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Comparison of the antihypertensive effect of maguey (*Agave salmiana)* sap concentrate extracts by *in vitro* and *in vivo* tests

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ABSTRACT

Introduction: The use of antihypertensive drugs causes side effects that lead to treatment discontinuation; therefore, plants containing polyphenols and saponins represent a viable alternative. Previous studies indicate that maguey sap concentrate (SC) contains these bioactive compounds. The objective of this study was to compare the antihypertensive effect of maguey SC extracts through *in vitro* and *in vivo* tests.

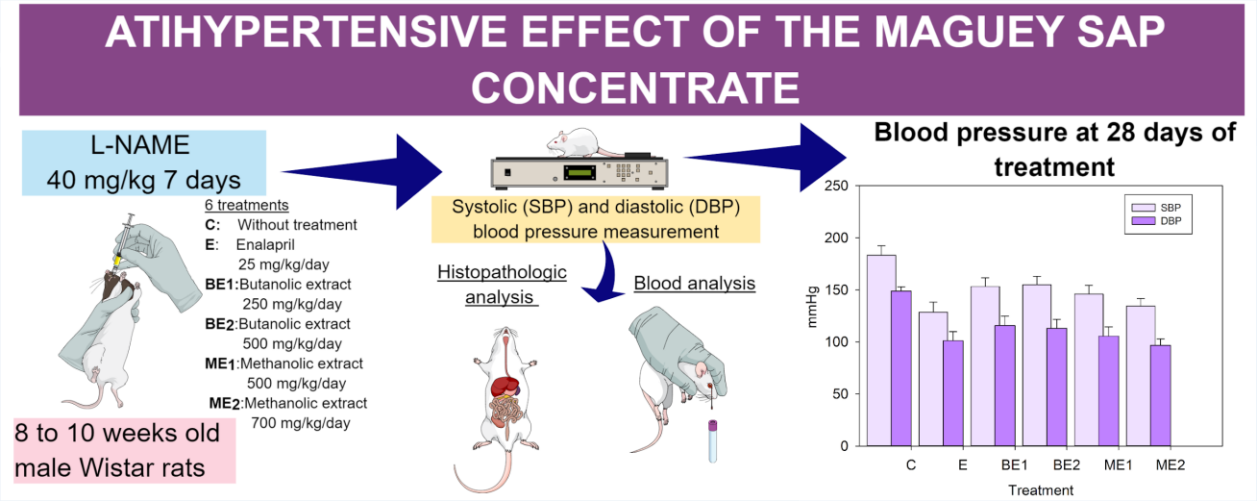
Methods: In the *in vitro* study, angiotensin-converting enzyme (ACE) inhibition was measured with butanolic and methanolic extracts of SC (100-250 ppm). For the *in vivo* tests, 48 male Wistar rats were divided into 6 groups: negative control (C), positive control (E): enalapril (25 mg/kg/day), BE_1 and BE_2: butanolic extracts (250 and 500 mg/kg/day) and ME_1 and ME_2: methanolic extracts (500 and 750 mg/kg/day). Their blood pressure was measured once a week for 28 days. In addition, blood samples were taken to determine glucose, creatinine, C-reactive protein, cholesterol, sodium,

potassium, calcium, and platelet count. At the end of the experiment, the animals were sacrificed, and their livers, kidneys, and hearts were preserved for histopathological analysis.

Results: Both extracts showed ACE inhibition greater than 90%. All treatments, except C, showed a decrease in blood pressure at 28 days; however, the ME_2 treatment had an effect like that of enalapril ($P>0.05$). Blood test results showed an increase in glucose levels, although within normal parameters, C-reactive protein levels are outside or above the maximum allowed limit except in those treated with enalapril. This research demonstrates for the first time the antihypertensive effect of methanolic and butanolic extracts obtained from maguey sap concentrate, opening the possibility of using them in the production of functional foods, as well as promoting the production and consumption of maguey sap, adding value to this natural resource.

Conclusion: The results of this study introduce the possibility of further exploring the bioactive compounds in aguamiel concentrate. Although the methanolic extract was able to lower blood pressure, it should only be considered as an adjunct to pharmacological treatments, and the possible toxicological effect of the extracts in the long term should be explored in greater detail.

Keywords: hypertension; sap; aguamiel; agave; saponins; antioxidants



Graphical abstract: Comparison of the antihypertensive effect of maguey (*Agave salmiana*) sap concentrate extracts by in vitro and in vivo tests

INTRODUCTION

Chronic non-communicable diseases such as cancer, chronic respiratory diseases, diabetes, and cardiovascular diseases are responsible for most deaths worldwide [1]. Hypertension is the main risk factor for the development of heart disease, kidney failure, stroke and even death, doubling the chances of a person suffering from these diseases, which has positioned it as

a serious public health problem worldwide [2–5]. The most recent statistics indicate that approximately 1.28 billion adults worldwide have arterial hypertension [6,7]. Generally, a person suffering from hypertension is treated with pharmacological therapies that must be taken for life, which can cause multiple side effects demeriting the quality of life of patients. That is why much of the research has focused on the study of plants, which are empirically known to have antihypertensive action, attributing this action to the presence of polyphenolic compounds and saponins [8].

The genus Agave is the largest and most diverse of the *Agavaceae* family; 75% of the 211 species of this genus are distributed in Mexican territory. The maguey is a plant belonging to this genus, widely used as a source of food, fiber, production of fermented or distilled alcoholic beverages, and even in herbal medicine [9,10]. It is also known for the bioactivity of its by-products, as they contain large amounts of saponins, phenolic compounds, and fructooligosaccharides [11,12].

The sap of the *Agave*, "aguamiel", is produced by removing the flower bud or scape of the plant, to form a cavity or hole in the center, where the sap accumulates. This translucent liquid, slightly yellow, herbaceous odor and sweet tasting [13], is considered a nutritional alternative for its nutritional attributes, as it provides proteins, amino acids, sugars, minerals, saponins, fructooligosaccharides and high antioxidant potential due to the presence of large amounts of phenolic compounds and vitamin C [9,14]. The main disadvantage of this product is its high instability, since it has a significant microbial load that, in a short time, causes the fermentation of the liquid, which is why the production of syrup has emerged as an alternative for its use. This is obtained by applying high temperatures to eliminate as much moisture as possible, remaining at approximately 60-80 °Brix, causing the final product to take on a brown to black color due to non-enzymatic darkening reactions [9,14]. Some authors have reported that this process causes a concentration effect in some components, such as saponins, and an increase in antioxidant capacity, since the products of the Maillard reaction can act as antioxidants [9,14,15], concentrated sap can have up to 10 times more nutrients than fresh sap [16].

