Antihypertensive effect of *Justicia spicigera* in L-NAME-induced hypertensive rats


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Abstract

The cardiovascular disorder of hypertension is one of the main non-communicable diseases worldwide, and is responsible for over 80% of the deaths in low- and middle-income countries. *Justicia spicigera* (muicle) is a plant used in Mexican traditional medicine for the treatment of various disorders, including hypertension. The principle aim of this work was to evaluate the effects on blood pressure of different extracts taken from the aerial parts of the muicle plant *Justicia spicigera* (Acanthaceae). To the best of our knowledge there are no such previous reports. For the purpose of this study, the extracts were applied in hypertensive L-NAME Wistar rats. The results show that hexane and methanol extracts did not lower blood pressure and the aqueous extract only decreased the diastolic blood pressure (from 163/148 ± 6.65/6.64 mmHg to 156/128 ± 11.8/4.51 mmHg) in the hypertensive rats, but not in the normotensive animals. However, the chloroform extract lowered the arterial blood pressure (180/164 ± 1.7/3.2 mmHg) to values similar to those of the normotensive rats (149/133 ± 4.0/3.7 mmHg). The extracts had no effect on the control animals and the vehicles did not modify arterial blood pressure in any of the animals. Finally, the chloroform extract of aerial parts of *J. spicigera* was further analyzed by high performance liquid chromatography (HPLC) to identify and quantify major bioactive flavonoid compounds profile. As revealed by the results, the chloroform extract yielded three major flavonoid compounds, identified as hesperidin, naringenin and kaempferol, metabolites that could be responsible of antihypertensive effects of *J. spicigera*.

KEY WORDS: HYPERTENSION, BLOOD PRESSURE, JUSTICIA SPICIGERA, ALTERNATIVE MEDICINE, FLAVONOIDS
Introduction

High blood pressure, also called hypertension, is a condition that afflicts almost 1 billion people worldwide, and is a leading cause of morbidity and mortality\textsuperscript{28,30}. It also represents a major risk factor for developing other diseases such as endothelial dysfunction, metabolic syndrome, diabetes, congestive heart failure, coronary artery disease, stroke and renal dysfunction\textsuperscript{29}. In fact, in México 30.8\% of individuals that are 20 years of age have hypertension; in other words, 17 million adults. More than both 50\% of men and 60\% of women over 60 years of age have hypertension and epidemiological studies have demonstrated that cardiovascular diseases are the principal cause of death in almost all countries, including Mexico (Norma Oficial Mexicana NOM-030-SSA2-2009, 2012)\textsuperscript{14}. Modern hypertension treatment is costly for many people in developing countries, and such medical expenses represent an additional burden\textsuperscript{20}. This population often relies on the alternative therapy of medicinal plants for the treatment of various disorders.

Hypertension treatment has been shown to prevent cardiovascular diseases, extending and enhancing life, but its inadequate management is widespread\textsuperscript{30}. The World Health Organization estimates that more than 80\% of people use traditional medicine, mainly plants, to treat the primary diseases, and about 85\% of traditional medicine involves the use of plant extracts\textsuperscript{31}. In Mexico, it is a common practice in the indigenous communities and correlates with the diversity of plants and the ethno-medical knowledge of the Mesoamericans. Notwithstanding, on a worldwide level, chemical validation of the pharmacological effects on biomedical practices has only been carried out in approximately 5\% of the vegetable species\textsuperscript{13}.

Muicle (\textit{Justicia spicigera}), or Mexican honeysuckle, is a medicinal plant that belongs to the Acanthaceae family and is used in Mexican traditional medicine for the treatment of various disorders, including hypertension. This family comprises \textit{Justicia}, an important genus in medicine that has species with significant biological activity such as \textit{J. secunda} Vahl, \textit{J. procumbens} Linn, \textit{J. pectoralis} Jacq, \textit{J. simplex} Vahl, and \textit{J. insularis}. All of these species synthesize types of cytotoxic molecules, justicidine A and B have anti-leukemic and analgesic activity and prostadiladine A, B and C produce antidepressive action\textsuperscript{12,17,18}. Muicle is a native Mexican plant that is used as a blood tonic, a stimulant and as anti-dysentery, anti-inflammatory, and antispasmodic agents. It also provides relief in menstrual disorders, anemia, nervousness, insomnia, bronchitis, intestinal disorders that include nausea, diarrhea and vomiting, and it is used to treat cancer and kidney infections. The aerial parts of \textit{Justicia spicigera} are used in Mexican traditional medicine\textsuperscript{1,24}.

