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Preclinical investigation of the cardiovascular actions induced by aqueous extract of *Pimpinella anisum* L. seeds in rats

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ABSTRACT

Ethnopharmacology relevance: *Pimpinella anisum* is used in traditional medicine because of its pharmacological properties which include cardiovascular action. However, no scientific information supports this use.

Aim of the study: This study investigated the effects of *Pimpinella* on arterial blood pressure (BP) and its pharmacological mechanism of action.

Material and methods: *Pimpinella* seeds were extracted with water, concentrated and freeze-dried yielding the aqueous extract (AE). A non-invasive BP assessment method was used (via the caudal artery) on Wistar, Wistar Kyoto, SHRs and rats that were submitted to high intake of dietary salt. Direct BP and heart rate were evaluated in Wistar rats in the absence or presence of atropine, L-NAME and angiotensin II. Spontaneous diuresis and the effect of AE on depolarized portal vein of Wistar rats was also examined.

Results: The data revealed that AE reduced BP in all groups evaluated and its effects were not due to diuretic, sympatholytic or parasympathomimetic actions. Additionally, it was shown that AE does not act as an angiotensin receptor blocker and does not induce hypotension by reducing vascular resistance induced by oxide nitric. In the depolarized portal vein, AE inhibited calcium influx, which indicates that AE acts as calcium channel blocker.

Conclusion: This study validates the cardiovascular actions of *Pimpinella* and characterizes the hypotensive effects of *Pimpinella* that are related to the blockade of calcium channels.

1. Introduction

Hypertension is a multifactorial disease that affects more than one billion people worldwide. Secondary hypertension, a high blood pressure condition caused by another pathology, accounts for around 10% of cases, and essential hypertension, a polygenic and multifactorial disease that is caused by the interaction of genetic determinants and environmental factors accounts for the remaining 90% (Mills et al., 2016; Rossier et al., 2017). Treatment and control of hypertension are critically important for the prevention of consequent cardiovascular and kidney diseases (Mills et al., 2016).

Conventional hypotensive drugs are usually associated with many side effects that adversely affect health and quality of life. Thus, there is a need to find products that have high efficacy and minimal adverse effects (Sultana and Asif, 2017; Tabassum and Ahmad, 2011). Over the last three decades, there have been concerted research efforts focused on plants as a possible sources of bioactive products with therapeutic values. 75–80% of the world population uses herbal medicines in primary health care. These uses are prevalent in low- and middle-income countries because, in many cases, they are well tolerated by the human body and have less frequent side effects than conventional medicines (Sultana and Asif, 2017; Tabassum and Ahmad, 2011).

One of the plants that is described as having hypotensive properties and is indicated by Brazilian traditional medicine for its ability to prevent and/or treat hypertension is *Pimpinella anisum (Apiacea)* (Teixeira et al., 2017; Oliveira and Araújo, 2007; Lopes et al., 2010), an annual herb and grassy plant with white flowers and very aromatic yellow seeds. Chemical analyses have revealed trans-anethole, estragole, γ-himachalene, p-anisaldehyde, and methylchavicol, and also coumarins, scopoletin, umbelliferone, estrols, terpene hydrocarbons, polyenes and polyacetylenes as the major compounds of the plant (Gülcın et al., 2003; Shojaii and Fard, 2012). Pharmacological studies

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Abbreviations

blood pressure BP
aqueous extract AE
spontaneously hypertensive rats SHRs
systolic blood pressure SBP
angiotensin converting enzyme ACE
angiotensin I Angio I
angiotensin II Angio II
nitric oxide NO

performed with *Pimpinella* demonstrated antimicrobial, antifungal, antiviral, antioxidant, muscle relaxant, analgesic, hypoglycemic, hypolipidemic, anticonvulsant and neuroprotective actions, which are in addition to its beneficial effects on dysmenorrhea and menopausal hot flashes (Shojaii and Fard, 2012). No evidence for the possible anxiety-related central action of *Pimpinella* seeds reported in traditional medicine (Gülcın et al., 2003; Tepe and Tepe, 2015) was found in a study by Gamberini et al. (2015) indicating that there is a need for using scientific protocols to assess claims made regarding this plant's therapeutic properties. There is also a need to properly interpret the observed pharmacological effects of this plant on its users. One hypothesis is that the perception of a tranquilizing action can be determined by a lower cerebral blood flow that is induced by hypotension. This response may include the cardiovascular action of the plant that is postulated by traditional medicine, but has not been demonstrated in a scientific study.

