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EXTRACTION AND CHARACTERIZATION OF ANDIROBA (*Carapa guianensis*) OIL AND ITS EFFECTS ON ECTONUCLEOTIDASES OF BLOOD SERUM OF RATS

PATOS DE MINAS – MG
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Artigo científico apresentado ao Instituto de Genética e Bioquímica da Universidade Federal de Uberlândia como requisito final para a obtenção do título de Bacharel em Biotecnologia.

Orientadora: Profa. Dra. Cristina Ribas Fürstenau

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EXTRACTION AND CHARACTERIZATION OF ANDIROBA (*Carapa guianensis*) OIL AND ITS EFFECTS ON ECTONUCLEOTIDASES OF BLOOD SERUM OF RATS

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Abstract

Carapa guianensis, popularly known as andiroba, is a tree that grows at Amazon Basin and is used in popular medicine in Brazil. Oil obtained from andiroba seeds is employed to treat inflammation, wound healing, as well as infections and insect repellent. Purinergic signaling helps to control cardiovascular functions which may be related to these pathologies. Thus, the aim of the present study was to evaluate the effects of *C. guianensis* oil on ectonucleotidase activities from rat blood serum. Oil extraction was performed using hexane as solvent. The procedure yielded 49.11 % of andiroba oil that was used for further characterization and functional assays. Peroxide index resulted in 7,916 ± 0,54 meq peroxide/ 1 Kg sample that signals to high levels of oxidants in the oil. However, low acidity was observed, evidencing that the oil was not in decomposition state. Total phenolic content and antioxidant activity by DPPH method had low results (24.99 mg EAG/ Kg of sample and negative values, respectively), showing that from this methodology, these molecules were not kept in the oil. Besides, hydrolyses of ATP, ADP and AMP in blood serum of rats were not affected by andiroba oil at different concentrations tested (0 μg mL^{-1}, 125 μg mL^{-1}, 250 μg mL^{-1}, 500 μg mL^{-1} and 1000 μg mL^{-1}). In conclusion, oil extraction from andiroba seeds was good in terms of yield; however, the quality of this andiroba oil was different from others obtained through different methodologies, which importantly impacted the expected effects upon antioxidant capacity of the oil and its effect on nucleotide hydrolysis.

Keywords: Meliaceae. *Carapa guianensis*. Oil Extraction. Oil Characterization. NTPDase. Ecto-5'-nucleotidase.

1. Introduction

Amazonian region presents the highest biodiversity in the world, including a great variety of exotic food and medicinal plants with distinct biological properties [1, 2]. Meliaceae family is among medicinal plants found in Amazonia. This family includes *Carapa guianensis* specimens, popularly known as andiroba, which is medium to large tree that normally grows at all extension of Amazon Basin and Central America [3]. Andiroba oil is extracted from the seeds of *C. guianensis* and is widely used because of its medicinal properties [2]. The oil is currently used in the treatment of arthritis, inflammation, wound healing, diarrhea, diabetes, ear infection, as a digestive stimulant, to treat uterine cancer and as insect repellent [4].

Biological activity of andiroba oil has been related to the presence limonoids, flavonoids, steroids, coumarins [2, 4], fatty acids and antioxidants. The occurrence of such molecules in the oil is closely related to its extraction process, which may thus significantly impact on the oil quality and the functionality of final product [5, 6].

Purinergic signaling is a common via of cellular communication mediated by purines that may influence different physiological and pathological processes [7]. The agonists of this signaling are extracellular adenosine-5'-triphosphate (ATP), and its breakdown products, adenosine-5'-diphosphate (ADP) and adenosine (ADO) that are present on a variety of biological processes, including inflammation, platelet aggregation, contraction of smooth muscle, neurotransmission and cell proliferation [8,9, 10].

Purines exert their function through the activation of specific receptors. There are two families of purinergic receptors, named P1, that are G-protein coupled receptors responsive to adenosine; and P2, that may bind ATP, ADP and others. P2 receptors are also divided into two subclasses: 1) P2X receptors, that correspond to ion-channels responsive to ATP; and 2) P2Y receptors, G-protein coupled receptors that may answer to ATP, ADP, UDP and UDP-glucose [11]. ATP co-released with norepinephrine (NE) from sympathetic nerve terminals can activate P2X receptors, causing vasoconstriction of smooth muscle cells [12, 13]. On the other hand, when binding to P2Y receptors, ATP may promote vasodilation with direct actions on vascular smooth muscle, or indirect effects on the endothelium, being a non-adrenergic non-cholinergic perivascular

neurotransmitter [12]. ADP through the activation of specific P2Y receptors may stimulate platelet aggregation and promote vasoconstriction [14, 15].

