



## Soursop (*Annona muricata* L.) Fruit Peels as Source of Phenolic Constituents and Annonacin with Biological Activities

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### Authors' contributions

This work was carried out in collaboration among all authors. Authors OVDS, ALDSDMA and ESF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors GDVV, JODLX and JLMC managed the analyses of the study. Author GDVV managed the literature searches. All authors read and approved the final manuscript.

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### ABSTRACT

**Aims:** In this study, chemical constituents and biological activities of the *Annona muricata* L. fruit peels were evaluated using methanol extract (MEAM) and hexane (HFAM), dichloromethane (DFAM), ethyl acetate (EFAM), and butanol (BFAM) fractions.

**Place and Duration of Study:** All the experiments were done in the Department of Pharmaceutical Sciences and Department of Biochemistry, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, 36026-900, Brazil, between January 2012 and July 2016.

**Methodology:** Phytochemical screening (specific chemical reactions), total phenolic and flavonoid contents (Spectrophotometric methods) and chemical compounds were assessed (High performance liquid chromatography analysis). The antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), beta-carotene, and thiobarbituric acid assays. The inhibitory effect against digestive enzymes (lipase,  $\alpha$ -amylase and  $\alpha$ -

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glucosidase) was measured by spectrophotometric assays and toxicity by the brine shrimp lethality bioassay.

**Results:** Tannins, flavonoids, coumarins, terpenes and steroids, saponins, and alkaloids were detected. EFAM had the highest values of total phenolic and flavonoids, while a similar compound to annonacin was found in MEAM by HPLC. EFAM was also more active in DPPH and FRAP assays, and HFAM was effective in inhibiting the linoleic acid oxidation and the malondialdehyde. MEAM and fractions blocked lipase,  $\alpha$ -amylase and  $\alpha$ -glucosidase, while HFAM and DFAM were toxic against *Artemia salina*.

**Conclusion:** The results showed that the *A. muricata* fruit peels have important biological effects, which can bring great benefits to human and animal health.

**Keywords:** *Annona muricata*; phenolic compounds; annonacin; antioxidant; anti-digestive enzyme; toxicity.

## 1. INTRODUCTION

A large number of chemical and biological investigations have been performed in fruits and vegetables, but only a few of them involve waste parts of fruits, as seeds and peels [1,2]. These products are usually thrown in the garbage, but they can be sources of bioactive compounds from extractive, nutritional and biotechnological processes [1]. Among the active constituents, vitamins, minerals, natural pigments and phenolic compounds present in fruits and vegetables are highlighted by their antioxidant activity, since they avoid the oxidation of metabolic reactions, acting both in the initiation stage and in the propagation of the oxidative process [3]. Besides, these constituents can be important in the prevention and treatment of diseases caused by free radical, such as neurodegenerative diseases, diabetes, dyslipidemia, cardiovascular disorders, obesity, and cancer, among others [4].

*Annona muricata* L. (Annonaceae), commonly known as "soursop", is a plant found in South and Central America, Africa and Asia [5]. The fruits are used as natural medicine [5] and consist of a white edible pulp containing protein, carbohydrate and vitamins B and C [6], as well as esters of aliphatic acids and mono- and sesquiterpenes [7]. Alkaloids (annonaine, nornuciferine and asimilobine, for example) [8], annonaceous acetogenin (epomusenin-A, epomusenin-B and epomurinin-B, among others) [9] and phenolic compounds (5-caffeoylquinic acid, dihydrokaempferol-hexoside and caffeic acid derivative, for example) [10] were also identified in the fruits, which have been related to several biological properties [5]. From a pharmacological point of view, anti-inflammatory and anti-nociceptive, antitumor, anti-arthritis, antibacterial, anticonvulsant, antidiabetic and

hypolipidemic, and antihypertensive activities [5], as well as relevant antioxidant action [5,11,12], have been reported for *A. muricata* using extracts from leaves.

Considering that the fruit peels are commonly discarded and may represent a strategy for the search for active compounds and nutrients and the oxidative mechanisms are associated with metabolic processes and toxicity, this study investigated the chemical potential and the biological properties of the *A. muricata* fruit peels.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

*Annona muricata* L. (Annonaceae) has been cultivated at the Medicinal Garden of the Faculty of Pharmacy, Federal University of Juiz de Fora, Juiz de Fora, localized in latitude 21° 41' 20" S and longitude 43° 20' 40" W, Minas Gerais, Brazil. A voucher specimen (CESJ n° 48236), identified by Dr. Fatima Regina Gonçalves Salimena, was deposited in the Herbarium Leopoldo Krieger, Juiz de Fora, MG, Brazil. Mature fruits, suitable for consumption, were collected (January to February 2012) and the peels were removed from the pulp.

