. *AmSO1.0*- animals receiving *AmPtO* dose of 1.0 mL/Kg body weight + 0.5% DMSO, orally. Liver relative weight: ratio liver weight/body weight. (##) p <0.01 and (###) p <0.001, compared to *CtrlAmSO* group. *One-Way* ANOVA and *Tukey test* a post-test.

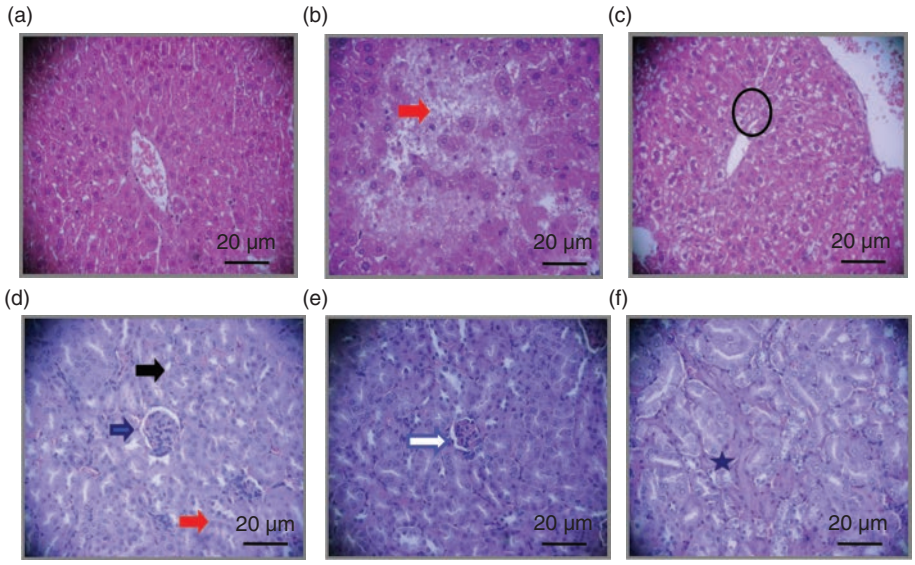

FIGURE 2. Liver and kidney histopathology *AmPtO* protocol in vivo toxicity. AmPtO- crude oil seeds *Annona muricata* L. (A, D) *CtrlAmPtO* group- animals receiving saline + 0.5% DMSO, orally. (B, E) *AmPtO0.5* group- animals receiving *AmPtO* dose of 0.5 mL/Kg body weight + 0.5% DMSO, orally. (C, F) *AmPtO1.0* group- animals receiving *AmPtO* dose of 1.0 mL/Kg body weight + 0.5% DMSO, orally. (B) Arrow: hemorrhagic area and necrosis. (C) Circle: hepatic steatosis. (D) Black Arrow: proximal convoluted tubule. Blue arrow: glomerulus. Gray arrow: distal convoluted tubule. (E) White arrow: glomerulus atrophied. (F) Asterisk: karyolysis and coagulative necrosis. 5 blades per group were examined. (Hematoxylin/eosin, 20µm- 400X).

4. DISCUSSION

A. muricata L. seeds have potential for use in human and animal diets, provided they are not toxic. Fasakin *et al*., (2008) also evaluated the composition of the seeds of soursop fruit from Nigeria, and identified a high content of oil and protein, which were low in toxins (tannins, phytate, and cyanide). These authors identified 22.57% lipids in the seed, and a total of 27.34% protein, which is higher than in the current study. These variations may due to the climatic and edaphic conditions in which the plants were grown (Marineli *et al.*, 2004). Therefore, it is important to characterize the fruits in different regions and countries. Elagbar *et al.,* (2016) identified 21.5% of lipids in *A. muricata* seeds' crude fixed oil on a dry weight basis. In the crude oil of the present study, a total of 18.3% lipids were identified in the *in natura* samples. Considering the presence of moisture (34.7%), it is probable that our seed sample had a higher yield of lipids and greater potential for oil extraction. Santos *et al.,* (2014) evaluated total phenolic compounds in four varieties of *Annona cherimola*, of Portuguese origin, and found values lower than 0.05 $mg \cdot g^{-1}$ of seed sample. In the current study, *A. muricata* seeds had a total of approximately 0.31 $mg \cdot g^{-1}$ of phenolics, hence, they can be considered a moderate source of phenolic compounds.

The Seed oil showed low antioxidant activity *in vitro*. Vegetable oils have a considerable content in tocopherols, which are potential antioxidants (Guinazi *et al.*, 2009). It is probable that *A. muricata* seeds oil can naturally have low antioxidant compounds or such compounds may be lost in the oil extraction process.