Ectonucleotidases are enzymes able to breakdown extracellular adenine nucleotides. They include members from nucleoside triphosphate diphosphohydrolase family (NTPDases), nucleotide pyrophosphate/ phosphodiesterase family, alkaline phosphatases and 5'nucleotidases [16]. These families of enzymes often act in a coordinated manner to terminate the signaling cascade initiated by nucleotides and allow the initiation of adenosine signaling. Thus, NTPDases hydrolyze ATP and ADP to AMP and 5'nucleotidase converts AMP to adenosine [17]. Both families of enzyme are ubiquitous in endothelial and hematopoietic cells, acting as important regulators of purinergic signaling in the blood [18]. Besides NTPDases and ecto-5'-nucleotidase are ectoenzymes anchored to plasma membrane and with their active site facing the extracellular medium, they may also appear in a soluble form, as is the case for ectonucletidases from blood serum. Thus, ATP and ADP can be metabolized by the action of NTPDases and 5'-nucleotidase in rat blood serum [19].

NTPDases family is constituted by enzymes that are anchored in the cell membrane and are capable to hydrolyse γ and β phosphates of di- and triphosphates of nucleotides [20]. Eight different genes are already described to the members of this protein family. NTPDases1, 2, 3 and 8 are located with the catalytic site facing the extracellular medium. NTPDases5 and 6 are placed intracellularly can be found in secreted forms. Finally, NTPDases4 and 7 are situated intracellularly, with the catalytic site turned to the lumen of cytoplasmic organelles [21].

Considering the importance of andiroba oil as a popular medicine used to treat several pathologies, including those of the cardiovascular system, as well as the importance of purines and purinergic signaling to the circulation homeostasis, the present study aimed to investigate the effects of *C. guianensis* oil on ectonucleotidases from rat blood serum rats.

2. Materials and methods

2.1 Sample preparation

Seeds of *Carapa guianensis* were collected in the Ouro Preto D'Oeste, Rondônia, Brazil (10º47' 16.99" S/62º10' 30.75" W) between January and July 2016. Plant identification was done by Dra. Rosana Romero and one sample was deposited at the Herbarium of Universidade Federal de Uberlândia (UFU, Brazil). After collection, seeds were kept immersed in water for 24 hours to eliminate *Hypsipyla grandella* by drowning and also to homogenize the water content of the seeds. After that period, seeds could dry at 40 ºC for 12 hours, further crushed using a knife mill (Willye 50) and stored at room temperature until use.

2.2 Oil extraction

Soxhlet extraction was carried out using 150 mL hexane (Alphatec) to extract around 5 g of *C. guianensis* seeds to extract the oil for 24 hours, performing this process 20 times. After this time, fixed oil was concentrated and hexane recovered under low pressure at 50 ºC in a rotary evaporator. The oil extraction yield was calculated from the equation:

$$( ) \quad \frac{( )}{( )}$$

2.3 Determination of total antioxidant activity by DPPH method

Antioxidant activity of *Carapa guianensis* oil was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) method as previously described by Lopes-Lutz et al. [22], with some modifications. Initially, an ethanol solution of DPPH (Sigma) was prepared at a concentration of 40 μg mL^{-1}. About 2.7 mL of the DPPH solution was added to each test tube, followed by 0.3 mL of Tween 20 (Dinâmica) dissolved oil at final

concentrations of 125 µg mL^{-1}, 250 µg mL^{-1}, 500 µg mL^{-1} and 1000 µg mL^{-1}. Butylated hydroxytoluene (BHT) (Sigma) standard was used as a positive control. The negative control was a solution containing all the reagents except the oil. The reaction was kept in the dark at room temperature for one hour. Absorbances were measured at 517 nm. Antioxidant Activity (AA%) was calculated by:

( )

