BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902019000117754

Original Article

Vasorelaxant activity and acute toxicity of the ethanolic extract of *Zanthoxylum rhoifolium* Lam leaves

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The study evaluated the vasorelaxant effect induced by the ethanolic extract of the leaves of *Zanthoxylum rhoifolium* Lam (EEtOH-Zr/leaves). Wistar rats were treated with the leaf extract containing a single dose of 2,000 mg / kg, v.o. After 14 days, the animals were anesthetized for blood collection and subsequent analysis of the biochemical parameters; they were then euthanized (sodium pentobarbital-100 mg/kg, i.p.) for the removal and morphological analysis of the heart, lung, liver and kidney. The vasorelaxation activity the and vascular reactivity of EEtOH-Zr/leaves were evaluated on artery mesenteric rings isolated from rats. The extract showed no signs of toxicity and no significant difference in the values of the biochemical parameters between the control group and the group of treated animals. In the evaluation of pharmacological activity in the smooth muscle, the EEtOH-Zr/leaves caused vasorelaxant effect on the tonic contraction induced by phenylephrine in mesenteric artery preparations in the presence (pD2=2.17±0.05 µg/mL; Emax=99.8±5.2%) and absence (pD2=2.14±0.05 µg/mL; Emax=95.3±6.4%) of the vascular endothelium. Oral administration of EEtOH-Zr/leaves reduced the contraction induced by the cumulative addition of PHE. It is concluded that the EEtOH-Zr/leaves promote vasorelaxation and reduce vascular reactivity of adrenergic alpha-1 agonist in the mesenteric artery. The results did not show toxic effects of the extract.

Keywords: Mesenteric arteries/ drug effects. Vasorelaxant. *Zanthoxylum rhoifolium*/ toxicity. Rats, Wistar. Blood Vessels/ drug effects.

INTRODUCTION

Cardiovascular disorders are the leading cause of morbidity and mortality worldwide (Hoyert, Xu, 2012). 25% of adults suffer from this disease and this number will reach 29% by 2025 (Kearney *et al.*, 2005). Lowering blood pressure greatly reduces the risks of developing heart failure, coronary diseases, renal damages and cerebral vascular diseases (Ezzati *et al.*, 2002). Most of these disorders are untreatable and the current pharmacological strategies only aim at the disease control (Canto, Kiefe, Greenland, 2012). Various biochemical compounds, especially those used in the treatment of arrhythmia and heart failure, have serious adverse events. Therefore, there is a growing trend towards using medicinal plants in health care in general medicine, particularly in cardiovascular medicine (Imanshahidi, Hosseinzadeh, 2008). The reputation of plants in saving human beings has a long history, cutting across different cultures in the world (Hosseinzadeh *et al.*, 2015). In the contemporary world of conventional medicine, the practice of herbal medicine has drawn more attention and is becoming accepted globally (Khan, Yadava, 2010). In Brazil, the use of medicinal plants by the population in order to treat diseases was always expressive, mainly due to the extensive and diverse flora (Pasa, 2011). Secondary metabolites extracted from these plants raise great interest due to their pharmacological activities and healing potential (Pereira, Cardoso, 2012).

The species *Zanthoxylum rhoifolium* Lam is a tree belonging to the Rutaceae Juss family (Costa *et al.*, 2014).

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Its botanical synonym is *Fagara rhoifolia* (Lam) (Weber, 2005) and it is included in the Rutales order. Popularly known as "maminha-de-porca", it is found in the Cerrado areas (Moreira, 1996; Salgado *et al.*, 1998), Atlantic forest and the Amazon (Pirani, 2005). In Northeastern Brazil, it is found in the states of Piauí and Ceará (Melo, Zickel, 2004). Some researches point out the medicinal potential of this species. Phytochemical screening of the ethanolic extract of *Zanthoxylum rhoifolium* stem barks suggested the presence of isoprene substances (pentacyclic triterpenes and steroids) and alkaloids (Freitas *et al.*, 2011). The presence of triterpenes in the species was confirmed by the isolation and identification of lupeol (Camelo *et al.*, 2007; Pereira *et al.*, 2010). The phytochemical profile of the leaves was not determined in this study. However, terpenoid constituents such as lupeol can also probably be found.