Despite the low antioxidant activity identified in the seeds, leaf extracts of the *A. muricata* are well characterized in the literature as having antioxidant properties. Lee *et al*., (2014) evaluated the antioxidant activity and components in dry extracts of the *A. muricata* leaves and reported DPPH IC_{50} at 98.9±9.1 mg/mg extract, and the total content of phenolics to be 86.5±14.8 mg GAE/g extract. Leaf extracts of *A. muricata* were identified as a rich source of antioxidant components such as carotenoids.

The high lipid content also enhances the use of *A. muricata* seeds or the production of pharmaceutical and industrial oils. The most representative fatty acids in the oil analyzed were oleic and linoleic, which are well characterized and imply benefits for cardiovascular health and a reduced risk of metabolic syndrome (Mayneris-Perxachs *et al.*, 2014). Elagbar *et al.* (2016) identified fourteen fatty acids in the fixed oil of *A. muricata* seeds extracted using hexane or diethylether and found a total of approximately 23% saturated, 40% monounsaturated and 36% polyunsaturated. In this study, oleic (39%) and linoleic (35%) acids were the main fatty acids identified. The results were similar to our study, despite the different methods of oil extraction.

Some studies suggest that in addition to the benefits of the fatty acids present in vegetable oils, the presence of minor components which are contained in the natural source of the oil act synergistically,

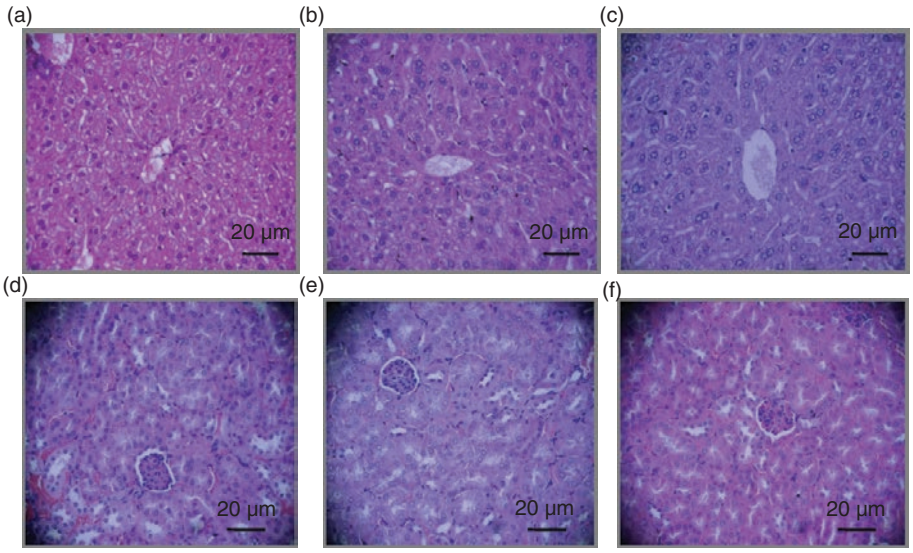

FIGURE 3. Liver and kidney histopathology *AmSO* protocol in vivo toxicity. *AmSO*- supernatant oil seeds *Annona muricata* L. (A, D) *CtrlAmSO* group- animals receiving saline + 0.5% DMSO, orally. (B, E) *AmSO0.5* group- animals receiving *AmSO* dose of 0.5 mL/Kg body weight + 0.5% DMSO, orally. (C, F) *AmSO1.0* group- animals receiving *AmSO* dose of 1.0 mL/Kg body weight + 0.5% DMSO, orally. 5 blades per group were examined. (Hematoxylin/eosin, 20µm- 400X).

favoring the inherent functional properties, for example in Chilean chia oil (Marineli *et al.*, 2014). Some alkaloids have been identified in *A. muricata* such as anonina, muricina and muricinina (Franzão and Melo, 2007) and acetogenins (Moghadamtousi *et al.*, 2015). Acetogenins are derived from long chain fatty acids (C35 and C39) and are found specifically in the Annonaceae family and have antitumor activity and apoptosis-inducing activity (Moghadamtousi *et al.*, 2015). Thus, it is important to identify the bioactive compounds in vegetable oils which have functional and pharmacological properties.

Alkaloids were not found in *AmSO* or *AmPtO* (data not shown). The principal spot was present in all the samples analyzed in the present study, mainly in the methanol fraction, and may be associated to the presence of annonacin, considering that acetogenin is the major compound in *A. muricata* seeds (Moghadamtousi *et al.*, 2015; Grzybowski *et al.*, 2012; Champy *et al.*, 2002). The strong absorption at 1745 cm^{-1} (lactone C=O), a strong aliphatic C-H absorption below 3000 cm^{-1} in the IR spectrum suggested the presence of an unsaturated lactone moiety in an annonacin-type acetogenin (Champy *et al.*, 2002). Le Ven *et al.*, (2012) identified anonacin as the main acetogenin in extracts of the manufactured *A. muricata* nectar. In this study, the authors identified nine compounds of Fragmentation of m/z 603.4797 ([C35H64O7Li] +), which corresponds to the isomers of annonacin.

