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Cardioprotective effects of *Baccharis trimera* (Less.) DC in a rodent model of hookah, alcohol, and energy drink exposure

[Efectos cardioprotectores de *Baccharis trimera* (Less.) DC en un modelo de roedor de exposición a narguile, alcohol y bebidas energéticas]

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Abstract: This study proposes an unpreceded model of cardiovascular disease by combining alcohol and energy drink intake with hookah smoking to investigate the cardiovascular effects of *Baccharis trimera* (Less.) DC., a medicinal plant used to treat dyslipidemia. For 10 weeks, Wistar rats (n=8) received alcohol (10% ad libitum) and energy drink (2 mL/kg) and/or were exposed to hookah smoke (1 hour/day). In the last 4 weeks, the animals received daily treatment with vehicle (filtered water) or ethanol soluble fraction of *B. trimera* (30, 100 and 300 mg/kg). Electrocardiography was performed. Systolic, diastolic, and mean blood pressure, heart rate, and plasmatic cholesterol, triglycerides, urea, creatine, aspartate, and alanine aminotransferase levels were determinate. The heart, aorta, and kidneys were histopathological evaluated. In isolation the risk factors altered all the evaluated parameters and when the risk factors were associated, a synergistic effect was observed. Treatment with *B. trimera* reversed these cardiovascular changes.

Keywords: Alcohol; *Baccharis trimera*; Carqueja; Cardiovascular diseases; Hookah; Tobacco

Resumen: Este estudio propone un modelo sin precedentes de enfermedad cardiovascular mediante la combinación de la ingesta de bebidas energéticas y alcohol con fumar narguile para investigar los efectos cardiovasculares de *Baccharis trimera* (Less.) DC., una planta utilizada para tratar la dislipidemia. Durante 10 semanas, las ratas Wistar recibieron alcohol (10%) y bebida energética y/o fueron expuestas al humo de narguile. En las últimas 4 semanas, los animales recibieron tratamiento con vehículo, fracción soluble en etanol de *B. trimera* (30, 100, 300 mg/kg). Se realizó electrocardiografía. Se determinaron los niveles de presión arterial sistólica, diastólica y media, frecuencia cardíaca, colesterol plasmático, triglicéridos, aspartato y alanina aminotransferasa, urea y creatina. El corazón, la aorta y los riñones fueron evaluados histopatológicamente. De forma aislada los factores de riesgo alteraron todos los parámetros evaluados y cuando se asociaron los factores se observó un efecto sinérgico. El tratamiento con *B. trimera* revirtió estos cardiovasculares cambios.

Palabras clave: Alcohol; *Baccharis trimera*; Carqueja; Enfermedades cardiovasculares; Narguile; Tabaco.

INTRODUCTION

Cardiovascular disease (CVD) is the largest contributor to the development of chronic disease worldwide (Hoyert & Xu, 2012). With an estimated 15 million people who have CVD (excluding hypertension), these numbers will grow as the population ages and therapies increase the lifespan of individuals (Heidenreich *et al*., 2011; Koene *et al*., 2016). Estimates indicate that CVD-related mortality could reach 23.3 million in 2030 (ESC, 2019). The high occurrence of CVD can be explained by a set of common risk factors to which the population is exposed, such as hypertension, being overweight, obesity, diabetes, dyslipidemia, smoking, and alcoholism (Appelman *et al*., 2015).

Mild and moderate alcohol consumption has a well-established cardioprotective effect (Ronksley *et al*., 2011). However, in CVD-free individuals, excessive alcohol intake leads to an increase in triglyceride levels and the occurrence of cardiovascular events (e.g., hypertension, atrial fibrillation, cardiomyopathy, and stroke), in addition to causing higher mortality from all causes (Teissedre *et al*., 2018; Day & Rudd, 2019). A very common habit among consumers, especially young people, is to combine alcohol with energy drinks, which potentiates the harmful effects of alcohol and energy drinks on the cardiovascular system (Marczinski *et al*., 2016; Somers & Svatikova, 2020).

Another well-established risk factor is smoking, a global epidemic that affects both the incidence and mortality of CVD through various mechanisms. Smoking decreases nitric oxide levels and causes vasomotor dysfunction and oxidative stress, leading to endothelial and structural changes, in addition to playing a highly thrombotic role in acute coronary events (Morris *et al*., 2015). However, despite the well-established cardiovascular risk and public policies for smoking cessation, approximately 8 million deaths are related to this risk factor annually, and hookah smoking is a growing practice among the population. The worldwide prevalence of daily hookah smoking is estimated to be 100 million, with alarmingly increasing rates of popularity among young people (OMS, 2018; Mohammad *et al*., 2019). Contrary to the popular misconception about the safety of hookah smoking, several studies have reported adverse effects on many organs, especially the cardiovascular and respiratory systems, where there are reports of coronary artery disease and chronic obstructive pulmonary disease, in addition to a higher risk of developing lung cancer (Jawad *et al*., 2019; Rezk-Hanna & Benowitz, 2019).

Despite the isolated impact of such risk factors as hookah smoking and alcoholic and energy drink consumption on the cardiovascular system, few animal models have been developed that combine these risk factors and evaluate possible synergistic effects, thus justifying the development of new models. Additionally, despite the existence of pharmacological therapies for CVD, many of them are expensive for patients and have adverse effects (Ramkumar *et al*., 2016; Thomas & Gregg, 2017). Thus, the search for new, less expensive, and safer therapeutics that can effectively treat CVD that is associated with multiple risk factors is necessary.

Baccharis trimera (Less.) DC., popularly known as "carqueja", is widely used in folk medicine to treat gastrointestinal disorders and as a hypocholesterolemic agent (Karam *et al*., 2013). Scientific studies in animal models have proven its anti-inflammatory (Nogueira *et al*., 2011), antiulcerogenic (Lívero *et al*., 2016a), antioxidant, hepatoprotective (Pádua *et al*., 2014; Lívero *et al*., 2016b; Barbosa *et al*., 2020), lipid-lowering, and cardioprotective (Souza *et al*., 2020) effects. Preclinical studies have also indicated that *B. trimera* extract is safe and does not cause toxicity (Lívero *et al*., 2016b). Considering the potential effects of *B. trimera* on the cardiovascular system and the absence of animal models that associate hookah smoking and the consumption of alcoholic and energy drinks, the present study sought to propose a model of CVD that associates these risk factors and investigate the biological effects of *B. trimera* on the cardiovascular system.

