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## ***Eugenia punicifolia* leaf extract has a hypotensive effect and inhibits angiotensin-converting enzyme activity in both *in vitro* and *in vivo* models**

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## Abstract

**Background:** *Chronic high blood pressure has for many years been considered a public health problem. Eugenia punicifolia is a plant used to treat diabetes by the local population, however its hypotensive effect has never been investigated.*

**Materials and methods:** *The effect of the 21-day treatment on systolic blood pressure was measured using the two kidneys, one clip (2K1C) model with hypertensive Goldblatt rats, which were treated with a repeated dose of 0.30 g/kg/day of extract by gavage (EEP group 300) and 0.15 g/kg/day (EEP group 150). Prior to pharmacological testing, acute toxicity tests were also performed on the rats. The activity of plasma angiotensin converting enzyme (ACE) during the experiment was determined spectrofluorometrically.*

**Results:** *The EEP, in concentrated form, inhibited ACE activity and a hypotensive effect in vivo. Systolic blood pressure (SBP) levels were significantly reduced in the EEP 150 and EEP 300 groups, respectively. ACE activity was inhibited in plasma.*

**Conclusion:** *Eugenia punicifolia appears to affect blood pressure significantly. Hypotension produced by the EEP extract can be related to the inhibition of ACE.*

**Keywords:** Angiotensin converting enzyme; Hypotension; Eugenia punicifolia leaves, Antihypertensive

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## BACKGROUND

Foods rich in sodium consumed without due control collaborate to increases in blood pressure and aggravate hypertension [1, 2]. Statistics suggest that, by 2025, 56 billion individuals will suffer from hypertension [2]. One of the particularities of hypertension is the action of the renin-angiotensin system, in the vasomotor center, which is responsible for the relaxation or constriction of blood vessels. This modulation process occurs because renin converts the angiotensinogen to angiotensin I. [3].

Angiotensin I is converted to angiotensin II, a powerful vasoconstrictor intermediated by the angiotensin-converting enzyme (ACE) [4]. This process causes, among other implications, inflammation of the blood vessels, autonomic alterations of the myocardium, and also reduction in the salt and water eliminated by the kidneys [6, 7, 8, 9]. Consequently, blood pressure is raised [10].

The literature relates the use of several plant extracts that have antihypertensive effects in humans and animals. Quedrago et al. (2011) [11] demonstrate the use of 0.01-10 mg/ml *Agelanthus dodoneifolius* in monortense rats. Similarly, González-Peña et al. (2014) [12] used 10% onion powder (*Allium cepa*) mixed with a diet supplemented with cholesterol. At the end of the studies, the researchers observed decreases in blood pressure in the supplemented groups when compared to the control group.

For hypertensive humans, Ashraf et al. (2013) [13] prescribed garlic capsules (*Allium Sativum* L) containing 300-1500 mg/day for 24 weeks. The study showed significant a decrease in both systolic and diastolic blood pressure in both dose and duration dependent manner. In other studies, the inhibitory effects on ACE using plant extracts have been investigated [14, 15, 16].

The species *Eugenia punicifolia*, which belongs to the Myrtaceae family and is known by the natives of the Amazon as the "insulin plant" [17, 18].

Previous studies have shown that this species presents anti-inflammatory effects [19], as well as antioxidant [18, 20], antidiabetic [21], gastroprotective [22], antibacterial [23] and antimicrobial effects

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[24]. These pharmacological properties are attributed to the high concentration of flavonoids and monoterpenes, and also the condensed tannins present in this plant [25, 26,].

However, the literature does not report studies regarding the effects of this species on blood pressure. Therefore, the aim of this study was to investigate the effects of *E. punicifolia* extract (EEP) on ACE inhibition *in vitro* and its hypotensive effect *in vivo*.

## MATERIALS AND METHODS

This experimental study was conducted in the state of Amazonas as result of the thesis of PhD in Biotechnology at the Federal University of Amazonas. It aimed to analyze biological activities *in vitro* and *in vivo* of the aqueous extract of the leaves of the *E. punicifolia* (EEP), emphasizing the inhibitory activity of digestive enzymes, enzyme angiotensin-converting (ACE), antioxidant, hypolipemiant, cytotoxic and antihypertensive effects

### Reagents

The chemical compounds and the following reagents were used in this experiment: Quinic acid (PubChem CID: 6508); Chlorogenic acid (PubChem CID: 1794427); Gallic acid (PubChem CID: 370); Protocatechuic acid (PubChem CID: 72); Vanillic acid (PubChem CID: 8468); Catechin (PubChem CID: 73160); Quercetin (PubChem CID: 5280343). All remaining reagents were of the highest purity available ( $\geq 98\%$ ).

