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Effects of *Psidium guajava* L. leaves extract on blood pressure control and IL-10 production in salt-dependent hypertensive rats

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ABSTRACT

Psidium guajava (guava) leaves extract displays anti-hypertensive properties by mechanisms not yet fully understood. Here, we investigated whether sympathetic drive and immune signaling mechanisms are involved with the antihypertensive effect of the guava extract in a model of salt-dependent hypertension. Raw guava extract (r*Ps*E) was characterized by colorimetric and UPLC-MS techniques. Two doses of r*Ps*E (100 and 200 mg/kg) were evaluated for anti-hypertensive effect using a suspension system (*Ps*E). Weaned male Wistar rats were put on a high-salt diet (HSD, 0.90 % Na^+) for 16 weeks and received gavages of *Ps*E for the last 4 weeks. Blood pressure (BP) was measured at the end of treatment in conscious rats. The neurogenic pressor effect was assessed by ganglionic blockade with hexamethonium. Autonomic modulation of heart rate was evaluated by spectral analysis. The effects of orally administered *Ps*E on lumbar sympathetic nerve activity (LSNA) were assessed in anesthetized rats. Blood IL-10, IL-17A, and TNF were measured. The increased neurogenic pressor effect of HSD rats was reduced by *Ps*E 100 mg/kg, but not by 200 mg/kg. *Ps*E (200 mg/kg) administration in anesthetized rats produced a greater fall in BP of HSD rats compared to standard salt diet (SSD) rats. *Ps*E hypotensive response elicited an unproportionable increase in LSNA of HSD rats compared to SSD rats. *Ps*E (200 mg/kg) increased plasma concentrations of IL-10 but had no effect on TNF or IL-17A. Our data indicate that the antihypertensive effects of *Ps*E may involve autonomic mechanisms and immunomodulation by overexpression of IL-10 in salt-dependent hypertensive rats.

1. Introduction

Psidium guajava L. is a plant that belongs to the Myrtaceae family, popularly known as guava tree. Aerial parts of the guava tree (leaves and bark) are reported to be used in folk medicine. Several studies carried out with extract prepared from *P. guajava* leaves and barks demonstrated biological activities, such as antidiarrheic [1], antimicrobial activity [2] hypoglycemic, and anti-hypertensive [3]. The leaf extracts of *P. guajava* have been shown to contain many biologically active molecules like phenolic compounds, flavonoids, tannins, and saponins [4], which displayed antimicrobial and anti-hypertensive properties [5].

Hypertension can be defined as a shift from normal (120/80 mmHg, systolic/diastolic) to high (> 140/90 mmHg, systolic/diastolic) levels in arterial BP [6]. It is a serious public health problem that has many causes

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Table 1
Phenolic compounds, flavonoids, and condensed tannins content in the raw extract (r*Ps*E) and the formulation (*Ps*E) of *Psidium guajava* L. leaves extract.

Assay	r*Ps*E	*Ps*E
Phenolic compounds (mg GAE/g of r*Ps*E)	170.5 ± 6.0	54.2 ± 4.8*
Flavonoids (mg QE/g of r*Ps*E)	11.7 ± 0.2	3.4 ± 1.7
Condensed tannins (mg CE/g of r*Ps*E)	0.204 ± 0.007	0.084 ± 0.002

Results are expressed as mean of triplicates ± standard error of the means. GAE: gallic acid equivalents, QE: quercetin equivalents, CE: catechin equivalents
* Different of rPsE; Student's t-test; $p < 0.05$.

Table 2
Compounds and structure of raw extract (r*Ps*E) obtained in UPLC-DAD-ESI-MS analysis.

Peak	Compound	RT (min)	UV (nm)	LC-MS [M - H]$^-$ (*m/z*)	LC-MS [M + H]$^+$ (*m/z*)	Ref.
1	Kaempferol	2.60	255; 351	286	288	[31]
2	Quercetin	3.01	255; 353	301	303	[32]
3	Myricetin	3.16	265; 346	317	319	[32]
4	Derivated of Myricetin	3.17	265; 346	317	319	[33]

associated with its development. Only 5–10 % of hypertensive patients have a defined diagnosis of the high BP cause as aldosteronism, gestation, or renal artery stenosis (secondary hypertension); on the other hand, 90–95 % of hypertensive patients have unknown or multiple causes for the high BP which has been usually termed essential hypertension [7–9]. Hypertension development and maintenance involve autonomic (central system nerves), vascular, and hormonal (renin-angiotensin-aldosterone systems – RAAS) mechanisms [6–9]. The major risk factor associated with hypertension includes obesity, genetic variation, sedentary lifestyle, alcoholism smoking, and excessive sodium intake [6,7,9]. The last one has been considered one of the most important factors associated with hypertension development in modern society [10–13]. Classically, high salt intake is associated with hydro mineral unbalance due to renal lesion, which leads to hypertension [14,15]. However, cumulative evidence has indicated a role for neurogenic mechanisms in the development of salt-dependent hypertension based on changes in autonomic regulation and in the increase of sympathetic drive to the cardiovascular system [16–25]. Yet, late evidence indicates that immune-mediated signaling originated in the gut (the gut-brain axis) may also be involved with the development of hypertension by high salt intake, placing the proliferation of T_H17 cells and higher production of interleukin(IL)-17 as key immune components in the sodium-dependent neurogenic hypertension [10,11]. In addition, other studies have also reported a role for interleukin-1β (IL-1β) [12], tumor necrosis factor-α (TNF-α) [13] and interleukin-6 (IL-6) [14] in neurogenic hypertension, suggesting that immune signaling mechanisms may excite important pressor descending pathways in the central nervous system (CNS).

The clinical management of hypertension focus on ameliorating BP, thereby preventing complications. To this end, hypertensive medication targets the major mechanisms regulating BP: total blood volume, mostly through the renin-angiotensin-aldosterone system, and blood flow, through neurogenic and vascular controlling systems [9]. However, the efficacy of conventional medication treatment for hypertension can vary from person to person, and some patients may have a lower response to the effects of medications or even be resistant or refractory to treatment [9]. Thus, there is a need to search and develop new strategies for medication treatment of hypertension and the use natural products has a suitable approach to this end, specially the products from *Psidium guajava*.

Based on the report conducted by Ojewole in 2005, which implies that guava leaf extract attenuates the pressure effect of noradrenaline [3], and that inflammatory mediators can lead to the development of hypertension [15], we hypothesized that bioactive molecules present in the guava leaves extract could modulate arterial BP of salt-dependent hypertensive rats by changes in autonomic and immune signaling mechanisms.

2. Methods

2.1. Leaves harvesting and ethanolic extraction of bioactive compounds

Leaves of *P. guajava* L. were harvested from trees grown spontaneously and undomesticated in an open field located in the rural area of Ouro Preto, Brazil (GPS coordinates: 20°17'15" S and 43°30'29" W) according to the National System of Management of Genetic Heritage and Associated Traditional Knowledge permissions (SisGen AEB4F09; A298B77; AE59080). A propagating sample of the plant was collected and deposited as an exsiccate at the José Badini Herbarium of the Federal University of Ouro Preto (OUPR27242) for species identification and documentation.