### 2.2 Chemicals and Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH•), linoleic acid,  $\beta$ -carotene, tween® 40, butylated hydroxytoluene (BHT), gallic acid, rutin, caffeic acid, 5-caffeoylquinic acid, acarbose, orlistat, pancreatic lipase, pancreatic  $\alpha$ -amylase, and  $\alpha$ -glucosidase (Sigma Chemical Co, St. Louis, MI, USA); aluminum chloride, calcium chloride, potassium chloride, magnesium chloride, potassium bromide, sodium sulfate, sodium

bicarbonate, dimethylsulfoxide, potassium ferrocyanide, ferric chloride, sodium chloride, dichloromethane, hexane, butanol, methanol, ethanol, pyridine, and sodium carbonate (Labsynth, Diadema, SP, Brazil) and Folin-Ciocalteu reagent, trichloroacetic acid, and ascorbic acid (Cromoline Química Fina, Diadema, SP, Brazil).

### 2.3 Extract Preparation

Dried and powdered peels (86 g) were extracted in methanol by static maceration for 3 weeks. The methanol extract (MEAM) was filtered and evaporated under a rotary vacuum evaporator (Rotavapor RII, Büchi, Flawil, Switzerland) at controlled temperature ( $50 \pm 1$  °C) and yielded 12.78 g. ME (9.45 g) was suspended in water: methanol (9:1) followed by liquid/liquid partition to obtain the hexane (HFAM), dichloromethane (DFAM), ethyl acetate (EFAM), and butanol (BFAM) fractions, which yielded 2.36, 1.07, 1.28 and 2.12g, respectively.

### 2.4 Phytochemical Screening

Chemical constituents (tannins, flavonoids, terpenes and phytosterols, saponins, coumarins, anthraquinones, and alkaloids) were investigated with specific reagents [13].

### 2.5 Total Phenolic Content Determination

As recommended by Sousa et al. [14], the total phenolic content was determined by Folin-Ciocalteu method using gallic acid (GA) as standard. The Folin-Ciocalteu reagent oxidized phenolic compounds present in the samples whose reaction was neutralized with sodium carbonate. After 60 min, in triplicate, the absorbance was measured at 765 nm (spectrophotometer Shimadzu®, UV-1800). The results were expressed as gram of gallic acid equivalent (g GAE/100g).

### 2.6 Total Flavonoids Content Determination

The total flavonoid content was evaluated spectrophotometrically using rutin (RU) as standard (from 2 to 30 µg/mL) [15]. After reaction in media containing acetic acid, pyridine:ethanol (2:8), 8% aluminum chloride, and distilled water at room temperature for 30 min, the absorbance was measured at 420 nm (Spectrophotometer Shimadzu®, UV-1800). The results, in triplicate,

were expressed as gram of rutin equivalent (g RUE/100g).

### 2.7 High Performance Liquid Chromatography (HPLC) Analysis

MEAM, EFAM and BFAM were dissolved in methanol (2 mg/mL) and filtered (0.45 µm filter) and HPLC analysis was performed using an Agilent Technologies 1200 Series composed of a quaternary pump with a PDA detector and an automatic injector. The column employed was a reversed phase C18 silica column Zorbax SB-18 (4.6x150 mm; 5 µm of particle size). The mobile phase was composed of ultrapure water (Solvent A), and methanol (Solvent B). After injection (20 µL), the extract was eluted in a gradient in which the concentration of eluent B was increased from 5% to 75% in 5 min, followed by a 15-minutes gradient increase from 75% to 100%. The final gradient condition was maintained for an additional 15 min. The elution flow was 0.8 mL/min, and the column temperature was kept at 25 °C. Detection was performed at 230 nm. Markers, as quercetin, rutin, gallic acid, caffeic acid and 5-caffeoylquinic acid, were used to identify compounds present in MEAM.

### 2.8 DPPH radical Sequestration Method

The antioxidant activity was determined following the DPPH method described by Mensor et al. [16]. In reaction medium, antioxidant compounds present in the samples neutralized the DPPH (0.03 mM in methanol) after 60 min of incubation. In triplicate, the absorbance was recorded at 518 nm (Spectrophotometer Shimadzu®, UV-1800). The percentage of antioxidant activity (%AA) and the half maximal inhibitory concentration (IC<sub>50</sub>) were determined. Rutin was used as standard.

### 2.9 Ferric Reducing Antioxidant Power Assay

The ferric reducing antioxidant power (FRAP) assay was done according to Oyaizu [17]. Samples and ascorbic acid were added to the medium containing phosphate buffer pH 6.6, and potassium ferrocyanide. After incubation, in triplicate, this mixture reacted with trichloroacetic acid. The supernatant was removed after centrifugation and mixed with distilled water and ferric chloride. The absorbance was recorded at 700 nm in a spectrophotometer (Shimadzu®, UV-1800). Absorbance 0.5 was considered as half-

maximal inhibitory concentration (IC<sub>50</sub>). Ascorbic acid was used as positive control.