The *Agave* genus, specifically species such as *A. americana* and *A. salmiana*, present saponins, derived from kammogenin, manogenin, gentrogenin and hecogenin, in concentrations of 179.0-478.3 µg/g of sap, a concentration that tends to decrease in the mead as the agave is capped in periods prior to the maturation of the plant [17,18]. Recent studies have focused on measuring and characterizing the antihypertensive properties of these compounds, an example of which is the work done by Liang et al. [19], who conducted a study on the identification of bioactive compounds present in the roots of *Panax notoginseng* and *Panax quinquefolium*, reported 1.028 bioactive ingredients, including 60 triterpenoid saponins and 178 flavonoids, among others. These authors also indicate that these bioactive compounds showed antioxidant activity with elimination of ABTS and DPPH radicals of 86.73% and 70.03%, respectively. Additionally, they showed inhibition of the angiotensin I-converting enzyme with an IC_{50} of between 1.37 and 3.50 mg/mL, indicating their antihypertensive activity. On the other hand, Astiani et al. [8] conducted a computer simulation study of the antihypertensive effect of triterpene saponins extracted from *Centella asiática*, concluding that these secondary metabolites have renin inhibitory activity and that the compound with the greatest inhibition was madecassoside. González Mosquera et al. [20] evaluated the antihypertensive effect of the aqueous extract and main flavonoid of the plant *Boldoa purpurascens* Cav. using a model of hypertensive rats. This research demonstrated that the extract at a dose of 80 mg/kg/day significantly reduced systolic blood pressure (8 mmHg) and diastolic blood pressure (5.2 mmHg) in a similar way to enalapril. Similarly, although to a lesser extent, the flavonoid

showed an antihypertensive effect (0.7 and 2.5 mmHg for systolic and diastolic pressure, respectively). The authors conclude that the antihypertensive effect observed is mainly mediated by phenolic compounds such as flavonoids.

For this reason, the present work proposes, through an experimental investigation, to compare the antihypertensive effect of methanolic and butanolic extracts of maguey sap concentrate, by means of *in vitro* tests measuring the inhibitory action of angiotensin converting enzyme and *in vivo* tests, with an animal model of hypertensive male Wistar rats.

MATERIALS AND METHODS

Obtaining the sample: The sap was collected from "maguey pulquero" *Agave salmiana* aged between 10 and 12 years, endemic to the Altiplano Hidalguense region, in Tlanalapa, Hidalgo, Mexico, located in the south territory of Hidalgo between parallels 19° 45' and 19° 54' north latitude; meridians 98° 30' and 98° 40' west longitude; with an altitude between 2500 and 3100 m above sea level. The sap was deposited in sterile glass jars and transported to the laboratory in a cold chamber to keep the temperature as low as possible and to avoid fermentation. It was then filtered to remove impurities and centrifuged to remove smaller suspended particles, after heat treatment at 60-63°C for 30 minutes to-hold microbial growth, and then it was canned and stored in the freezer (-18°C) for further use. To obtain the concentrate, the sap was heated to boiling, stirring constantly until most of the water evaporated and 60 to 65° Brix was obtained. This product was bottled when the product reached a temperature between 75 and 80°C, in plastic bottles with screw caps (previously disinfected with 0.05% sodium hypochlorite solution) and protected from light until its subsequent use.

Preparation of extracts: Methanol (ME) and butanol (BE) extracts were obtained from the sap concentrate. The methodology used was according to Santos-Zea et al. [20], 2.5 g of sample were taken and mixed with 12.5 mL of methanol (Meyer, 0410, Mexico), the mixture was kept in agitation for 15 minutes, then stored for 24 hours under refrigeration to finally centrifuge at 5000 rpm for 20 minutes, the supernatant was evaporated using a rotary evaporator until the methanol was completely removed. The extract rich in antioxidant compounds were freeze-dried and stored in the dark. To obtain the extract of saponins, the methodology reported by Leal-Díaz et al. [17], was applied with slight modifications. A 1:1 mixture of sample with n-butanol (Meyer, 0385, Mexico) was homogenized for 30 min at 250 rpm and 37°C, subsequently, samples were centrifuged for 5 min at 5000 rpm. The organic phase was collected and dried at 50°C for 24 h in a water bath. Once all the solvent was evaporated, the resulting solids were dissolved in the minimum amount of water and finally submitted to lyophilization.

Antioxidant capacity: For the methanolic extracts, the antioxidant capacity was measured by inhibition of the ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-difenil-1-picrilhidrazilo) radical and the concentration of phenolic compounds was also measured. For the ABTS technique, the technique reported by Donoso-Bustamante et al. [22] was used with some modifications. Activation of the radical (Sigma-Aldrich, A1888, USA) (7 mM) was performed with 2.45 mM potassium persulfate (J. T. Baker, 3238-01, Mexico) for 16 hours. The active radical was diluted to an initial absorbance of 0.7± (0.01) at 754 nm. A standard curve was performed, with Trolox (Sigma-Aldrich, 238813, USA) at concentrations of 50 - 300 µmol/L. Sixty µL of each dilution or sample and 980 µL of ABTS were mixed and allowed to stand for 7 min for subsequent absorbance measurement. For the DPPH technique, a solution was prepared with 7.4 mg of DPPH (Sigma-Aldrich, 238813, USA) in 100 mL of ethanol. A standard Trolox curve was

used, in concentrations of 50- 300 µmol/L. From each dilution or sample, 100 µL and 500 µL of DPPH were taken, the mixture was shaken and allowed to stand for 1 h, and finally the absorbance of the sample was taken at 520 nm. The concentration of total phenols was measured using Folin-Ciocalteu (Sigma-Aldrich, T9252, USA) diluted in water (1:10). A standard curve was made with gallic acid at a concentration of 50-300 mg/L. From each dilution or sample 100 µL were taken and mixed with 500 µL of Folin solution and 400 µL of sodium carbonate (7.5%), the mixture was shaken and allowed to stand for 30 min before reading the absorbance at 765 nm. All measurements were performed in a BioTek microplate reader, Model MQX200R, using deionized water as blank [23].

Quantification of saponins: A standard solution of *Saponin quillaja* (Sigma-Aldrich, 1003529117, USA) (10 mg/mL), an 8% solution of vanillin (Sigma-Aldrich, V1104, Germany), and finally a 72% solution of sulfuric acid (J. T. Baker, 9681-05, USA) were prepared. For the standard curve, saponin solutions were prepared at concentrations of 2-10 mg/mL, from which 50 µL were taken to mix with 50 µL of the vanillin solution, shaken and placed in an ice bath, in order to add 500 µL of sulfuric acid, then placed in a water bath at 60 °C for 10 min. Each solution was cooled in ice for 30 minutes to take absorbance readings at 460 nm. The same procedure was performed for the butanolic extract samples [24].

