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Pharmacological safety of *Plinia cauliflora* (Mart.) Kausel in rabbits

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ABSTRACT

Fruit peels of *Plinia cauliflora* (Mart.) Kausel are widely used in Brazilian traditional medicine, but no studies have proved the safety of its pharmacological effects on the respiratory, cardiovascular, and central nervous systems. The present study assessed the safety pharmacology of *P. cauliflora* in New Zealand rabbits. First, an ethanol extract (EEPC) was selected for the pharmacological experiments and chemical characterization. Then, different groups of rabbits were orally treated with EEPC (200 and 2000 mg/kg) or vehicle. Acute behavioral and physiological alterations in the modified Irwin test, respiratory rate, arterial blood gas, and various cardiovascular parameters (i.e., heart rate, blood pressure, and electrocardiography) were evaluated. The main secondary metabolites that were identified in EEPC were ellagic acid, gallic acid, *O*-deoxyhexosyl quercetin, and the anthocyanin *O*-hexosyl cyanidin. No significant behavioral or physiological changes were observed in any of the groups. None of the doses of EEPC affected respiratory rate or arterial blood gas, with no changes on blood pressure or electrocardiographic parameters. The present study showed that EEPC did not cause any significant changes in respiratory, cardiovascular, or central nervous system function. These data provide scientific evidence of the effects of this species and important safety data for its clinical use.

1. Introduction

Safety pharmacology studies are essential for the development of new medicines [1]. This type of study aims to investigate the probable undesirable pharmacodynamic effects of new compounds on physiological functions, using doses to the therapeutic range and above [2]. One of the reasons that lead to pharmacological safety studies is due to serious adverse effects, especially on the central nervous system, respiratory rate, arterial blood gas (GAC), and cardiovascular parameters, including heart rate, blood pressure and cardiac electrical activity [3,4].

The Brazilian population, similar to populations worldwide, uses different vegetal species as important sources of food and medicine. Despite the widespread use of these agents, safety pharmacology studies are relatively restricted and mainly limited to compounds with broad industrial use. One example in Brazil is *Plinia cauliflora* (Mart.) Kausel (Myrtaceae). This species is endemic in South America. Its fruits are found in the most diverse Brazilian biomes, such as Cerrado, Caatinga, Atlantic Forest, Amazon Forest, and Pampa [5].

Popularly known as “jabuticaba,” the fruit is consumed fresh or used

Abbreviations: ABG, Arterial blood gas; ANOVA, One-way analysis of variance; ASE, Accelerated solvent extraction; BB, Buffer Base; BE, Base Excess; BE_{ecf}, Base excess in the extracellular fluid compartment; Ca^{++}, Calcium; $_cHCO_3$, Bicarbonate concentration; Cl, Chloride; CNS, Central nervous system; $_{ct}CO_2$ (B), Concentration of total carbon dioxide of whole blood; $_{ct}CO_2$ (P), Concentration of total carbon dioxide in plasma; $_{ct}O_2$, Concentration of total oxygen; DBP, Diastolic blood pressure; ECG, Electrocardiography; EEPC, Ethanol extract of *Plinia cauliflora*; GAE, Gallic acid equivalent; H^+, Hydrogen ion dissociated; Hct, Hematocrit; HHb, Deoxyhemoglobin; K^+, Potassium; LA, Left arm; LC-DAD-MS, Liquid chromatography coupled to a diode array detector and mass spectrometer; LL, Left leg; MAP, Mean arterial pressure; Na^+, Sodium; Na_2CO_3, Sodium carbonate; O_2Hb, Oxyhemoglobin; P50, Half of the maximum hemoglobin saturation; PCO_2, Partial pressure of carbon dioxide; pH, Potential of hydrogen; PO_2, Partial pressure of oxygen; RA, Right arm; RL, Right leg; S.E.M, Standard error of the mean; SBP, Systolic blood pressure; SO_2, Level of hemoglobin-saturation by oxygen; tHb, Hemoglobin; UFLC, Ultra fast liquid chromatograph

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for the production of liqueur, vinegar, wine, juice, jam, and jelly [6]. Several phenolic compounds, including flavonoids and anthocyanins, are present in the fruit peel of *P. cauliflora* that exert numerous biological effects [7]. Several pharmacological studies have been conducted using different extracts that were obtained from fruit peels of this species, highlighting its antioxidant [8], hypotensive [9], anti-obesity [10], anti-inflammatory [11], hypolipidemic [12], and antibacterial [13] activity.

Despite the widespread use of *P. cauliflora* fruit peel in the production of different bioactive materials, no data are available in the literature on the safety pharmacology of this preparation. The present study sought to optimize extraction procedures for *P. cauliflora* fruit peel and perform a detailed phytochemical analysis. We also performed a detailed pharmacological safety study to evaluate respiratory, cardiovascular, and central nervous system effects in rabbits.

2. Material and methods

2.1. Phytochemical study

2.1.1. Plant material

Plinia cauliflora fruits were collected in Esperança Nova, Paraná, Brazil (-23.719864, -53.802104), in September 2017. A voucher specimen (no. 5983) was authenticated by Dr. Zefa Valdivina Pereira and deposited in the Herbarium of the Federal University of Grande Dourados (UFGD). The fruit peels were manually removed and dried by forced air circulation for 5 days. The dried peels were then pulverized in a knife mill and stored in plastic bags under refrigeration (2–8 °C) until use.

2.1.2. Extraction procedures by accelerated solvent extraction

The extract was obtained from peels by accelerated solvent extraction (ASE; Dionex) using the solvents acetone: water (1: 1, v/v), ethanol, ethanol: water (7: 3, v/v), and water. Nitrogen was used for ASE. The following parameters were applied and repeated three times: 125 °C temperature, 4 min static extraction time, 100% washing volume, 1500 psi pressure, and 60 s purge. The solvents were evaporated by a rotary evaporator (Büchi R-3, Flawil, Switzerland) under reduced pressure and lyophilized to yield the extracts. All of the extracts were analyzed by liquid chromatography-diode array detector-mass spectrometry (LC-DAD-MS), and the total phenol and tannin contents were determined.