### 2.10 β-carotene/linoleic Acid Assay

Due to the presence of lipophilic compounds in the samples, the linoleic acid and β-carotene system was used to determine the lipid peroxidation inhibition [18]. In this assay, in triplicate, β-carotene (in chloroform), linoleic acid, and tween 40 were mixed into a rotaevaporation flask. After removal of the solvent, distilled water was added to form an emulsion. The assay was performed in a microplate reader (ThermoPlate<sup>®</sup>, TP-Reader) and the absorbance was recorded at 492 nm every 15 minutes during 105 minutes. The graph of decay (absorbance x time) and the inhibition of lipid peroxidation (ILP) were determined. Butylated hydroxytoluene (BHT) was used as standard.

### 2.11 Thiobarbituric Acid Method

As recommended by Zeb and Ullah [19], the thiobarbituric acid method was used in this test. From 25 g of low-fat ground beef, a homogenate was prepared with distilled water, and 7.5, 15, or 30 mg of each sample with heating. The homogenate was transferred to the test tubes containing BHT, phosphoric acid, and thiobarbituric acid. After cooling in an ice bath, butanol was added to each tube and centrifuged. The formation of the chromogenic complex was measured at 535 nm in a spectrophotometer (Shimadzu<sup>®</sup>, UV-1800). The concentration of the thiobarbituric-malonaldehyde acid complex was calculated from the standard malonaldehyde (MDA) curve.

### 2.12 Pancreatic Lipase Assay

As described by Souza et al. [20], the anti-lipase activity was evaluated using orlistat as control positive. Solution of porcine pancreatic lipase (Sigma<sup>®</sup>, 10 mg/mL) in Tris-HCl buffer (0.05 mol/L, pH 8.0) containing CaCl<sub>2</sub> (0.010 mol/L) and NaCl (0.025 mol/L) was prepared, while the p-nitrophenolpalmitate (substrate) was dissolved in Triton-X 100 (0.5%, p/v) and the samples and orlistat (Sigma<sup>®</sup>) were prepared at increasing concentrations (10 - 1,000 µg/mL). Microplates containing of enzyme solution (100 µL), substrate (50 µL) and sample/orlistat (50 µL) were incubated were incubated in a water bath (37 °C) for 10, 20, 30 and 40 minutes. The reaction was stopped with an ice bath and

absorbances were measured in a microplate reader (Thermoplate<sup>®</sup>, TP-Reader) at 405 nm.

### 2.13 Pancreatic α-amylase Assay

Pancreatic α-amylase inhibitory activity was performed according to the methodology proposed by Freitas et al. [21] with some modifications. Porcine pancreatic α-amylase (Sigma<sup>®</sup>, 1 mg/mL) in TRIS-HCl buffer (pH 7.0, 0.05 mol/L) containing CaCl<sub>2</sub> (0.010 mol/L) was prepared while starch (substrate, 1%) was made. Samples and acarbose (10 – 1,000 µg/mL) were solubilized in DMSO. Microplates containing α-amylase (50 µL), samples or acarbose (50 µL) and substrate (50 µL) were preincubated in a water bath at 37 ° C for 10 minutes. After this time, the substrate (100 µL) was added to the medium and the reaction was incubated in a water bath at 37 ° C for 10, 20, 30 and 40 minutes. The reaction was stopped with an ice bath and absorbances were measured in a microplate reader (Thermoplate<sup>®</sup>, TP-Reader) at 405 nm.

### 2.14 α-Glucosidase Assay

α-Glucosidase inhibitory activity was determined according to the methodology proposed by Chelladurai and Chinnachamy [22], with some modifications. Solutions of α-glycosidase (Sigma<sup>®</sup>, 2 U/mL) and p-nitrophenyl-α-D-glycopyranoside substrate (5 mmol/L) were solubilized in citrate phosphate buffer (pH 7.0, 0.1 mol/L), while samples and acarbose (Sigma<sup>®</sup>) were prepared at increasing concentrations (15.62 - 500 µg/mL) and solubilized in DMSO. Microplates containing α-glucosidase (100 µL), samples or acarbose (50 µL) and substrate (50 µL) were incubated in a water bath at 37 ° C for 10, 20, 30 and 40 minutes. The reaction was stopped with an ice bath and absorbances were measured in a microplate reader (Thermoplate<sup>®</sup>, TP-Reader) at 405 nm.

### 2.15 Determination of Inhibition percentage (%) and Half-maximal Inhibitory Concentration (IC<sub>50</sub>)

Absorbance values obtained from digestive enzyme assays were used to calculate the inhibition percentage (%) by linear regression using the least square method according to the equation (1) below.

$$I\% = 100 \times \frac{(A - a) - (B - b)}{(A - a)}$$

Where: *A*: Angle coefficient value of enzyme-only reading (enzyme + substrate); *a*: value of the angular coefficient of the reading without enzyme and without sample (substrate); *B*: angular coefficient value of enzyme plus inhibitor reading (enzyme + substrate + inhibitor); *b*: Angle coefficient value of reading without enzyme and inhibitor.

The half-maximal inhibitory concentration ( $IC_{50}$ ) was determined by linear least square regression using samples (10 – 1,000  $\mu\text{g/mL}$ ) and inhibition percentage (*I*%).