ACE inhibition: ME and BE were used to perform *in vitro* ACE inhibition tests. Hypuryl Histidyl Isoleucine (HHL) (Sigma-Aldrich, H4884, USA) at 0.3% in 0.1M borate buffer pH 8.3 with 300 mM sodium chloride was used as substrate. ACE (Sigma-Aldrich, A6778, USA) was prepared at 0.2 UN/mL in deionized water. To obtain the percentage inhibition, readings were taken from the following samples: Tube A = 100 µL of HHL + 40 µL of H_2O + 20 µL of ECA (Control); Tube B = 100 µL of HHL + 60 µL of H_2O (blank); Tube C = 100 µL of HHL + 40 µL of sample + 20 µL of H_2O + 20 µL of ECA. Each of the tubes was placed at 37°C for 1 hour in a water bath, then 1 mL of 1 N hydrochloric acid (Realyt´s, RAI500, Mexico) was added to stop the enzymatic reaction, then, with 1 mL of concentrated ethyl acetate (Meyer, 1305, Mexico) the extraction of the hippuric acid produced was performed. Subsequently, the samples were centrifuged at 1500 rpm for 10 minutes keeping the supernatant, which was brought to 85°C for 15 minutes until the complete evaporation of the solvent. The residue was suspended in 1 mL of deionized water and readings were taken at 228 nm. Equation 1 was used to calculate the percent inhibition [25, 26]. Percent inhibition was plotted against the concentration of each extract and the mean inhibitory concentration (IC_{50}) was calculated using a nonlinear regression model (equation 2).

1. % inhibition= (Abs $_{control}$ (Tube A) - Abs_{sample} (Tube C)) / ($Abs_{control}$ (Tubo A) - Abs_{blank} (Tube B)) x 100
2. $y=m(1-e^{-bx})$

Animal model: Fifty male Wistar rats between 8 to 10 weeks of age and weighing between 200-250 g, provided by the "Biotherium of the Autonomous University of the State of Hidalgo" with production certificate, were used. The animals were kept in polycarbonate boxes with stainless steel lids under natural light/dark cycle conditions and a constant temperature of 22°C, water and Rodent Laboratory Chow 5001 LabDiet feed [27], with water ad libitum and 20 g of feed per day throughout the experiment. These were divided into six groups (8 rats per group): control rats (C): hypertensive, untreated; positive control rats (E): hypertensive animals given enalapril (25 mg/kg/day) as control drug; hypertensive rats administered with butanolic extract at 250 and 500 mg/kg/day (BE_1, BE_2); rats given methanolic extract at 500 and 700 mg/kg/day (ME_1, ME_2); the two

remaining rats were sacrificed to obtain blood and organs as control (healthy rats). On the day they were received, the animals were randomly assigned to each of the treatments, with identification marking and were kept for two weeks prior to the start of the experiment for acclimatization to the blood pressure measuring equipment (CODA®), to make handling easier and minimize animal stress. The administration was done intragastrically with an administration cannula using distilled water as a vehicle. All procedures were performed under the Mexican Official Standard NOM-062-ZOO-1999 "Technical specifications for the care and use of laboratory animals", United Kingdom Animals Act 1986, European Union Directive 2010/63/EU for animal experiments and the National Research Council's Guide for the Care and Use of Laboratory Animals. To induce hypertension, L-NAME was used at a dose of 40 mg/kg intragastric route for 7 days, administered to all animals before performing the division by group. Once the animals were declared hypertensive, the administration of enalapril and the extracts at different concentrations was started for 28 days, so that the total duration of the experiment was 45 days.

Blood pressure (BP) measurement: Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with plethysmographic equipment for small animals, a non-invasive CODA® method (Kent Scientific Corporation, USA), which consists of a cage that allows the animal to be restrained with the least movement and without stress, and also uses a sensor (cuff, bracelet or ring) that is placed on the animal's tail, which measures the differences in blood volume. Measurements were performed once a week for 28 days, before the administration of L-NAME and at time zero (once the rats are already hypertensive). Each measurement was performed with prior vasodilatation of the animal's tail veins, keeping the animal at 37°C/15-20 minutes. The results were expressed in millimeters of mercury (mmHg). Measurements were taken at the same time but taking animals of different order each time. Because the measurements in this investigation are performed by the experimenter there is no need to perform a blinding process.

Blood analysis: Blood samples were taken by decapitation under anesthesia (Zoletil 50 mg/kg), on days 0, 15, and 30 of administration of the extracts, determining, C-reactive protein (Rat CRP/C-Reactive Protein ELISA Kit, RAB0097, USA) glucose, creatinine, cholesterol, sodium, potassium and calcium (Exigo C200 Clinical Chemistry Analyzer, Sweden), as well as platelet count (Hematology Analyzer HEMALYZER 2000 HBL). At the end of the experiment, the animals were sacrificed with carbon dioxide in a euthanasia chamber.

Histopathological analysis: At the end of the experiment, the animals were sacrificed using carbon dioxide in a euthanasia chamber, necropsied and liver, heart and kidney were extracted for histopathology analysis. Each organ was placed in 10% formaldehyde for 24 hours, then the previously cut tissue sections were fixed with 10% buffered formaldehyde, embedded in kerosene to form histological blocks and subsequent 4.0 µm thick sections to finish with staining using hematoxylin and eosin. Microscopic evaluation was performed by reviewing the histological sections of the tissues described above using an Olympus, BX41, Japan clinical microscope, 4x/0.10, 20x/0.25 and 40x/0.65 objectives.

Statistical analysis: The sample size for the analysis with Biomodels was calculated using the GRANMO Version 7.12 Online calculator, indicating: 6 groups or treatments, a significance value of 5% and 20% loss percentage [28]. The results are reported as the average with its standard deviation, the data were analyzed to verify normality (Shapiro-Wilk) and homoscedasticity (equality of variances) to subsequently determine

significant differences between treatments using a one-way analysis of variance (ANOVA), followed by a comparison of means by Tukey, with a reliability value of 95%. The type of treatment was taken as the independent variable and the SBP and DBP values as the response variable. The statistical software Sigma Plot for Windows version 12.0 was used for the analysis. Build 12.2.0.45 Copyright© 2011 Systat Software, Inc.