The aim of this study is, therefore, to investigate the cardiovascular actions of *Pimpinella anisum* seeds by analysing the effects of the plant on arterial blood pressure in rats. For this, experimental protocols *in vivo* and *ex vivo* were performed to identify any possible hypotensive action and, if present, investigate the pharmacological mechanism(s) responsible for the biological response.

2. Material and methods

2.1. Animals

A total of 89 three-month-old Wistar rats, 15 three-month-old Wistar Kyoto rats and 15 three-month-old spontaneously hypertensive rats (SHRs) that weighed between 220 and 300 g, were used in this study. Animals were housed in groups of five and maintained in a temperature controlled environment (22 ± 2 °C), a 50% humidity level and a 12 h light-dark cycle (lights on at 06h00). Rats had free access to food and water. All procedures were performed in strict accordance with the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA, Brazilian Committee on Animal Research Ethics), and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NHI Publications 80–23).

2.2. Plant material

The dried seeds of *Pimpinella anisum* L. (www.theplantlist.org) were purchased from a commercial source in São Paulo (Brazil) and kept at 8 °C. For confirmation of botanical plant identity, a sample of the seeds were grown in the Instituto Botânico de São Paulo, Secretaria do Meio Ambiente do Estado de São Paulo, Brazil. Experts in Botany of the same Institute were responsible for the botanical identification and providing of the registration (SP 468 443).

The seeds (100 g) were extracted with 1L of distilled water (70 °C, 30min), concentrated and then freeze-dried to produce an aqueous extract (AE) with a 5% yield according to the protocol described by Gamberini et al. (2015).

2.3. Bioassays

2.3.1. Indirect measurement of systolic blood pressure in conscious animals

The systolic blood pressure (SBP) of the Wistar, Wistar Kyoto, SHRs and Wistar rats submitted to high intake of dietary salt was determined using the non-invasive method (Alexander, 1957; Ishii et al., 1980). Initially, the animals were treated daily with distilled water (5 mL/kg, p.o.) and their blood pressure was measured on alternate days using the tail-cuff method. The animals were placed in rat holders with the tail remaining free for the fitting of a pressure cuff coupled to a sphygmomanometer. Prior to taking the measurements, the animals were allowed 5min to adapt to the system to avoid pressure variations (Fregly, 1963). After allowing a few days for blood pressure stabilization, the rats were divided into three groups of five animals each to assess the effect of AE (0.5–2.0 g/kg, p.o.). The aqueous extract was solubilized in distilled water for oral administration (gavage). The first group (control) was given water (5 mL/kg/day, p.o.) throughout the experiment and the second group received AE 0.25 g/kg for 1–2 weeks, and subsequently AE 0.5 g/kg for the same period. The third group received AE 1.0 g/kg for 1–2 weeks, and subsequently AE 2.0 g/kg for the same period. Systolic blood pressure was assessed 30min and 24 h after administration of the water and the AE. The same protocol was used for the Wistar, Wistar Kyoto, SHRs and Wistar rats submitted to high intake of dietary salt. For this last group, normotensive Wistar rats were submitted to an excessive amount of sodium to induce elevated blood pressure. Shortly after weaning, 15 normotensive rats were fed a diet containing excess sodium (Yamamoto et al., 2008). To this end, the chow used for animal feed ("Bio base") with 0.2% sodium content was enriched to contain 8% sodium. In order to determine if the cardiovascular effects were caused by the high intake of dietary salt (sodium), a group (n = 5) that received a balanced diet (0.2% sodium) was maintained throughout the experiment. After one month of consuming the modified diet (8% sodium), blood pressure measurements were taken on alternate days until stable values of systolic blood pressure were obtained. Throughout the experimental period, the weight and consumption of water and feed of the rats was monitored.

2.3.2. Direct measurement of systolic blood pressure in anesthetized animals

After anesthesia (Equitezim 0.4 mL/100 g weight), Wistar rats were fixed in dorsal decubitus on cork plates and the femoral vein (for drug injection) and the carotid artery (to record the systolic, diastolic and mean arterial blood pressure and heart rate) were cannulated (Rees et al., 1989). The parameters used were recorded through a pressure transducer connected to a computerized recording system. After stabilization, increasing doses of AE were injected and variations in the parameters of the animals were recorded for 5min. To investigate the possible mechanisms of action of AE, a non-selective cholinergic antagonist, atropine (1 mg/kg), the nitric oxide synthase inhibitor, L-NAME (20 mg/kg), and angiotensin II (Angio II 0.7 µg/kg) were employed. The aqueous extract and the drugs were solubilized in 0.9% saline solution for intravenous administration.