### 2.16 Brine Shrimp Lethality Bioassay

The brine shrimp lethality bioassay was performed according to Meyer et al. [23]. In this assay, five concentrations (10 - 1,000  $\mu\text{g/mL}$ ) of MEAM, HFAM, DFAM, EFAM and BFAM, and thymol (positive control) were prepared in artificial seawater and transferred to the test tubes. Then, ten shrimps (*Artemia salina* Leach) were placed in each tube ( $n = 4$ ). After 24 hours of exposure, the surviving larvae were counted and the 50% lethal concentration ( $LC_{50}$ ) was determined by the probit method. This assay was used to assess the toxicity of acetogenins present in plants of the Annonaceae family.

### 2.17 Statistical Analysis

The results were expressed as mean  $\pm$  standard error mean (S.E.M.). Analysis of variance (ANOVA) followed by Tukey's HSD (honest significant difference) test was applied to measure the degree of significance for  $p < 0.05$ . Graphpad Prism 5.0<sup>®</sup> Software was used in this analysis.

## 3. RESULTS AND DISCUSSION

Tannins, flavonoids, coumarins, terpenoids and steroids, saponins and alkaloids were revealed in MEAM. According to the polarity of the solvent, these chemical classes were also detected in HFAM (terpenes and steroids), DFAM (terpenes and steroids, and alkaloids), EFAM (tannins, flavonoids, coumarins and alkaloids) and BFAM (tannins, flavonoids, coumarins, saponins and alkaloids). Considering the quantification of constituents, total phenolic varied from  $0.40 \pm$

$0.03$  to  $8.45 \pm 0.05$  g/100 g and flavonoid ranged from  $1.35 \pm 0.07$  to  $2.74 \pm 0.06$  g/100 g (Table 1). EFAM exhibited the highest phenolic and total flavonoid contents. In addition, after HPLC analysis, the markers (quercetin, rutin, gallic acid, caffeic acid and 5-caffeoylquinic acid) were not identified. However, considering the retention time ( $R_t = 22.193$  min) and UV spectrum, a compound with similar characteristics to annonacin, an acetogenin from Annonaceae, was detected (Fig. 1). Due to the lack of standard compound, it was not possible to confirm the authenticity of this substance.

Using DPPH assay, the  $IC_{50}$  values of the samples were statistically different ( $P < 0.05$ ) and ranged from  $20.03 \pm 0.08$  to  $204.50 \pm 1.12$   $\mu\text{g/mL}$ , while FRAP varied from  $16.35 \pm 0.04$  to  $249.70 \pm 2.54$   $\mu\text{g/mL}$  (Table 2). According to the Table 1, EFAM ( $20.03 \pm 0.08$  and  $16.35 \pm 0.04$   $\mu\text{g/mL}$ ) was more active in both methods, respectively, when compared to the other fractions and extract. Based on inhibition of lipid peroxidation (ILP), the tested products produced values between 23.02 to 75.82% (Table 2). Among these products, HFAM (68.26%) and DFAM (51.83%) were the most active. These data are also seen in Fig. 2, which shows the decay of absorbance with respect to time.

The  $IC_{50}$  values of MEAM, HFAM, DFAM, EFAM and BFAM on pancreatic lipase were  $192.13 \pm 2.31$ ,  $248.67 \pm 1.28$ ,  $232.51 \pm 3.50$ ,  $131.14 \pm 1.70$ , and  $159.32 \pm 1.70$ ,  $\mu\text{g/mL}$ , respectively (Fig. 4a). In this Figure, these values were significantly different ( $P < 0.001$ ) when compared to orlistat ( $289.07 \pm 3.65$   $\mu\text{g/mL}$ ) and these extracts were more potent in inhibiting pancreatic lipase.

Acarbose, a synthetic pancreatic  $\alpha$ -amylase inhibitor, produced  $IC_{50}$  of  $225.14 \pm 4.11$   $\mu\text{g/mL}$ , being significantly ( $P < 0.001$ ) less potent than MEAM ( $160.60 \pm 2.29$ ), EFAM ( $142.42 \pm 1.95$ ), and BFAM ( $120.43 \pm 3.88$ ) (Fig. 4b). HFAM ( $286.02 \pm 4.08$ ) and DFAM ( $257.1 \pm 3.63$ ) also inhibited pancreatic  $\alpha$ -amylase, but were less active than positive control (Fig. 4b).

Regarding  $\alpha$ -glucosidase inhibition, acarbose produced  $IC_{50}$  of  $389.3 \pm 4.01$   $\mu\text{g/mL}$ , while MEAM ( $191.1 \pm 2.67$   $\mu\text{g/mL}$ ), DFAM ( $264.4 \pm 2.51$   $\mu\text{g/mL}$ ), EFAM ( $157.3 \pm 2.64$   $\mu\text{g/mL}$ ) and BFAM ( $109.3 \pm 1.76$   $\mu\text{g/mL}$ ) were more active (Fig. 4c). Although HFAM ( $432.02 \pm 3.39$   $\mu\text{g/mL}$ ) inhibited  $\alpha$ -glucosidase, it was significantly ( $P < 0.001$ ) less potent than acarbose and other extracts (Fig. 4c).