RESULTS AND DISCUSSION

Antioxidant capacity and quantification of saponins: The antioxidant capacity of the methanolic extract was 129.6 ± 1.43 and 121.5 ± 0.10 µmol ET/g for ABTS and DPPH radical inhibition, respectively; the concentration of total phenols was 103.2 ± 5.07 mg EAG/g. For the butanolic extract, it was found that the concentration of saponins was 833.30 ± 17.9 mg/g of extract. Castro-Díaz & Guerrero-Beltrán, [29], indicate for freshly harvested mead of *A. salmiana* a total antioxidant capacity by DPPH of 5.01 mg ET/100 mL, for the same *Agave* variety, but from a different geographical area, Tovar-Robles et al. [27], report 1.09 mmol ET/g, both values lower than those obtained for sap concentrate extracts. Likewise, these same authors report lower concentrations of total phenols 0.34 and 0.153 mg EAG/g. The results found in DPPH, on the contrary, agree with those reported by Chagua-Rodríguez et al. [15], who carried out a study on the kinetics of degradation of the antioxidant capacity of sap from *A. americana* during the pasteurization process at 80 °C, the study revealed that after 10 minutes of treatment the antioxidant capacity (percentage of inhibition) decreases by two percentage points, since the antioxidant effect depends on the destruction of the antioxidant components, thus the authors recommend a heat treatment of less than 30 minutes in order to optimize the content of antioxidants in the mead. On the other hand, Santos-Zea et al. [21] report for commercial mead concentrate from the region of Monterrey, Nuevo León, Mexico, values of antioxidant capacity by ABTS of 7.7, 12.7 and 13.2 µmol ET/g, which is lower than that found in the present investigation. Hernandez-Ramos et al. [9], assert that, in the production of honey from the evaporation of water, a concentration effect of all components is obtained, enhancing the functional properties such as the antioxidant capacity, which in addition to this, is increased by the formation of certain chemical species, caused by the same heating process. An example of this is the melanoidins, which are formed during the heating of the sap, these compounds are pigments consisting of complexes of high and low molecular weight with antioxidant effect that are formed by the interaction of phenolic compounds and carbohydrates with proteins. These compounds, in the same way as free phenolic compounds, can react with the Folin-Ciocalteu reagent. Other compounds with antioxidant effects found in sap are β-carotene and vitamin C, in concentrations of 30.82 mg/g and 13.5 mg/100 g, respectively [15]. According to studies on the characterization of saponins present in *Agave* sap, the saponins mostly present in this are kmmogenin, manogenin, gentrogenin and hecogenin [21], in concentrations of 179.0 - 478.3 µg/g of mead, respectively; concentration that tends to decrease in mead as agave capping is carried out in periods previous to the maturation of the plant [30]. Other studies show that mead contains saponins in concentrations of 1.17 g/100 g dry base [31] and 65.85 mg/g saponin extract [32], data that are below those found in this work. The *Agavaceae* family is known for its high content of steroidal saponins, mainly in the leaves. However, the amount of saponins found in this study is high despite being quantified in the sap [33].

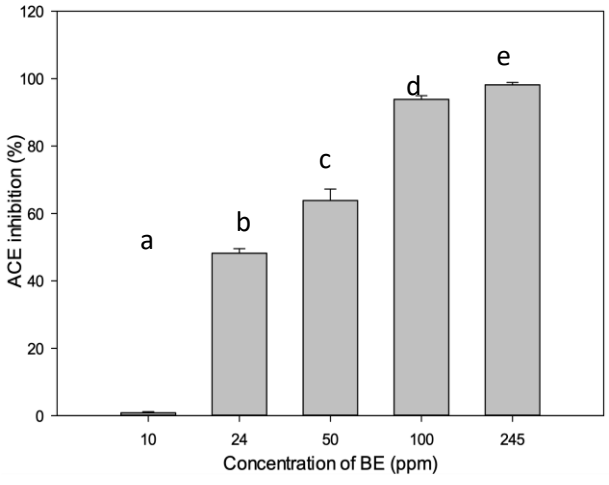
ACE inhibition: In Figure 1 the results of percentage inhibition for extracts a) BE and b) ME, respectively, are shown. Statistical analysis indicated that there are significant differences ($P<0.05$) in the percentage inhibition for all concentrations in both extracts. With BE, the highest percentage of inhibition (98.18%) was achieved with the concentration of 245 ppm. On the other hand, for ME, the highest percentage of inhibition (90.63%) was observed at 156 ppm, which is almost 50%

less than the concentration of compound required with BE.

The value of the r coefficient for both extracts was 0.96 and 0.98, thus affirming that the nonlinear model used is suitable. On the other hand, the r^2 coefficient allows identifying whether the regression, and therefore the calculated parameters, can be used for the calculation of the IC_{50}. Likewise, as in the previous case, the r^2 value for both extracts (0.93 and 0.96 BE and ME, respectively) is acceptable. In the case of the butanolic extracts (saponins) the IC_{50} was 32.61 ppm and for the methanolic extracts (antioxidants) an IC_{50} of 44.109 ppm was obtained.

ACE is membrane-anchored and is essential for regulating blood pressure and maintaining electrolyte homeostasis. Synthetic ACE inhibitors constitute the main treatment for arterial hypertension, heart failure, myocardial infarction and diabetic nephropathy [29,34]. The ACE inhibition results for both extracts (BE 98.18% and ME 90.63%), are higher than reported by Cú-Cañetas et al. [35] using peptide fractions obtained from the legume *Vigna unguiculata*, these authors indicate 77.78 to 79.57% of maximum inhibition in peptide fractions of low molecular weight (< 1 kDa), in the same way Vera-Valle, [36], applied whey peptides in yogurt, reporting that such yogurt manages to present in *in vitro* tests, maximum values of 70% inhibition of ACE activity. In the work of Zhou et al. [37] it is mentioned that fermented Persimmon juice has high antihypertensive potential showing ACE inhibition percentages of 6.88% and 42.14%, in unfermented juice and fermented juice respectively, the authors attribute this increase in inhibition to the production of gamma-aminobutyric acid (GABA) due to the action of the microorganisms involved in the fermentation process. Qiu et al. [38], studied the inhibitory activity of cheddar cheese on ACE, finding that it was higher after 6 months of maturation with 68.4% inhibition. They isolated six peptides with inhibitory activity, and the authors assert that the type of inhibition is competitive, non-competitive, and mixed depending on the type of peptide. Regarding the IC_{50} values, the data found in this research for BE (32.61 ppm) and ME (44.109 ppm), are lower than those reported for the drug lisinopril (709. 36 ppm) [39], those found by Vera-Valle, [36], in yogurt enriched with antihypertensive peptides (313 ppm), and that found by Wang et al. [40] for peptides in pasteurized and frozen orange juice (310 to 1930 ppm). On the other hand, the results are higher compared to the data published by Forero et al., [39] using bioactive compounds from lulo (*S. quitoense*) (1.08 ppm) and Díaz et al. [41], for sweet cucumber (3.72 ppm).