Fresh leaves were washed under running water to remove dust and assessed for removal of those with signs of deterioration. Selected fresh whole leaves were weighed and immediately immersed in 98 % ethanol (LS Chemical) in a 3:2 proportion. The maceration process was allowed for 10 days, after which the ethanolic fluid was drained and repeated two times more when complete discoloration of the leaves was observed. Ethanolic fluid was pooled together, and the ethanol was removed by rotaevaporation, at low pressure (80 mbar), and 40 ℃. The remaining fluid extract was then further dried at 40 ℃ until a raw pasty extract (r*Ps*E) was obtained.

2.2. Guava leaves extract formulation (PsE)

The r*Ps*E was conveyed in a formulation as described in the patent deposited under registration BR10201900148. The formulation (*Ps*E) was prepared in two different doses of the r*Ps*E (*Ps*E 100: 100 mg/kg and *Ps*E 200: 200 mg/kg) for orogastric administration.

2.3. Extract characterization

2.3.1. Phenolic compounds, flavonoids, and tannins quantification

The colorimetric quantification of total phenolics, flavonoids, and condensed tannins content was performed in r*Ps*E and *Ps*E samples. Total phenolics were quantified by the Folin-Ciocalteu method, adapted from Singleton [16]. Gallic acid was used as a standard for the calibration curve and the content of total phenolics was expressed as mg of gallic acid equivalents mg (GA)/g of r*Ps*E. Flavonoid content was quantified by the reaction of these flavonoids with the $AlCl_3$ method, adapted from Maksimovic et al. [17]. Quercetin was used as a standard for the calibration curve and the content of flavonoids was expressed as mg of quercetin equivalents mg (Q)/g of r*Ps*E. The content of tannin in the r*Ps*E was quantified as described elsewhere [18]. Catechin was used as a standard for the calibration curve and the content of tannins was expressed as mg of catechin equivalents mg (CE)/g of r*Ps*E.

2.3.2. Ultra-performance liquid chromatography coupled with mass spectrometry (UPLC – MS) analysis

A sample of the r*Ps*E was resuspended in methanol (10 mg/mL). The analysis of the extract components was performed on a UPLC Acquity Waters® liquid chromatography coupled with an electron capture mass spectrometer (Agilient®) and atmospheric pressure chemical ionizer. Chromatographic separations were performed using an Acquity UPLC chromatographic system (Waters, Millford, MA) equipped with a binary pump system as well as an Acquity BEH C18 column (1.7 mm × 50 mm,

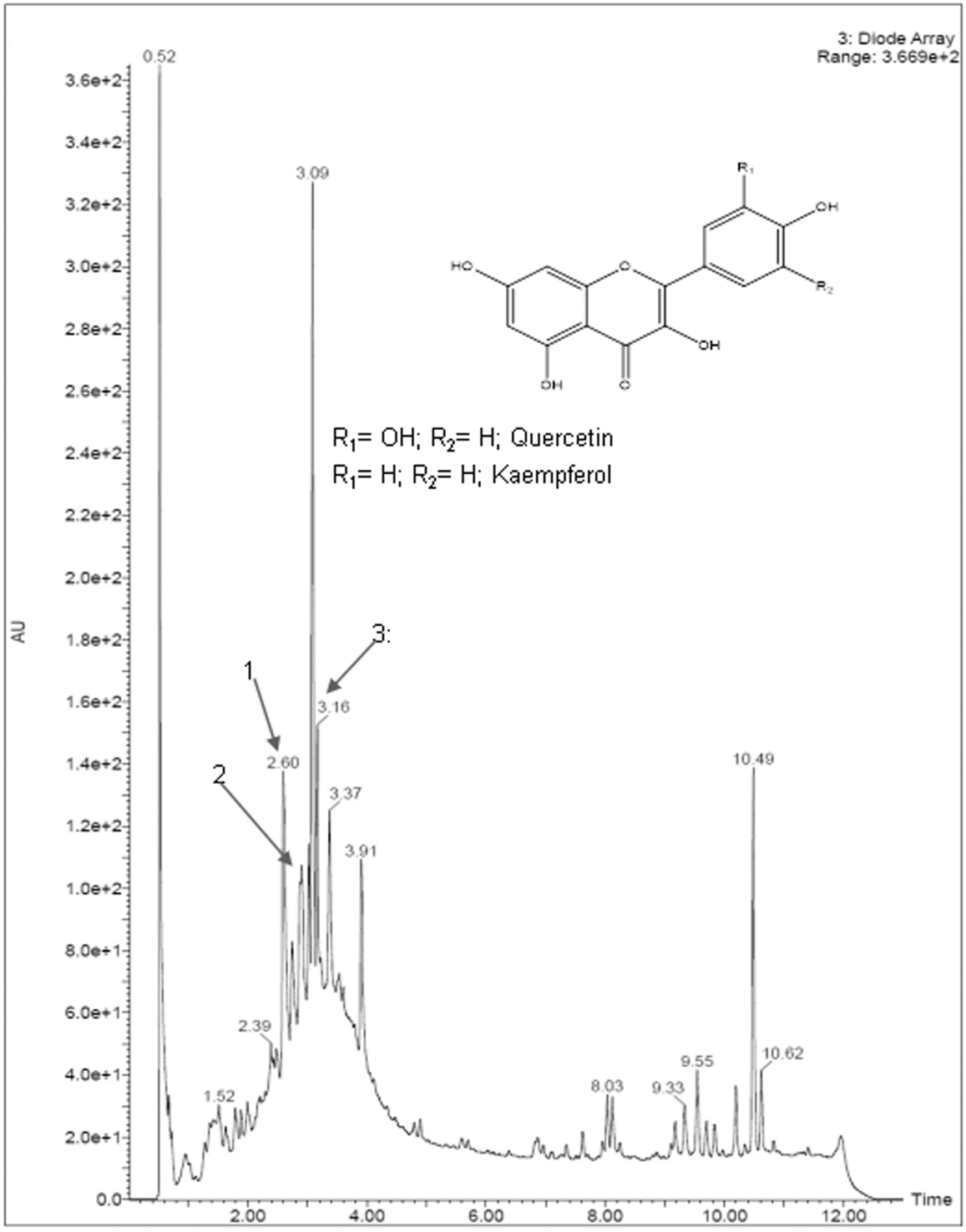

Fig. 1. UPLC–DAD/QTOF-MS base peak chromatograms and structures of: (1) Kaempferol, (2) Quercetin, (3; 4) Myricetin and Derivate of Myricetin for r*Ps*E.

2 μm i.d.), also from Waters Corporation. The UPLC system was coupled to a mass spectrometer (MS) using a Z-spray electrospray ionization (ESI) source. The mobile phase consisted of acetonitrile (A) and water slightly acidified with formic acid (B). Elution followed a linear gradient starting with 95 % of A plus 5 % of B and finishing with 5% of A plus 95% of B in a total time of 11 min. The ionization source conditions were: 5 kV capillary voltage, 320 ℃ source temperature, 5 mA electric current, nitrogen carrier gas, and pressure of 27 psi. Data were processed using the MassLynx v4.1 software, according to the retention time, and compared with data from compounds already identified in the literature by the same technique, independent of the matrix.