2.3.3. Quantification of spontaneous diuresis of rats

Wistar rats were kept without access to food or water for 2 h prior to the experiment in individual cages. After this period, and 30min before being placed in the metabolic cages, the animals received water (5 mL/kg - control group) and AE, which was solubilized in distilled water (0.5–2.0 g/kg, p.o.). The spontaneously voided urine was collected for 3 h and the excreted volume of urine was measured (Leander, 1983).

2.3.4. Depolarized portal vein preparations

Depolarized portal vein in 80 mM KCl and no calcium nutritive solution contracted to bath application of $CaCl_2$. The portal veins that were isolated from Wistar rats were placed in a Krebs solution with the composition (in mM): NaCl 119.0, KCl 4.6; $MgCl_2$ 1.2; $NaHCO_3$ 15.0; $CaCl_2$ 1.5; NaH_2PO_4 1.2 and glucose 11.0, gassed continuously with

95% O_2 and 5% CO_2 and maintained at pH 7.4 and 37 °C. After rapid dissection, segments of the portal vein were mounted under a resting tension of 0.5 g in a 3 mL organ bath containing Krebs solution. Isometric contractions of the organ were recorded using a force displacement transducer (Grass FT03) on an "Ugo Basile" recorder. After 60min, the normal Krebs solution was replaced by a Ca-depleted high KCl (80 mM) solution and cumulative concentration-response curves were constructed to $CaCl_2$ (10 µM–100 mM) (Torres et al., 2000) prior to and after 15min exposure to different concentrations of AE that was solubilized in depolarizing Krebs.

2.4. Statistical analysis

Data are expressed as mean ± standard error. Parameters were evaluated using analysis of variance (ANOVA) followed by Tukey's test with a significance threshold of $p < 0.05$.

3. Results

In normotensive Wistar (Fig. 1A) and Wistar Kyoto (Fig. 1B) rats, the SBP of non AE-treated rats did not change during the experimental period. The administration of AE (0.25–2 g/kg/day, p.o.) 30min before the measurement of SBP showed a dose-dependent reduction in pressure values (Fig. 1A and B). In SHRs, SBP values were significantly increased compared to normotensive rats (Fig. 1C). Non-AE-treated rats showed a constant and increased SBP during the experimental period. Similarly to the normotensive rats, the administration of AE (0.25–2 g/kg/day, p.o.) 30min before the measurement of SBP showed a dose-dependent reduction in pressure values (Fig. 1C). The ingestion of excessive amounts of salt (8% sodium) in the diet of rats after weaning led to an increase in SBP of approximately 10% compared to animals that received a balanced diet (0.2% sodium) (Fig. 1D). In both groups, non-AE-treated rats had constant SBP values during the experimental period. In this protocol, AE (0.25–2 g/kg/day, p.o.) was administered to rats submitted to the ingestion of excessive amounts of salt and again, a reduction in SBP was measured, although there was no dose-dependent effect.

Measurements 24 h after administration of the AE revealed that SBP returned to its pretreatment value in all groups evaluated (data not shown).

The recording of direct BP in Wistar rats also confirmed the hypotensive effect of AE and demonstrated that it also induced bradycardia. Administration of 100 mg/kg of AE (n = 6) reduced mean arterial pressure by 43% in relation to the control group (110 ± 7.3 mmHg, n = 12) due to changes in diastolic arterial blood pressure (Fig. 2) and increases in the intervals between heart beats (by 129%) in relation to the control group (0.14 ± 0.02s). Both responses were immediately reversed (Figs. 3 and 4) suggesting the occurrence of physiological mechanisms that control BP, such as the baroreflex. This result indicates that the cardiovascular responses induced by AE are not related to the blockade of adrenergic receptors.

Previous administration of atropine 1 mg/kg (n = 5) (Fig. 3) or L-NAME 20 mg/kg (n = 4) (Fig. 4) did not alter the response to AE, thus indicating that a parasympathomimetic action and the participation of nitric oxide (NO) are not involved in the hypotensive responses to AE. The evaluation of the possible interference of AE in the action of Angio II (0.7 µg/kg, n = 3) revealed that AE was unable to alter the response of this agonist (Fig. 5).