2.1.3. Total phenolic content

The phenolic content determination was based on the methodology of Herald et al. (2012) [14] with minor modifications. A 96-well microplate was used. To each well were added 75 µl of methanol and 75 µl of sample or standard (gallic acid), which were used for serial dilutions. Folin-Ciocalteu reagent (1:1 v/v, deionized water) was then added to the wells. After 6 min, 75 g/l (100 µl) Na_2CO_3 was added and mixed again. After 90 min, the samples were measured at 765 nm using a spectrophotometric microplate reader. The analyses were performed in triplicate. The results are expressed as milligrams (mg) of gallic acid equivalent (GAE) per gram (g) of extract.

2.1.4. Total tannin content

The extracts were solubilized at a concentration of 4 mg/ml using methanol and water (1:1, v/v), and skin powder (20 mg) was added and stirred for 60 min. After centrifugation, the supernatants were used for total phenol content determination as described previously. Total tannin content was calculated as the difference between the concentration of total phenols and non-tannin phenols. The results are expressed as milligrams of GAE per gram of extract.

2.1.5. Antioxidant activity determined by DPPH assay

The DPPH assay was performed according to Fukumoto and Mazza (2000) [15] with minor modifications. A 96-well microplate was used. A 150-µM solution of DPPH was prepared in methanol: water (8: 2 v/v). For each well was added 200 µl of DPPH solution, with the exception of blank wells, to which only methanol: water (8: 2 v/v) was added. Samples were analyzed in triplicate for each concentration (0–500 µg/ml). In control, only DPPH solution and methanol: water (8:2 v/v) were added. Quercetin was applied as the standard. The plate was covered and left in the dark at room temperature. After 6 h, absorbance was read at 520 nm in a spectrophotometric microplate reader. Absorbance decay of the samples (Aam) that is correlated with absorbance decay of the control (Ac) results in a percentage of free radical sequestration (% FRS):

$$\% \text{ FRS} = (\text{Ac} - \text{As}) / \text{Ac} \times 100$$

The data were used to calculate the IC_{50} (the concentration that is able to sequester 50% of free radicals).

2.1.6. Identification of constituents in the extracts determined by LC-DAD-MS

A UFLC Prominence Shimadzu LC device coupled to a DAD and MicrOTOF-Q III mass spectrometer (Bruker Daltonics, Billerica, MA, USA) was used for the analyses. A Kinetex C18 column (2.6 µm, 150 mm × 2.1 mm, Phenomenex) was applied, with a 1 µl injection volume, 0.3 ml/min flow rate, and 50 °C oven temperature. For the mobile phase, water (A) and acetonitrile (B) were used, to which 0.1% formic acid (v/v) was added. The gradient elution profile was the following: 3% B for 0–2 min, 3–25% B for 2–25 min, 25–80% B for 25–40 min, and 80% B for 40–43 min, followed by subsequent reconditioning conditions (5 min). The analyses were performed in negative and positive ion modes. Nitrogen was applied as the nebulizer gas at 4 bar and drying gas at 9 L/min. The capillary voltage was 3.5 kV. All of the extracts were prepared at 1 mg/ml, filtered (Millex0.22 µm, PTFE, Millipore), and injected in the chromatographic system.

The compounds were identified based on ultraviolet spectra, accurate mass, and fragmentation profile and compared with data in the literature. The molecular formulas were determined by accurate mass, considering errors up to 8 ppm and mSigma < 25.

2.2. Safety pharmacology study

2.2.1. Animals

Male New Zealand rabbits (Twenty-week-old) were obtained from Universidade Federal do Paraná (UFPR, Brazil) and housed in the vivarium of UFGD under controlled temperature (20 °C ± 2 °C) and humidity (50% ± 10%) and a 12 h/12 h light/dark cycle with ad libitum access to food and water. The Institutional Ethics Committee of the Universidade Federal da Grande Dourados previously approved all procedures employed in this study (UFGD, Brazil; protocol no. 11/2018; approved March 16, 2018).

2.2.2. Effects on the central nervous system

The effects on the central nervous system were performed according to the modified Irwin test [16]. After a 6-h fasting, two doses of EEPC (200 and 2000 mg/kg) [17] were administered in different groups of male rabbits (n = 6/group) by oral gavage. Filtered water was administered in the control group (1 ml/kg; n = 6). Food was only given to the rabbits 1 h after treatment. The effects of the treatments were evaluated 0-15 min, 15, 30, 60, 120, and 180 min, and 24 h after the administrations. To observe possible behavioral and physiological changes, the following parameters were recorded: piloerection, stereotypes (i.e., chewing, sniffing, and head movements), scratching, catalepsy, locomotor activity, reactivity to touch, akinesia, head-twitches, tremors, jumping, aggression, gait (rolling, tip-toeing), fear-related behavior, motor coordination, convulsions, grasping, traction, writhing, analgesia, exophthalmia, mydriasis, ptosis, myosis, salivation,

lacrimation, diarrhea, defecation, respiration, hyperthermia, and hypothermia.

2.2.3. Cardiovascular and respiratory evaluation

The effects on the cardiovascular and respiratory systems were evaluated according to the adapted protocol of Grahan and Li (1973) [18]. After 6-h fasting, two doses (200 and 2000 mg/kg) of EEPC (17)] were administered in different groups of rabbits (n = 6/group) by oral gavage. Vehicle (filtered water) was administered in the control group (1 ml/kg).