2.4 Determination of total phenolic content

Phenolic compounds of the samples were prior treated with methanol and hexane to extract the bioactive phytochemicals [23,24], with some modifications. About **2.5** g of oil was dissolved in 5 mL of hexane and phenolic compounds were extracted by 5 mL of a methanol/water solution (60:40, v/v) under agitation for 2 minutes. Phases of hexane and methanol/water were separated from each other by centrifuging the solution at 3500 rpm for 10 min. A volume of 0.2 mL of the methanolic phase and was transferred to a flask and completed with deionized water to 5 mL. Folin-Ciocalteau (Sigma) reagent (0.5 mL) was further added to the mixture. After 3 minutes, 1 mL of sodium carbonate (35%) was also incorporated to the reaction that was finally completed with water until 10 mL. The reaction was incubated for 2 hours at room temperature and the absorbance was then read at 725 nm using a spectrophotometer (Gehaka, UV-340G). Total phenolic content was expressed in mg equivalent of gallic acid (Sigma) per kilogram of oil (mg GAE/ Kg).

2.5 Oil characterization

2.5.1 Determination of acidity

Oil samples were homogenized and completely liquid. A sample of 2 g of oil was dissolved into 25 mL of neutral ether-alcohol (2:1) solution mixture and titrated against 0.1 M or 0.01 M sodium hydroxide standard using phenolphthalein solution (2 drops) as an indicator, until a visible pink colour appeared, which should remain for 30 seconds [25].

Calculations:

number in mL of 0.1 M sodium hydroxide solution titration expenses.

factor of sodium hydroxide solution.

number of grams of the sample.

2.5.2 Determination of the peroxide index

Five grams of oil were mixed to 30 mL of an acetic acid-chloroform solution (3:2 v/v) and let under agitation dissolution of the sample. KI (0.5 mL) solution was added to the previous mixture and allowed to stand in the dark for exactly one minute. After that, 30 mL of distilled water was also added and the reaction was titrated with 0.1 N or 0.01 N sodium thiosulfate solution under constant agitation. Titration was continued until the yellow color had disappeared. The indicator starch solution (0.5 mL) was added to the mixture and allowed to titrate until the completely disappearance of blue color. Blank tube was prepared under the same conditions and holder [25].

Calculations:

( )

A = number in mL of 0.1 N or 0.01 N sodium thiosulfate solution spent in the titration of the sample.

B = number in mL of 0.1 N or 0.01 N sodium thiosulfate solution spent in the titration of blank.
N = normality of the sodium thiosulfate solution.
f = factor of sodium thiosulfate solution.
P = number of g of the sample.

2.6 Isolation of blood serum fraction

Blood serum obtention was performed as previously described by Yegutkin [26], with little modifications. Male *Wistar* rats, 60 days old, weighting around 250 grams, were euthanized by decapitation. Blood samples were drawn into tubes and allowed to coagulate at room temperature for approximately 30 minutes. Next, blood was centrifuged (5000 *g*, 5 minutes, room temperature) and serum samples were transferred to another tube. Serum samples were frozen at -80 ºC until use for enzyme activities determination. All protocols used will be previously approved by the Committee for Ethics in the Use of Animals (CEUA) of UFU.

2.7 Assays of E-NTPDase and ecto-5'-nucleotidase activities

ATP, ADP and AMP hydrolysis were determined using a modification of the method described by Yegutkin [26]. Reaction mixture (final volume of 0.2 mL) containing 3.0 mM ATP (Sigma), ADP (Sigma) or AMP (Sigma) as substrate, 112.5 mM Tris-HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37ºC for 40 minutes. Different concentrations of andiroba oil (0 $\mu g\ mL^{-1}$ – control group, 125 $\mu g\ mL^{-1}$, 250 $\mu g\ mL^{-1}$, 500 $\mu g\ mL^{-1}$ and 1000 $\mu g\ mL^{-1}$) were added prior to the incubation time. Reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid (TCA) (Sigma) and the amount of Pi released was measured by the colorimetric method previously reported by Chan et.al. [27]. All enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after the addition of TCA were used to correct nonenzymatic hydrolysis of

the substrates. Enzyme activities were expressed as nanomoles of phosphate released per minute per milligram of protein. Tween 20 (Dinâmica): water solution (1:4, v/v) was used to solubilize the oil used in enzyme assays. A vehicle control was tested in the highest concentration and did not alter enzyme activity.

2.8 Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [28].