\textit{Justicia spicigera} appears to have a broad range of molecules that modify cell function and some studies have reported on its antioxidant activity, total phenolic content, and total flavonoid content in the stem, leaves and flowers. Two types of extracts have been used; aqueous extract and methanol, and the results suggest that muicle is a rich source of antioxidants, which lends support to its use as an anti-inflammatory agent against various free radical–related disorders\textsuperscript{26}.

Therefore, we studied the acute effects of muicle extracts on blood pressure in L-NAME hypertensive rats. Accordingly, we were able to address whether or not the extracts (hexane, chloroform, methanol or aqueous extract) had an effect on blood pressure. The metabolites that could be responsible of antihypertensive effects of \textit{J. spicigera} as well were studied.

Material and Methods

Animals

We divided male Wistar rats (200-250g) into 12 groups of 3-5 rats each. 1 Control, 2 Control + Vehicle (DMSO 0.5\%), 3 Control + Hexane extract, 4 Control + Chloroform extract, 5 Control + Methanol extract, 6 Control + Aqueous extract, 7 L-NAME, 8 L-NAME + Vehicle, 9 L-NAME + Hexane extract, 10 L-NAME + Chloroform extract, 11 L-NAME + Methanol extract and 12 L-NAME + Aqueous extract. All of
them had free access to tap water and food.

**L-NAME rats**

Male Wistar rats were used and maintained under standard laboratory conditions with free access to food and water. All animal procedures were conducted in accordance with the Mexican Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, México) and were approved by the Institutional Committee of the Universidad Michoacana de San Nicolás de Hidalgo, for the Use and Care of Animals.

Experimental hypertension was induced by chronic administration of N-nitro-L-arginine methyl ester (L-NAME) in the drinking water (75 mg/kg/day) over a period of 18 days. All the extracts were administrated by orally through oropharyngeal probe using a single dose (150 mg/kg/w) and the blood pressure was measured 3 h after. Blood pressure was measured by tail cuff plethysmography using an LE 5007 automatic blood pressure recorder (Letica, PanLab, Barcelona Spain).

**Plant material and extraction**

*Justicia spicigera* aerial parts were collected in October 2009 in El Letrero, Michoacán, México. Voucher specimen (No. HFB11JS) has been deposited in the Herbarium of the Biology Faculty, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México. Briefly, the dried plant material was pulverized and extracts were prepared by successive maceration with hexane, chloroform, methanol and aqueous extract (5 days at room temperature), adding 10 mL of solvent per 1 g of plant material. After filtration, the extracts were evaporated to dryness in rotary evaporator with reduced pressure at 40°C and dissolved in DMSO (0.5%) to a final concentration of 150 mg/mL. The extracts were stored at 4°C until use. Table 1 shows yield quantities of different treatments.

**HPLC analysis**

An aliquot (1 mL) of chloroform extract was filtered through a small column of C18 silica (55-105 mm, 0.50 g) and the column eluted with 9 mL of methanol. The volume of the eluent was completed to 10 mL to obtain a concentration of 1 mg/mL, 20 µL of this solution was injected to HPLC analysis.

The analysis was achieved by RP-HPLC (Agilent Technologies Model 1200) (Palo Alto, CA, USA) apparatus equipped with an autosampler G1329C, a pump G1312A, a diode array detector G1315B and an analytical C18 column Zorbax Eclipse XDB (150 X 4.6 mm, 5 µm i.d.), at constant temperature (25°C) and at 1 mL/min flow rate. The mobile phase consisted of methanol/acetonitrile/water (40:15:45) (v/v) with acetic acid 1% under isocratic conditions. The chromatograms were recorded at 282 nm to hesperidin, quercetin and naringenin\(^3\) and at 368 nm to kaempferol\(^4\).