### Preparation of *E. punicifolia* extract (EEP)

Dry *E. punicifolia* leaves were supplied by Embrapa-Amazons Occidental. The extraction was performed using infusion for 15 minutes in a proportion of 7.5% (w/v) inordance with Galeno et al. (2014) [17]. The dry product was obtained by spray drying (Mini Spray Dryer/MSD 1.0, Labmaq, São Paulo, Brazil).

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### **Bioassay procedure for ACE inhibition**

The EEP inhibition activity assay was adapted from Serra et al. (2005) [27]. The ACE solution was obtained from an extract of rabbit lung acetone (Sigma) at a concentration of 100 mg/mL in phosphate buffer (5 mM, pH: 8.0).

The ACE inhibition assay was determined by inhibiting the release of the dipeptide L-leukin-L-histidine (His-Leu) formed in the hydrolysis of the synthetic tripeptide substrate Hippuryl-L-histidine L-leukin (Hip-His-Leu) (5 mM) by ACE.

The His-Leu of dipeptide formed was coupled to a fluorescent substance, orthophthaldialdehyde (2% in methanol; 10 mL) and the resulting fluorescence was quantified by the fluorimeter and read in the ELISA reader (DTX 800 Multimodal Detector, Beckman Coulter) The calculation of the inhibition percentage was as follows: % inhibition = 100 - (sample absorbance/control absorbance) X 100.

### **Chemical composition analyses**

Liquid chromatography (LC-MS/MS, Shimadzu SPDM10) coupled to a Bruker Amazon-trap ion mass spectrometer was used. The electrospray (ESI) was used to explore the phenolic composition of *E. punicifolia* extract.

Chromatographic separation was performed in a C18 column (Shimadzu, 5 µm, 250 x 4.6 mm) using a binary mobile phase. The temperature was maintained at 20 °C with an injection volume of 10 µL. For structural elucidation, the MS/MS spectral data which were obtained were compared with data from previous literature [28, 29].

### **Animals and ethical considerations**

Wistar rats (200-350 g) were kept under controlled conditions (temperature: 23±1 °C and light: 08:00h to 20:00h), and received food *ad libitum*.

The procedures were approved by the Ethics Committee at the Universidade Federal do Amazonas, (Approval N°.CEEA-UFAM 77/2012) and were conducted in accordance with the Declaration of Helsinki.

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### **Evaluation of EEP acute toxicity**

After 2 hours of fasting, the animals (n = 12) were divided into two groups: Group 1 – animals that received a single oral dose of EEP (2 g/kg) and Group 2 – control animals that received saline solution (0.9%), under the same experimental conditions.

Subsequently, the rats were observed for 60 minutes in individual cages and during the first 48 h until the 14<sup>th</sup> day for any indication of toxic shock syndrome or mortality [30, 31]. At the end of the 14 days, the animals were anesthetized with ketamine and xylazine to obtain plasma and evaluate the biochemical parameters.

### **Effects of EEP on blood pressure**

After anesthesia, the animals were submitted to a laparotomy in order to expose the renal peduncle. A small silver clip (2 x 5 cm) with 0.2 mm was placed over the left renal artery. The control group was submitted to the same surgical procedure, but without partial occlusion of the renal artery.

After surgery, 30 rats were randomly distributed into five groups: normotensive (2K or SHAM), hypertensive (2K1C), enalapril hypertensive (ENAL), hypertensive and receiving 150 mg/kg/day of extract (EEP150) and hypertensive and receiving 300 mg/kg/day extract (EEP 300), both orally.

The effects of EEP on blood pressure (BP) were indirectly evaluated by tail-cuff plethysmography. BP was measured before and after surgery until the sixth week. During the treatment period, the systolic blood pressure was measured three times a week. Animals with systolic blood pressure above 160 mmHg were considered hypertensive.

### **Treatment**

The experimental treatment was started 21 days after the third week of continuous clamping of the renal artery or false surgery.

Treatment was via gavage with EEP (0.150 g/kg or 0.300 g/kg), the vehicle was saline (1 mL/kg) or enalapril (20 mg/kg). The

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control animals (Group 2K and 2K1C) received the same volume of vehicle.

### **Blood sampling**

At the end of the experiment, about 1 mL of blood was drawn via heart puncture from all rats of the 5 groups.