2.9 Statistical analysis

Data were analyzed by one-way ANOVA, followed by the Scott-Knott to DPPH method and Tukey test to nucleotides hydrolyses. $P<0.05$ was considered statistically different. All analyses were performed using the GraphPad Prism version 7.03.

3. Results and discussion

3.1 Extraction and Characterization

Yields of andiroba oil obtained from extraction using hexane as solvent were 49.11 % (m/m). This result was calculated from twenty independent extractions, using a total of 100 g of seeds, performed in Soxhlet apparatus, as described before. This yield demonstrates that seeds may have components with affinity by hexane and that Soxhlet extraction method may be appropriated to obtain large concentrations of the oil. Costa-

Silva et al. [29] also performed the extraction of oil from andiroba seeds using hexane as the solvent and found a yield of 34 %. Those authors, however, performed extractions with shorter periods of time and used a 95% greater amount of seeds we have used in the present study. Also, Mendonça and Ferraz [30] used the traditional extraction of oil subdivided in (1) collection and selection of the seeds; (2) seeds mass preparation; and (3) oil extraction using typical press of Amazonia, and showed results with lower yield than our results, using about 11 Kg/ L of seeds.

Chemical characterization is important to determine the suitability of *Carapa guianensis* oil to be used by the population. Results are expressed in Table 1. Quality of plant material directly influences the characteristics of the oil extracted. Peroxide index shows the substances in terms of milliequivalents of peroxide for 1000 g of sample which oxidize potassium iodide in the test conditions. Our results (7,916 ± 0,54 meq peroxide/ 1 Kg sample, n=3) reached high levels of oxidants when compared to 3,84 meq.Kg^{-1} of oil presented by Cavalcante et al. [31]. Acidity value can provide an important clue in the evaluation of conservation state of the oil. Decomposition, like hydrolysis, oxidation or fermentation, may change the concentration of hydrogen ions in the oil sample. As shown in Table 1, low acidity was observed in the present study in comparison to those of Cavalcante et al. [31], evidencing differences in the method of oil extraction in both studies.

Parameters of acidity and peroxide show up the degree of seeds deterioration in terms of oxidative and hydrolytic rancidity by peroxide index and acidity, respectively. In accordance with Ibrahim et al. [32], values higher than 5 meq.Kg^{-1} for peroxide index were designated as enzyme inhibitors. Peroxide index of andiroba oil obtained in this study ranged from 7.60 to 8.54 meq.Kg^{-1}, indicating that this oil may not be appropriated to be used in enzymatic reactions.

Table 1 – Chemical characterization of *C. guianensis* oil.

Parameter	Mean ± SD	Method[1]
Peroxide Index	7,916 ± 0,54	326/IV
Acidity	1,073 ± 0,01	325/IV

[1]*Adolfo Lutz Institute. (IAL). Analytical Standards of Adolfo Lutz Institute – Chemical and Physical Methods for Food Analysis. 4th ed. Brasília: 2005.*

3.2 Antioxidant capacity and total phenolic content

Antioxidant activity is commonly evaluated by the DPPH test. One of the mechanisms used to measure it is through the scavenging of free radicals by antioxidants [33]. Figure 1 demonstrates the inhibition of DPPH radical by the andiroba oil and the standard antioxidants at different concentrations. According to the results, at any concentration test, andiroba oil samples are very divergent of the BHT values, showing that the oil does not present antioxidant activity. Using n-butane for oil extraction, Novello and colleagues observed antioxidant activity, at a concentration of 320 µg mL^{-1}, analyzed by DPPH in different temperature and pressure conditions [6].