(a)



(b)

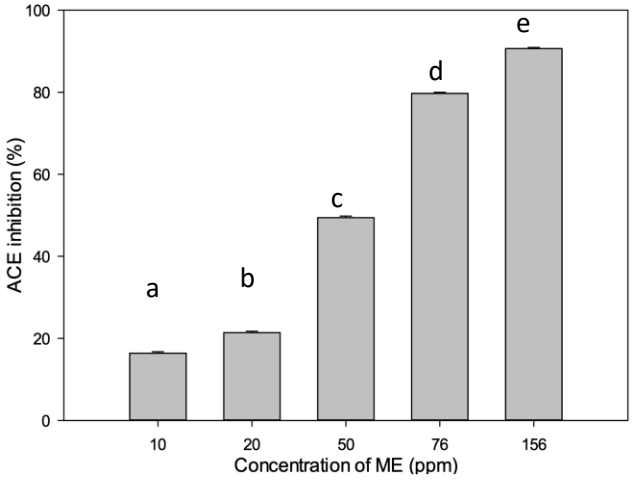


Figure 1. Percent inhibition of angiotensin-converting enzyme in the presence of extracts a) Butanolic extract and b) Metanolic extract. Letters represent significant difference in percent inhibition (P<0.05). One-way ANOVA and Tukey mean comparison.

Some authors mention that the inhibition of ACE enzyme activity is dependent on the concentration of the inhibitor [39, 42], which differs from what was observed in this study, since the linear dependence between the inhibition percentage and the inhibitor concentration does not obey a linear model, but rather an exponential growth model with a maximum peak that then remains constant.

Generally, ACE inhibitors act on the renin-angiotensin-aldosterone system by competitively, specifically, and reversibly inhibiting the enzyme [35,36]. Inhibition is carried out by the binding of a chemical group characteristic of each inhibitory substance to the Zn^{+2} atom of the convertase, which gives rise to a complex that subsequently dissociates. Similarly, molecular simulation studies have shown that bioactive compounds bind to key residues of the enzyme's active site through hydrogen bonds and hydrophobic interactions, causing ACE inhibition [43]. The characteristics of this process determine the duration of action of the inhibitors and the dose to be used [36]. On the other hand, the experiments carried out by Qiu et al. [38] indicate that ACE inhibitors act through competitive, non-competitive, and mixed inhibition. These authors report that increasing the concentration of antihypertensive peptides can cause the mechanism to change from competitive to non-competitive or mixed.

The results of this work show that BE and ME extracts have potential antihypertensive activity, which creates new opportunities for further research, focusing on the purification and identification of the phytochemical compounds that are responsible for this effect. Recent studies have focused on measuring and characterizing the antihypertensive properties of saponins, an example of this is the work carried out by Guerrero et al. [44], who carried out an investigation in Wistar rats, which allowed them to establish the vascular effects exerted by two monodesmosidic triterpene saponins: 3-O-β-D-glucopyranosylleanolic acid and 3-O-[β-D-glucopyranosyl-(1→2) -β-D-glucopyranosyl] oleanolic acid, isolated from the ethanolic extract of *Passiflora quadrangularis* leaves. The authors mention that in general, the extract of this plant has sedative and anxiolytic effects, as well as prevention of hypertension induced by nitric oxide deficit. However, Astiani et al. [8] conducted a computer simulation study of the antihypertensive effect of triterpene saponins extracted from *Centella asiatica*, concluding that these secondary metabolites have renin inhibitory activity and that the compound with the greatest inhibition was madecacoside. Alternatively, it has been reported that foods rich in phenolic compounds have hypotensive and antioxidant pharmacological properties [37]. Phenolic compounds manage to inhibit ACE combating arterial hypertension, preventing heart disease and demonstrating that it is an alternative to drugs for the same purpose [41]. The work of Farida et al. [45] indicates that the ethanolic extract of propolis from the stingless bee *Tetragonula sapiens* is rich in bioactive compounds such as saponins, flavonoids, essential oils, and alkaloids, among others, which are responsible for the ACE inhibitory activity proven in an in vitro study, with an IC_{50} of 115.06 ppm.

Antihypertensive effect. In vivo model: Blood pressure (BP) was monitored for 28 days, once a week, systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were recorded, showing a decreasing trend over time in all treatments except for treatment C (Figure 2), indicating an antihypertensive effect of all the extracts administered. At 28 days, control rats had higher SBP (183.25 mm Hg) and DBP (148.85 mm Hg), indicating that L-NAME administration was effective in inducing hypertension. BE_1 and BE_2 rats had a decrease in BP, however, they failed to match the effect of enalapril, on

the contrary, ME_1 and ME_2 rats recorded values closer to those obtained with enalapril.

Table 1 shows the SBP and DBP results at 28 days of administration. Statistical analysis indicated that there is no significant difference in SBP and DBP values between the animals of treatment E and ME_2, tending to normotensive rats at 28 days. On the contrary, the results observed with the BE_1 and BE_2 treatments, although they show a decrease in BP values, these are much higher than those found with rats administered enalapril, and in the specific case of SBP, no significant differences were found with respect to the C treatment.

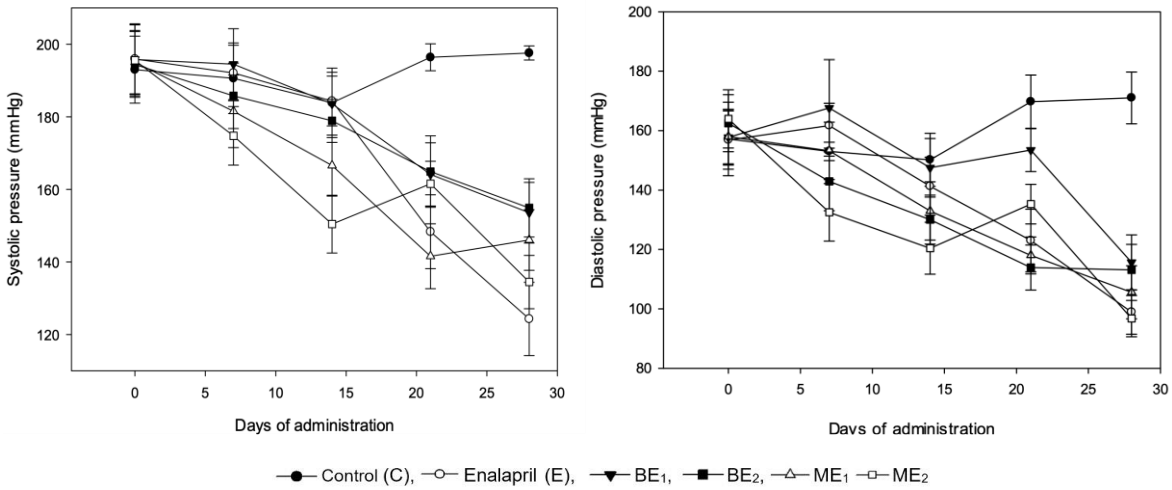


Figure 2. SBP and DBP values during 28 days of administration of extracts and the control drug. Mean values are shown with their standard deviation. C: control, untreated hypertensive rats; E: hypertensive rats, treatment with enalapril at 25 mg/kg; BE_1 and BE_2: hypertensive rats, treatment with butanolic extract at 250 and 500 mg/kg; ME_1 and ME_2: hypertensive rats, treatment with methanolic extract at 500 and 700 mg/kg.