The plasma was separated by centrifugation (10 min, 3000 rpm, 10°C) and stored at 20 °C, until the time of determination of enzymatic activity and biochemical analysis.

Analysis was performed using the automatic Cobas Mira® analyzer using BIOCLIN® kits. The animals were submitted to a previous fasting of 12 hours. Relative fluorescence was determined in all groups for ACE activity [32].

### **Statistical analysis**

Data with a normal distribution were assessed using univariate analysis of variance (either one-way ANOVA or two-way ANOVA), followed by the Bonferroni or Tukey post-test. Data were expressed as mean ± SEM.

In all cases, differences were considered significant when the p value was < 0.05. All analyses were performed with *GraphPad* Software, USA, for Windows 7. *In vitro* results were presented as the means ± standard deviations of measurements in triplicate.

The 50% inhibitory concentration (IC<sub>50</sub>) values were obtained by non-linear regressions of concentration–response curves using the program *Microcal*™ *Origin*® version 6.0 (*Microcal Software* Inc.).

## **RESULTS**

In the present study, for the first time, the ACE inhibitory activity of *Eugenia punicifolia* extract was demonstrated. EEP (0.3 mg/mL) was able to inhibit 70.8% of enzyme activity. Captopril inhibited 83.6% at 0.003 mg/m.

As shown in Figure 1A, EEP inhibited ACE activity in a concentration-dependent manner showing an IC<sub>50</sub> value of 24.3 ± 0.7

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µg/mL, while captopril has an IC<sub>50</sub> value of  $0.9 \pm 0.1$  µg/mL (Figure 1B).

In an attempt to identify biologically active compounds related to ACE inhibition activity, the phenolic composition of EEP was analyzed by LC-MS/MS (Figure 2).

The fragments found were compared with fragmentation previously described in the literature [17, 29]. The phenolic compounds in Figure 2 have been previously reported and the fragment data belong to the large class of phenolic compounds (Table 1)

Before starting the evaluation of biological activities of EEP, acute toxicity tests were performed *in vivo*, using EEP at a dose of 2 g/kg.

No neurotoxic effects were observed in the groups treated with EEP, when compared to the control group. Subsequently, the effects of EEP and enalapril on blood pressure were evaluated in the hypertensive animals for 21 days after the establishment of hypertension, as can be seen in Figure 3.

Systolic blood pressure (SBP) was evaluated for six weeks. Hypertensive rats were treated with two different doses of EEP (150 mg/kg/day and 300 mg/kg/day), or enalapril (20 mg/kg/day), after confirmation of hypertension (3<sup>rd</sup> week).

For the EEP 150 group, SBP values decreased from  $215.4 \pm 2.6$  mmHg to  $168.7 \pm 0.3$  mmHg (a reduction of 21.7%). Additionally, when compared to untreated hypertensive rats (2K1C), the EEP 150 group presented a significant drop in SBP only after 5 weeks of treatment ( $p < 0.001$ ).

Moreover, the observed drop in SBP did not reach the values observed in normotensive animals (2K), which were treated with saline solution (0.9 %).

In the EEP 300 group, SBP values decreased from  $173.1 \pm 3.3$  mmHg to  $144.0 \pm 1.3$  mmHg (16.8 %), resulting in significantly lower SBP values when compared to the 2K1C group between the fourth and sixth weeks of treatment ( $p < 0.001$ ).



In the enalapril group (20 mg/kg/day), SBP decreased from  $202.2 \pm 0.7$  mmHg to  $146.4 \pm 3.2$  mmHg (38.1%), compared to the 2K1C group in the 4th week of treatment ( $p < 0.001$ ). Finally, the EEP 150 group showed a hypotensive effect similar to the enalapril group in the sixth week of treatment ( $p > 0.05$ ).

As show in Figure 4, ACE activity was measured in all the experimental groups. The quantification of ACE in the EEP 300 group did not differ from the normotensive group (2K) ( $p > 0.05$ ), though it was statistically different from the hypertensive group (2K1C) ( $p < 0.05$ ).

From these results, we can infer that the inhibition of ACE was a result of EEP treatment, since the enzymatic levels were high in the untreated hypertensive animals. The activity of ACE in the enalapril group was similar to that of the hypertensive group (2K1C).

At the end of the experiments, biochemical parameters were also evaluated (Table 2). The total cholesterol (C) values were lower in both of the EEP treatments (150/300), as well as in the enalapril group ( $p < 0.001$ ).