2.4. Evaluation of the anti-hypertensive effect of the PsE

2.4.1. Animal model

A model of salt-dependent hypertension (HS12W) that mimics characteristics reported in human sodium-sensitive hypertension [19] was used here. In summary, weaned male Wistar rats with 21 days old were split into two groups: 1) standard salt group (SSD) that received regular sodium (0.27 % Na^+ w/w) powdered chow (NuviLab, Paulínea,

Table 3
Food intake, fluid balance and sodium balance assessment for SSD and HSD rats before and after *Ps*E treatment.

	Before treatment		After treatment			
	SSD	*HSD*	*SSD*	*HSD*		
			Vehicle	Vehicle	*Ps*E 100	*Ps*E 200
Food intake (g/100 gBW/24 h)	4.5 ± 0.17 (n = 47)	5.0 ± 0.11 (n = 53)	4.4 ± 0.34 (n = 17)	4.6 ± 0.24 (n = 23)	4.3 ± 1.46 (n = 15)	4.1 ± 0.32 (n = 12)
Na^+ balance (mmol/100 gBW/ 24 h)	0.14 ± 0.02 (n = 47)	0.29 ± 0.04 (n = 53)	0.05 ± 0.07 (n = 17)	0.08 ± 0.12 (n = 23)	-0.37 ± 0.55 (n = 15)	0.21 ± 0.15 (n = 12)
Water balance (mL/100 gBW/ 24 h)	1.82 ± 0.28 (n = 47)	4.16 ± 0.32[a] (n = 53)	1.5 ± 0.32 (n = 17)	3.36 ± 0.30 (n = 23)	1.37 ± 0.57[b] (n = 15)	2.17 ± 0.38 (n = 12)

[a] Different of SSD group.
[b] Different of HSD group.

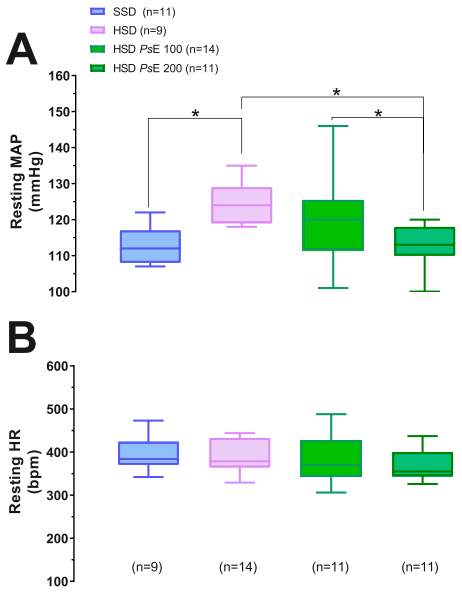

Fig. 2. Resting mean arterial BP (MAP; panel A) and heart rate (HR; panel B) of SSD (control group) and HSD rats treated for four weeks with vehicle (hypertension model) or guava leaf extract formulation (100 mg/kg; PsE 100 or 200 mg/kg; *Ps*E 200) by orogastric gavage. Data was graphed as 95 % of the confidence interval. One-way ANOVA followed by Tukey's post-hoc test; *p < 0.05.

Brazil) for 12 weeks after weaning, and 2) high salt group (HSD) that received high-sodium (0.90 % Na^+ w/w) powdered chow (NuviLab, Paulínea, Brazil) for 12 weeks after weaning as described elsewhere [19]. Both groups had free access to tap water and their respective diets. All animals used in this study were held under controlled temperature (22–24 °C), humidity (40–60 %), and light/dark cycle (12 h:12 h). All experimental procedures were conducted according to the National Council for the Control of Animal Experiments (CONCEA) guidelines and approved by the Institutional Ethics Commission on Animal Use (CEUA-UFOP # 2017-60 and 8880090719). Animal experiments also complied with the ARRIVE guidelines.

2.4.2. Animal treatment with PsE

After 12 weeks under the respective diets, SSD and HSD rats were treated with *Ps*E (100 mg/kg or 200 mg/kg) [3] or vehicle by orogastric gavage, in a volume of 1 mL, once a day, and for 4 more weeks. The *Ps*E concentration in the formulation was adjusted daily according to the rat gain weight but the gavage volume was kept constant over the 4 weeks. The respective diets were kept during the entire treatment period.

2.4.3. Food intake, Na^+, and water balance

At the 12th and 16th weeks after weaning, SSD and HSD rats were housed in metabolic cages (Tecniplast SPA, Buguggiate, Italy). For adaptation, rats were placed in the cages 24 h prior to the start of measurements. After the adaptation period, water intake, and food intakes, and urine volume were gravimetrically measured after a 24 h period. Urine samples were set apart and stored at − 20 °C for sodium and potassium quantification. The water balance was calculated by subtracting the volume of water ingested from the urinary volume and sodium balance was calculated by subtracting sodium excretion from sodium intake. Sodium and potassium concentrations in the urine were measured by flame photometry as described elsewhere [19,20].

2.4.4. Blood pressure measurements in non-anesthetized, freely moving rats

At the end of treatment, SSD and HSD rats were anesthetized with a mixture of ketamine (80 mg/kg, *i.p.*) plus xylazine [21] (7 mg/kg, *i.p.*) after preemptive analgesia with tramadol (12 mg/kg, *s.c.*) [22] for cannulation of the left femoral artery and vein. Procedures are described elsewhere [19,20]. Rats were allowed to recover from surgery for 48 h after and the cardiovascular parameters were monitored by connecting the arterial catheter, previously heparinized, to a pressure transducer (model MLT0699, ADInstruments Pty Ltd, Australia) connected to an amplifier (ML224 Quad Bridge Amp, PowerLab 4 series 35; ADInstruments Pty Ltd, Australia) for approximately 20–30 min, always in the morning period.

2.4.5. Pharmacological evaluation of sympathetic tonus

Hexamethonium (20 mg/kg) [23], a nicotinic cholinergic antagonist that acts as a ganglionic blocker, was given intravenously, and the cardiovascular signals were recorded for 10 min before finishing the BP and HR recording. The magnitude of the fall in BP after hexamethonium was used as an index to evaluate the contribution of the sympathetic tone to the resting BP in all groups.

2.4.6. Time and frequency domain analysis of the heart rate variability

Cardiac interval (CI) and systolic blood pressure (SBP) time series were obtained from 1500 to 2100 consecutive heartbeats (5 min of continuous recording), extracted from a period where animals were quiet. The CI and SBP time series were exported to the CardioSeries software v2.4 (kindly provided by Daniel Penteado Martins Dias) and processed to generate a spectrum of the CI using a fast Fourier transform algorithm as described elsewhere [19,20]. Three oscillatory components of the resulting spectra were then compared between groups: very-low-frequency band (VLF; 0–0.20 Hz), the low-frequency band (LF; 0.20–0.75 Hz) and high-frequency band (HF; 0.75–3.00 Hz), as well as the LF/HF ratio and the root mean square of the successive differences (RMSSD) for time-domain variability assessment of the CI. The power density of HF and LF bands per se and the relationship between them have been widely used as a parameter to describe sympathetic/parasympathetic relationship (autonomic balance) that governs cardiovascular rhythms [24]. Spontaneous baroreflex sensitivity was computed using the sequence method, as described elsewhere [25]. Linear regressions of individual data were performed to assess slopes, and all computed slopes were averaged together to obtain mean spontaneous baroreflex sensitivity (SBrS).