The standards of hesperidin, kaempferol, naringenin and quercetin were purchased from Sigma-Aldrich (Toluca, México). All HPLC grade solvents were purchased from Merck (México City, México). The calibration curves were constructed for each flavonoid in the range of sample quantity 0.01 – 10 µg.

**Statistical analysis**

Statistical analysis and graphs were made using the software Graph Pad Prism 5.01 software (San Diego, California, USA). The results were arithmetic averages of individual values assigned to the standard error of the mean (m ± SE). Differences were considered to be statistically significant when p <0.05.

**Results and Discussion**

L-NAME ingestion caused a large rise in the resting mean arterial pressure (MAP) (175 ± 5 mm Hg) and heart rate (HR) (440 ± 17 beats per minute) compared with the untreated control rats (resting MAP: 112 ± 2 mm Hg and HR: 345 ± 8 beats per minute) when the dose was high (100mg/kg/day)\(^7\). Tail-cuff pressure rose progressively in the L-NAME rats (60-70 mg-100 ml\(^{-1}\)), reaching 168 ± 7 mmHg by treatment day 30, compared with 108 ± 5 mmHg in the untreated controls (p<0.05)\(^9\). The hypertension
model of chronic L-NAME administration is characterized by endothelial dysfunction, thereby causing vasoconstriction. It also affects the glomerular filtration rate and activation of the renin angiotensin system, which are features that are also described in primary hypertension in humans. Furthermore, this model is characterized by the development of cardiac hypertrophy and renal damage. In the present study using a single dose (150 mg/kg/w), we evaluated the antihypertensive effect of Justicia spicigera (muicle) in a rat model of hypertension, L-NAME rats. There are no studies about the toxicity of muicle, even though, the limited dose generally accepted is 1000 mg/kg in rodents (National Centre for Replacement, Refinement and Reduction of Animals in Research), but previous reported the use of less than 500 mg/kg to evaluate antihypertensive effects. Also, it was decided to measure blood pressure 3 h later because we wanted to avoid pressure variations due to stress generated when extracts were administered. Besides, taking into account that Justicia spicigera contains flavonoids and these have been involved in lowering blood pressure in a dose-dependent manner and its effect persists up to 12 h.

In Mexican traditional medicine, Justicia spicigera plant leaves have been used in the treatment of circulation-related disorders. The whole plant and its aerial parts are typically used in folk medicine. In this context, the aerial parts (stem, flowers and leaves) of the muicle plant were employed to obtain four extracts (hexane, chloroform, methanol and aqueous extract) in order to determine if any of them modified blood pressure.

In our results we found that chloroform extract showed the best effect antihypertensive effect; it lowered the blood pressure in L-NAME rats that had values of 180/164± 1.7/3.2 mmHg (systolic/diastolic) to values of 149/133 ± 4.0/3.7 mmHg (systolic/diastolic) with P < 0.05 (Figure 1). Because blood pressure values vary markedly within 24 hours due to day-night changes, and there can also be variations at different hours, minutes, and even in adjacent heart beats, all measurements were carried out at the same hour. The control animals were treated with the vehicle and extract under the same conditions as the L-NAME rats, and blood pressure was not affected (Figure 2).

No changes were observed in the blood pressure of the L-NAME rats treated with hexane or methanol extracts (data not shown) and the aqueous extracts only modified diastolic blood pressure, going from values of 148 ± 6.6 mmHg (diastolic) to 128 ± 4.5 (diastolic) (Figure 3). Data from overviews suggest that a 2 mmHg reduction in diastolic blood pressure would result in an important decrease in the prevalence of hypertension and the risk of stroke.