**Table 1. Total phenolic and flavonoid contents of *A. muricata***

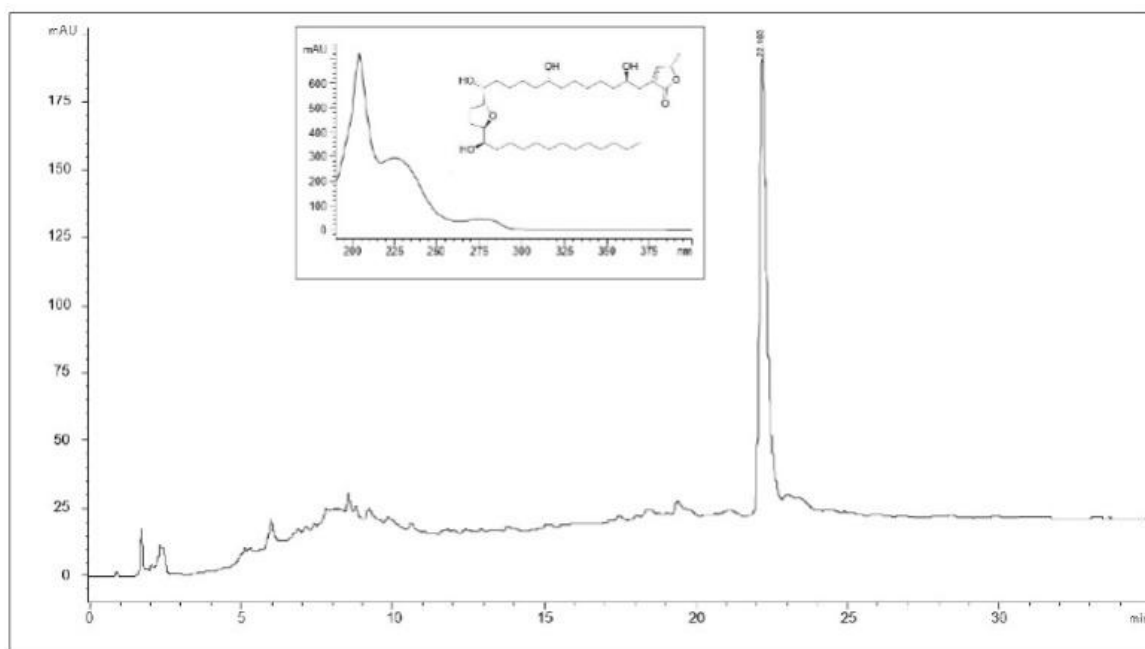
Tested product	Total phenolic (g GAE/100g)	Total flavonoid (g RUE/100g)
MEAM	6.57 ± 0.16 <sup>a</sup>	1.35 ± 0.07 <sup>a</sup>
HFAM	0.40 ± 0.03 <sup>b</sup>	-
DFAM	1.38 ± 0.02 <sup>c</sup>	-
EFAM	8.45 ± 0.05 <sup>d</sup>	2.74 ± 0.06 <sup>b</sup>
BFAM	4.54 ± 0.06 <sup>e</sup>	1.75 ± 0.05 <sup>c</sup>

Mean ± S.E.M. (n = 3). Different letters, there was significant difference between the means (P < 0.05) after ANOVA - Tukey's test. (-) Not detected or not quantified

**Table 2. Antioxidant activity of *A. muricata***

Tested product	IC <sub>50</sub> (µg/mL)		ILP (%)
	DPPH	FRAP	
MEAM	67.57 ± 0.58 <sup>a</sup>	81.75 ± 0.78 <sup>a</sup>	37.70 ± 1.10 <sup>a</sup>
HFAM	204.5 ± 1.12 <sup>b</sup>	249.7 ± 2.54 <sup>b</sup>	68.26 ± 0.41 <sup>b</sup>
DFAM	82.26 ± 0.13 <sup>c</sup>	171.50 ± 0.84 <sup>c</sup>	51.83 ± 0.48 <sup>c</sup>
EFAM	20.03 ± 0.08 <sup>d</sup>	16.35 ± 0.04 <sup>d</sup>	33.30 ± 0.60 <sup>d</sup>
BFAM	31.61 ± 0.46 <sup>e</sup>	25.96 ± 0.11 <sup>e</sup>	23.02 ± 1.29 <sup>e</sup>
Rutin	10.58 ± 0.14 <sup>f</sup>	-	-
Ascorbic acid	-	7.45 ± 0.10 <sup>f</sup>	-
BHT	-	-	75.82 ± 0.92 <sup>f</sup>

Mean ± S.E.M. (n = 3). Different letters, there was significant difference between the means (P < 0.05) after ANOVA - Tukey's test. (-) Not detected or not quantified

**Fig. 1. HPLC chromatogram and UV spectrum of MEAM showing a similar compound to annonacin**

After the *Artemia salina* toxicity test, MEAM, HFAM and DFAM were cytotoxic producing LC<sub>50</sub> values lower than 1,000 µg/mL (Table 3). According to the Table 3, HFAM (LC<sub>50</sub> = 184.30 µg/mL) and DFAM (LC<sub>50</sub> = 149.53 µg/mL) fractions were more active than thymol (LC<sub>50</sub> =

433.23 µg/mL), used as reference substance. Thus, due to its lower LC<sub>50</sub>, DFAM is the most toxic among the tested products.