Investigation of the acute effect of AE on spontaneous diuresis of Wistar rats showed that a single administration of AE 0.25 (n = 5); 0.5 (n = 5) and 1 g/kg p.o. (n = 5) by gavage did not change the volume of urine excreted within 3h compared with control animals treated with 5 mL/kg p.o. (n = 5) (Fig. 6).

The responses of AE were tested on rat portal vein preparations depolarized with an 80 mM KCl Ca2 + -free physiological solution. Depolarization of the vascular smooth muscle cells with a high-KCl medium allowed the influx of Ca + 2 through L-type channels and triggered a contractile response. Cumulative addition of CaCl2 (0.01–10 mM) to depolarized rat portal vein preparations (n = 9) caused concentration-related contractions of the smooth musculature with an EC50 of 0.15 mM (95% Confidence limits: 0.12–0.18 mM). Incubation of AE 3 mg/mL (n = 3), 10 mg/mL (n = 3) and 30 mg/mL (n = 3) shifted the concentration-response curves to CaCl2 to the right, 1.4, 6.4 and 12.6-fold, respectively (Fig. 7).

4. Discussion

The data obtained in this study validates (scientifically) the information regarding the cardiovascular actions of *Pimpinella anisum* (Teixeira et al., 2017; Oliveira and Araújo, 2007; Lopes et al., 2010) that have been proposed by traditional medicine. Here we characterized the hypotensive action of the aqueous extract of seeds, the form in which this agent is customarily consumed. In addition, we identified the mechanism of action for the cardiovascular responses caused by this extract.

The assessment of SBP using the non-invasive method via the caudal

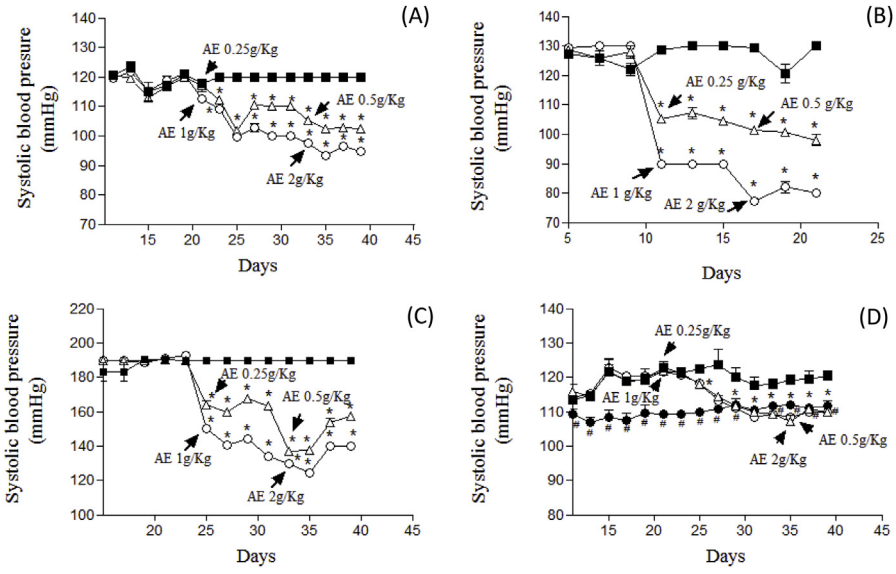


Fig. 1. Systolic blood pressure (SBP) of conscious Wistar rats (A), Wistar Kyoto rats (B), spontaneously hypertensive rats (SHRs) (C) and Wistar rats submitted to a high intake of dietary salt (D). After stabilization of SBP, rats were treated with an aqueous extract of *Pimpinella anisum* seeds AE 0.25–0.5 g/kg/day, p.o. (-Δ-, n = 5) or AE 1–2 g/kg/day, p.o. (-○-, n = 5). The control group (-■-, n = 5) received water 5 mL/kg/day, p.o. throughout the experiment. Measurements of SBP were obtained 30min after administration of AE and water. Each point represents a mean value and the bar indicates S.E.M. * significant difference between control and AE-treated rats ($p < 0.05$).# significant difference between control and rats submitted to balanced diet ($p < 0.05$).