2.2.3.1. Respiratory rate and ABG analysis. One hour after treatment, all of the rabbits remained conscious in the ventral decubitus position. Respiratory rate was determined using a Kofranyi-Michaelis respirometer [19]. For ABG analysis, arterial blood samples were obtained from the central artery of the ear and immediately processed. All of the parameters below were determined in a Cobas b 221 blood gas system (Roche Diagnostics, Rotkreuz, Switzerland): pH, PCO_2 (mmHg), PO_2 (mmHg), SO_2 (%), Hct (%), tHb (g/dl), Na^+ (mmol/l), K^+ (mmol/L), Ca^{2+} (mmol/L), Cl^- (mmol/L), glucose (mg/dl), lactate (mmol/L), O_2Hb (%), HHb (%), P50 (mmHg), H^+ (nmol/L), BE (nmol/L), BE_{ecf} (nmol/L), BB (mmol/L), $cHCO_3$ (mmol/L), $ctCO_2$ (B) (mmol/L), $ctCO_2$ (P) (mmol/L), and ctO_2 (vol%).

2.2.3.2. Electrocardiography. After evaluating the respiratory system, all of the rabbits were intramuscularly anesthetized with 10-mg/kg diazepam plus 115-mg/kg ketamine and kept in the dorsal decubitus position. Four alligator electrodes (RL, RA, LL, and LA) were positioned in the folds of both knees and elbows. A small amount of conductive gel was applied to each electrode for better electrical conduction. In addition, 6 (V1-V6) precordial electrodes were also connected. The V1 was placed in the fourth intercostal space, on the right margin of the sternum. The V2 was placed in the fourth intercostal space, on the left margin of the sternum. The V3 is halfway between the electrodes V2 and V4. The V4 in the fifth left intercostal space, in the hemiclavicular line. The V5 is at the same level as the electrode V4, in the anterior axillary line. And the V6 was placed on the same level as the electrodes V4 and V5, on the mid-axillary line. After 5 min for acclimation, the electrocardiographic waves were recorded for 5 min in an ECG recorder (WinCardio, Micromed, Brasília, Brazil).

2.2.3.3. Effects on blood pressure. After electrocardiography, all of the rabbits subcutaneously received a bolus injection of heparin (50 IU). A tracheotomy was then performed to allow the animals to breathe spontaneously. The left carotid artery was then isolated, cannulated, and coupled to a pressure transducer connected to a PowerLab recording system. Chart 4.1 (ADI Instruments, Castle Hill, Australia) was used to register diastolic blood pressure (DBP), systolic blood pressure (SBP), and mean arterial pressure (MAP). Changes in SBP, DBP, and MAP were recorded for 20 min.

2.3. Statistical analysis

The mean ± standard error of the mean (SEM) is shown. Differences between groups were assessed using analysis of variance (ANOVA), followed by Dunnett's post hoc test. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism Mac 6.0 was used to draw the graphs and for statistical analysis.

3. Results

3.1. Optimization of extraction procedures

The extracts were obtained by ASE using the solvents ethanol, ethanol:water (7:3), acetone:water (1:1), and water. All of the extracts were analyzed by LC-DAD-MS (Fig. 1), and total phenol content, tannin content, and antioxidant activity were determined (Table 1).

The extracts that were obtained with acetone:water (1:1) and ethanol:water (7:3) had the highest total phenol content and tannin content and high antioxidant activity, with an IC_{50} of 13.08 ± 2.03 µg/ml and 11.54 ± 0.20 µg/ml, respectively. The extracts that were obtained with acetone:water (1:1) and ethanol:water (7:3) had total phenol content of 313.86 ± 1.73 mg GAE/g and 299.60 ± 4.26 mg GAE/g, respectively, and tannin content of 169.64 ± 6.74 mg GAE/g and 179.46 ± 1.76 mg GAE/g, respectively. The chromatograms of the extracts revealed chemical similarities (Fig. 1), but ion peak intensities presented some differences. More chromatographic peaks were observed with the ethanol:water (7:3) extract. Overall, the extracts that were obtained with acetone:water (1:1) and ethanol:water (7:3) presented the best results. The ethanol:water extract was selected for the safety pharmacology study because this solvent composition is widely used because of its low toxicity.

3.2. Identification of extract constituents determined by LC-DAD-MS

The ethanol:water (7:3) extract (EEPC) was analyzed by LC-DAD-MS, and 12 compounds were identified (Table 2, Fig. 2). Compounds **1** and **2** showed intense ions at m/z 341.1083 and 191.0181 $[M\text{-}H]^-$, which were putatively identified as di-hexoside and citric acid, respectively. Peak **3** revealed a band at a wavelength of 270 nm in the ultraviolet spectrum and also an ion at m/z 169.0121 $[M\text{-}H]^-$, which was confirmed as gallic acid by injection of the authentic standard. This compound has been previously reported in *P. cauliflora* (13).

Compounds **7**-**10** showed bands in the ultraviolet spectra at wavelengths of 260 and 360 nm, suggesting a chromophore group relative to ellagic acid. Compound **9** presented the molecular formula $C_{14}H_6O_8$ (from ions m/z 301.9979 and 303.0157) and fragment ions at m/z 283, 257, and 229, which resulted from losses of water, CO_2, and CO molecules. This fragmentation pathway is similar to the ellagic acid profile [20,21], which was confirmed by injection of the authentic standard. Compounds **7** (m/z 465.0677, $C_{20}H_{17}O_{13}^+$), **8** (m/z 435.0575, $C_{19}H_{15}O_{12}^+$), and **10** (m/z 449.0729, $C_{20}H_{17}O_{12}^+$) presented fragment ions at m/z 303 from losses of hexosyl (162 *u*), pentosyl (132 *u*), and deoxyhexosyl (146 *u*) groups. Compounds **7**, **8**, and **10** were identified as *O*-hexosyl ellagic acid, *O*-pentosyl ellagic acid, and *O*-deoxyhexosyl ellagic acid, which have been previously reported in *Plinia* species (*P. trunciflora*, *P. caulifora*, *P. jaboticaba*, and *P. phitrantha*) [20].