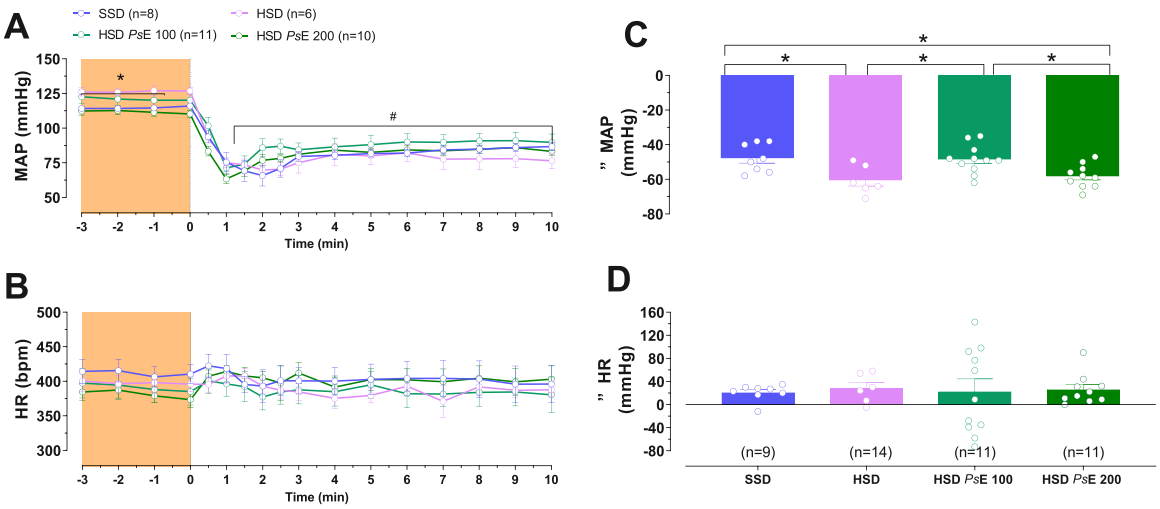

Fig. 3. Cardiovascular effects of intravenous injection of hexamethonium chloride (20 mg/kg) in rats treated for four weeks with vehicle, and guava leaf extract formulation containing 100 mg/kg (*Ps*E 100) or 200 mg/kg (*Ps*E 200) in unanesthetized, freely moving rats (mean arterial pressure – MAP, panel A and heart rate – HR, panel B). Hexamethonium (20 mg/kg) injection is indicated by vertical dashed lines. Maximal changes in mean arterial pressure (MAP) and heart rate (HR) due to hexamethonium were calculated and expressed as ΔMAP (C) and ΔHR (D) respectively. The open circles (panels C and D) represent individual values and bars represent average group data (mean ± SEM). Panels A and B: two-way ANOVA followed by Dunnetś post-hoc test; #different from baseline (before hexamethonium injection). *different from SSD vehicle, $p < 0.05$. Panels C and D: one-way ANOVA followed by Tukey's post-hoc test; $p < 0.05$.

Table 4
Frequency and time domain variability results of the cardiac interval assessed in SSD, HSD and HSD rats treated with *Ps*E.

	SSD vehicle		HSD vehicle		HSD *Ps*E 100		HSD *Ps*E 200	
	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	n
RMSSD (ms)	12.4 ± 4.43	8	8.4 ± 0.97	9	6.8 ± 1.13	8	8.9 ± 1.39	9
VLF (% de total Power)	41.1 ± 4.33	8	54.1 ± 4.75	9	51.6 ± 3.58	8	34.8 ± 5.55[a]	9
LF (% de total Power)	17.2 ± 2.44	8	21.7 ± 5.49	9	12.3 ± 1.63	8	19.5 ± 3.97	9
HF (% de total Power)	41.3 ± 4.09	8	23.8 ± 2.30[b]	9	35.8 ± 3.60	8	45.2 ± 5.94[a]	9
LF/HF ratio	0.4 ± 0.07	8	1.1 ± 0.33	9	0.4 ± 0.08	8	0.5 ± 0.1	9
SBrS (ms/mmHg)	2.3 ± 0.41	8	1.9 ± 0.15	9	1.9 ± 0.48	8	1.9 ± 0.37	9

Very low frequency (VLF), low frequency (LF) and high frequency (HF) bands ranged from 0.0 to 0.2 Hz, 0.2 to 0.75 Hz and 0.75 to 2.50 Hz respectively. RMSSD = root mean square of the successive differences was used to assess time-domain heart rate (HR) variability.

[a] Different of HSD Vehicle. Two-way ANOVA followed by Tukey's post-hoc test.

[b] Different of SSD group.

2.4.7. Cerebrospinal fluid (CSF) and blood sampling

Twelve hours after the end of the cardiovascular measurement experiments, plasma and CSF were sampled from fasted animals. In ketamine/xylazine anesthetized rats, CSF sampling (80–100 μL) was carried out through a 30 G needle inserted into the Cisterna Manga with the aid of a Stoelting stereotaxic Instrument (Stoelting, Illinois, USA). Blood sampling was carried out by cardiac puncture, after euthanasia with anesthetic overdose. In a group of rats, without any surgical intervention, blood sampling was also carried out by cardiac puncture. Plasma samples were set apart, frozen in liquid nitrogen and stored at − 80 ℃ for cytokines levels determination. Na^+ and K^+ concentrations were measured by flame photometry technique as described elsewhere [19, 20] using a flame photometer (MicroNal B462, Tecnal Equipamentos para Laboratórios Ltda., Piracicaba, Brazil).

2.4.8. Plasma cytokines

Plasma concentrations of the cytokines IL-10, IL-17A, and TNF-α were measured according to the commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, BT Lab, China for IL-10 and IL-17A; PeproTech, London, UK for TNF-α) following the manufacturers' instructions. In addition, the ratio TNF/IL-10 was used to index systemic inflammation [26].