The bark extract has also demonstrated analgesic activity, popularly mentioned for toothache and earache, as well as fungicidal activity (Pereira, *et al.*, 2010; Carotenuto, *et al.*, 2015). Furthermore, Jullian *et al.* (2006) were able to prove the antimalarial potential of the shell. Silva, Figueiredo, Yano (2007a, b) evaluated the essential oil of the leaves of *Z. rhoifolium* and observed the cytotoxic effect against tumor cells, thus suggesting a possible therapeutic action against such cells. Freitas *et al.* (2011) demonstrated that the ethanolic extract of the species bears significant gastric protection because it inhibits the formation of gastric lesions using different models. Costa *et al.* (2008) identified antibacterial and larvicidal effects in the essential oil extracted from the fruit. *Z. rhoifolium* bark extract showed analgesic activity, popularly mentioned for toothache and earache, as well as fungicidal activity (Pereira, *et al.*, 2010; Carotenuto, *et al.*, 2015). The study by Jullian *et al.* (2006) was able to prove the antimalarial potential of the shell. Silva, Figueiredo, Yano (2007a, b) evaluated the essential oil of the leaves of *Z. rhoifolium* and analyzed the cytotoxic effect against the tumor cells, thus suggesting a possible therapeutic action against such cells. Freitas *et al.* (2011) demonstrated that the ethanolic extract of the species exhibits significant gastric protection because it inhibits the formation of gastric lesions using different models. Costa *et al.* (2008) identified antibacterial and larvicidal effects in the essential oil extracted from the fruit. In addition, a previous study showed that the ethanolic extract from the *Z. rhoifolium* stem bark has antihypertensive effect in spontaneously hypertensive rats (SHR) and vasorelaxant effect in the mesenteric artery of normotensive rats (Ferreira-Filho *et al.*, 2013), and how the vascular tone is directly involved in the regulation and maintenance of blood pressure (Sonkusare *et al.*, 2006). However, no report on the effect of the leaf extract was found, what allows the present study to contribute to the knowledge of the pharmacological actions of the *Z. rhoifollium* Lam species. In order to shed light to its therapeutic potential for the treatment of cardiovascular diseases, this study is to evaluate the effect of the extract of *Z. rhoifollium leaves* on vascular smooth muscle and its possible action toxicity in rats.

MATERIAL AND METHODS

Preparation of plant extracts

The botanical identification was performed by Prof. Dr. Roseli Farias Melo de Barros. The voucher specimen (TEPB 13870) was deposited at the Graziela Barroso Herbarium of the Federal University of Piauí (UFPI). The leaves from EtOH-Zr were provided by Prof. Dr. Mariana Helena Keys, Department of Chemistry, Natural Sciences Center, Federal University of Piauí. Dried and powdered leaves of *Z. rhoifolium* (1000g) were extracted at room temperature exhaustively with ethanol. The solvent was removed by evaporation under reduced pressure using a Hedolph Rotary Evaporator to yield the EEtOH extract (75.0 g, 7.5%).

Drugs and reagents

The composition of Tyrode´s solution used was (mM): NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0; and glucose, 5.6 mM (Tanaka *et al.*, 1999), sodium thiopental (Cristália) and sodium salt of heparin (Roche). In order to prepare the stock solutions of the drugs, all substances were dissolved in distilled water and diluted to the appropriate concentrations. The extract was dissolved in Tyrode's solution for the *in vitro* protocols and in brine for the *in vivo* protocols using Cremophor (0.1% w / w) as the eluent. All solutions were stored at 0° C.

Animal study

Wistar rats (*Rattus norvegicus*), weighing between 250 and 300 grams, from the Research Center of the Vivarium on Medicinal Plants of the Federal University of Piauí, kept at 24 ± 2 ° C and under light-dark cycle of 12 hours with water and feed *ad libitum*. All protocols were approved by the Ethics Committee on Animal Experiment of the Federal University of Piauí (EAEC Nº008/12). The animal euthanasia procedure is in accordance with the Resolution No. 1000 from 2012 of the Federal Council of Veterinary Medicine.

Experimental protocols

In vivo toxicity of EEtOH-Zr/leaves

The oral toxicity study was performed using the fixed dose method (OECD guideline no. 420). The animals (n=6) were weighed, identified and treated with the ethanolic extract obtained from the leaves of *Zanthoxylum rhoifolium* Lam (EEtOH-Zr/leaves), orally (rigid orogastric tube) with a single dose of 2000 mg/kg, in a volume of 10 mL/kg. During the treatment, each animal was observed for 14 days to check the occurrence of possible indicative signals of a pharmacological and/or toxicity effect (tremors, convulsions, hypoactivity, ataxia, lethargy, and others) (Gazda *et al.*, 2006). Control group animals received isovolumetric doses of saline (0.9% NaCl) via oral route. After 14 days of treatment, the animals were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and submitted to blood collection by puncturing the abdominal artery. The biochemical parameters were determined in a Biopluss 2000® analyzer to test glucose, AST (aspartate aminotransferase), ALT (Alanine aminotransferase), cholesterol, triglycerides, GSH (glutathione) according to Cartágenes (2009), modified.