Table 1. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) values, at 28 days of administration.

Treatment	SBP (mm Hg)	DBP (mm Hg)
C	183.25 ± 9.1^{c}	148.85 ± 4.1^{c}
E	128.636 ± 9.6^{a}	101.111 ± 8.7^{a}
BE_1	153.25 ± 8.3^{bc}	115.667 ± 9.2^{b}
BE_2	154.9 ± 8.2^{bc}	113.105 ± 8.6^{b}
ME_1	146.053 ± 8.3^{b}	105.571 ±8.9^{ab}
ME_2	134.476 ± 7.3^{a}	96.727 ± 6.1^{a}

Mean data ± standard deviation. ^{abc}Different letters in the same column indicate significant difference (P<0.05), One-way analysis of variance (ANOVA) and Tukey's comparison of means. C: control, untreated hypertensive rats; E: hypertensive rats, treatment with enalapril at 25 mg/kg; BE_1 and BE_2: hypertensive rats, treatment with butanolic extract at 250 and 500 mg/kg; ME_1 and ME_2: hypertensive rats, treatment with methanolic extract at 500 and 700 mg/kg.

The results obtained in this research agree with those reported by various authors for extracts obtained from different plant sources. Arroyo et al. [46] investigated the antihypertensive activity of the hydroalcoholic extract of purple corn in rats, finding that the reduction in BP was dose-dependent, with the greatest effect at a dose of 1000 mg/kg. Condorhuamán et al. [47] mention that the administration of methanol extracts of the plant *Calceolaria myriophylla* to hypertensive mice for 20 days decreased BP to normal values by dosing 600 mg/kg of the extract, the authors attribute this effect to the high content of phenolic

compounds, flavonoids, tannins, alkaloids and quinones, likewise Carrera-Lanestosa et al. [48] demonstrated that ethanolic extracts obtained from the *Stevia rebaudiana Bertoni* plant, rich in flavonoids, had a high antihypertensive effect in Wistar rats after a study for four weeks. The authors claim that the extracts of the leaves of this plant are rich in flavonoids (quercetin, apigenin and luteolin) and steviosides, with high effectiveness in lowering systolic blood pressure by up to 30%. This is attributed to the fact that these compounds are potent inhibitors of zinc-dependent enzymes such as ACE. Jacas et al. [2] reports that passionflower tincture administered at a dose of 20 drops three times a day in combination with pharmacological treatment, is effective in maintaining normal blood pressure values, not so with patients who were only administered their usual medications. The sedative, cardiotonic, hypotensive, myorelaxant and spasmolytic action of the tincture causes patients with hypertension to evolve more rapidly to normal blood pressure values. In this research the hypotensive effect is attributed to the presence of saponins in the butanol extracts (833.30 ± 17.9 mg/g) and to the high antioxidant capacity of the methanolic extract (129.6 ± 1.43µmol ET/g), which was observed in previous studies. Finally, like this study, where an antihypertensive effect similar to enalapril was observed in rats administered extracts with high antioxidant activity, González-Mosquera et al. [20] indicates that the aqueous extract of *Boldoa purpurascense* Cav. shows antihypertensive activity in rats induced to hypertension due to the presence of flavonoids. Although these authors conducted their experiment over 15 days, they observed a decrease in blood pressure when administering doses of 80 mg/kg/day.

Blood test: Table 2 shows the results of the biochemical parameters in blood serum at 28 days of administration. According to the reference values, it is observed that the values of C-reactive protein (CRP), calcium and potassium are outside the range considered as normal in some treatments. The highest CRP values are shown in treatments C and BE_1 (12 mg/dl), followed by treatments BE_2, ME_1 and ME_2 with 10 mg/dl, in E rats the concentration was 4 mg/dl the same as for healthy rats (HR). Calcium concentration in all treatments is higher (5.6 - 9.8 meq/L) compared to reference values, except for HR (5.6 meq/L). Sodium and potassium concentrations are within normal parameters.

Table 2. Biochemical parameters in serum of the animals at 28 days of administration.

PARAMETER	HR	C_{T0}	C	E	BE_1	BE_2	ME_1	ME_2	R.V.*
Glucose (mg/100 mL)	139±2.7	155±5.65	200±2.66	207±0.3	195±2.6	199±1.9	203±5.1	200±6.1	**134-219**
Creatinine (mg/100 mL)	0.35±0.07	0.45±0.07	0.4±0.05	0.4±0.07	0.4±0.06	0.5±0.09	0.4±0.07	0.49±0.06	**0.5-0.6**
C-reactive protein (mg/100 mL)	4±0.02	12.3±0.42	12±0.08	4±0.1	12±0.3	10±0.11	10±0.2	10±0.05	**3-10**
Homosysteine (µmol/L)	6.99±0.02	8.6±0.34	7.28±1.6	7.29±0.34	8.95±0.13	9.07±0.2	7.14±0.4	8.11±0.1	**5–15****
Cholesterol (mg/100 mL)	58±2.8	56±1.41	58±1.7	55±0.9	52±0.3	60±1.9	60±0.93	55±0.9	**54-74**
Sodium (meq/L)	142±1.16	139±7.01	143±5.2	143±2.9	142±3.3	151±4.1	142±5.9	143±2.9	**141-150**
Calcium (meq/L)	5.6±0.23	9.7±0.9	9.7±1.1	9.8±0.5	9.6±0.61	7.15±0.7	9.4±0.54	6.78±0.66	**4.2-6.10**
Potassium (meq/L)	5.7±0.1	5.1±0.7	5.6±0.6	5.5±0.2	5.2±0.51	9.7±0.8	4.9±0.1	9.84±0.73	**9.5-11**
Platelets/ µL (x10^3)	567	576	466	828	595	290	185	502	**379-967**

Average values ± standard deviation. *Reference values Charles River Laboratories International, [49]. **Reference values Hermann & Sitdikova, [50]. HR: healthy rats, C_{T0}: control, hypertensive rats at time zero, C: untreated hypertensive rats, E: hypertensive rats administered with enalapril as control drug,

BE_1 and BE_2: hypertensive rats administered with butanolic extract at 250 and 500 mg/kg, ME_1 and ME_2: hypertensive rats administered with methanolic extract at 500 and 700 mg/kg.