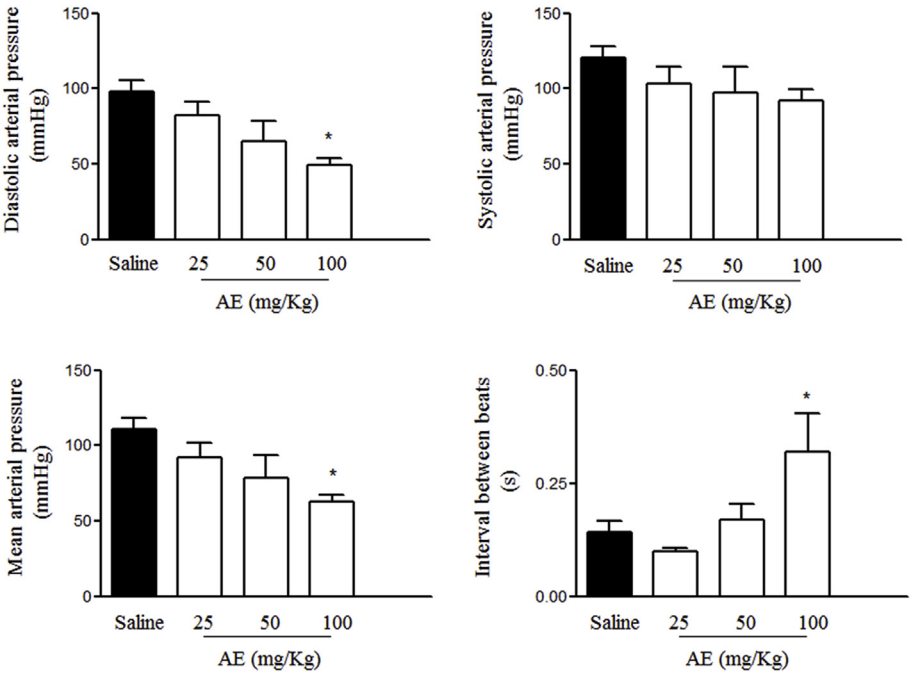


Fig. 2. Acute cardiovascular effect of the aqueous extract of *Pimpinella anisum* seeds (AE) in normotensive anesthetized Wistar rats. Diastolic, systolic, mean arterial blood pressure and the change in heart rate were recorded after the single intravenous injections of vehicle (saline 0.9%, n = 12) or AE 25 mg/kg (n = 3), 50 mg/kg (n = 3) and 100 mg/kg (n = 6). Each column represents a mean value and the bar indicates S.E.M. * different to vehicle ($p < 0.05$).

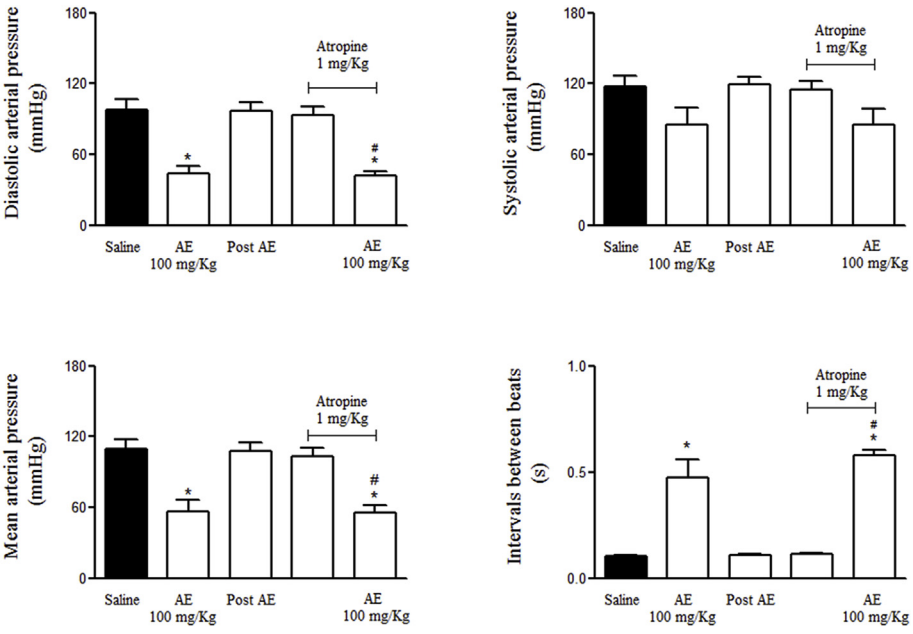


Fig. 3. Effect of the aqueous extract of *Pimpinella anisum* seeds AE 100 mg/kg (n = 4) on the blood pressure and heart rate of Wistar rats anesthetized in the absence and presence of atropine 1 mg/kg (n = 4). Cardiovascular responses of AE were immediately reversed (Post AE) demonstrating the participation of physiological mechanisms responsible for control of blood pressure.* different to vehicle ($p < 0.05$).# different to atropine ($p < 0.05$).