MATERIAL AND METHODS

Plant material and extract preparation

Aerial parts of *Baccharis trimera* were collected in February 2018 in the Medicinal Plants Garden of Paranaense University (UNIPAR), which is located 430 m above sea level (S23°46'11.3"–W53°16'41.2"). A voucher specimen (no. 07) was deposited in the herbarium of UNIPAR. An ethanol-soluble fraction of *B. trimera* was prepared at the Laboratory for Pre-Clinical Research and Natural Products at UNIPAR according to previously described methods (Souza *et al*., 2020). The phytochemical characterization of the prepared extract has been previously reported by our group (Barbosa *et al*., 2020; Souza *et al*., 2020).

Experimental animals

The choice of the animal species, sample size, and extract doses were based on Mendes *et al*. (2021). The model was developed in male Wistar rats, weighing between 200 and 250 g, that were obtained from the State University of Maringá (UEM). The animals were housed in the vivarium of the Laboratory for Pre-Clinical Research of Natural Products at UNIPAR with free access to a liquid and solid diet. They were housed under controlled environmental conditions (20°C ± 2°C, 50% ± 10% relative humidity, 12 h/12 h light/dark cycle) with environmental enrichment. The experimental protocol was approved by the Research Ethics Committee Involving Animal Experimentation (protocol No. 36948/2020) and performed according to international guidelines on animal welfare and research ethics. The reporting of animal investigations conformed to Animal Research Reporting of *in vivo* experiments (ARRIVE) guidelines (Percie du Sert *et al*., 2020).

Experimental design and treatments

Three risk factors for CVD were used: the consumption of alcoholic and energy drinks and exposure to hookah smoke. During 10 weeks of the experiment, the rats (n = 8/group) received standard commercial chow, a liquid diet with 10% alcohol *ad libitum* as proposed by Lívero *et al*. (2016b), and an energy drink (Red Bull®, 32 mg/dL caffeine + 400 mg/dL taurine, 2 mL/kg, once daily, orally by gavage) as proposed by Reis *et al*. (2017), and were exposed to hookah smoke (1 h/day, 5 days/week) as proposed by Khabour *et al*. (2018), with minor modifications. The hookah smoke exposure device consisted of a collection and release system, in which the smoke was released vertically from top to bottom, reaching the animals and spreading throughout the box. At the end of the exposure period, the animals were treated with vehicle (filtered water; negative control [C-] group) or the ethanol-soluble fraction of *B. trimera* (30, 100, and 300 mg/kg) once daily by gavage during the last 4 weeks of the experiment. A separate group of animals that was subjected only to hookah smoke (Hookah group) or the alcoholic and energy drinks (AED group) was treated only with vehicle (by gavage). A group of rats that were not exposed to these risk factors and were treated with vehicle served as the basal group. The body weight of the animals and consumption of water and alcohol were controlled weekly over 10 weeks.

Evaluation of cardiovascular parameters

Electrocardiography

Electrocardiography (ECG) was recorded using a 12-lead ECG recorder (WinCardio, Micromed, Brasilia, Brazil) according to Romão *et al*. (2019). After intramuscular anesthesia with ketamine and xylazine (100 and 20 mg/kg, respectively), using four alligator clips, the electrodes were positioned on the animal's two forelimbs and two hindlimbs. An acclimatization period of 5 min elapsed, and electrocardiography waves were recorded for 5 min.

Heart rate and blood pressure measurements

After the total treatment period, the animals were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) intramuscularly. A subcutaneous injection of heparin (30 IU) was administered as a bolus before the surgical procedure. The left carotid artery was then isolated, cannulated, and connected to a pressure transducer coupled to a computerized recording system to evaluate heart rate (HR) and systolic, diastolic, and mean blood pressure (BP) for 20 min.

Renal and vascular reactivity

The mesenteric vascular bed (MVB) and left kidney were isolated and prepared for perfusion according to previously described methods (Schaedler *et al*., 2018). After renal artery and superior mesenteric artery cannulation, the kidney and MVB were removed, isolated, coupled to a perfusion system, and continuously perfused with physiological saline solution (PSS; 119 mM NaCl, 4.7 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11.1 mM dextrose, and 0.03 mM ethylenediaminetetraacetic acid) that was aerated with 95% O_2 and 5% CO_2 at a constant flow rate of 4 mL/min. The PowerLab system and Chart 7.1 software (ADInstruments, Castle Hill, Australia) was used to record changes in perfusion pressure (PP; mmHg). After a stabilization time of 30 min, tissue integrity was assessed with a bolus injection of KCl (120 mmol). Different doses of phenylephrine (Phe) were then administered in the MVB (30, 100, and 300 pmol) and kidney (1, 3, and 10 nmol) preparations. Angiotensin II (Ang II) was perfused only in the kidney (1, 3, and 10 pmol). After a new 30-min stabilization period, the renal and mesenteric bed tissues were continuously perfused with PSS plus 3 µM Phe to induce a prolonged increase in PP. After stabilization of the contractile process, vascular reactivity to acetylcholine (Ach) in the MVB (1, 3, and 10 nmol) and kidney (10, 30, and 100 nmol) was

evaluated. Finally, vascular reactivity to sodium nitroprusside (SNP) was investigated in the MVB (10, 30, and 100 nmol) and kidney (30, 100, and 300 nmol). A 10 min period elapsed between each drug administration.

Sample collection and biochemical profile

After the measurement of cardiovascular parameters, blood was collected from the carotid artery, using heparinized syringes, and plasma was separated by centrifugation at 1,500 × *g* for 10 min. The rats were then euthanized by deep anesthesia by puncture of the diaphragm while under anesthesia and the heart, aorta artery, and kidneys were removed and weight. Samples were rapidly separated and frozen in liquid nitrogen to evaluate oxidative stress and perform biochemical analyses. Other organ samples were stored in 10% formalin solution for further histological analysis. Cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and urea were evaluated in plasma using commercial kits and an automated analyzer (Quick Lab®).

Oxidative stress evaluation

The heart and kidney samples were homogenized in a 1:10 dilution of potassium phosphate buffer (0.1 M, pH 6.5) to investigate the antioxidant system. Afterward, 100 µL was separated, suspended in 80 µL of trichloroacetic acid (12.5%), vortexed, and centrifuged at 6000 rotations per minute (rpm) for 15 min at 4ºC for the analysis of reduced glutathione (GSH) levels according to Sedlak & Lindsay (1968). The remaining homogenate was centrifuged at 9700 rpm for 20 min at 4ºC for the determination of superoxide dismutase (SOD) activity and lipoperoxidation (LPO) levels according to Gao *et al*. (1998) and Jiang *et al*. (1992), respectively.