The presence of tannins, flavonoids, alkaloids, terpenes and steroids and saponins in the extract

and fractions from the *A. muricata* fruit peels showed a wide possibility of compounds [5]. These compounds may be separated with solvents of increasing polarities and have been detected by specific reagents [13]. For example, the chemical structure of cholesterol is identified by the Libermann-Burchard reaction [24], while the color reaction with 3,5-diinitrobenzoic acid depends upon the presence of an  $\beta$ -unsaturated lactones, as occurs in annonaceous acetogenins [5]. Positive reactions to alkaloids confirm the presence of these compounds in *A. muricata*, as well as phenolic compounds [5,6]. In particular, the extraction in ethyl acetate allows the removal of free flavonoids, tannins, and xanthenes, while the butanol extracts glycosylated flavonoids and tannins [13]. In this study, these solvents extracted 12.99 and 4.49% of total phenols and flavonoids, respectively (Table 1), confirming previously described data [10]. In addition, annonacin (Fig. 1), an acetogenins found in fruits

of *A. muricata*, has been related to the toxic effects on the nervous system [5], and this may be a limitation on the use of *A. muricata* fruit peels for food. However, the fractionation process performed in this study may be an alternative for the consumption of part of the fruit peels as food and herbal medicines, especially EFAM and BFAM that are rich in phenolic compounds.

Although the antioxidant effect has been studied in different parts of soursop [5], this property has not been explored in *A. muricata* fruit peels. MEAM, EFAM and BFAM exhibited significant IC<sub>50</sub> values in DPPH and FRAP assays, which can be explained by the high content of phenolic and flavonic substances (Table 1). These compounds have ability to donate hydrogen or electrons and prevent the oxidation in biological media [25], which can bring great benefits to human health.

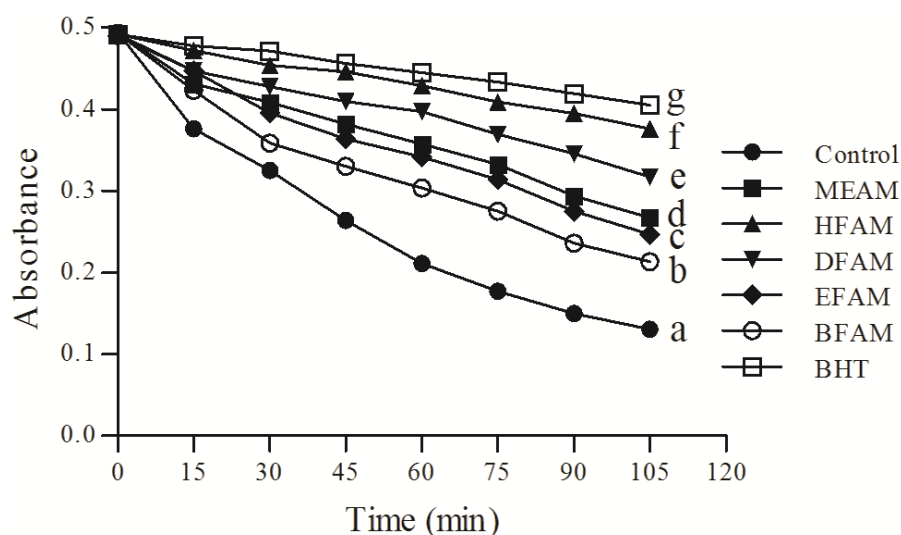


Fig. 2. Decay of absorbance versus time by the co-oxidation of the  $\beta$ -carotene/linoleic acid method