CRP functions as a direct mediator of various pathological processes, it is considered an independent predictor of future cardiovascular disease [51, 52], so elevated serum levels (even within values considered normal) predict the development of coronary events [53]. Some studies in rats show that CRP is not only used as a marker of vascular inflammation but can also induce atherosclerosis [54]. In general, when values are less than 1 mg/dl, the presence of mild inflammatory processes or vigorous exercise can be assumed; with values between 1-10 mg/dl, there is a possibility of acute myocardial infarction; values higher than these indicate the presence of severe acute bacterial infections, major trauma or systemic vasculitis [53]. All this indicates that, although the BE_2, ME_1 and ME_2 treatments show a decrease in blood pressure and CRP concentration, they are still susceptible to suffer a cerebrovascular event, which is not present in those animals that were administered with enalapril; however, it can be suggested that this effect cannot be observed due to the time of administration, so a second experiment is contemplated as a later stage, taking a longer period to verify whether the downward trend in these values is dependent on the time of administration. High serum calcium levels can create calcium deposits in arterial walls, increasing arterial stiffness and increasing arterial pressure [55]. On the other hand, it is important to mention that high serum calcium is associated with a less rapid deterioration of renal function as opposed to what is observed when serum calcium is low [56]. Homocysteine levels remained within normal parameters for all treatments, which is favorable, since homocysteine is a cytotoxic nonessential amino acid, and high blood levels are considered a risk factor for cardiovascular disease and stroke closely related to hypertension [57]. Regarding glucose levels, although all treatments-maintained levels within normal parameters, it is important to note that a slight increase was observed, especially in animals administered with methanolic extract. It is important to verify that prolonged consumption of this extract does not interfere with the treatment of hypertension. High blood glucose levels cause type 2 diabetes, which brings with it various complications such as cardiovascular disease. In fact, it is known that patients with type 2 diabetes have a higher risk of cardiovascular death [58].

Histopathological analysis: After processing each sample, as described in the methodology section, the samples were examined microscopically, observing in kidney, tubular degeneration only in control rats; interstitial edema in C, E and BE_1 (Figures 3a and 3b). In the heart, myomuscular degeneration, perivascular lymphoplasmacytic infiltrate and interstitial lymphoplasmocytic infiltrate were found only in group E (Figure 3c, 3d). Finally, in liver the findings were: mild hemorrhage except in groups BE_2 and ME_2; lobular edema in group E and periportal hepatocellular degeneration in group E (Figure 3f). In general, the lesions observed were moderate to mild; the organs of the rats administered with enalapril showed the greatest number of lesions in all cases, and some of these were only observed in this group.

The damage shown in the liver is reported to a lesser degree, compared to that in the kidney; among treatments, the rats that received methanolic extract at a dose of 700 mg/kg show the least damage, which may represent a regenerative and protective effect of the extracts due to the presence of compounds with antioxidant capacity. This effect could rule out toxicological effects from the solvent used, although the extracts were evaporated until the methanol or butanol was eliminated. It is important to highlight the periportal hepatocellular degeneration and lobular edema observed only in rats administered with enalapril. The

latter is indicative of cell damage and alteration of cell membrane permeability, causing fluid accumulation.

Hypertension produces harmful effects on the arterial system causing the kidney to be one of the most affected organs, developing long-term renal hypertrophy and chronic renal failure [59–61]. The histopathological study of the kidneys in this study revealed the presence of mild interstitial edema in the groups C, BE_1 and E with the highest levels of hypertension which is related to the low levels of potassium in the blood, shown in the control rats, since the elevated blood pressure causes fluid to leave the bloodstream, which accumulates to form edema. It is important to point out that the animals treated with extracts at a higher dose did not present this alteration, thus attributing a regulatory effect by the bioactive compounds present in the extracts administered. It is important to highlight that several authors attribute the presence of edema and, in general, renal damage as adverse effects due to the consumption of angiotensin-converting enzyme inhibitor (ACEI) drugs such as enalapril. Lin et al. [62] indicates that chronic consumption angiotensin-converting enzyme inhibitors (ACEIs), may be associated with reduced angiotensin II and aldosterone concentration with a considerable increase in bradykinin, which causes cough and edema as a side effect. Sychev et al. [63] mentions that, although ACEIs are widely used in the treatment of hypertension or some cardiac diseases, 19% to 30% of patients taking enalapril discontinue treatment due to edema from the first week of treatment.

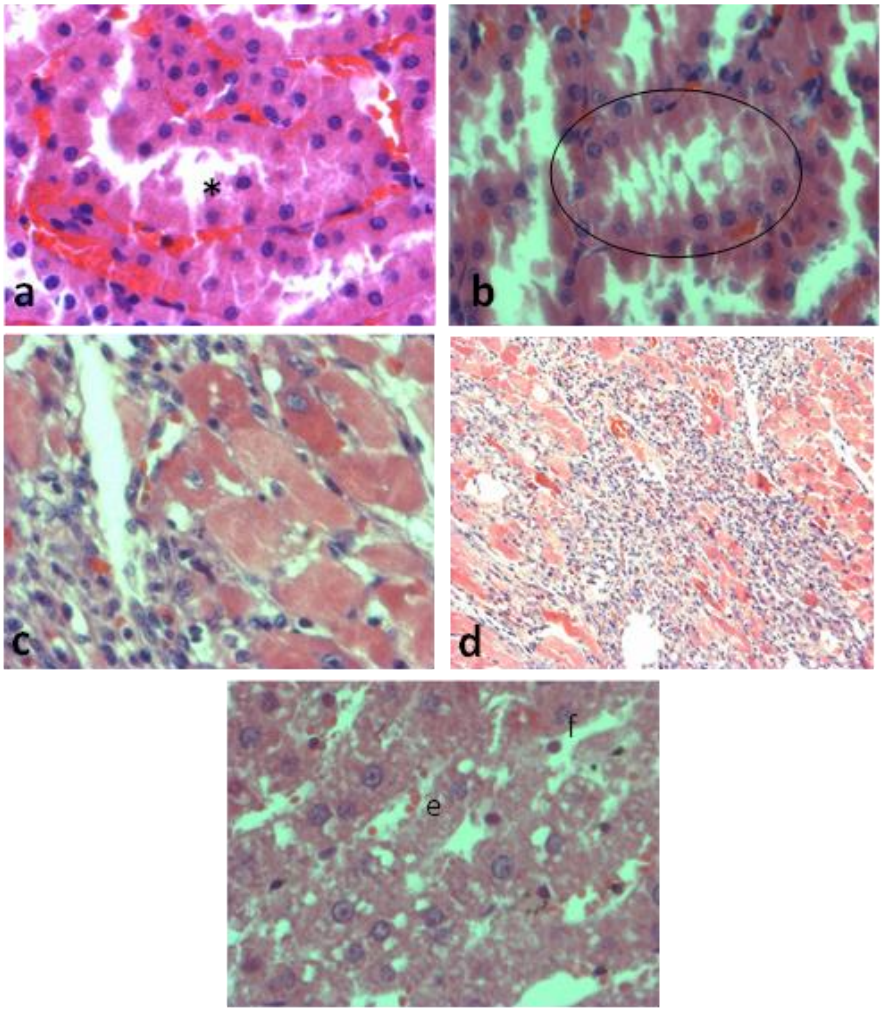


Figure 3. Histopathology of kidney, heart and liver in rats induced to hypertension L-NANME 50 mg/kg Findings in kidney of untreated rats treated with enalapril and butanolic extract 25 mg/kg, a: interstitial edema (*); b: tubular degeneration. Findings in heart

of rats treated with enalapril 25 mg/kg, c: muscle degeneration, d: interstitial and perivascular lymphoplasmacytic infiltrate. Findings in liver of rats treated with enalapril 25 mg/kg, e: cellular degeneration, f: lobular edema. H-E staining, 40x scale.