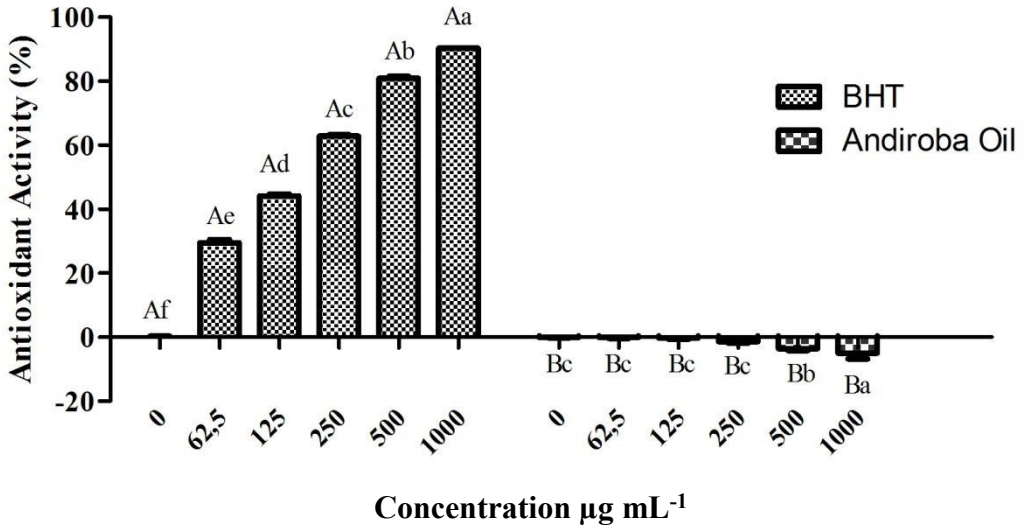

Fig. 1. Percentage of the antioxidant activity of andiroba oil by DPPH radical inhibition method. Mean values by the same letter in uppercase to compare the concentration in the sample (oil or BHT) analyzed and in lowercase for comparison between the concentrations of the same sample do not differ significantly at 5% probability Scott-Knott test.

Total phenolic content indicates the presence of potential antioxidants molecules in the sample, since these compounds are classified as free radical scavengers, preventing auto-oxidation [34]. Phenolic content of andiroba oil resulted in 24.99 mg EAG Kg^{-1} of sample, which was low when compared to other extract methodologies like pressurized fluid extraction, where the value ranged from 11.15 to 35.67 mg EAG 100 g^{-1} of dry sample [6]. Phenolic components may be related to the stability of the oil

and may be associated with antioxidants properties [24]. Thus, the low results obtained regarding total phenolic content in andiroba oil was kind of expected since antioxidant activity was observed in the DPPH test. Some authors reported that plants from Meliaceae family exhibit total phenolic content, evaluated by compounds, extracts by the Folin-Ciocalteu method, ranging from 89.82 to 500.24 mg EAG g^{-1} of sample at different fractions (ethyl acetate fraction and aqueous fraction, respectively) [6, 35].

3.3 Hydrolysis of ATP, ADP and AMP in blood serum rats

Purinergic signaling consists in a large network of cell-to-cell communication constituted by agonists (purines), cell-surface receptors, and ectoenzymes. Thus, ectonucleotidases are able to regulate extracellular levels of adenine nucleotides (ATP, ADP and AMP) and nucleoside (ADO) that participate on a variety of biological processes including contraction of smooth muscle, inflammation, platelet aggregation, and cell proliferation [36, 37, 38]. *Carapa guianensis* oil was tested in different concentrations (0 μg mL^{-1}, 125 μg mL^{-1}, 250 μg mL^{-1}, 500 μg mL^{-1}, 1000 μg mL^{-1}) upon nucleotide hydrolysis.

Results are presented in Figure 2 and demonstrated that andiroba oil did not interfere in the hydrolysis of ATP (Figure 2A), ADP (Figure 2B) and AMP (Figure 2C) at any tested concentration in blood serum of rats. These assays were performed before chemical analysis of the oil. ATP and ADP exhibit vasoactive properties as well as andiroba oil does. Besides, ATP is involved in wound healing as well as andiroba oil does. Thus decided to check if purines could contribute to the vasoactive and wound healing effects of andiroba oil. So far, the experiments performed with blood serum, a fraction containing soluble nucleotidases seem to not be altered by *C. guianensis* fixed oil. The high values of peroxide index in andiroba oil may explain in part the lack of response of enzyme activity to such oil [39]. Furthermore, this study was performed only with total seeds oil, not at other levels, like isolated limonoids or other components. In this sense, a previous study already showed that components isolated from andiroba oil were able to significantly reduce triglyceride levels in hepatocytes of high glucose-pretreated human hepatocellular carcinoma cell line [32]. Also, bioactive

compounds in *C.guianensis* oil can influence and have effects in anti-allergic and anti-hyperalgesic activities, which are closely mimicked by theirs constituents [40].