Compounds **6** and **11** showed characteristic ultraviolet spectra of anthocyanin (λ_{max} = 279 and 512 nm) and flavonols (λ_{max} = 265 and 358 nm; Markham, 1982) [22]. Ions at m/z 449.1098 $[M]^+$ and 449.1097 $[M+H]^+$ confirmed the molecular formulas $C_{21}H_{21}O_{11}^+$ and $C_{21}H_{20}O_{11}$, respectively. The loss of 162 *u* indicated their hexosyl substituents. Compounds **6** and **11** were identified as *O*-hexosyl cyanidin and *O*-deoxyhexosyl quercetin, respectively, which was similar to data reported by Calloni et al. (2015) [23] and Neves et al. (2018) (20) from *Plinia* species.

3.3. Effects on the central nervous system

The toxic effects of EEPC on behavioral and physiological status in male rabbits are shown in Table 3. During the 24 h observation period, none of the experimental animals were inactive or refused to consume food or water. No significant changes in behavior or physiological status were observed until the end of 24 h (i.e., convulsions, tremors, locomotor activity, jumping, fear-related behavior, reactivity to touch, aggression, head-twitches, stereotypies [i.e., head movements, chewing, sniffing], scratching, catalepsy, akinesia, gait [rolling, tiptoeing], motor coordination, traction, grasping, writhing, analgesia, ptosis, exophthalmia, myosis, mydriasis, piloerection, defecation, diarrhea, salivation, lacrimation, respiration, hypothermia, and hyperthermia).

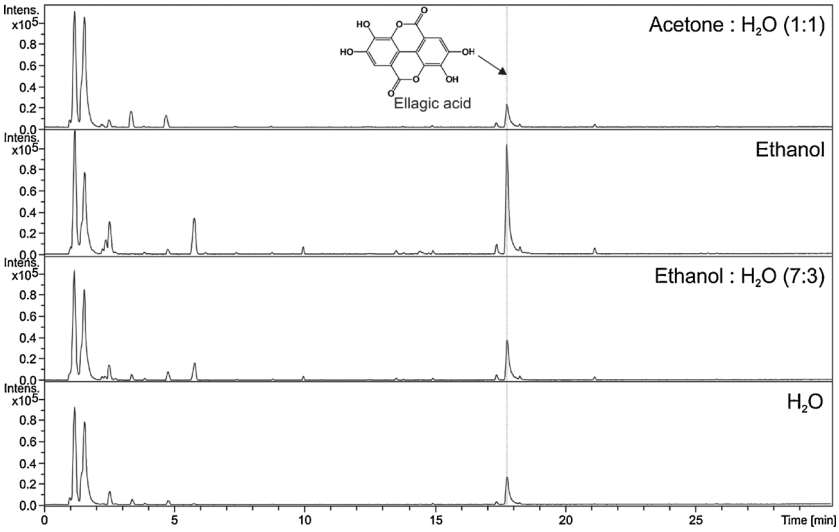

Fig. 1. Base peak chromatograms (negative ion mode) from the extracts of peels obtained by ASE with the extractor solvents acetone:water (1:1 v/v), ethanol and ethanol:water (7:3 v/v) and water.

Table 1
. Yield, phenolic content, tannin content and IC_{50} value for free radical scavenging activity by DPPH method from the extracts.

Extract	Yield (%)	Phenolic content (mg GAE g^{-1})	Tannin content (mg GAE g^{-1})	DPPH IC_{50} (μg/mL)
Acetone:water (1:1)	27.9	313.86 ± 1.73	169.64 ± 6.74	13.08 ± 2.03
Ethanol	24.5	197.39 ± 5.71	69.05 ± 5.23	16.25 ± 1.18
Ethanol:water (7:3)	22.6	299.60 ± 4.26	179.46 ± 1.76	11.54 ± 0.20
Water	16.8	181.42 ± 3.67	136.13 ± 6.58	23.75 ± 0.72
Quercetin	–	–	–	1.73 ± 0.10

GAE: gallic acid equivalent (GAE) g^{-1} (per gram) of extract.

Table 2
Identification of the constituents from ethanol and water (7:3) extract (EEPC) of *P. cauliflora* by LC-DAD-MS.

Peak	RT (min)	Compound	MF	UV (nm)	Negative mode (*m/z*)		Positive mode (*m/z*)	
					MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
1	1.2	di-hexoside	$C_{12}H_{22}O_{11}$	–	341.1083	191	–	–
2	1.6	Citric acid	$C_6H_8O_7$	–	191.0181	–	–	–
3	2.5	Gallic acid*	$C_7H_6O_5$	270	169.0121	–	–	–
4	3.0	NI	$C_6H_{10}N_6O_4$	–	–	–	231.0842	
5	5.8	NI	$C_8H_{12}O_7$	–	219.0492	–	221.0659	–
6	12.5	*O*-hexosyl cyanidin	$C_{21}H_{21}O_{11}^+$	279, 512	447.0933	284, 255, 162, 147	449.1098	287
7	14.9	*O*-hexosyl ellagic acid	$C_{20}H_{16}O_{13}$	255, 360	463.0513	–	465.0677	303
8	17.3	*O*-pentosyl ellagic acid	$C_{19}H_{14}O_{12}$	265, 360	433.0411	301	435.0575	303
9	17.7	Ellagic acid*	$C_{14}H_6O_8$	255, 362	301.9979	283, 257, 229, 185	303.0157	275, 257, 229, 201
10	18.3	*O*-deoxyhexosyl ellagic acid	$C_{20}H_{16}O_{12}$	265, 365	447.0577	301	449.0729	303
11	21.1	*O*-deoxyhexosyl quercetin	$C_{21}H_{20}O_{11}$	265, 358	447.0929	300, 271, 255, 243, 178,163	449.1097	303
12	35.9	NI	$C_{13}H_{18}O_4$	279	237.1134	221, 206, 166	–	–

RT: retention time; MF: molecular formula; NI: non-identified; *confirmed by the authentic standard.