Moreover, the samples from the EEP 300 group showed a greater reduction (48%), when compared to the samples from the EEP 150 group (37.95%), as well as those from the enalapril group (42.9%).

Triglycerides (TG) and VLDL-cholesterol values were also reduced during treatment, and the EEP at the highest dose was able to reduce these levels by approximately 52% ( $p < 0.001$ ) compared to the enalapril group 40% ( $p < 0.01$ ).

In relation to HDL-c, the LDL-c values increased by 161.8% after hypertension induction ( $p < 0.01$  vs. normal blood pressure) and were reduced by 55.7%, 39 4% and 51.4% after EEP300, EEP150 and enalapril administration, respectively. The EEP did not alter the blood glucose levels, and the values were within the normal range [32].

The anthropometric measurements did not change significantly between the groups. However, weight gain was less pronounced in the EEP 150 (9%) and EEP 300 (6%) groups when

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compared to the 2K (11.8%), 2K1C (11.9%), and ENAL (21.8%) control groups.

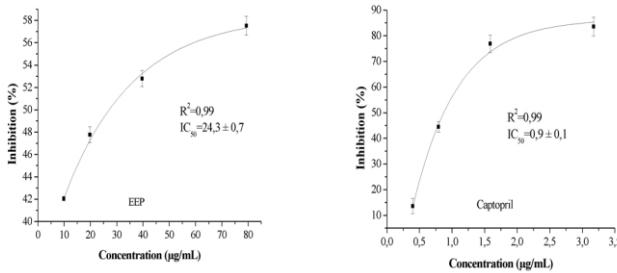


Figure 1. Angiotensin converting enzyme (ACE) inhibitory activity of *E. punicifolia* extract (A) and captopril (B). Data were expressed as IC<sub>50</sub> (mean ± standard deviation of triplicate values). The *E. punicifolia* concentrations (EEP)/captopril were diluted according to the final volume of the microplate well.

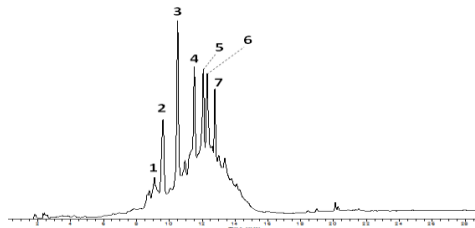


Figure 2. Representative chromatogram (LC-MS/MS) obtained from EEP extract showing the following compounds: (1) quinic acid, (2) chlorogenic acid, (3) gallic acid, (4) protocatechuic acid, (5) vanillic acid, (6) catechin, and (7) quercetin.

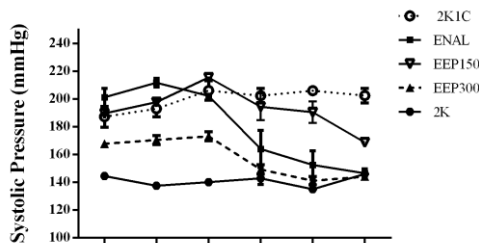


Figure 3. Evolution of systolic blood pressure (SBP) between groups. Data were expressed as mean ± standard error of the mean (n = 5-7). Multiple comparisons were done by two-way ANOVA followed by the Bonferonni post-

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test vs. Hypertensive group (2K1C) \*\*\* (p <0.001). Normotensive group (2K); Hypertensive group (2K1C); Group hypertension Enalapril 20 mg/kg/day (ENAL); Group hypertensive extract 150 mg/kg/day (EEP150) and Group hypertensive extract 300 mg/kg/day (EEP300).

Peak	Rt (min)	m/z, [M-H] <sup>-</sup>	Fragment ions	identification
1	8.89	191	127, 109, 93	Quinic acid
2	9.22	353	191	Chlorogenic acid
3	11.09	169	125	Gallic acid
4	11.80	153	109	Protocatechuic acid
5	12.05	167	139, 111	Vanillic acid
6	12.46	289	191, 189	Catechin
7	12.94	301	179, 151	Quercetin

Table 1. Phenolic compounds identified by LC-MS/MS

RT(min), retention time; (m/z, [M-H]<sup>-</sup>) deprotonated molecule (m/z, [M-H]<sup>-</sup>); MS/MS, fragments.