artery showed that administration of AE reduced SBP values in normotensive rats (Wistar and Wistar Kyoto) and in rats with elevated SBP, genetically determined in SHRs (Doris, 2017; Pravenec and Kurtz, 2010) or induced by a high intake of dietary salt (Yamamoto et al., 2008).

A consistent finding in SHRs is elevated total peripheral resistance. This increase in peripheral vascular resistance appears to be largely related to small arteries, arterioles and possibly to precapillary sphincters. Active and structural processes are believed to be responsible for the increased peripheral resistance (Mar et al., 2013; Yan et al., 2004; Fazan et al., 2001). Using the same experimental protocol employed in normotensive animals, it was possible to reduce blood pressure to values close to those recorded in normotensive rats.

Many international agencies have acknowledged the role of diet, in particular sodium intake, on blood pressure levels (Santos et al., 2017). Diets high in salt are recognized as one of the leading risks to cardiovascular health worldwide. These diets result in increased blood pressure in both children and adults (Campbell et al., 2012). It is well established that one of the factors that contributes to the development of hypertension is an imbalance between sodium intake and its renal excretion (Weinberger, 1996). In the present study, the mode by which high blood pressure was induced involved changes in the control of blood pressure by the “renal-body fluid” mechanism, whereby the physiological equilibrium point at which water and salt output is equal to intake was modified. This modification was promoted through a high sodium diet. Subsequently, the effect of AE was determined in animals whose systolic blood pressure was significantly higher than animals that were given a balanced diet. This allowed a measure of the magnitude of the plant's action to be determined for the different mechanisms underlying hypertension. Using this model, the effectiveness of the AE in correcting the pressure values was also confirmed. The administration of AE reduced SBP, although no dose-dependent

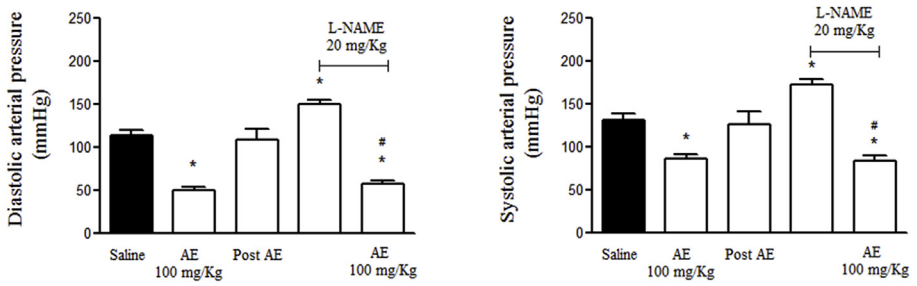
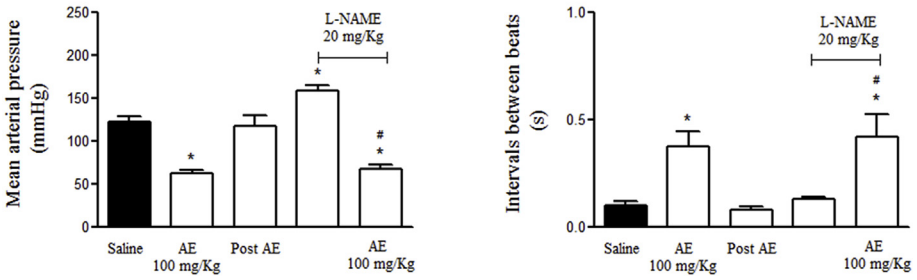


Fig. 4. Effect of the aqueous extract of *Pimpinella anisum* seeds AE 100 mg/kg (n = 4) on blood pressure and heart rate of Wistar rats anesthetized in the absence and presence of L-NAME 20 mg/kg (n = 4). Cardiovascular responses of AE were immediately reversed (Post AE) revealing the physiological mechanisms responsible for the control of blood pressure.* different to vehicle ($p < 0.05$).# different to L-NAME ($p < 0.05$).