Histopathological analysis of the kidneys, heart, and aorta

Segments of the thoracic aortic artery were carefully collected, washed with ice-cold saline, and packed in 10% buffered formalin. After standard histological processing, the sections were stained with hematoxylin and eosin and analyzed histopathologically. The heart and kidney samples were fixed, sectioned, and stained with hematoxylin and eosin to assess cellular changes that resulted from the different treatments. The slides were analyzed by a veterinarian pathologist under an optical microscope (Leica DM 2500) for cellular changes that were caused by exposure to hookah smoke, the alcohol and energy drink liquid diet, the *B. trimera* extract, and vehicle.

Statistical analyses

The data were analyzed for homogeneity of variance and a normal distribution. One-way analysis of variance (ANOVA) followed by the Newman-Keuls *post hoc* test was used to determine differences between means. The data are expressed as the mean ± standard error of the mean (SEM). The level of significance was set at 95% ($p<0.05$).

RESULTS

Water and alcohol consumption and body and organ weight

The weekly consumption of water in the basal and hookah groups was 360.00 ± 36.86 mL and 336.7 ± 52.39 mL, respectively. The consumption of alcohol was 181.70 ± 24.55 mL in the AED group, 205.00 ± 35.00 mL in the C- group, 226.70 ± 38.33 mL in the 30 mg/kg *B. trimera* extract group, 201.70 ± 38.44 mL in the 100 mg/kg *B. trimera* extract group, and 180.00 ± 46.46 mL in the 300 mg/kg *B. trimera* extract group. The median body weight of the rats in the basal group at the end of the experiment was 346.5 ± 4.26 g. No differences were observed in body weight between groups. For organ weights, it was observed an increase in the relative weight (%) of the heart in the C- group compared with the basal group (0.37% ± 0.01%). This increase was not observed in the AED group (0.40% ± 0.01%), hookah group (0.38% ± 0.01%), or *B. trimera* extract group (0.36% ± 0.01%, 0.37% ± 0.01%, and 0.36% ± 0.01% for 30, 100, and 300 mg/kg, respectively). No differences were observed in the relative weight of the kidneys between groups (basal value: 0.36% ± 0.01%).

Baccharis trimera normalized electrocardiograms in rats

The electrocardiograms are shown in Figure No. 1. An increase in the PR segment was observed in the AED, hookah, and C- groups compared with the basal group (47.25 ± 1.68 ms; Figure No. 1A). An increase in the QRS segment was observed only in the C- group compared with the basal group (36.38 ± 1.08 ms; Figure No. 1B). Treatment with the *B. trimera* extract (30 and 100 mg/kg) reversed these alterations. No significant changes were found in the amplitude of the P-, Q-, R-, or S-waves between experimental groups (Figure No. 1).

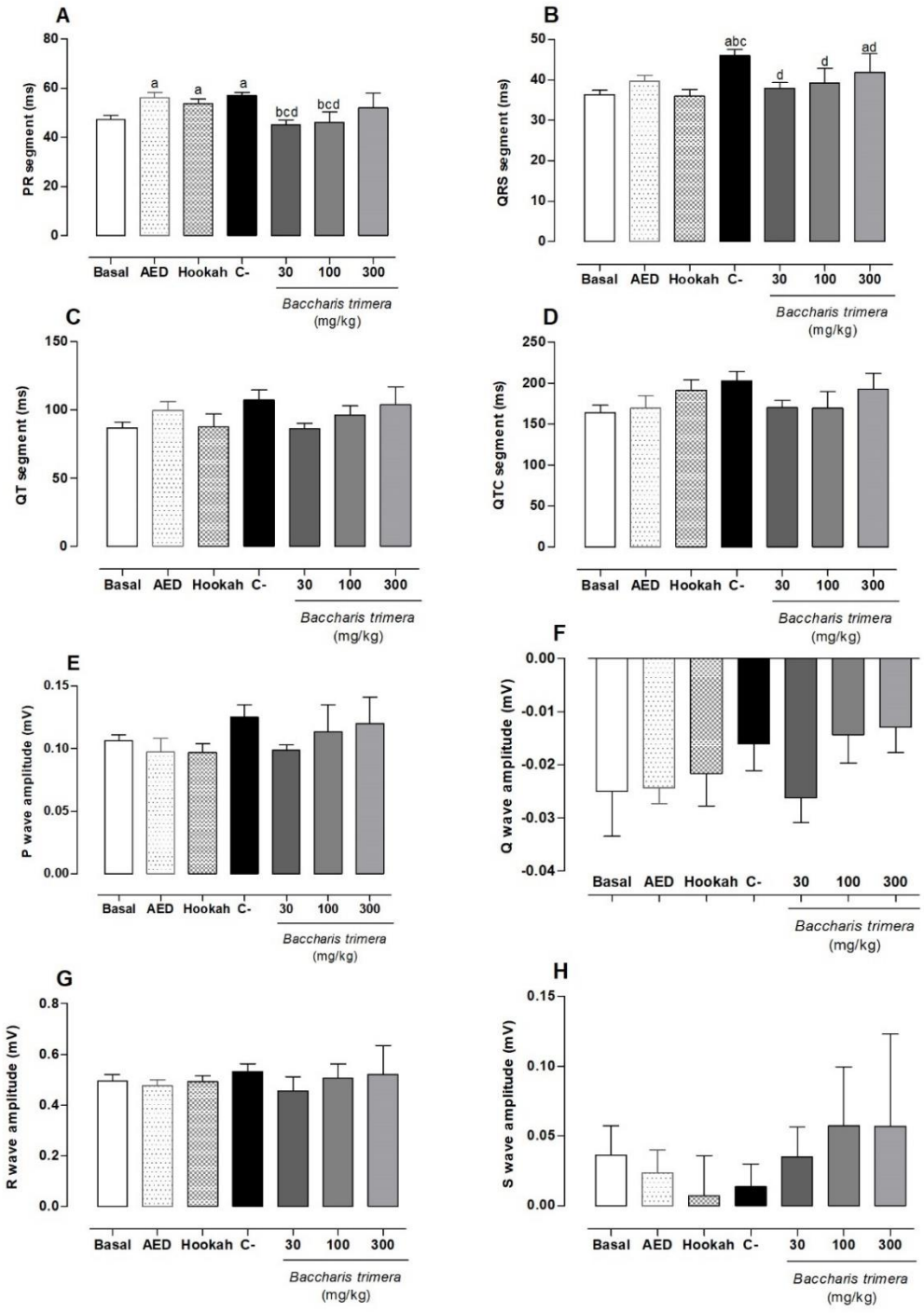

Figure No. 1

Quantitative electrocardiographic data on the PR (A), QRS (B), QT (C), and QTC (D) segments and P (E), Q (F), T (G), and T (H) wave amplitudes in Wistar rats that were not exposed to alcohol, the energy drink, or hookah smoke (basal group) and rats that were exposed to alcohol + energy drink (AED), hookah smoke (hookah group), and alcohol + energy drink + hookah smoke and treated with vehicle (C-) or *Baccharis trimera* (30, 100, and 300 mg/kg). The data are expressed as mean ± SEM. $^{a}p<0.05$, *vs.* basal group; $^{b}p<0.05$, *vs.* AED group; $^{c}p<0.05$, *vs.* Hookah group; $^{d}p<0.05$, *vs.* C- group (one-way ANOVA followed by Newman-Keuls *post hoc* test)