Variables	2K	2K1C	EEP150	EEP300	ENAL
CT (mg/dL)	59,2 ± 3,5 <sup>a</sup>	119,2 ± 16,4 <sup>b</sup>	74 ± 13,3 <sup>a</sup>	61 ± 6,8 <sup>a</sup>	68 ± 8,7 <sup>a</sup>
TG (mg/dL)	43,2 ± 5,6 <sup>a</sup>	63,5 ± 8,0 <sup>b</sup>	52,5 ± 13,0 <sup>a,b</sup>	30 ± 10,4 <sup>a</sup>	38,0 ± 10,1 <sup>a</sup>
VLDLc (mg/dL)	8,6 ± 1,1 <sup>a</sup>	12,7 ± 1,8 <sup>b</sup>	11,9 ± 1,4 <sup>a,b</sup>	6,1 ± 2,0 <sup>a</sup>	7,6 ± 2,0 <sup>a</sup>
HDLc (mg/dL)	26,8 ± 3,4 <sup>a</sup>	28,8 ± 2,0 <sup>a</sup>	31,8 ± 4,7 <sup>a</sup>	28,3 ± 2,6 <sup>a</sup>	30,4 ± 4,0 <sup>a</sup>
LDLc (mg/dL)	24,9 ± 1,3 <sup>a</sup>	65,2 ± 20,7 <sup>b</sup>	39,5 ± 6,9 <sup>a</sup>	28,9 ± 8,9 <sup>a</sup>	31,7 ± 4,2 <sup>a</sup>
GL (mg/dL)	111,3 ± 21,2 <sup>a</sup>	94,5 ± 10,6 <sup>a</sup>	90,3 ± 7,4 <sup>a</sup>	99,4 ± 15,0 <sup>a</sup>	63,5 ± 9,6 <sup>b</sup>

Table 2. Biochemical variables among hypertensive and normotensive groups

CT, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol ratio; LDL-c, low-density lipoprotein cholesterol; GL, Glucose; VLDL-c, very low-density lipoprotein cholesterol. Different letters mean that there were significant differences between the groups (p <0.05) and identical letters indicated there were no significant differences (p >0.05).

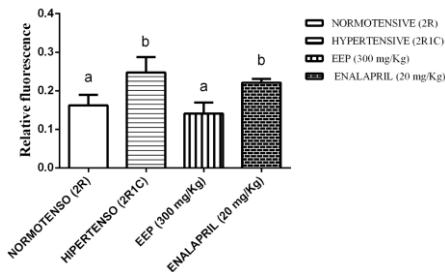


Figure 4. Relative Fluorescence values between groups. Data were expressed as mean ± standard deviation (n = 3-7). Multiple comparisons were made by two-way ANOVA followed by Tukey post-test. Different letters mean that there were significant differences between the groups (p <0.05) and identical letters indicated there were no significant differences (p >0.05).

## DISCUSSION

Studies have shown that plant extracts, such as *Tribulus terrestris* [33], *Phyllanthus urinaria* [34], Egyptian *Tropaeolum majus* L. [35] and *Moringa oleifera* Lam. (Moringaceae) [36], are potent ACE inhibitors. In this study, the inhibitory action of *Eugenia punicifolia* extract (EEP) in a concentration-dependent manner ( $IC_{50}$   $24.3 \pm 0.7$   $\mu\text{g/mL}$ ) was also evidenced *in vitro* when compared to captopril as a control.

This is an important discovery and, as far as we know, it is the first time that an extract of the Myrtaceae family has been shown to inhibit this enzyme. This inhibition may have occurred due to the high concentrations of phenolic compounds found in the EEP extract (Table 1).

The phenolic compounds interact with bradykinin-mediated nitric oxide and characterize the hypotensive effects [37]. These effects lead to an increase in renal blood flow and diminish the vascular resistance caused by the blockage of the calcium inflow, which leads to a reduction in cardiac output [38, 39].

Additionally, the literature describes several compounds derived from plants which have the capacity to inhibit the activity of ACE *in vitro*, such as proanthocyanidins, tannins, peptides, xanthenes, terpenoids, and quercetin [40, 41, 42, 43]. Quercetin together with quinic acid, chlorogenic acid, gallic acid were identified in this study (Figure 2) and are among those cited in the literature for their anti-hypertensive action [44, 45, 46].

Studies have demonstrated the action of quercetin on ACE and its reduction of the pressure effect of angiotensin I, similar to the effect of captopril [47] Quercetin exerts an antioxidant effect on endothelial dysfunction, improving vascular function. Synergic effects of acids, such as quinic acid, chlorogenic acid, gallic acids may have contributed to the ACE inhibition result found in this study [48, 49].