The active principles of andiroba oil have been analyzed using solvent extraction, and interfered at quality of this oil. Fractions and pure substances of *C. guianensis* have been submitted to pharmacological assays in order to demonstrate their anti-inflammatory effects and other properties. Test of crude andiroba oil by pharmacologists have produced evidence of its anti-inflammatory and analgesic properties. The anti-inflammatory properties of andiroba oil are probably due to the presence of limmonoids (non-saponifiable fraction) that are soluble in the unsaturated fraction of the oil [41, 42].

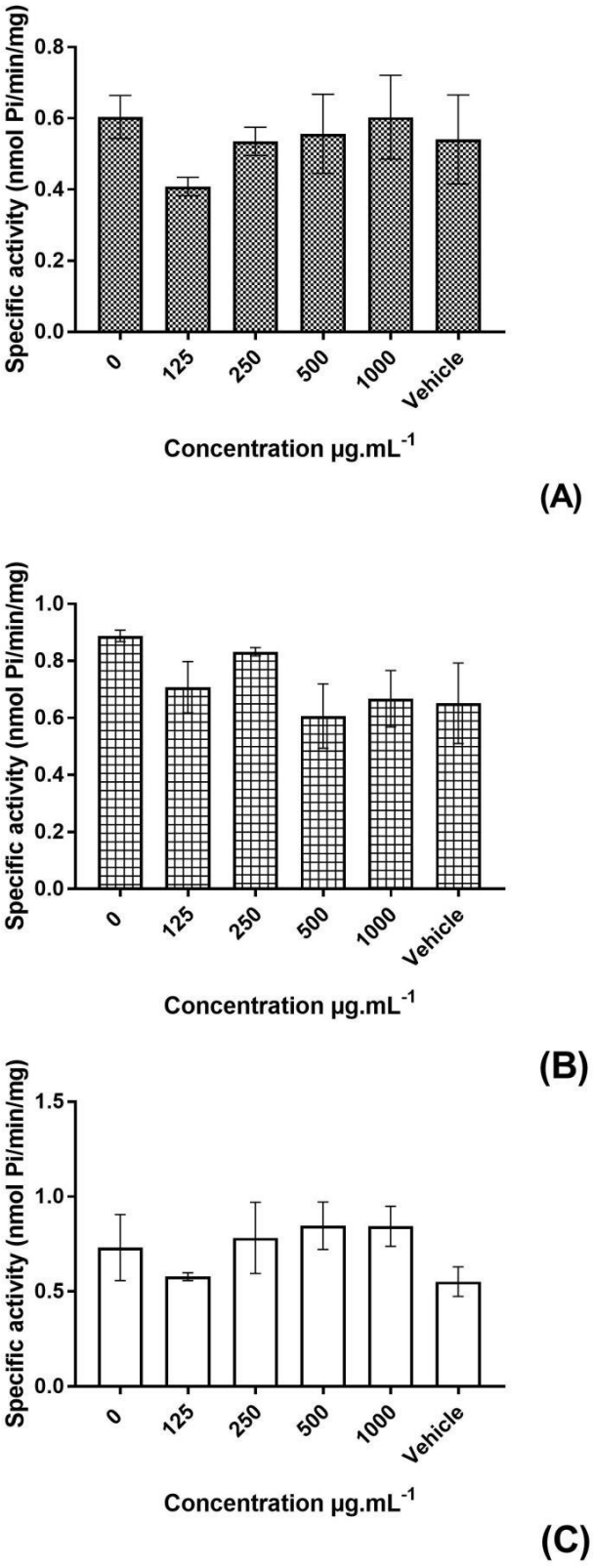

Fig. 2. Effect of *C. guianensis* oil on ATP (A), ADP (B) and AMP (C) hydrolysis of rats blood serum. Bars represent mean ± SD for six independent experiments. Results are expressed as nmol Pi released per min per mg of protein. Vehicle is the negative control of the emulsifier Tween 20. Data were statistically analyzed by one-way analysis of variance (one-way ANOVA), followed by Tukey test.

4. Conclusions

According to the results obtained, the extraction method using hexane as solvent in Soxhlet apparatus seemed to be efficient in the acquirement of high amounts of andiroba fixed oil. Results of the peroxide and acidity index contributed to show the degree of oxidation and the conservation of the oil. The method of extraction, however, influenced the quality of the oil that was poor in antioxidant molecules and was consequently unable to influence circulating ATPase, ADPase and AMPase activities. Other studies are necessary to test the crosstalk between wound healing property of andiroba oil, mostly used for this purpose, and purinergic participation in such effect.

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