2.4.9. Biopotential recording of the sympathetic nerve activity

Here, the effects of a single dose of *Ps*E on sympathetic nerve activity were evaluated in untreated rats under HSD or SSD diets for 16 weeks. Rats were deeply anesthetized with isoflurane (2.0–3.0 % in oxygen 100 %; Cristália, São Paulo, Brazil) [27]. The femoral artery and vein were cannulated with polyethylene cannulas (from PE-50 connected to PE-10; Clay Adams, Parsippany, NJ, USA) for blood pressure recording and drug administration, respectively. After cannulation, anesthesia was switched to urethane (1400 mg/kg; Sigma, SP, Brazil) given intravenously [28]. Next, a stainless-steel cannula was implanted in the animal's trachea to improve spontaneous ventilation. The lumbar sympathetic nerve (LSN) was located and dissected through lateral and retroperitoneal access, removing adjacent tissues. The nerve bundle was perpendicularly and carefully suspended from adjacent tissue on a bipolar electrode constructed with two stainless steel wires (0.127 mm, A-M Systems Inc., Carlsborg, WA; USA). Surrounding fluid was removed and a self-curing polyvinylsiloxane silicone resin (Kwik-Cast, WPI, Sarasota) was quickly applied at the site to involve and isolate the nerve bundle and electrode from the rest of the tissues and extracellular fluids. Signal quality was assessed by a signal/noise ratio higher than 3 times the noise level. The sympathetic signal was amplified 10,000 times and filtered in a 100–1000 Hz bandwidth by a DP-311 differential amplifier (Warner Instruments, USA). Sympathetic and BP signals were digitized using a computerized biological signal recording system (PowerLab 4 series 35; ADInstruments Pty Ltd, Australia) at 10,000 Hz and 1000 Hz (temporal resolution) in a digitizing window of 1000 mV and 20 mV (special resolution), respectively. Raw lumbar sympathetic nerve activity (LSNA) signal was further processed in the LabChart 8.1 software for Windows (PowerLab 4 series 35, ADInstruments, Pty Ltd, Australia)

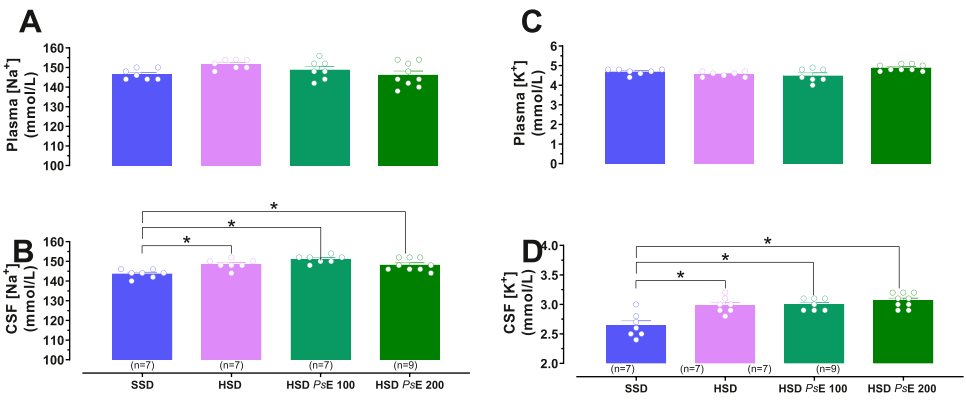

Fig. 4. Cerebrospinal fluid (CSF) and plasma concentration of sodium and potassium. Measurements were performed in samples drawn from HSD and SSD rats on the 16th weeks after wearing, after 4 weeks of intragastric administration of guava leaf extract formulation containing 100 mg/kg (*Ps*E 100), 200 mg/kg (*Ps*E 200) or vehicle (distilled water) through orogastric gavage. The open circles represent individual values and bars represent average group data (mean ± SEM). One-way ANOVA followed by Tukey's post hoc test; *$p < 0.05$.

in order to rectify and integrate (0.1 s time window) the signal to generate the integrated lumbar sympathetic nerve signal, which was quantified.

After completing the instrumentation, animals were allowed to rest for 15–25 min. Then, baroreflex was evoked with intravenous injection of phenylephrine (40 μg/kg) [29] and sodium nitroprusside (100 μg/kg) [30]. The baroreflex reactivity index of the LSNA was assessed by the ratio between the maximal ∫LSNA change as a function of the maximal mean arterial pressure change. After baroreflex evaluation, baseline recordings started.

Before baseline recording, a polyurethane tube (⌀ 2.6 mm; Mark Med, Brazil) was inserted into the stomach through the oral cavity and esophagus to intragastric administration (1 mL) of *Ps*E (200 mg/kg) or vehicle. BP and LSNA were recorded for 2 h after *Ps*E or vehicle administration. Rats were euthanized with urethane overdose and the small intestine (duodenum and jejunum) was inspected *postmortem* for visual confirmation of stomach extract emptying into the intestine. Only rats with the presence of *Ps*E in the intestine were considered for data analysis.

2.5. Statistical analysis

Data were expressed as mean ± standard error of the mean. The comparisons between the groups were performed by analysis of variance (ANOVA) one or two-way (when considering two factors: diet type and time after oral administration of the extract) when applicable. Tukey's post-test was used for multiple comparisons between pairs of means after one ANOVA detected differences in variances between groups. Dunnett's post-test was used for multiple comparisons between pairs of means after two ANOVA detected differences in variances between groups over time. All data were analyzed statistically using the GraphPad Prism 9.0 software for Windows (GraphPad Software, San Diego California, USA). Differences between pairs of means were considered significant when the probability of type I error was less than 5 % ($p < 0.05$).

3. Results

3.1. Extract characterization

3.1.1. Phenolic compounds, total tannins, flavonoids quantification

The processed yield of the extract was 7 %. The total phenolic compounds, flavonoid contents, and condensed tannins of *Ps*E and r*Ps*E are expressed in Table 1.

3.1.2. UPLC – MS analysis

Compounds and structures identified in the r*Ps*E assessed by the UPLC-DAD-ESI-MS technique are descript in Table 2 and the chromatogram from the UPLC is shown in Fig. 1.

3.2. In vivo biological effects

3.2.1. Effects of PsE on sodium intake and water balance

Food intake and Na^+ balance were not different between SSD and HSD rats, even after the treatment with *Ps*E (Table 3). On the other hand, water balance was increased in HSD rats, which was reduced by the *Ps*E (100 mg/kg) oral treatment as assessed in the 4th week (Table 3). The high *Ps*E dose (200 mg/kg) did not significantly reduced water balance of HSD rats (Table 3).

3.2.2. Effect of PsE on blood pressure

The HSD produced an increase in the resting mean arterial BP (MAP) of HSD rats (SSD + vehicle: 113 ± 1.5 mmHg vs. HSD + vehicle: 125 ± 1.9 mmHg; one-way ANOVA; $p < 0.05$). Results also showed that the inferior limit of the confidence interval (95 %) of MAP for HSD rats (120–129 mmHg) was above the superior limit of the confidence interval (95 %) of MAP for SSD rats (109–116 mmHg) suggesting that at least 95 % of the HSD rats were hypertensive when compared with the SSD rats. HSD rats treated with *Ps*E 200 mg/kg had a significant reduction in resting BP when compared with those treated with vehicle (HSD + vehicle: 125 ± 1.9 mmHg vs. HSD + *Ps*E200: 111 ± 2.4 mmHg; $p < 0.05$) as shown in Fig. 2 (panel A). On the other hand, average BP levels of HSD rats treated with *Ps*E100 (100 mg/kg) were not different from the HSD + vehicle or SSD rats (HSD + vehicle: 125 ± 1.9 mmHg vs. HSD + *Ps*E100: 120 ± 2.9 mmHg; one-way ANOVA; $p > 0.05$) as shown in Fig. 2. Resting heart rate was not different among groups (Fig. 2 panel B).