The present study demonstrated that supplementation of EEP 150 mg/kg/day and EEP 300 mg/kg/day led to a decrease in blood pressure.

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Furthermore, evidence indicates a positive association between the ingestion of diets rich in phenolic compounds and the control of platelet aggregation, in the reduction in the oxidation of LDLc, and greater release of nitric oxide from the endothelium [50].

Chen et al. [51] describe a vasorelaxant effect in a compound that is also found in the EEP extract. These effects help in myocardium perfusion [52].

Moreover, in this study, it has been shown for the first time that EEP has a hypotensive effect *in vivo* (Figure 4). Similar results found by Suveren et al. [53], while testing an extract from *Viscum album* L., confirmed once again the hypotensive potential of EEP, as found in our research.

Similarly, Zhou et al. [54] used an ethanolic extract from *Cydonia oblonga* in normotensive rats, and confirmed an enzymatic inhibition (ACE) of 50%, soon after the administration of the extract, which reached its peak (120 %) after 1 hour.

This effect was shown to be prolonged after 12 hours of treatment. Another beneficial effect found in the study by Zhou et al. [54] was the hypocholesterolemic action of this extract.

Some authors consider that ACE inhibitors have the effect of significantly reducing lipid profile markers [55,56]. Ramalingam et al. [57] point out that ACE inhibitors can act on gene expression in adipocytes by increasing the adiponectin profile, leading to an increase in HDLc and reducing the plasma levels of triglycerides.

Considering that the hypotensive effect of EEP observed in this study was induced in rats through the installation of renovascular hypertension, it is recommended that new studies involving other models of hypertension be performed in order to further confirm the hypotensive effects of *E. punicifolia*.

## CONCLUSION

*In vitro*, the *E. punicifolia* extract showed angiotensin-converting enzyme (ACE) inhibition activity. *In vivo*, the oral treatment using *E.*

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*punicifolia* extract caused hypolipidemic and hypotensive effects in renal hypertensive rats.

Since this is the first study to test *E. punicifolia* extract in the inhibition of ACE, the results presented here corroborate the popular use of *E. punicifolia*, which can be a prominent ally in the treatment of hypertension and cardiovascular diseases, mainly due to the presence of antioxidant compounds, such as gallic acid, catechins, chlorogenic acid and quercetin.

#### **Abbreviations.**

(ACE): angiotensin converting enzyme; (SBP): systolic blood pressure; (HIS-LEU): dipeptide L-leukin-L-histidine; (MSD): mini spray dryer; (HIP-HIS-LEU): hippuryl-L-histidine L-leukin; (LC-MS/MS): liquid chromatography-MS/MS; (ESI): electrospray; (BP): blood pressure; (IC<sub>50</sub>): the 50% inhibitory concentration; (EEP): *Eugenia punicifolia* extract; (VLDL-c): very low-density lipoprotein cholesterol; (HDL-c): high-density lipoprotein; (LDL-c): low-density lipoprotein cholesterol; (C):cholesterol; (TG): triglycerides.

#### **DECLARATIONS**

##### **Ethics approval and consent to participate**

Approved by the Ethics Committee at the Universidade Federal do Amazonas, (Approval nº CEEA-UFAM 77/2012) and were conducted in accordance with the Declaration of Helsinki.

##### **Competing interests**

The authors declare that they have no conflicts of interests.

##### **Availability of data and materials**

All data generated or analysed during this study will be available to public without restrictions

##### **Authors' contribution**

Denise Morais Lopes Galeno; Rosany Piccolotto Carvalho, Emerson Silva Lima: designed and performed the experiments related to aqueous extracts preparation; Máira Soares Biváqua de Araújo; Fernando Villalaz Chiong Neto; Raphael Holanda Santos: designed and performed the experiments related to methanolic extracts preparation; Toga Abdalazim Fadlalla; Zinalton Gomes de Andrade; José Wilson do Nascimento Corrêa; Felipe Moura Araújo da Silva: analyzed and interpreted the data; Hector Henrique Ferreira Koolen; Carlos Cleomir Pinheiro: drafted the manuscript; Denise Morais Lopes Galeno; Emerson Silva Lima; Ozanildo Vilaça do Nascimento: revised the manuscript critically and Denise Morais Lopes Galeno; Rosany Piccolotto Carvalho, Emerson Silva Lima; Francisco Célio Maia Chaves and Tatiane Pereira de Souza: conceived and supervised this study. All authors read and approved the final version of manuscripts

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