Baccharis trimera reversed the increases in heart rate and blood pressure

Increases in systolic BP (Figure No. 2A), diastolic BP (Figure No. 2B), and mean BP (Figure No. 2C) were observed in the C- group compared with the basal group (89.90 ± 2.92, 59.75 ± 1.93, 68.95 ± 3.21 mmHg, respectively). Treatment with *B. trimera* (30 mg/kg) reversed these changes. Treatment with 100 mg/kg *B. trimera* extract partially reversed these changes, whereas treatment with 300 mg/kg *B. trimera* was ineffective. An increase in HR was observed in the C- group and AED group compared with the basal group (183.3 ± 5.37 beats per minute [BPM]). Treatment with 30 and 100 mg/kg *B. trimera* normalized HR. Treatment with 300 mg/kg *B. trimera* was ineffective (Figure No. 2D).

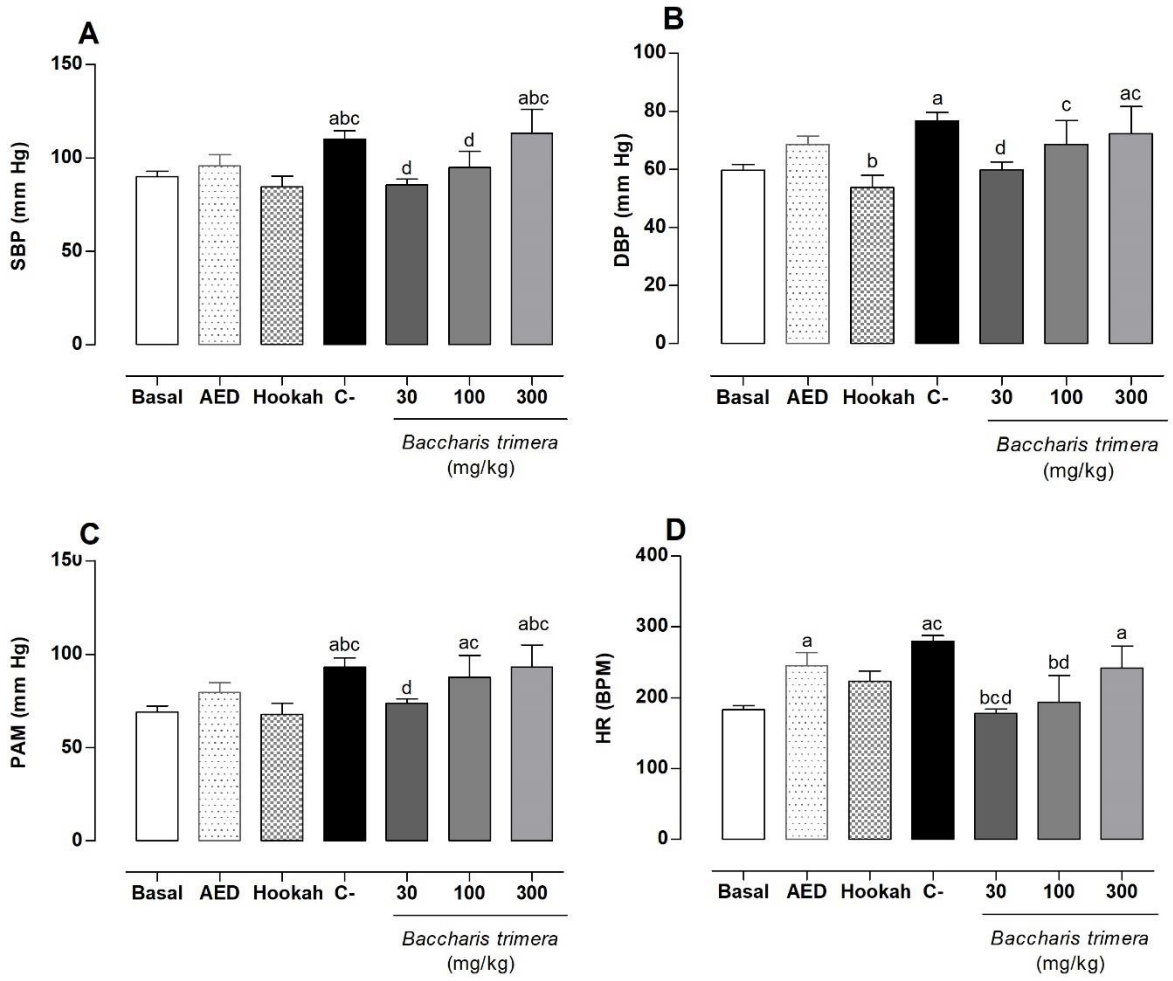

Figure No. 2

Systolic blood pressure (A), diastolic blood pressure (B), mean blood pressure (C), and heart rate (beats per minute [BPM]) (D) in Wistar rats that were not exposed to alcohol, the energy drink, or hookah smoke (basal group) and rats that were exposed to alcohol + energy drink (AED), hookah smoke (hookah group), and alcohol + energy drink + hookah smoke and treated with vehicle (C-) or *Baccharis trimera* (30, 100, and 300 mg/kg). The data are expressed as mean ± SEM. $^{a}p<0.05$, *vs.* basal group; $^{b}p<0.05$, *vs.* AED group; $^{c}p<0.05$, *vs.* hookah group; $^{d}p<0.05$, *vs.* C- group (one-way ANOVA followed by Newman-Keuls *post hoc* test)

Baccharis trimera recovers vascular reactivity

Renal perfusion with phenylephrine (3 nmol) decreased PP in the C- group compared with the basal group. Angiotensin II infusion (1 and 3 pmol) also decreased PP in the C- group compared with the basal group. The infusion of acetylcholine (100 nmol) increased PP in the C- group compared with the basal group. Treatment with *B. trimera* (30 mg/kg) prevented these alterations in rats. No differences in kidney PP were observed with the SNP infusion between groups. With regard to vascular reactivity, the phenylephrine infusion did not induce alterations of PP between groups. The infusion of acetylcholine (10 nmol) decreased PP in the C- group compared with the basal group. The infusion of SNP (300 nmol) decreased PP in the AED group, hookah group, and C- group compared with the basal group. Treatment with *B. trimera* (30 mg/kg) restored vascular reactivity in the MVB (Table No. 1).