3.2.3. Pharmacological evaluation of sympathetic tonus

The intravenous injection of hexamethonium hydrochloride (20 mg/kg) was used as a pharmacological approach to assessing sympathetic tonus in freely moving rats. Fig. 3C shows a greater fall in BP of HS rats (HSD + vehicle: − 61 ± 3 mmHg) when compared to SSD rats (SSD + vehicle: − 48 ± 3 mmHg; one-way ANOVA; $p < 0.05$). Only the treatment with *Ps*E 100 mg/kg was able to attenuate the fall in BP in HSD rats when compared to the vehicle control group (HSD + vehicle: − 61 ± 3 mmHg vs. HSD + *Ps*E 100: − 48 ± 2 mmHg; one-way ANOVA; $p > 0.05$; Fig. 3A and C), but not the *Ps*E 200 mg/kg dose (HSD + vehicle: − 61 ± 3 mmHg vs. HSD + *Ps*E 200: − 58 ± 2 mmHg; one-way ANOVA; $p < 0.05$) as shown in Fig. 3C. Heart rate responses to hexamethonium injection were not different among groups (Fig. 3B and D).

3.2.4. Time and frequency domain variability of the cardiac interval and spontaneous baroreflex sensitivity

The HSD, as well as the treatment with both *Ps*E doses, did not affect cardiac interval time-domain variability (RMSSD), as shown in Table 4. Also, the percentage of power densities of the VLF and LF oscillatory components of the CI spectra were not affected by the diet or by the *Ps*E

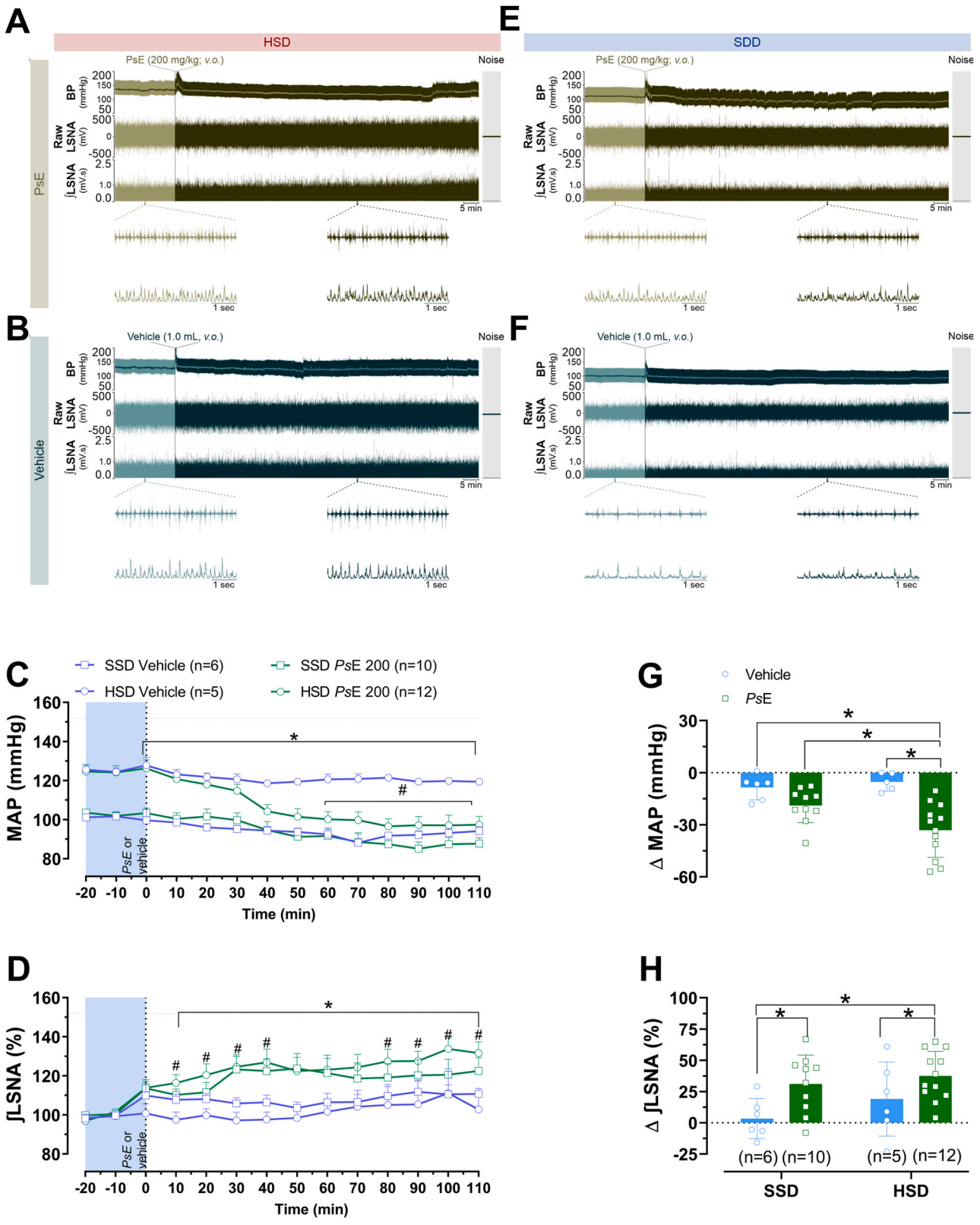

Fig. 5. Blood pressure (BP) and lumbar sympathetic nerve activity (LSNA) effect of intragastric administration of a single dose of *Ps*E (200 mg/kg) or vehicle in untreated, anesthetized SSD and HSD rats. Panels A, B, E and F show trace recordings of BP and LSNA (raw and integrated signal) over 130 min. Dashed lines indicate the moment of the intragastric administration of the *Ps*E (200 mg/kg) or vehicle administration. Maximal changes in mean arterial pressure (MAP) and lumbar sympathetic nervous activity (LSNA) due to *Ps*E were calculated and expressed as ΔMAP (panel G) and Δ ∫LSNA (panel H), respectively. For panels G and H, scattered squares and circles represent individual values and bars represent average group data (mean ± SEM). Panels C and D: two-way ANOVA followed by Dunnet's post-hoc test; $p < 0.05$, #different from baseline (before *Ps*E administration); *different from SSD vehicle. Panels G and H: two-way ANOVA followed by Tukey's post-hoc test; $p < 0.05$.