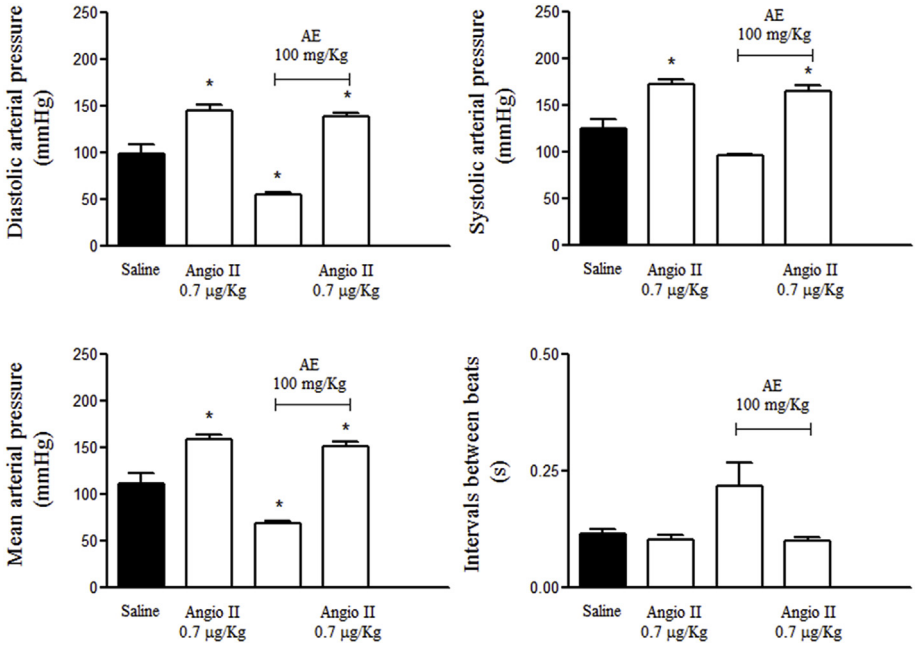


Fig. 5. Effect of angiotensin II (Angio II 0.7 µg/kg, i.v.) on blood pressure and heart rate of Wistar rats anesthetized in the absence (n = 3) and presence of the aqueous extract of *Pimpinella anisum* seeds AE 100 mg/kg (n = 3). * different to vehicle ($p < 0.05$).

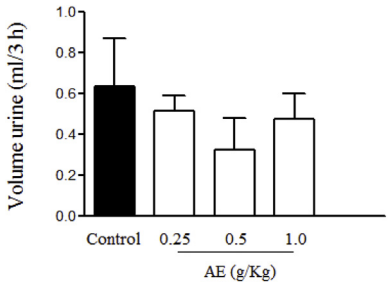


Fig. 6. Spontaneous diuresis of Wistar rats treated with water 5 mL/kg (control group, n = 8) and aqueous extract of *Pimpinella anisum* seeds AE 0.25 g/kg (n = 5), 0.5 g/kg (n = 7) and 1.0 g/kg (n = 8), given by single dose gavage. Each column represents mean value and the bar indicates S.E.M.

relationship was evident.

Arterial hypertension is a highly prevalent disease worldwide that affects young people, adults and the elderly of all ethnicities, gender and social backgrounds. Arterial hypertension is occasionally caused by another specific disease, but in most cases this disorder appears to be linked to heredity and dietary habits (Mills et al., 2016; Rossier et al., 2017; Campbell et al., 2012). When persistently high or uncontrolled, blood pressure can lead to more serious problems such as heart diseases, loss of vision, kidney failure and stroke, all of which are more difficult to treat and have serious consequences. Although the disease is often incurable, it can be properly controlled through drug treatment (Wright et al., 2018).