On the other hand, the tubular degeneration observed only in the control group stands out, which suggests that it is a secondary effect of hypertension, since the groups of rats that received treatment and at the end of the experiment had a decrease in blood pressure did not present this damage. Gutiérrez-Pérez et al. [59], mention that in hypertensive rat models by administration of L-NAME for 4 weeks, the main cellular changes observed during hypertension are related to endothelial dysfunction and increased vascular resistance, with intense renal vasoconstriction, which causes a marked reduction in renal blood flow and a slight reduction in the glomerular filtration rate. This model of hypertension caused histopathological changes associated with chronicity: sclerotic lesions in the blood vessels and in the renal glomerulus, reduction in the number of glomerular endothelial cells and in the peritubular capillaries, tubular damage and interstitial fibrosis in the renal medullary zone.

The lesions in liver can be attributed to the hepatotoxic effect of enalapril, which is mentioned by several authors; Cano-Paniagua et al. [64], indicate that antihypertensive drugs such as enalapril elevate liver enzyme levels and produce jaundice as well as structural changes in the liver; García-Cortés et al. [40], indicate that, in general, ACE inhibitor antihypertensive drugs such as captopril, enalapril, lisinopril, and fosinopril can produce cholestasis, cholestatic hepatitis and hepatocellular injury, additionally, Cruz-Cruz, [65], classifies enalapril within the drugs that cause idiosyncratic hepatotoxicity and mentions that one of the mechanisms causing hepatocellular injury is the alteration of intracellular calcium homeostasis, which leads to the disarrangement of the actin fibrils existing on the surface of the hepatocyte, causing the cell membrane to be modified leading to rupture and lysis. Although the findings reported in the present investigation are mild or moderate, it should not be ruled out that chronic administration of this drug can lead to irreversible damage. In general, the results of the histopathological analysis of the present investigation agree with the study performed by Navarro-Arámbulo, [60] who administered parsley extract at 100 mg/kg in rats with L-NAME-induced hypertension, indicating that the histopathological analysis of the liver revealed fewer lesions in blood vessels and hepatic parenchyma. The latter explains that rats administered with methanolic extracts (rich in antioxidants) will show less organ damage.

The analysis of the heart revealed lesions such as muscular degeneration, perivascular and interstitial lymphoplasmacytic infiltrate (Figure 1c, 1d) only in rats that received treatment with enalapril, so their presence is attributed to the consumption of this drug: this agrees with what was stated by Ito et al. [66], who observed muscular hypertrophy from the second week of administration of ACE inhibitor drugs such as captopril and enalapril in mice. However, what was observed in this research suggests that extracts of mead concentrate, show a preventive effect on the appearance of these lesions, since, Paredes et al. [67], indicate that the administration of natural extracts rich in antioxidant compounds (lemon, orange and grapefruit extract) in addition to significantly reducing blood pressure, caused improvement in vascular damage of organs, such as cardiac infarcts, hyaline arteriopathy and fibrinoid necrosis of coronary arteries. The presence of lymphocytes is usually attributed to infections, however, in the present investigation and because of elevated blood pressure, it can be attributed to an inflammatory process due to the observed muscle degeneration.

This research provides valuable information on the antihypertensive effect of extracts obtained from *Agave salmiana* sap concentrate in an *in vitro* and *in vivo* model, which had not been explored previously. The results

obtained open a research gap in the purification and characterization of the extracted compounds to determine a correlation between the antihypertensive effect and the types of compounds obtained from the methanolic extracts that showed the greatest effect in this study. In addition, the mechanisms of action can be explored, which will allow it to be enhanced and thus offer a natural option that can act in synergy with medications and reduce side effects due to excessive and chronic consumption. In addition, the use of agave sap as a functional food is being promoted, contributing not only to the control but also to the prevention of hypertension, a disease with a high incidence worldwide that has a major economic impact on health services.

CONCLUSION

Methanolic and butanolic extracts from maguey sap concentrate showed more than 90% inhibition of ACE at a concentration of (146 and 256 ppm respectively), which was confirmed by *in vivo* studies. The blood pressure of male Wistar rats for all treatments decreased, with a considerable effect observed after 15 days of administration. However, the best treatment compared to treatment E (enalapril) was ME_2. These results indicate effective antihypertensive activity that places these extracts as a feasible option in the alternative treatment of hypertension or as an adjuvant to pharmacological treatments. However, it is important to mention that the biochemical parameters in blood serum show alterations in CRP values indicating imminent danger of cerebrovascular events. In the second stage of this research, the regenerative effect of the extracts on organs considered as targets of high blood pressure should be explored, considering a longer period of administration.

Abbreviations: SC, sap concentrate; ACE, angiotensin converting enzyme; C, negative control; E, positive control; BE_1, butanolic extracts 250 mg/kg; BE_2, butanolic extracts 500 mg/kg; ME_1 methanolic extracts 500 mg/kg; ME_2, methanolic extracts 750 mg/kg; ME, menthanol extract; BE, butanol extracts; ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-difenil-1-picrilhidrazilo; HHL, Hypuryl histidyl isoleucine; L-NAME, Nω-Nitro-L-arginine methyl ester; SBP, systolic blood pressure; DBP, diastolic blood pressure; ANOVA, analysis of variance; ET, Trolox equivalents; EAG, gallic acid equivalents; IC_{50}, mean inhibitory concentration ; GABA, gamma-aminobutyric acid; BP, blood pressure; CRP, c-reactive protein; HR, healthy rats; C_{T0}, control, hypertensive rats at time zero; ACEI, angiotensin-converting enzyme inhibitor; ACEIs, angiotensin-converting enzyme inhibitors.

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Author Contributions: BM and EA conceptualization, Funding acquisition and Visualization; BM methodology and Investigation; BM, EA and AGE writing – review and editing; EA Project administration and Supervision; ZGC and GA software, formal analysis and validation.

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