3.4. Respiratory rate and ABG analysis

The mean respiratory rate in rabbits that were treated with vehicle alone was 57 ± 6.01. No increase or decrease in respiratory rate was observed after acute EEPC administration (200 mg/kg EEPC: 56 ± 7.58; 2000 mg/kg EEPC: 55 ± 6.59). The ABG analysis indicated that none of the doses of EEPC altered pH, PCO_2 (mmHg), PO_2 (mmHg), SO_2 (%), Hct (%), tHb (g/dl), Na^+ (mmol/L), K^+ (mmol/L), Ca^{2+} (mmol/L), Cl^- (mmol/L), glucose (mg/dl), lactate (mmol/L), O_2Hb (%), HHb (%), P50 (mmHg), H^+ (nmol/L), BE (nmol/L), BE_{ecf} (nmol/L), BB (mmol/L), $cHCO_3$ (mmol/L), $ctCO_2$ (B) (mmol/L), $ctCO_2$ (P) (mmol/L), or ctO_2 (vol%) compared with control animals (Table 4).

3.5. Electrocardiography

Fig. 3A-G shows representative electrocardiograms and quantitative data for rabbits that were treated with 200 and 2000 mg/kg EEPC or vehicle. We did not observe any significant changes in electrocardiographic characteristics (PR, QRS, or QT segment) between experimental groups, with no alterations of the amplitude of P-, R-, or T-waves.

3.6. Effects on blood pressure

Basal SBP, DBP, and MAP that were recorded after the 15-min stabilization period were 102 ± 6.6 mmHg, 62 ± 3.5 mmHg, and 71 ± 4.1 mmHg, respectively, in control animals. Oral administration

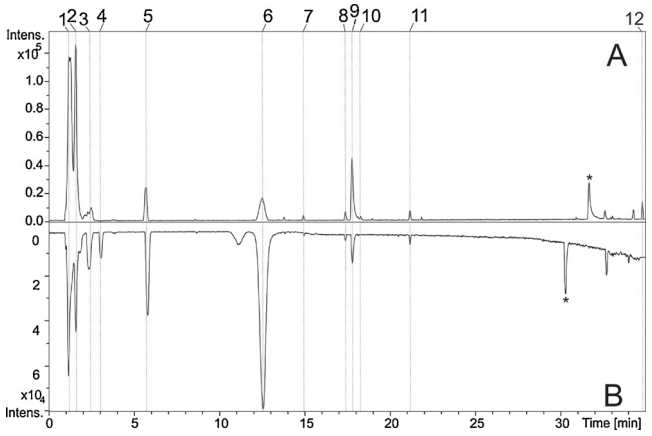

Fig. 2. Base peak chromatograms obtained by negative (**A**) and positive ion modes (**B**) from ethanol:water (7:3) extract (EEPC). (*chromatographic peaks are not of the analyzed sample).

Table 3
Effects of EEPC acute treatment on behaviors and clinical signals observed in Irwin modified test.

Category	Symptoms	Control	EEPC (200 mg/kg)	EEPC (2000 mg/kg)
Excitation	Convulsion	(-)	(-)	(-)
	Tremor	(-)	(-)	(-)
	Increased activity	(-)	(-)	(-)
	Jumping	(-)	(-)	(-)
	Increase fear	(-)	(-)	(-)
	Increased reactivity to touch	(-)	(-)	(-)
	Aggression	(-)	(-)	(-)
Stereotypy	Head-twitches	(-)	(-)	(-)
	Stereotypies (head movements)	(-)	(-)	(-)
	Stereotypies (chewing)	(-)	(-)	(-)
	Stereotypies (sniffing)	(-)	(-)	(-)
	Scratching	(-)	(-)	(-)
Motor	Catalepsy	(-)	(-)	(-)
	Akinesia	(-)	(-)	(-)
	Abnormal gait (rolling)	(-)	(-)	(-)
	Abnormal gait (tip-toe)	(-)	(-)	(-)
	Motor incoordination	(-)	(-)	(-)
	Loss of traction	(-)	(-)	(-)
	Grasping	(-)	(-)	(-)
Sedation	Decreased activity	(-)	(-)	(-)
	Decreased fear	(-)	(-)	(-)
	Decreased reactivity to touch	(-)	(-)	(-)
Pain	Writhing	(-)	(-)	(-)
	Analgesia	(-)	(-)	(-)
Autonomic	Ptosis	(-)	(-)	(-)
	Exophthalmia	(-)	(-)	(-)
	Myosis	(-)	(-)	(-)
	Midriasis	(-)	(-)	(-)
	Piloerection	(-)	(-)	(-)
	Defecation	(-)	(-)	(-)
	Diarrhea	(-)	(-)	(-)
	Salivation	(-)	(-)	(-)
	Lacrimation	(-)	(-)	(-)
Others	Increased respiration	(-)	(-)	(-)
	Decreased respiration	(-)	(-)	(-)
	Hypothermia	(-)	(-)	(-)
	Hyperthermia	(-)	(-)	(-)

The evaluation time was 0–15 min, 15, 30, 60, 120, 180 min and 24 h after the treatments acute administration. (-): Absence of the symptom.

of EEPC (200 or 2000 mg/kg) did not significantly change SBP, DBP, or MAP compared with the control group (Fig. 4A-C). Heart rate was not significantly different between experimental groups. The mean values

Table 4
Effects of EEPC acute treatment on respiratory rate, blood gases, electrolytes, and metabolites parameters.