Table No. 1
Variation in perfusion pressure induced by phenylephrine, angiotensin II, acetylcholine and sodium nitroprusside in isolated kidney and mesenteric vascular bed from Wistar rats exposed to risk factors for cardiovascular disease and perfused under different conditions

	Basal	AED	Hookah	C-	*Baccharis trimera*		
					30 mg/kg	100 mg/kg	300 mg/kg
Kidney reactivity							
Phe 1 nmol	81.7±24.0	27.9±8.3	39.3±14.4	68.6±36.8	80.1±24.8	40.5±20.8	37.2±20.1
Phe 3 nmol	286.6±42.0	208.6±33.2	171.7±47.0	138.3±21.5[a]	237.0±14.0[d]	153.5±18.4[a]	152.8±30.3[a]
Phe 10 nmol	340.2±32.1	308.3±80.4	205.9±54.4	229.9±53.5	274.9±77.2	379.2±73.8	225.8± 40.0
Ang II 1 pmol	112.3±20.6	94.0±26.8	67.3±12.4	15.8±10.7[a]	76.4±13.8[d]	37.9±6.8	60.5±23.6
Ang II 3 pmol	126.1±19.2	76.2±22.6	70.4±14.7	33.1±10.7[a]	88.8±13.7[d]	58.5±19.8	59.2±15.4
Ang II 10 pmol	60.1±15.5	47.9±10.2	25.5±6.8	27.6±7.9	56.6±14.3	49.1±10.9	40.3±11.9
Ach 10 nmol	-21.3±4.5	-26.1±3.6	-31.6±7.9	-36.4±8.4	-17.3±3.7	-19.3±3.5	-21.3±4.8
Ach 30 nmol	-12.3±2.5	-23.2±4.3	-29.5±4.4	-31.2±8.1	-19.4±3.7	-19.6±4.0	-22.4±5.8
Ach 100 nmol	-12.6±1.7	-22.6±3.4	-26.7±5.7	-37.0±6.6[a]	-12.5±2.4[d]	-22.3±5.2	-22.0±4.8
SNP 30 nmol	-5.7±1.5	-9.0±1.3	-4.6±1.5	-9.5±0.8	-4.3±1.3	-4.8±1.6	-7.39±1.8
SNP 100 nmol	-11.8±1.7	-11.9±2.6	-11.6±1.3	-12.9±2.7	-8.6±1.8	-6.4±1.6	-9.7±0.9
SNP 300 nmol	-11.0±2.8	-11.3±1.9	-15.0±4.4	-11.6±3.3	-14.4±4.4	-12.2±3.5	-12.0±2.6
Mesenteric vascular bed reactivity							
Phe 30 pmol	2.0±0.7	0.6±0.3	1.4±0.9	0.6±0.2	0.5±0.5	0.2±0.4	0.3±0.0
Phe 100 pmol	0.2±0.3	0.3±0.2	-0.04±0.3	0.7±0.2	0.5±0.3	0.1±0.2	0.5±0.4
Phe 300 pmol	0.3±0.4	-0.02±0.2	0.2±0.7	0.3±0.2	0.3±0.1	0.5±0.3	0.5±0.4
Ach 1 nmol	-30.2±5.6	-31.2±4.0	-20.1±4.2	-29.1±3.4	-31.4±6.3	-27.4±6.9	-39.8±4.8
Ach 3 nmol	-25.7±4.8	-36.8±4.9	-27.0±4.2	-41.1±4.1	-41.6±11.7	-36.5±8.5	-42.0±5.8
Ach 10 nmol	-42.6±5.1	-32.3±4.3	-35.6±3.7	-16.3±1.9[a]	-51.1±8.1[d]	-43.0±6.2	-30.1±7.9
SNP 30 nmol	-23.5±9.7	-16.4±4.9	-15.8±3.3	-7.9±1.7	-19.2±3.8	-13.5±3.5	-14.9±2.2
SNP 100 nmol	-14.6±3.1	-10.9±3.2	-10.2±2.1	-6.4±1.5	-15.8±2.9	-11.2±3.1	-14.4±2.1
SNP 300 nmol	-17.2±2.3	-8.0±1.2[a]	-5.0±2.0[a]	-5.5±1.1[a]	-15.9±0.9[bcd]	-9.9±2.0[a]	-10.3±2.7[a]

AED: alcohol + energy drink. C-: negative control group. Kidney reactivity: Phenylephrine (Phe) and angiotensin II (Ang II) increased perfusion pressure (%), acetylcholine (Ach) and sodium nitroprusside (SNP) decreased perfusion pressure (%). Mesenteric vascular bed reactivity: Phenylephrine increased perfusion pressure (mmHg). Acetylcholine and NPS decreased perfusion pressure (mm Hg). The data are expressed as mean ± SEM. [a]*p*<0.05, *vs.* basal group; [b]*p*<0.05, *vs*. AED group; [c]*p*<0.05, *vs*. Hookah group; [d]*p*<0.05, *vs*. C- group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Baccharis trimera exerts lipid lowering activity

Alcohol + energy drink and hookah smoking increased plasma triglyceride and cholesterol levels by 144.59% and 217.28%, respectively, compared with the basal group (41.13 ± 2.26 and 33.44 ± 1.61 mg/dL, respectively, Figure No. 3A and Figure No. 3B). These risk factors also increased AST and ALT levels by 301.39% and 159.39%, respectively, compared with the basal group (32.86 ± 2.13 and 33.64 ± 1.20 U/L, respectively, Figure No. 3C and Figure No. 3D). Alcohol + energy drink increased all biochemical parameters evaluated, whereas Hookah smoking only AST, ALT, urea, and creatine levels. Treatment with 30 mg/kg *B. trimera* completely reversed the increase in cholesterol, triglycerides, AST and ALT levels and restored urea and creatinine levels that were altered by these risk factors (basal group: 32.38 ± 1.50 and 0.33 ± 0.009 mg/dL, respectively, Figure No. 3E and Figure No. 3F), whereas 100 and 300 mg/kg *B. trimera* only partially reversed these changes (Figure No. 3).