Ex vivo protocols

Biochemical tests

The parameters were evaluated with three different methods using commercial kits. The enzymatic methods assessed glucose, urea, creatinine, cholesterol and triglycerides. The kinetic methods assessed AST, ALT, catalase and GSH (data not shown). The animals were euthanized with a lethal dose of thiopental (100 mg/kg, i.p.) and organs (heart, lung, liver and kidneys) were removed, weighed and morphologically and macroscopically examined (Cartágenes, 2009; Sabino *et al.*, 2013).

In vitro protocols

Preparation of rat superior mesenteric artery rings

The superior mesenteric arteries were quickly removed and cleaned of adherent connective tissues and fat. Mesenteric rings (2–3 mm length) were obtained and suspended by cotton threads in an organ bath containing 10 mL of Tyrode's solution, maintained at 37 ºC and gassed with a 95% O_2+5% CO_2 mixture (pH 7.4). The rings were stabilized with a resting tension of 0.75 g for at least 60 min, with replacement of Tyrode's solution every 15 min to prevent the accumulation of metabolites that could cause the results to be biased and thus misinterpreted (Altura, Altura, 1970). Isometric tension was recorded by a force-displacement transducer coupled to a data acquisition software (AECAD 1604, AQCAD 2.0.5; AVS Projetos, SP). When necessary, endothelium was removed by gently rubbing the intimal surface of the vessels with a thin stainless wire and endothelial functionality was assessed through the ability of acetylcholine (10 μM) to induce more than 70% of the relaxation associated with the phenylephrine (PE 10 μM) tonus (Furchgott, Zawadzki, 1980). The absence of relaxation following acetylcholine administration was taken as an evidence that the rings were functionally denuded of endothelium.

Effect of EEtOH-Zr/leaves PE-induced tonic contractions in endothelium-intact and endothelium-denuded rat mesenteric rings

After the verification of the endothelium integrity and during the tonic component of a second response to the PE (10 μM), **EEtOH-Zr/leaves** (0.1 – 750 μg/mL) was cumulatively added to the bath in different preparations. The relaxation was expressed as the reversal percentage of the initial contraction elicited by contractile agents, and pD_2 values (Anti-log concentration of a substance that produces 50% of its maximum effect) were obtained with nonlinear regression from **EEtOH-Zr/leaves** concentration–response curves of rat mesenteric rings with both endothelium-intact and endothelium-denuded mesentery.

Effects of EEtOH-Zr/leaves on the vascular tone of mesenteric artery rings

For the assessment of vascular reactivity in a series of experiments, phenylephrine was added to the tank (10^{-9}-10^{-5} M) to obtain a control curve. In other preparations with the arteries of the animals treated for seven days with EEtOH-Zr/leaves (50 mg/kg/7 days) orally, a study for the vascular reactivity to the cumulative addition of phenylephrine took place (10^{-9}-10^{-5} M).

Statistical analysis

Values are expressed as mean ± standard error of the mean. We used the "t" test for unpaired samples. The pD_2 (negative logarithmic effect of EC_{50}) values were calculated by nonlinear regression curves drawn from the percentages of the responses obtained by the substances tested for in vitro experiments. Emax refers to the maximum relaxation value. The level of significance was $p < 0.05$ and the GraphPad Prism 6.0 software was used.

RESULTS

Acute toxicity and biochemical parameters of EEtOH-Zr / leaves

The ethanolic extract of the leaves of *Z. rhoifollium* (EEtOH-Zr/leaves) at a dose of 2,000 mg/kg (orally) showed no obvious toxicity signal and did not cause death of the animals within 30 to 240 minutes, 24 hours and up to 14 days after the administration. Also, there were no significant differences between the groups treated for motor activity parameters, respiration, corneal reflexes and amount of dung (Table I). In relation to the body weight of the animals, there was no significant difference between the control groups and EEtOH-Zr/ leaves (Figure 1). The analysis of organs (lung, heart, liver, spleen and kidneys) failed to detect changes or gross weight change in any of the control and EEtOH-Zr/ leaves groups (Table II). There was also no significant difference between the values of biochemical parameters observed in the serum of the control group (saline) compared with the group treated with EEtOH-Zr/leaves (Figure 5).