	Control	EEPC (200 mg/kg)	EEPC (2000 mg/kg)
Respiratory rate	57 ± 6.01	56 ± 7.58	55 ± 6.59
Blood gases			
pH	7.25 ± 0.02	7.28 ± 0.04	7.25 ± 0.03
PCO_2 (mmHg)	55.90 ± 1.70	56.85 ± 2.42	56.42 ± 2.21
PO_2 (mmHg)	80.53 ± 3.13	71.83 ± 4.76	75.44 ± 4.51
SO_2 (%)	82.65 ± 6.31	88.78 ± 1.75	86.71 ± 1.88
Hct (%)	37.98 ± 2.71	38.25 ± 2.36	37.44 ± 2.51
tHb (g/dl)	13.85 ± 0.88	13.45 ± 1.13	13.55 ± 1.11
Electrolytes			
Na^+ (mmol/l)	143.10 ± 0.82	144.40 ± 0.85	142.23 ± 0.79
K^+ (mmol/l)	4.31 ± 0.12	4.35 ± 0.22	4.32 ± 0.18
Ca^{++} (mmol/l)	1.07 ± 0.02	1.04 ± 0.01	1.06 ± 0.02
Cl^- (mmol/l)	103.30 ± 0.68	104.50 ± 0.53	103.45 ± 0.61
Metabolites			
Glucose (mg/dl)	295.30 ± 18.93	303.80 ± 20.88	299.91 ± 21.31
Lactate (mmol/l)	1.05 ± 0.11	1.22 ± 0.22	1.12 ± 0.21
C)-oximetry			
O_2Hb (%)	88.75 ± 1.82	82.67 ± 3.59	85.13 ± 2.88
HHb (%)	14.15 ± 1.22	11.50 ± 1.59	13.20 ± 1.33
Calculated values			
P50 (mmHg)	38.03 ± 2.02	40.35 ± 0.99	39.22 ± 1.18
H^+ (nmol/l)	53.18 ± 5.71	52.58 ± 3.72	51.47 ± 2.99
BE (nmol/l)	−3.70 ± 0.47	−3.47 ± 0.28	−3.61 ± 0.33
BE_{ecf} (nmol/l)	−3.07 ± 0.77	−3.10 ± 0.14	−3.13 ± 0.41
BB (mmol/l)	43.80 ± 0.54	43.90 ± 0.63	43.65 ± 0.59
$cHCO_3$ (mmol/l)	93.68 ± 0.61	90.50 ± 1.78	92.45 ± 0.99
$_{ct}CO_2$ (B) (mmol/l)	23.63 ± 0.39	24.98 ± 0.92	23.44 ± 0.57
$_{ct}CO_2$ (P) (mmol/l)	21.55 ± 0.73	22.95 ± 0.76	21.35 ± 0.71
ctO_2 (vol%)	15.83 ± 1.04	15.38 ± 0.59	15.54 ± 0.85

Statistical analyses were performed using one-way ANOVA followed by Dunnett post hoc test. PCO_2: partial pressure of carbon dioxide, PO_2: partial pressure of oxygen, SO_2: level of hemoglobin-saturation by oxygen, Hct: hematocrit, tHb: hemoglobin, Na^+: sodium, K^+: potassium, Ca^{++}: calcium, Cl^-: chloride. O_2Hb: Ox hemoglobin, HHb: Deoxyhemoglobin, P50: half of the maximum hemoglobin saturation, H^+: hydrogen ion dissociated, BE: base excess, BE_{ecf}: base excess in the extracellular fluid compartment, BB: buffer base, $cHCO_3$: bicarbonate concentration, $_{ct}CO_2$ (B): concentration of total carbon dioxide of whole blood, $_{ct}CO_2$ (P): concentration of total carbon dioxide in plasma, ctO_2: concentration of total oxygen.

in the negative control and EEPC (200 and 2000 mg/kg) groups were 272 ± 11 bpm, 295 ± 23 bpm, and 284 ± 17 bpm, respectively (Fig. 4D).

4. Discussion

Natural products have been used as sources of food and as raw material for the production of medicines throughout history. Medicinal or fructiferous plants have been and remain to be used worldwide because of their economic and curative potential [24]. Although numerous pharmacological studies have investigated a wide range of new potential drugs, toxicological studies are not always carefully performed. Several data have reported potential side effects and systemic toxicity of several natural products [25]. In this sense, studies that evaluate pharmacological safety are important to ensure safe use and therapeutic rationalization. Thus, we obtained an ethanol extract from *Plinia cauliflora* fruit peels and mapped its main active metabolites. We found that the EEPC did not have significant deleterious effects on biological systems that are most affected by active molecules (i.e., cardiovascular, respiratory, and central nervous systems).

Different physiological systems of the body are subject to several deleterious effects of natural products. The respiratory, cardiovascular, and central nervous systems are key systems where such effects can occur, possibly with greater severity. Because of the high vascularization of these organs, biological agents can readily access them and reach target tissues rapidly and at high concentrations. Moreover, the