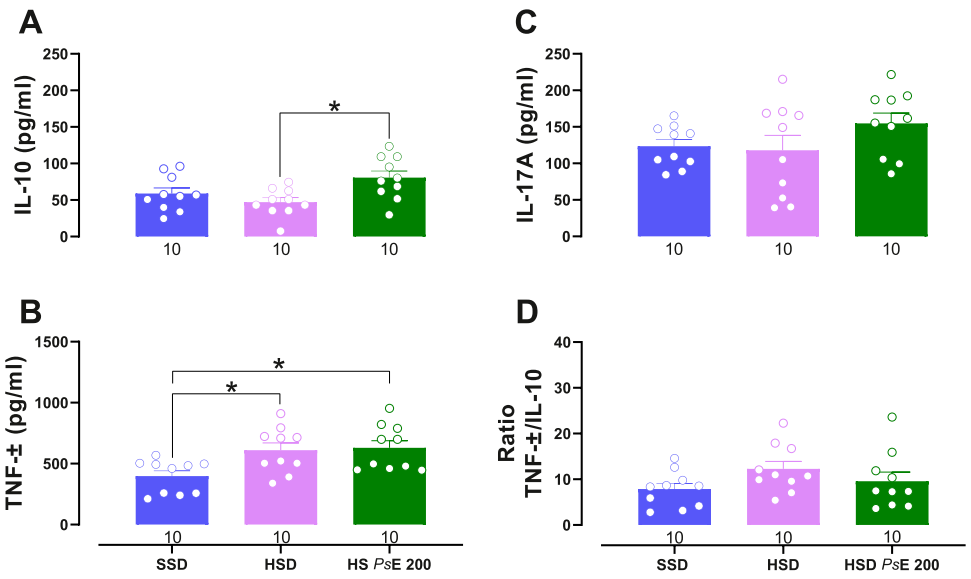

Fig. 6. Plasma concentrations of IL-10 (A), TNF-α (B) and IL-17A (C), and TNF-α/ IL-10 concentration ratio for control (SSD), model (HSD-vehicle) and *Ps*E treated (HS-treated) rats. Measurements were performed in plasma samples drawn from HSD and SSD rats after the 4 weeks of intragastric administration of *Ps*E 200 mg/kg or vehicle through orogastric gavage. Scattered squares and circles represent individual values and bars represent average group data (mean ± SEM). One-way ANOVA followed by Tukey's post-hoc test; *$p < 0.05$.

treatment (Table 4). However, a high-sodium diet diminished the power density of the HF oscillatory component of the CI spectra. Such effect of the high-sodium diet was abrogated by the *Ps*E (200 mg/kg) treatment, which reestablished the HF power density of HSD rats (Table 4). In this study, the LF/HF ratio was not different among groups, nor the spontaneous baroreflex sensitivity (Table 4).

3.2.5. Effects of PsE on Na^+ and K^+ concentration in the plasma and CSF

The four weeks treatment with both doses of *Ps*E did not reverse the increase in [Na^+] in the CSF of HSD rats, as shown in Fig. 4B. The high-sodium diet also increased [K^+] in the CSF of HSD rats, which was not affected by the *Ps*E treatment (Fig. 4D).

3.2.6. Effects of the acute administration of PsE on BP and LSNA

The acute administration of *Ps*E (200 mg/kg) in non-treated rats through an orogastric probe elicited a long-lasting fall in BP accompanied by an increase in LSNA of both, HSD and SSD urethane-anesthetized rats (Fig. 5C and D). The fall in BP was greater in HSD rats when compared to SSD rats (SSD + *Ps*E 200: − 18.9 ± 3.1 mmHg vs. HSD + *Ps*E 200: − 33.1 ± 4.4 mmHg; two-way ANOVA; $p < 0.05$). The *Ps*E-elicited fall in BP was accompanied by an increase in LSNA that was not different between groups (SSD + *Ps*E 200: 31 ± 7 % vs. HSD + *Ps*E 200: 37 ± 6 %; two-way ANOVA; $p > 0.05$) as shown in Fig. 5H. Therefore, the increase in sympathetic drive in HSD rats was unproportionable compared to SSD rats. Baroreflex reactivity index assessment showed that high sodium diet for 16 weeks was not effective in changing the baroreflex control of ∫LSNA when baroreflex was tested with phenylephrine (SSD: − 0.04 ± 0.005 %/mmHg; HSD: − 0.04 ± 0.007 %/mmHg) or sodium nitroprusside (SSD: − 0.97 ± 0.19 %/mmHg; HSD: − 1.05 ± 0.18 %/mmHg).

3.2.7. Effects of the PsE on IL-10, IL-17A and TNF plasma concentrations

HSD rats treated with *Ps*E (200 mg/kg) had a 70 % increase in the plasma IL-10 concentrations in relation to vehicle-treated HSD rats (Fig. 6A). Regarding TNF and IL-17 plasma concentrations, HSD rat showed a 53 % increase in the plasma TNF-α concentrations in relation to SSD rats, which was not decreased by the treatment with *Ps*E (Fig. 6B). Also, no significant changes were detected in the TNF/IL-10 ratio (Fig. 6D). Plasma concentrations of IL-17 in HSD + vehicle and HSD + *Ps*E200 were not different of those found in SSD rats (Fig. 6C).

4. Discussion and conclusion

In this study, we showed that the antihypertensive effects of the *Ps*E seems to rely, at least in part, on a neurogenic component with the involvement of the sympathetic nervous system mechanisms regulating BP levels. In addition, the administration of the guava leaves extract also increased the plasma concentration of the anti-inflammatory cytokine IL-10 in HSD rats.

The chemical analysis showed that the raw dried extract displayed contents of 17.1 % for total phenolic compounds and 1.2 % for flavonoids. As for the total phenolic compounds, the content in the r*Ps*E falls within the range reported in the literature when either, water or ethanol, are used as extracting solvents [34–38]. However, the flavonoids content in the ethanolic extract used here was smaller than the flavonoid content found in extracts obtained by other authors [36]. Also, the tannins content in was lower in the guava leaf extract used in this study then reported in other studies [39,40]. When tannins content in the diet is higher than 1 %, it may impair nutrient metabolism [41], what exclude nutritional problems related to the treatment and that could affect the result found here. Another important finding here is the lower content of phenolic compounds in the *PsE* compared to the r*PsE*, did not compromise the effectiveness of the guava extract in producing its anti-hypertensive effects in HSD rats. The UPLC-DAD-MS analysis allowed us to identify four compounds: compound 1 was proposed as kaempferol (flavonol), according to the peak [M– H]$^-$ at *m/z* 286 [31]; compound 2 was proposed as quercetin (flavonol), by the peak [M– H]$^-$ at *m/z* 301 [42]; compound 3 that was proposed as myricetin (flavonol), by the peak [M– H]$^-$ at *m/z* 317 [33]; and compound 4 was proposed as derivate of myricetin, by the peak [M– H]$^-$ at *m/z* 317 [43]. All these compounds were previously identified in *P. guajava* leaves extracts and, therefore, the molecules identified in the ethanolic guava extract used in this study are already commonly known as constituents of *P. guajava* L.

The chronic treatment of HSD rats with 200 mg/kg of *Ps*E was effective in lowering BP to the normal range, but the 100 mg/kg was not. Sodium and water balance was not affected by the treatment with 200 mg/kg of *Ps*E, indicating that the anti-hypertensive effect could not be associated with diuretic effect. Previous studies showed that guava leaf extract is an effective anti-hypertensive in Dahl-salt sensitive hypertensive rats, since the intravenous administration of aqueous guava leaf extract (50–800 mg/kg) elicited a significant reduction the blood pressure [3]. The effect was not associated with negative chronotropic activity in the heart because the hypotensive effect was not affected by systemic administration of atropine [3]. According to the Ojewole

findings, guava extract also inhibited or even abrogated the pressor effect of *i.v.* administration of noradrenaline [3], suggesting that the vascular sympathetic component or adrenergic-activated pressor signaling mechanisms on smooth muscle cells could be involved with the anti-hypertensive effects of guava extract. Data in the literature shows that guava leaf extracts reduce contractility and myogenic contractile amplitude of rat́s portal vein rings as well as the contractile responses induced by noradrenaline and norepinephrine plus serotonin of aortic rings [44–46]. The remaining question relies on: would be vascular contractile mechanism the only one by which guava extract could produce anti-hypertensive effects? As for the toxicity of the guava extract, previous studies reported a LD_{50} value of 1458 ± 63 mg/kg for mice [3], far above the doses used in this study.