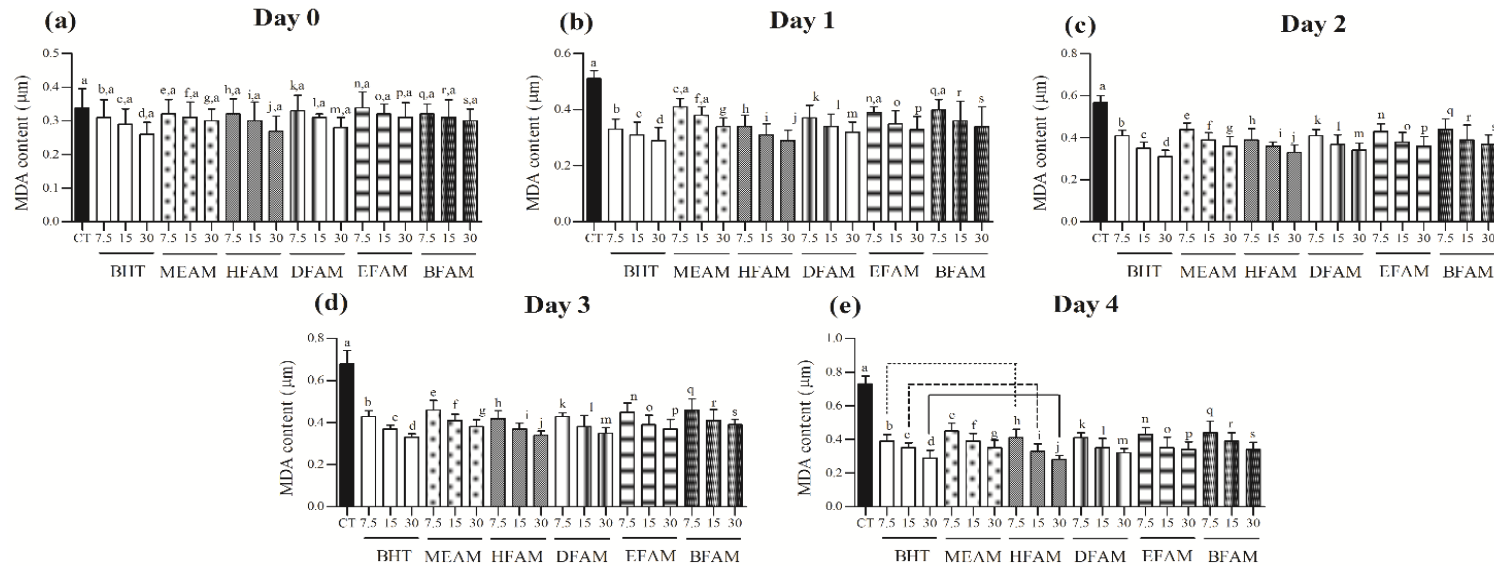
Mean  $\pm$  S.E.M. (n = 3)

Table 3. Toxicity of the methanol extract, fractions and thymol on the *Artemia salina*

Tested product	Concentrations ( $\mu\text{g/mL}$ )	LC <sub>50</sub> ( $\mu\text{g/mL}$ )	Confidence interval (95%)
MEAM	10, 50, 100, 500 and 1,000	385.24	264.68 – 560.74
HFAM	10, 50, 100, 500 and 1,000	184.30	129.45 – 262.40
DFAM	10, 50, 100, 500 and 1,000	149.53	103.48 – 216.07
EFAM	10, 50, 100, 500 and 1,000	> 1000	-
BFAM	10, 50, 100, 500 and 1,000	> 1000	-
Thymol	10, 50, 100, 500 and 1,000	433.23	305.88 – 613.59

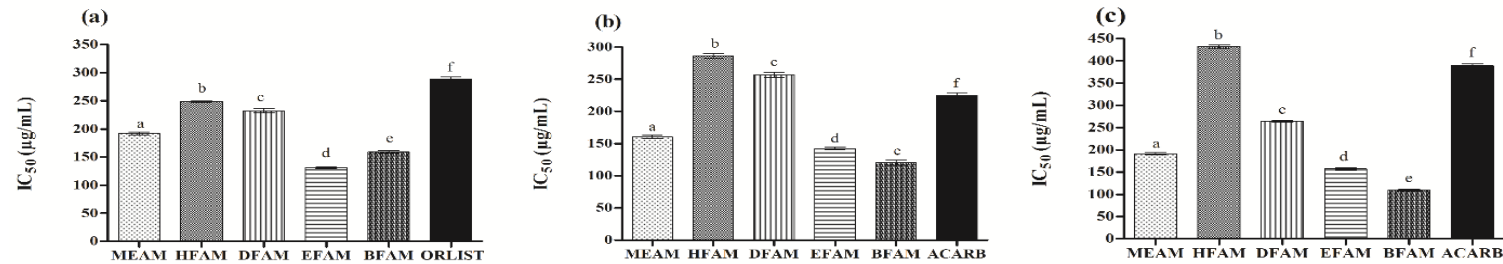
(-) Not detected or not quantified.





**Fig. 3. Effect of the methanol extract, fractions and BHT on the concentration of malonaldehyde**

Mean ± S.E.M. (n = 3) after treatment with 7.5, 15 and 30 mg of the sample. Same letters indicate that there was no significant difference between the means (P < 0.05) after ANOVA - Tukey's test when compared to control group (CT)



**Fig. 4. Inhibitory effects of the methanol extract, fractions and orlistat or acarbose on digestive enzymes**

Mean ± S.E.M. (n = 3). Different letters, there was significant difference between the means (P < 0.05) after ANOVA - Tukey's test. (a) Pancreatic lipase; (b) Pancreatic α-amylase; (c) α-Glucosidase. ORLIST: Orlistat; ACARB: Acarbose.