Champy *et al.*, (2002) analyzed annonacin in extracts obtained from *A. muricata*. In all preparations, fruit pulp, nectar, infusions and decoctions of leaves, the quantification showed that annonacin represented approximately 70% of all acetogenins. The analysis in the present study showed that *A. muricata* seed extracts and oil contained acetogenins and annonacin was the major compound. Annonacin was characterized by its neurotoxic properties and as an inhibitor of mitochondrial complex I (Yu *et al.*, 1992; De Pedro *et al.*, 2013). However, acetogenins can be related to the etiology of neurodegenerative diseases, such as Guadeloupean atypical Parkinsonism and perturbations in the protein *Tau* due to depletion ATP supply and interrupt the transport of mitochondria to the sum of neural cells (Yu *et al.*, 1992). The pharmacokinetic mechanisms of annonacin are still to be known.

The mechanical filtration process enabled a reduction in the presence of acetogenin, as shown in Figure 1-A. Other methods may be implemented to reduce toxic components, and thereby facilitate the use of *A. muricata* seed oil in the human diet.

Acute exposure to different doses of seed oil (*AmPtO* and *AmSO*) was performed for assessing toxicological effects. The process of oil filtration was important to differentiate the protocols, with crude oil (with precipitate) and liquid oil (oil supernatant). The survival rate of less than 50% in the *AmPtO1.0* group was high and implies considerable toxic effects of crude oil. In *AmSO* protocols, the *AmSO0.5* and *AmSO1.0* groups received filtered oil and remained alive throughout the experiment, suggesting that the mortality identified in the *AmPtO1.0* group was linked to the precipitated consumption in the oil, which was depleted after filtration.

Some biochemical and physical parameters evaluated confirmed the toxic effect of the crude oil *(AmPtO)*. The *AmPtO1.0* group showed increased blood glucose, a negative weight variable, toxicity signs and histopathological changes in hepatic and renal tissue (Table 2 and Figures 2 and 3). Animal groups that received *AmSO* showed no toxicity signs or histopathological changes, which indicates that the precipitate in *AmPtO* may be related to the toxicity observed in this group. This can probably be associated to the presence of acetogenins, more concentrated in the crude oil and its fractions (Figure 2).

Acetogenins, especially annonacin, act as mitochondrial complex I inhibitors and are implicated in neurological disturbances, arising from the decreased ATP available to neural cells (Yu *et al.*, 1992; De Pedro *et al.*, 2013). The mitochondrial respiratory chain can be separated into 4 enzyme complexes, with complex I representing the NADH-ubiquinone reductase. This enzyme plays a crucial role in the generation of ATP and its role in the performance of neurodegenerative diseases has been considered (Schapira, 2010). The hepatotoxic and nephrotoxic effects in the AmPtO consumption identified in this study may also be associated with the inhibitory action of the mitochondrial enzyme complex in these specific organs.

AmSO showed nutritional quality and no toxicity. However, a reduction in the liver weight of the *AmSO0.5* and *AmSO1.0* groups compared to *CtrlAmSO* group was observed (Table 2). The hepatoprotective effect has been identified in coconut oil, the study of Zakaria *et al.,* (2011). Treatment with virgin coconut oil reduced the hepatotoxicity induced by acetaminophen, reduced the dosages of liver enzymes and improved liver morphology. A reduction in the levels of creatinine also was observed in the *AmSO1.0* group in compared to *CtrlAmSO*, but without statistical significance. Hepatoprotective effects and renal metabolism can be exploited in other *in vivo* studies on the pharmacological potential of *AmSO*.

Furthermore, the identification of acetogenins in the oil suggests that this product may have pharmacological potential since these compounds mainly have anticancer activity. *Annona muricata* possess a wide spectrum of biological activities, and the use of their by-products or derived products, such as oil, as pharmaceutical or food ingredients is promising.

5. CONCLUSIONS

A. muricata seed oil (liquid fraction) (*AmSO*) has potential for use in food, especially due to its contents in oleic and linoleic fatty acids. *AmSO* showed no relevant antioxidant activity and moderate phenolic compounds.

The oil precipitate (AmPtO) showed a great amount of acetogenins and was considered toxic according to the parameters evaluated in the experimental protocol *in vivo*. The acetogenin identified in the oil was anonacin. The toxic property of the precipitate can be associated with the presence of the acetogenin. The liquid fraction of oil (AmSO) showed no toxic properties.

Further studies on toxic action mechanisms, as well as studies concerning biological activities and pharmacological properties can be conducted to better characterize the seed oil of soursop and encourage its use in medicine.

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