Effects of Baccharis trimera on cardiac and renal redox state

The combination of alcohol, energy drink and hookah smoking induced cardiac and renal oxidative stress in rats (Table No. 2). In isolation, the alcohol + energy drink and hookah smoking promoted a decrease in GSH levels and SOD activity, as well as an increase in LPO. When these risk factors were associated (C- group), a synergistic effect was observed on the oxidative parameters. Decreases in cardiac and renal GSH levels were observed compared with the basal group (143.60 ± 8.60 and 135.80 ± 5.54 µg GSH/g tissue, respectively). These risk factors decreased cardiac and renal SOD activity compared with the basal group (1509.0 ± 51.29 and 937.3 ± 26.85 U SOD/g tissue, respectively). Increases in cardiac and renal LPO levels were observed in the C– group compared with the basal group (77.35 ± 3.75 and 72.15 ± 3.06 mmol LPO/min/g tissue, respectively). Treatment with 30 and 100 mg/kg *B. trimera* completely reversed these changes, whereas 300 mg/kg *B. trimera* partially reversed these changes.

Table No. 2
Cardiac and renal redox status from Wistar rats exposed to risk factors for cardiovascular disease

	Basal	AED	Hookah	C-	*Baccharis trimera*		
					30 mg/kg	100 mg/kg	300 mg/kg
				Cardiac			
GSH	143.6±8.6	94.5±1.9[a]	111.1±5.0[a]	81.1±4.0[ac]	157.1±10.4[bcd]	115.7±3.2[ad]	98.5±2.8[a]
SOD	1509±51	1243±29[a]	1328±32[a]	1166±22[ac]	1502±71[bcd]	1348±26[ad]	1246±30[a]
LPO	77.3±3.7	139.5±4.0[a]	114.20±6.2[ab]	185.2±5.9[abc]	71.5±3.3[bcd]	120.10 ± 6.2[abcd]	156.0±7.2[abcd]
				Renal			
GSH	135.8±5.5	87.9±2.4[a]	100.6±2.6[a]	71.0±3.3[abc]	141.5±5.6[bcd]	112.0±3.6[abcd]	88.1±3.4[ad]
SOD	937±26	787±22[a]	819±21[a]	714±21[a]	945±25[bcd]	880±21[bd]	768±20[a]
LPO	72.1±3.0	102.1±2.2[a]	92.1± 3.1[a]	122.5±7.0[abc]	73.4±3.6[bcd]	92.0±3.6[ad]	105.1±2.5[ad]

AED: alcohol + energy drink. C-: negative control group. GSH: Reduced glutathione, SOD: superoxide dismutase, LPO: lipoperoxidation. The data are expressed as mean ± SEM. [a]*p*<0.05, *vs.* basal group; [b]*p*<0.05, *vs*. AED group; [c]*p*<0.05, *vs*. Hookah group; [d]*p*<0.05, *vs*. C- group (one-way ANOVA followed by Newman-Keuls *post hoc* test)

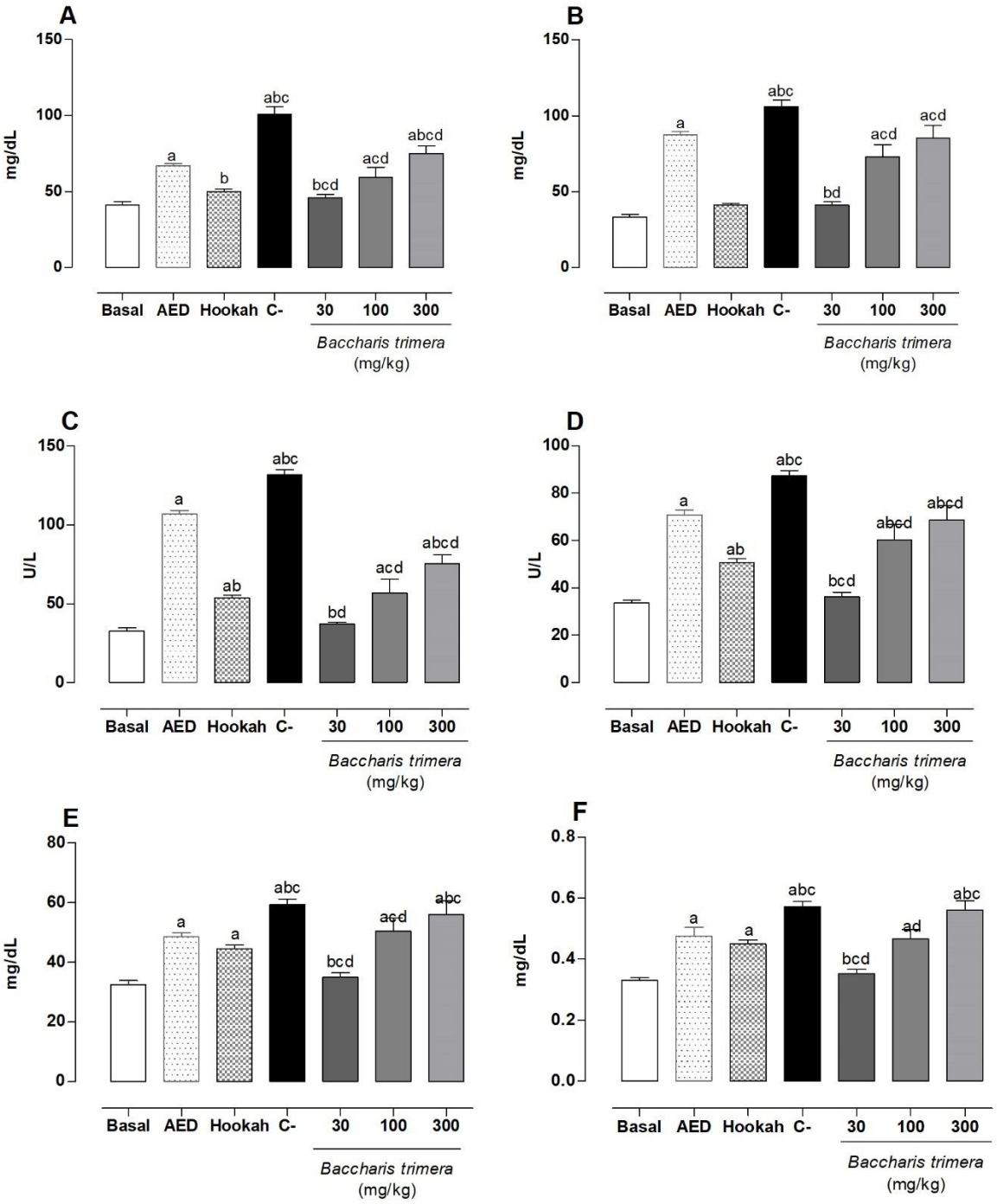

Figure No. 3

Cholesterol (A), triglycerides (B), aspartate aminotransferase (C), alanine aminotransferase (D), urea (E), and creatine (F) levels in Wistar rats that were not exposed to alcohol, the energy drink, or hookah smoke (basal group) and rats that were exposed to alcohol + energy drink (AED), hookah smoke (hookah group), and alcohol + energy drink + hookah smoke and treated with vehicle (C-) or *Baccharis trimera* (30, 100, and 300 mg/kg). The data are expressed as mean ± SEM. [a]*p*<0.05, *vs*. basal group; [b]*p*<0.05, *vs*. AED group; [c]*p*<0.05, *vs*. hookah group; [d]*p*<0.05, *vs*. C- group (one-way ANOVA followed by Newman-Keuls *post hoc* test)