Lipid peroxidation is related to oxidative degradation of lipids in which free radicals capture electrons in cell membranes and initiates a chain reaction mechanism [26]. Based on this phenomenon, the linoleic acid forms the peroxy radical that reacts with beta-carotene resulting in the loss of coloration [19]. Our results showed that HFAM and DFAM were more effective than MEAM, EFAM and BFAM, since they present decay closer to BTH (Table 2 and Fig. 2). Probably, this effect might be due to the presence of nonpolar compounds that interact with the lipid emulsion by inhibiting the peroxy radical. In addition to these data, thiobarbituric acid assay showed that all the samples were active in reducing the formation of free radicals from the day 2 (Fig. 3), which corroborated the results showed in Table 1 and Fig. 2. These findings are relevant, since the lipid peroxidation assays mimic cellular alterations related to different pathophysiological mechanisms [27].

On the other hand, the adipose tissue secretes adipokines that generate reactive oxygen species (ROS) and leads the oxidative stress (OS) [28]. In cases of obesity, several mechanisms, as mitochondrial and peroxisomal oxidation of fatty acids, produce OS and are capable of generating ROS, which promote cardiovascular diseases, among others [28]. Natural products that inhibit the pancreatic lipase constitute an important therapeutic alternative in the treatment of obesity and oxidative stress [29]. MEAM, HFAM, DFAM, EFAM and BFAM were able to inhibit the pancreatic lipase, especially HFAM and DFAM that were more potent in inhibiting this enzyme (Figure 4a). This effect may be related to the presence of different compounds, mainly phenolics [30], and confirm the antioxidant effect (Table 1, and Figs. 2 and 3). In addition, docking studies have revealed that the possible binding sites of polyphenolic compounds with pancreatic lipase were located close to the enzyme active site, serine<sup>153</sup> (Ser<sup>153</sup>), aspartic acid<sup>176</sup> (Asp<sup>176</sup>) and histidine<sup>263</sup> (His<sup>263</sup>) [31].

Pancreatic  $\alpha$ -Amylase and  $\alpha$ -glucosidase are the main enzymes involved in carbohydrate digestion, such as dietary starch, releasing oligosaccharides that are later degraded to glucose to be absorbed. Inhibition of these enzymes is considered a promising strategy to lower serum glucose levels and manage the symptoms of diabetes-related diseases [22,32]. Our results indicated that methanol extract and

fractions of *A. muricata* fruit peels are efficient inhibitors of pancreatic  $\alpha$ -amylase and  $\alpha$ -glucosidase activity (Figs. 4b and 4c). As for pancreatic lipase, EFAM and BFAM were more effective in inhibiting both enzymes. As shown in Table 1, these fractions are rich in phenolic compounds that may be related to inhibition of these digestive enzymes [33,34]. Currently, one of the therapeutic approaches for the treatment of type 2 diabetes is the reduction of postprandial hyperglycemia by preventing glucose absorption by inhibiting  $\alpha$ -amylase and  $\alpha$ -glucosidase in the digestive tract [35]. Among the inhibitors of these enzymes that slow carbohydrate digestion, causing a reduction in the rate of glucose absorption and thereby attenuating the postprandial increase in plasma glucose, acarbose, miglitol and voglibose are clinically used [36]. Another important aspect is that inhibitors help in the management of obesity, which may also contribute to the treatment of metabolic syndrome [37].

To assess the toxicity of MEAM and fractions, we opted for the Brine shrimp (*Artemia salina* Leach) method that is considered a simple bioassay for natural product research [23], which determines LC<sub>50</sub> values. In addition, brine shrimp could be a valuable tool in the search for compounds that are protective against damage by superoxide or other active oxygen species [38]. MEAM, HFAM and DFAM were toxic against *A. salina* and were more active than thymol (positive control) (Table 3). Among the compounds that can be found in *A. muricata*, acetogenins, as annonacin, have been studied for their cytotoxic potential [5]. Several annonaceous acetogenin (annonacin, anomuricin A, anomuricin B, anomuricin C, annohexocin, muricatocin C, corosolone, among others) of *A. muricata* exhibited toxicity against brine shrimp [5]. However, as chemical markers of Annonaceae species, the alkaloids have also been considered in toxicity studies [39]. Although the toxicity of MEAM and fractions can be an impediment to the use of fruit peels as food and medicine, our findings may be relevant for safety studies, since the EFAM and BFAM fractions were not toxic on *A. salina* in the tested concentrations.

Our results are in accordance with Chel-Guerrero et al. [40], which showed a valuable source of bioactive compounds from the tropical fruit peels using *Annona squamosa*, *A. reticulata*, *Chrysophyllum cainito*, and *Melicoccus bijugatus*. Thus, fruit peels of *A. muricata* can be used for the development of new therapeutic products.

#### 4. CONCLUSION

The results indicate that *A. muricata* fruit peels are a source of phenolic compounds and other chemical classes (acetogenins and alkaloids), which they may be related to their antioxidant, anti-lipase, anti-amylase and anti-glucosidase, and toxic properties. In addition, the peels could play a crucial role in the prevention and treatment of oxidative and metabolic disorders.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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