Several different classes of medications are available to reduce blood pressure. The six main drug classes are thiazide diuretics, beta-

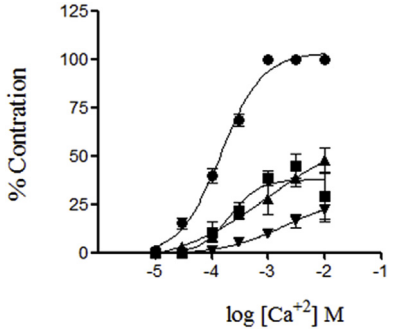


Fig. 7. Effect of the aqueous extract of *Pimpinella anisum* seeds (AE) on contractions evoked by calcium in KCl-depolarized portal vein. Preparations were pre-incubated in calcium-free physiological solution, depolarized in calcium-free KCl-rich solution, and then incubated with increasing calcium concentrations in the absence (-●-, n = 9) or in the presence of AE 3mg/mL (-■-, n = 3), 10 mg/mL (-▲-, n = 3) or 30 mg/mL (-▼-, n = 3). Responses are expressed as a percentage of the maximal contraction that is evoked before the addition of AE. Each point represents the mean value and the bar indicates S.E.M.

blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, calcium channel blockers and alpha blockers (Wright et al., 2018). These different classes of hypotensive drugs have different mechanisms of action. *Thiazides* act on the kidney, blocking the thiazide-sensitive sodium-chloride symporter which causes the reabsorption of sodium (Na^+) and chloride (Cl^-) ions from the distal convoluted tubules to be inhibited. The mechanism of action by which thiazide diuretics lower blood pressure in the long term is uncertain. It may involve effects on the whole body, renal autoregulation, or direct vasodilator actions. *Beta-blockers* are competitive antagonists that block the adrenergic beta-receptors (β1, β2, and β3), thus interfering with cardiac, vascular and renal responses induced by catecholamines. *ACE inhibitors* inhibit the conversion of Angio I to Angio II, and thus decrease the vascular, cardiac and renal actions of Angio II. Besides these decreases, *ACE inhibitors* induce increases in bradykinin because ACE is also responsible for the inactivation of this kinin. *Angiotensin receptor blockers* block the activation of Angio II by AT1 receptors that cause vasodilation, reduced secretion of vasopressin and the production and secretion of aldosterone. *Calcium channel blockers* inhibit calcium ion influx into vascular smooth muscle and myocardial cells. They reduce blood pressure through vasodilation, by reducing the force of contraction of the heart, and by slowing the heart rate. *α1-Adrenergic receptor blockers* inhibit the binding of catecholamines to the α1 receptors on vascular smooth muscle cells inducing vasodilation, which decreases blood pressure (Wright et al., 2018).

Considering these mechanisms of action that are already well-established for the reduction of blood pressure, we proceeded to investigate which mechanism was involved in the cardiovascular actions of AE.

The results obtained in the present study discarded the diuretic, sympatholytic and parasympathomimetic actions of AE. Additionally, it has been shown that AE does not act as an angiotensin receptor blocker, nor does it induce blood pressure reduction by reducing peripheral vascular resistance that is induced by nitric oxide, a potent vasodilator agent synthesized by endothelial cells (Fulton et al., 1999; Liu et al., 2014). However, in depolarized portal vein preparations, AE inhibited the calcium influx, thus demonstrating that it acts as calcium channel blocker.

Ca^{2+} entering the cell through voltage-gated Ca^{2+} channels serves as the second messenger of electrical signaling, initiating many different cellular events (Catterall, 2011). The activation of Ca^{2+} channels in cardiac and vascular smooth muscle cells initiates contraction by increasing cytosolic Ca^{2+} concentration and by activating calcium-dependent calcium release via ryanodine-sensitive Ca^{2+} release channels in the sarcoplasmic reticulum (Catterall, 2011).

In addition to the parameters that distinguish the different types of Ca^{2+} currents, which include the positive voltage-dependence of activation, the large single channel conductance, slow voltage-dependent inactivation, and their regulation by cAMP-dependent protein phosphorylation pathways, different Ca^{2+} channel antagonist drugs specifically inhibit distinct types of Ca^{2+} channels (Godfraind, 2017). The inhibitory effects of calcium channel blockers on cardiac cells in the sinoatrial and atrioventricular nodes are well established. This inhibition results in a slowing of cardiac conduction and contractility, and also causes peripheral vasodilation in vascular smooth muscle cells. These effects contribute to the correction of blood pressure during hypertension (Catterall, 2011; McKeever and Hamilton, 2018).

Based on this information, we hypothesized that the cardiovascular actions of AE (from *Pimpinella*) are related to the block of calcium influx in cardiac and vascular smooth muscle cells. This mechanism would explain the bradycardia and blood pressure reduction found in treated animals.

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