The healing properties of plants are known to be associated with the production of secondary metabolites, highly active compounds that give them the ability to interact with their environment. Different mechanisms regulate blood pressure and the secondary metabolites from plants have the potential to modify cell biology; the following are some examples: the effects of methanol extracts from Laelia anceps were mediated by calcium-channel antagonism, aqueous avocado seed extract (Persea americana Mill.) reduced blood pressure and heart rate of Sprague-Dawley rats, and there are others plants whom metabolites has been identified like tilianin, isolated from Agastache mexicana are mediated by NO/cGMP pathway and potassium channel opening. The chloroform extracts of different plants has been used in hypertensive models to clarify the vasorelaxant effects such as Kaempferia galanga (Zingiberaceae), Muntingia calabura (Tiliaceae) and Spilanthes acmella (Asteraceae).

The HPLC chromatogram from J. spicigera chloroform extract showed three major peaks with retention times (tR) of 1.88, 3.9 and 5.3 min, each peak corresponded to a single component, and were identified as hesperidin, naringenin and kaempferol, respectively (Figure 4). Those were corresponding with tR of standard flavonoids (Figure 5). The quercetin flavonoid (tR=2.16) was not of the major compounds present in the J.
**spicigera** extract. The quantification using a standard curve showed a greater amount of hesperidin (1.8 mg/g dry weight plant), followed by naringenin (0.06 mg/g dry weight plant) and of kaempferol (0.003 mg/g dry weight plant).

Flavonoids had been reported to possess hypotensive and vasodilator action $\text{Ca}^{2+}$ antagonism. As well, flavonoids appear to be a potential candidate as antihypertensive compounds, since these compounds are known to exert NO-dependent vasorelaxation. A number of flavonoids have been reported to dilate vascular smooth muscle and then reduce blood pressure in various animal models of hypertension. The oral administration of hesperidin and quercetin has been shown to exert potent antihypertensive effects.

Our observations of a hypotensive effect of *J. spicigera* provide evidences for potential therapeutic applications of this plant extract, and that the antihypertensive effect could be due to flavonoids.

**Conclusions**

This is the first time the effect of *Justicia spicigera* on blood pressure, a characteristic of hypertension, has been demonstrated in vivo. The chloroform extract had a potential antihypertensive effect due to the presence of three flavonoids, metabolites with antihypertensive properties, justifying the traditional use of this plant in hypertension treatment. Further studies are needed to determine the pharmacological profile. However, it should be stressed that the results cannot be extrapolated to humans or replace the current hypertension drug therapy.

**Acknowledgments**

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**References**

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Table 1: Yield quantities under different treatments.
Figure 1. Effects of chloroform extract (150 mg/kg/w) on blood pressure L-NAME-rats. Blood pressure was measured 3 h after administration orally through oropharyngeal probe. Vehicle (Vehic) employed was DMSO 0.5%. The results show the mean (n=5) and SE with P values $P<0.0098$ (systolic, Sys,***) and $0.0184$ (diastolic, Dia,*) compared with hypertensive animals. DMSO 0.5% does not modify the blood pressure.

Figure 2. Effects of chloroform extract (150 mg/kg/w) on blood pressure control rats. Blood pressure was measured 3 h after administration orally through oropharyngeal probe. Vehicle (Vehic) employed was DMSO 0.5%. The results show the mean (n=5) and SE. Diastolic (Dia); Systolic (Sys). There were no significant differences between control rats and treated with vehicle or extract.

Figure 3. Effect of aqueous extract (150 mg/kg/w) on blood pressure L-NAME-rats. Blood pressure was measured 3 h after administration through orally oropharyngeal probe. Vehicle employed was water. The results show the mean (n=5) and SE with P values $0.0145$ (*) (diastolic, Dia) blood pressure compared with diastolic blood pressure in hypertensive animals. There were no significant differences between systolic blood pressure L-NAME-rats and systolic (Sys) blood pressure treated with extract. Water does not modify the blood pressure.
Figure 4. Flavonoids in HPLC chromatogram of chloroform extract of *J. spicigera*: (1) Hesperidin ($t_R=1.877$), (2) Naringenin ($t_R=3.989$) and (3) Kaempferol ($t_R=5.280$).

Figure 5. Chromatogram of four flavonoids standards: 1) Hesperidin ($t_R=1.896$); (2) Quercetin ($t_R=2.160$); (3) Naringenin ($t_R=3.991$) and (4) Kaempferol ($t_R=5.279$).