Histopathological evaluation of the kidney, heart, and aorta

Kidney, heart, and aorta artery samples that were stained with hematoxylin/eosin revealed the absence of cellular alterations in rats that were not exposed to the three risk factors and were treated with vehicle (basal group), rats that were exposed to alcohol and the energy drink (AED group), rats that were exposed to hookah smoke, and rats that were exposed to alcohol, the energy drink, and hookah smoke and treated with vehicle (C-) or *B. trimera* (30, 100, or 300 mg/kg; Figure No. 4).

DISCUSSION

The experimental model that was proposed in this study associated multiple cardiovascular risk factors, such as exposure to hookah smoke, alcohol, and an energy drink, which generated surprising results. These risk factors have already been scientifically evaluated in isolation. Previous preclinical and clinical studies reported solid evidence that they exert effects on the cardiovascular system. However, few animal models have associated combinations of these cardiovascular risk factors. This highlights the importance of this study, in which it was established the effects of all three risk factors in various combinations on the cardiovascular system. A synergistic cardiovascular effect was found between exposure to hookah smoke and the consumption of alcohol and the energy drink.

Strong scientific evidence indicates that smoking alters the balance of the autonomic nervous system, leading to activation of the sympathetic autonomic nervous system, resulting in acute changes in BP and HR, and contributing to chronic elevations of BP through its renal actions on the structure of vessels and suppression of the baroreflex (Oakes *et al*., 2018; Price & Martinez, 2019). However, the literature shows that the effects of nicotine from cigarette smoke increases BP and HR. Few studies have evaluated the effects of hookah smoking. To date, more information is available about the constituents of hookah smoke and its toxic effects than about its effects on the cardiovascular system (Rezk-Hanna & Benowitz, 2019). Hookah users are exposed to many toxic compounds and products compared with cigarette users and at considerably higher levels. Hookah smoking may thus have more serious negative health effects, especially on the cardiovascular system (Qasim *et al*., 2019). In the present study, rats were exposed to nicotine from hookah smoke, and an opposite effect was observed, with decreases in BP and HR. These results are consistent with epidemiological studies of the effects of nicotine on the cardiovascular system, which paradoxically showed that BP in smokers was the same or even lower than in non-smoking individuals (Primatesta *et al*., 2001; Niskanen *et al*., 2004). It is important to highlight that in the present study, hookah smoking increased AST, ALT, urea, and creatinine levels, as well as promoted oxidative stress, in accordance with previous studies that described hepatic and renal toxicity of this substance (Qasim *et al*., 2019; Badran & Laher, 2020; Kadhum, 2021).

Another emerging trend that has become popular among young people is to mix alcohol with energy drinks (Thombs *et al*., 2006; O'Brien *et al*., 2008). One reason for consuming this mixture is predominantly the desire to achieve immediate and social pleasure (Vester *et al*., 2018). The isolated effects of alcohol on the cardiovascular system are particularly complex. Research has described both beneficial and adverse effects, depending on the amount and type of alcohol consumed and the particular CVD considered (Nguyen *et al*., 2019). Chronically and in large quantities, alcohol acts as a toxin and promotes cardiovascular injury that can progress to heart failure and eventual death. Additionally, preexisting heart disease, such as hypertension and cardiomyopathy, may worsen (Gardner & Mouton, 2015). With regard to the isolated effects of energy drinks on the cardiovascular system, their short- and long-term effects have been controversial. The short-term effects of caffeine (i.e., one component of energy drinks) include positive modulation of the cardiovascular system, such as the activation of adenosine monophosphate-activated protein kinase (a key enzyme for cell energy homeostasis) and sympathomimetic effects, leading to an increase in cardiac activity and BP (Higgins *et al*., 2010; Zheng *et al*., 2014).

Taurine is another component of energy drink. It interacts with phospholipids and stabilizes cell membranes, indirectly regulating oxidative stress in the myocardium. It also positively regulates the kinetics of calcium, protein kinases, and phosphatases in cardiomyocytes, maintaining cardiac contractile function and reducing blood pressure (Schaffer *et al*., 2010; El Idrissi *et al*., 2013).