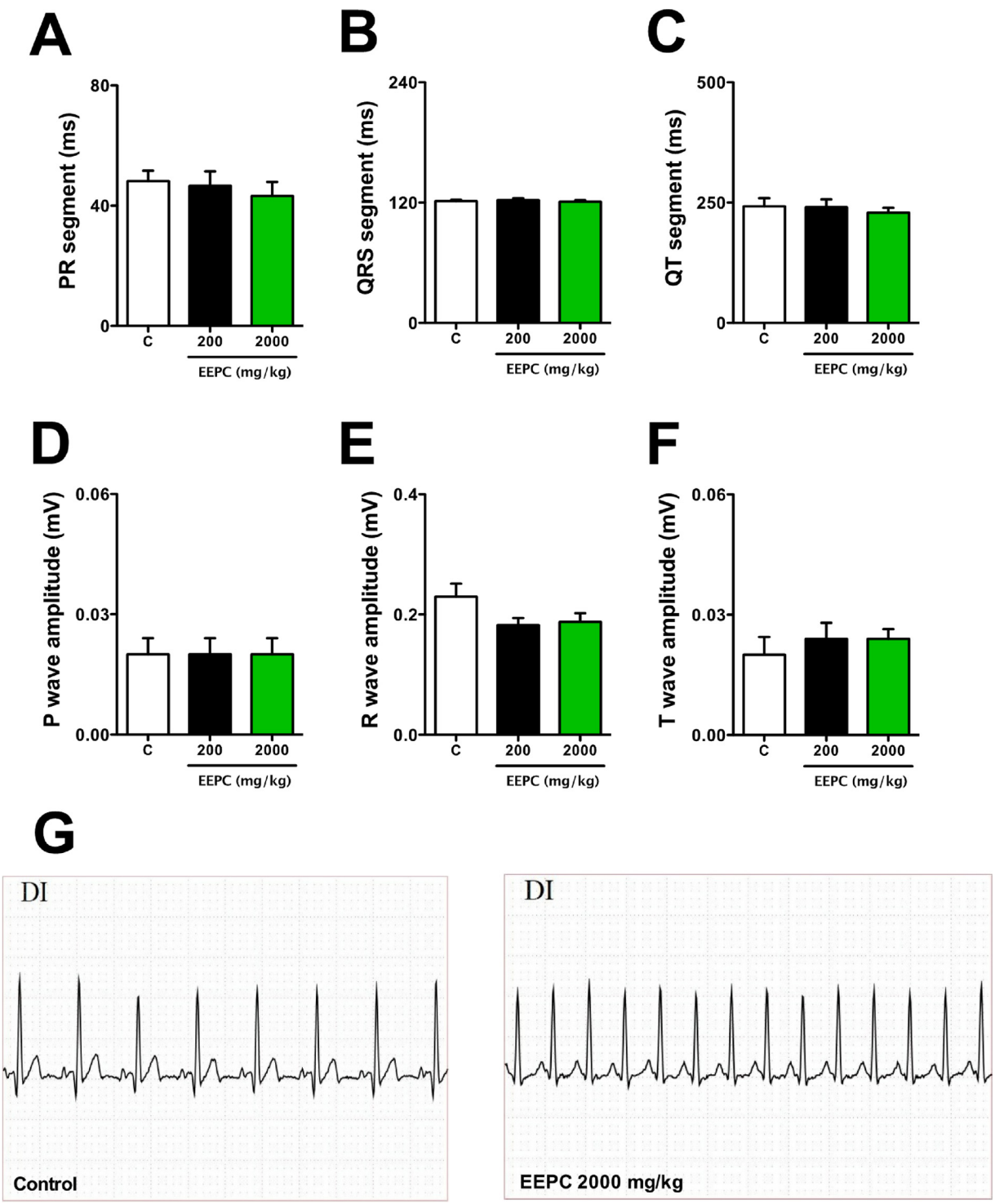

Fig. 3. Electrocardiographic quantitative data of rabbits treated with EEPC or vehicle in the PR (A), QRS (B), and QT-segments (C) and P (D), R (E) and T-waves (F) amplitude. Representative electrocardiographic records carried out in limb lead I also are shown (G). Statistical analyses were performed using one-way ANOVA followed by Dunnett *post hoc* test. The results are expressed as mean ± standard error of the mean (S.E.M.) and *p*-value of less than 0.05 was considered statistically significant. C: control (vehicle); EEPC: ethanol extract from *Plinia cauliflora*.

lungs, heart, and brain regulate highly sensitive bodily functions. Any changes that occur acutely in these organs can have significant consequences on body homeostasis [26].

When conducting preclinical safety pharmacology studies, an important factor to be considered is the animal model that is employed. Although rodents are a key pharmacological tool, they are not always a viable model for such pharmacological safety studies. Rats can be considered an acceptable option for studies of substances with potential effects on the central nervous system [27], including behavioral studies [28]. However, rats may be considered inadequate for evaluating effects on the cardiovascular system because the electrocardiographic tracing and arrhythmogenic potential of some drugs can have different profiles between rodents and humans [29,30]. The use of lagomorphs has gained prominence in this field. In addition to relatively easy maintenance and rapid reproduction, rabbits also possess physiological functions that are similar to humans.