The salt-induced hypertension model used in this study produced a stage 1 hypertension related to neurogenic mechanisms as previously showed by our group [19]. Therefore, we hypothesized that a neurogenic mechanism could also be involved with the anti-hypertensive effects of the *Ps*E in HSD rats. The fall in blood pressure after ganglionic blockade with hexamethonium has been used as an index of neurogenic pressor effect in many experimental studies [19,20,23]. Indeed, the increased neurogenic pressor activity found in HSD rats was restored to normal by the 100 mg/kg of *Ps*E, but not by the 200 mg/kg dose. Despite the discrepancy in the effects of both doses on BP and neurogenic pressor activity, it seems clear that the guava extract at 100 mg/kg dose can affect autonomic mechanisms regulating blood pressure in conscious HSD rats when neurogenic pressor effect is used as an index. On the other hand, the results from the spectral analysis of the cardiac interval, which has also been used to index autonomic balance, further support the idea that guava leaf extract may influence autonomic control of BP since the HF oscillatory component fo the spectra was restored to normal by the 200 mg/kg dose. One of the hypotheses that may explain why 200 mg/kg of *Ps*E did not reduce the neurogenic pressor activity index in HSD rats, but reduced BP levels, may involve a direct action of the *Ps*E on vascular motor tonus. We speculate that high doses of *Ps*E may lead to vascular-dependent hypotension, somehow compensated by a baroreflex-mediated increase in sympathetic drive. When the sympathetic outflow was interrupted by the hexamethonium, the fall would be more intense, resulting in a greater neurogenic pressor activity index. In fact, the results from direct measurement of the sympathetic nerve activity showed that oral administration of 200 mg/kg of *Ps*E resulted in an increase of the sympathetic drive for both groups, SSD and HSD.

Once antihypertensive effect of the *Ps*E was detected only for the 200 mg/kg dose, sympathetic drive and immune signaling assessments were conducted only in animals from the group receiving that dose. When it comes to direct assessment of the sympathetic activity by direct measurement of nerve biopotential, our data have shown that oral administration of *Ps*E in anesthetized rats increased the LSNA compared to animals that received only vehicle. Remarkably, the increase in LSNA was similar in SSD and HSD rats. However, the fall in BP produced by the *Ps*E administration was greater in HSD rats, which raised an important caveat: a greater fall in BP, as observed in HSD rats, should, at least in part, produce a greater increase in the sympathetic drive due to baroreflex unloading since baroreflex sensitivity was not different between SSD and HSD rats. The fact that LSNA did not increase as expected in HSD rats relative to the fall in BP may indicate that compounds present in the guava extract could attenuate the increase in the sympathetic drive. In such a case, this effect would further contribute to the drop in BP, in addition to the possible direct effect of guava extract on vascular motor tonus. Along with the neurogenic pressor activity evaluation and with the spectral analysis of the cardiac interval, the direct sympathetic nerve activity data allows us to stand for the hypothesis that part of the anti-hypertensive effects of *Ps*E may involve autonomic mechanisms regulating blood pressure in HSD rats. Some limitations must be considered when interpreting the results such as the background effects of the urethane anesthesia and the differences in *Ps*E emptying from stomach to intestine and consequent rates of extract compounds absorption.

Over the past decades, several findings have indicated that an increase in CSF sodium content is, somehow, associated with the development of high BP [19,20,47,48–51]. It is still under debate whether CSF high-sodium content is, indeed, a cause for autonomic dysfunction that leads to neurogenic hypertension. However, its correlation with high BP might constitute an important assessment of the *Ps*E treatment effectiveness on central mechanisms governing the control of BP, especially when the subjects are under a high-sodium intake regimen. The effects of *Ps*E treatment on BP did not involve a reduction in CSF sodium and potassium concentration of HSD rats, indicating that diet-induced changes in CSF sodium and potassium content are either not involved with high-blood pressure development or are not a target for the *Ps*E bioactive components.

Much evidence has supported the hypothesis that chronic and low-grade inflammation may be involved with the mechanisms leading to hypertension, especially in the sodium-dependent hypertension [10]. On the other hand, many studies indicate that the anti-inflammatory cytokine IL-10 has also anti-hipertensive proprieties through mechanisms associated with the angiotensin II signaling [52,53]. Furthermore, *P. guajava* extracts have shown anti-inflammatory properties through mechanisms involving increased production of the anti-inflammatory cytokine IL-10. Kumar and cols. (2020) showed that hydroalcoholic extract of *P. guajava* leaves increased tissue (liver) levels of IL-10 in mice exposed to radiation, suggesting that *P. guajava* have anti-inflammatory properties by inducing production of IL-10 [54]. In our study, the treatment with PsE increased the plasma concentration of IL-10 and, as suggested by previous studies, may suggest that the anti-hypertensive effects of the *Ps*E could involve an IL-10 signaling pathway. Data in the literature has indicated that TNF and IL-17 signaling pathways are involved with BP control and hypertension development, especially the sodium-dependent hypertension, through mechanisms associated with neuroinflammation and immune signaling in the central nervous system [11,55,56]. However, data in this study doesnt confirm the involvement of circulating IL-17 or TNF in our hypertension model. Also, the treatment with *Ps*E (200 mg/kg) had no significant effect on the circulating IL-17 concentration, suggesting that the anti-hypertensive effects of *Ps*E also doesnt seem to be directly related to circulating IL-17.

In conclusion, our data strongly suggest that guava extract may act as an effective anti-hypertensive agent through the autonomic control of the cardiovascular system and that part of the mechanism could also involve the overproduction of the IL-10. Moreover, our data provide evidence in favor of the use of medicines based on guava leaves extract as a potential (or promising) adjuvant for the treatment of hypertension.

CRediT authorship contribution statement

Daiane Cristina de Assis Braga: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Paula Magalhães Gomes:** Investigation. **Marcos Adriano Carlos Batista**: Investigation. **Jaqueline Aparecida de Souza**: Investigation**. Juliana Cristina Santos Almeida Bastos**: Investigation. **Rosana Gonçalves Rodrigues-das-Dôres:** Conceptualization, Investigation. **Andreia Carvalho Alzamora:** Resources, Writing – review & editing. **Gustavo Henrique Bianco de Souza:** Resources, Writing – review & editing. **Sandra Aparecida Lima de Moura**: Writing – review & editing. **André Talvani:** Formal analysis, Writing – review & editing. **Vagner Roberto Antunes**: Writing – review & editing. **Leonardo Máximo Cardoso**: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

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Conflict of interest statement

No conflicts of interest, financial or otherwise, are declared by the authors.

Data Availability

The data supporting the findings reported here are available from the corresponding author, upon reasonable request.

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