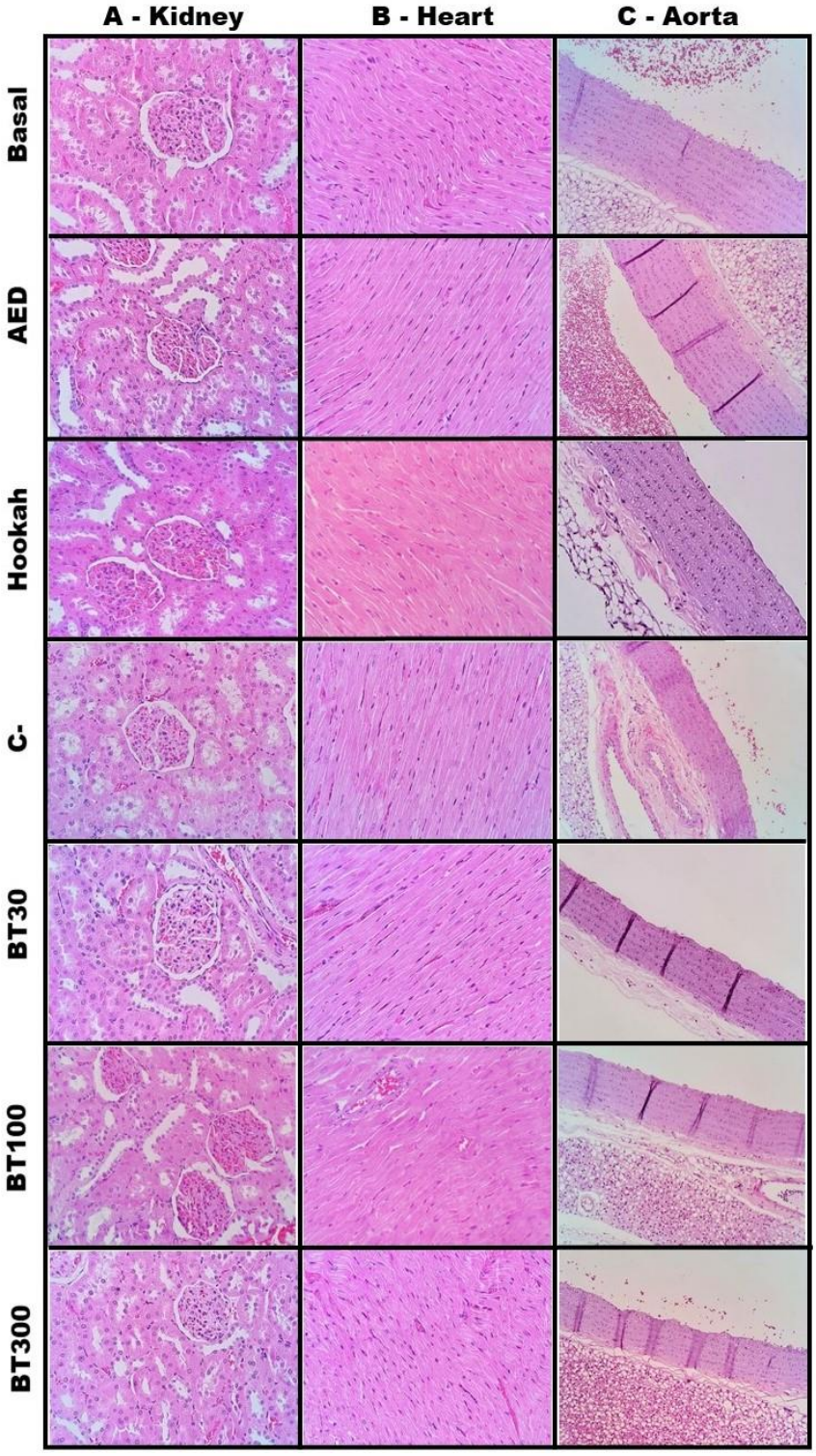

Figure No. 4

Histopathological analysis of the kidney, heart, and aorta artery in Wistar rats that were not exposed to alcohol, the energy drink, or hookah smoke (basal group), rats that were exposed to alcohol + energy drink (AED group), rats that were exposed to hookah smoke (hookah group), and rats that were exposed to alcohol + energy drink + hookah smoke that were treated with vehicle (C-) or *Baccharis trimera* (30, 100, and 300 mg/kg). Hematoxylin/eosin staining. Kidney and heart: 40× magnification. Aorta artery: 20× magnification

In addition to evidence that the combination of alcohol and energy drinks influences cardiovascular parameters in humans (Miles-Chan *et al*., 2015; Somers & Svatikova, 2020), the present study found an increase in HR in rats, with no changes in systolic, diastolic, or mean BP. Also, in accordance with another studies, it was observed an increased in cholesterol, triglycerides, AST, ALT, urea, and creatinine levels, as well as cardiac and renal oxidative stress. Other preclinical evidence in rats indicates that the cardiovascular system is impacted by the combination of alcohol and energy drinks. Male Wistar rats that received an energy drink and alcohol (1.5 mL and 0.486 mg/100 g of body weight, respectively) for 4 weeks exhibited a significant increase in cardiac glucose and glycogen concentrations and plasma levels of AST and ALT and a decrease in plasma levels of total cholesterol (Munteanu *et al*., 2018). The effects of alcohol + energy drink on oxidative stress were also described (Reis *et al*., 2017). Finally, Costa-Valle *et al*. (2018) described that Wistar rats orally acutely treated with energy drink + alcohol 20% presented significant hepatic and nephrotoxicity.

Given the increasing exposure to cardiovascular risk factors and high morbidity and mortality of CVD, strategies need to be developed to effectively control and reverse CVD with fewer adverse effects. Medicinal plants, in addition to monotherapies, may be alternatives that can be used as adjunct therapies for the treatment of CVD. The biological effects of several medicinal species on the cardiovascular system are widely described (Rouhi-Boroujeni *et al*., 2017; Lock & Rojas, 2019; Méndez-Bolaina *et al*., 2021; Zago *et al*., 2021). *B. trimera* is one such species. Several preclinical studies have investigated its ethnopharmacological and biological activity in models of CVD. In the present study, *B. trimera* reversed alterations of arterial pressure and vascular reactivity that were induced by alcohol, the energy drink, and hookah smoke. These effects may be attributable to the main metabolites of *B. trimera*, such as phenolic compounds and chlorogenic, monocaffeoylquinic and dicaffeoylquinic acids (Barbosa *et al*., 2020; Souza *et al*., 2020).

The biological effects of *B. trimera* in the present study are in accordance with previous studies with this species in animal models of hepatic and cardiovascular diseases. In diabetic, dyslipidemic, and smoking Wistar rats, Souza *et al*. (2020) reported that treatment with *B. trimera* (30 mg/kg) reversed oxidative stress, the increase in biochemical parameters, and thickening of the wall of the abdominal aorta. It also restored HR, systolic BP, diastolic BP, mean BP, and vascular reactivity. In hypertensive, dyslipidemic and smoking Wistar rats, treatment with *B. trimera* (30 mg/kg) reversed changes in blood pressure, lipid profile, and biomarkers of heart, liver, and kidney damage (Mendes *et al*., 2021). The authors partially attributed these effects to the lipid-lowering actions of *B. trimera* and the inhibition of free radical generation. These antioxidant and lipid-lowering effects of *B. trimera* were also observed in the liver, plasma, and feces in mice that were subjected to a model of alcoholic fatty liver disease (Lívero *et al*., 2016b), and diabetic Wistar rats that were exposed to smoke and subjected to dyslipidemia and treated with 30 mg/kg *B. trimera* (Barbosa *et al*., 2020).

Finally, it is important to highlight that an ideal animal model must generally be viable, accessible, and representative of human disease (Leong *et al*., 2015). Animal models provide evidence of disease-related behavior in humans and may open perspectives on novel pathways and outcomes that were not previously considered by establishing mechanistic and predictive validity (Sjoberg, 2017). One interesting effect that was observed in the present study was the synergistic effects of the three risk factors. The isolated risk factors induced slight changes in the analyzed parameters, whereas the combination of all three risk factors (hookah smoke, alcohol, and energy drink) significantly altered cardiovascular, biochemical, and oxidative parameters. These results reinforce the need to develop new models that associate multiple risk factors to achieve a better picture of the human condition.

CONCLUSION

Treatment with the *B. trimera* extract reversed cardiovascular alterations that were induced by the combination of hookah smoke, alcohol, and an energy drink in rats. However, more studies are necessary to explore the mechanisms underlying this biologic effect and to investigate whether the cardioprotective effects of *B. trimera* could be improved by the concomitant administration of a classic cardioprotective drug.

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