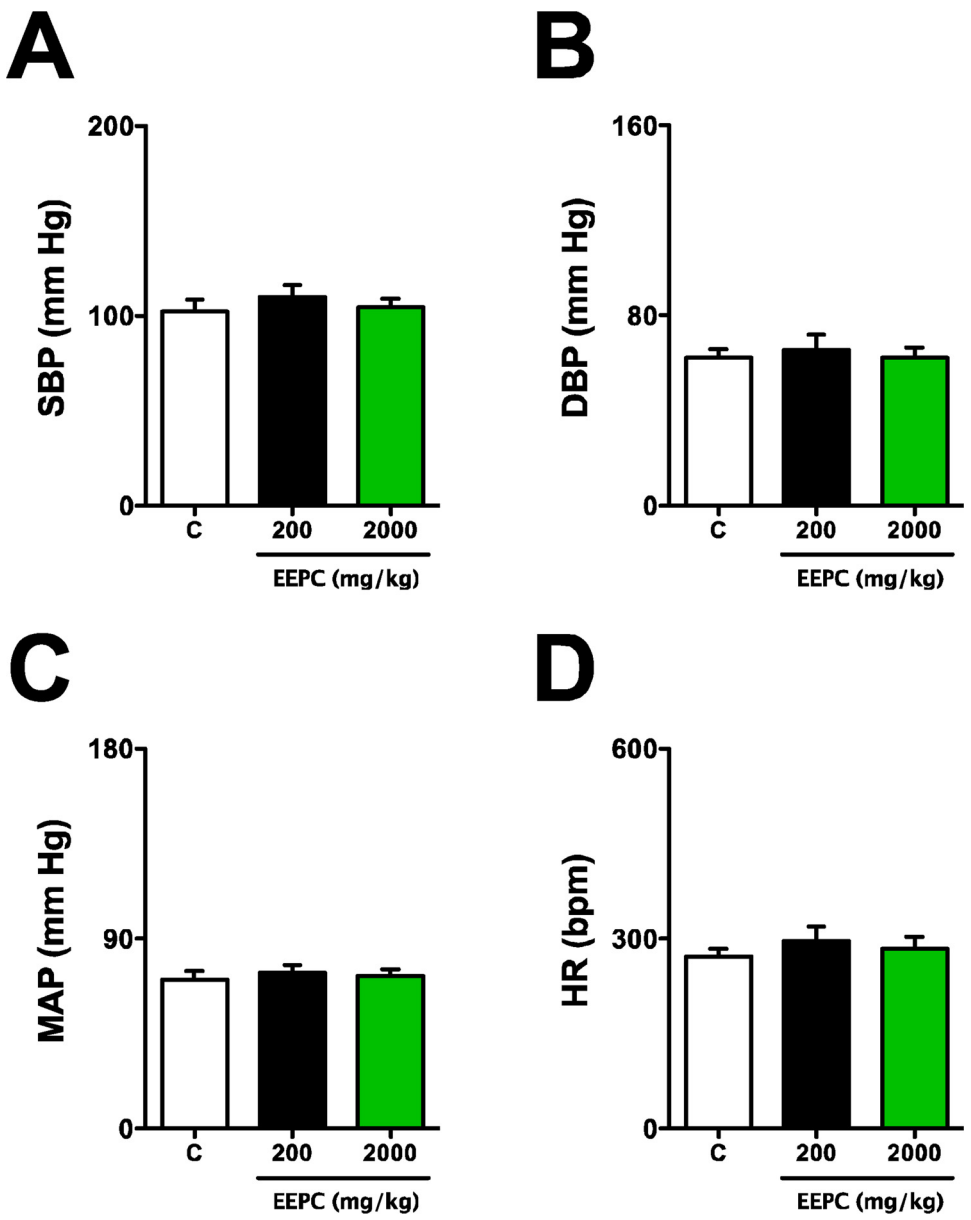

Fig. 4. Acute oral administration of EEPC obtained from *P. cauliflora* does not affect SBP, DBP, MAP, and HR in New Zealand rabbits. Statistical analyses were performed using one-way ANOVA followed by Dunnett *post hoc* test. The results are expressed as mean ± standard error of the mean (S.E.M.) and *p*-value of less than 0.05 was considered statistically significant. C: control (vehicle); DBP: diastolic blood pressure; EEPC: ethanol extract from *Plinia cauliflora*; HR: heart rate; MAP: mean arterial pressure; SBP: systolic blood pressure.

Several herbal medicines have significant action on the central nervous system. Many of them have also become important sources of modern drugs, including atropine (*Atropa belladonna* L.), morphine (*Papaver somniferum* L.), caffeine (*Coffea arabica* L.), and ephedrine (*Ephedra sinica* Stapf) [31–35]. Data also show that some species are still used as an abuse drug or due their recreational potential (e.g., *Erythroxylum coca* Lam. and *Cannabis sativa* L. [36–38]. Moreover, poisoning by natural products is not uncommon because of the sensitivity of the central nervous system to these agents [39,40]. Thus, the Irwin test is employed to evaluate the effects of a new drug on physiological and behavioral functions [41]; and may alert us to potential safety concerns, including seizure potential, sedation, and motor changes [42].

Another important fact to consider is the respiratory depressant potential of some natural products. In the United States alone, a significant increase in cases of opioid-induced fatal respiratory depression has been observed in recent decades [43,44]. In addition to more severe cases, deleterious effects on the respiratory system may present more subtly and become severe in the long term. Cases of respiratory acidosis or low oxygen saturation in red blood cells may be considered important indicators of systemic toxicity [45,46].

Cardiovascular effects are one of the least explored areas during safety evaluations of natural products. Although effects on blood pressure have been systematically evaluated, electrocardiographic profiles that are observed after treatment with different natural products have been quite unusual [47–49]. This can be concerning when considering the fact that some natural molecules are classic blockers of sodium channels (e.g., quinidine) in cardiac muscle [50,51]. Some studies have shown the ability of some drugs to affect the duration of the cardiac action potential. In fact, several drugs induce prolongation of the Q-T interval and may precipitate ventricular arrhythmias [52]. Any substance that is intended for long-term use, such as polyphenol-rich antioxidant preparations, should be evaluated for cardiovascular safety.

In recent years, consumers have sought treatments that are able to slow aging or prevent cardiovascular diseases. *P. cauliflora* has been used in Brazil for this purpose [53]. Their fruit peels are rich in polyphenols, such as anthocyanin, flavonoids, and ellagic acid derivatives; and these molecules are classically known as antioxidants [6–8,54,55]. Although we found that the EEPC was relatively safe with regard to its effects on the respiratory, cardiovascular, and central nervous systems, we do not exclude the possibility that other species with a similar phytochemical profile may present deleterious effects. In fact, several isolated secondary metabolites that are present in the EEPC have important effects on the central nervous system and different ion channels [56–60]. Thus, different extraction processes result in unique phytochemical profiles, and safety pharmacology studies need to be performed with each preparation of interest to guarantee their safety.

5. Conclusion

The present study found that the EEPC that was obtained from *Plinia cauliflora* fruit peels did not cause any significant changes in respiratory, cardiovascular, or central nervous system function. These findings provide important scientific knowledge about the species and safety data for its clinical use.

Author's contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; RACP, LPG, and PVMR conducted the experiments; DBS and SRN were involved with the preparation and chemical analysis of extract; ELBL, RACP and AGJ was responsible for data discussion, manuscript correction and AGJ was the senior researcher responsible for this work. All authors read and approved the final manuscript.

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