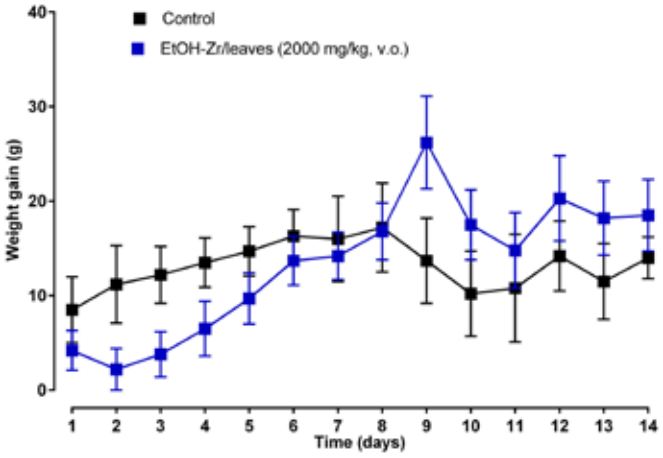

FIGURE 1 - Evolution of the weight of the animals of the control groups (■) and those treated with EEtOH-Zr / leaves 2,000 mg / kg (■). Data represented by mean ± standard error, n = 6 animals.

Vasorelaxant effect EEtOH-Zr/leaves on rat mesenteric artery

The EEtOH-Zr/leaves induced concentration-dependent vasorelaxant effect on the tonic contraction induced by phenylephrine in the mesenteric artery preparations in the presence (pD_2=2.17 ± 0.05 mg/mL; E_{max}= 99.8 ± 5.2 %) and absence (pD_2=2.14 ± 0.05 mg/mL; E_{max}=95.3 ± 6.4%) of the vascular endothelium (Figure 2).

TABLE I - Acute toxicity of EEtOH-Zr/leaves at the oral dose of 2,000 mg/kg: (0) No effect; (-) diminished effect; (+) increased effect

Activities	Time (min)				
	30	60	120	180	240
STIMULANT					
Hyperactivity	0	0	0	0	0
Agressiveness	0	0	0	0	0
Tremors	0	0	0	0	0
Convulsion	0	0	0	0	0
Piloeration	0	0	0	0	0
DEPRESSOR					
Eyelid ptosis	0	0	0	0	0
Sedation	0	0	0	0	0
Anesthesia	0	0	0	0	0
Ataxia Reflection of straightening	0	0	0	0	0
Catatonia	0	0	0	0	0
Analgesia	0	0	0	0	0
Loss of eyelid reflex	0	0	0	0	0
Loss of the atrial reflex	0	0	0	0	0
Eyelid ptosis	0	0	0	0	0
AUTONOMOUS NERVOUS SYSTEM					
Diarrhea	0	0	0	0	0
Cold	0	0	0	0	0
Tearing	0	0	0	0	0
Salivation	0	0	0	0	0
Cyanosis	0	0	0	0	0
OTHER BEHAVIORS					
Ambulance	0	0	0	0	0
Self-cleaning	0	0	0	0	0
Rise	0	0	0	0	0
Climb	0	0	0	0	0
Vocalization	0	0	0	0	0
Abdominal contortions	0	0	0	0	0
DEATH	0	0	0	0	0

Figure 3 shows the original record of the vasorelaxant effect of the EEtOH-Zr/leaves.

Effect of the prolonged treatment with EEtOH-Zr/leaves on vascular reactivity in rat mesenteric artery rings

In rat mesenteric artery rings, prolonged administration of the EEtOH-Zr/leaves (50mg/kg/7 days)

TABLE II - Effect of EEtOH-Zr/leaves (2,000 mg/kg, v.o.) on mean relative organ weight (%) in male rats.

Organs (g)	Saline	EtOH-Zr/folhas (2000 mg/kg)
Lungs	0.708 ± 0.032	0.778 ± 0.040
Heart	0.324 ± 0.012	0.332 ± 0.010
liver	2.881 ± 0.153	2.871 ± 0.170
Spleen	0.355 ± 0.021	0.361 ± 0.020
Kidneys	0.669 ± 0.027	0.669 ± 0.010

Values are expressed as Mean ± SEM (n = 6)

attenuated the contraction induced by the addition of phenylephrine (E_{max}= 0.99 ± 0.02 g/f **, p <0.05) when compared with control animal rings (E_{max}=1.17 ± 0.03 g/f), indicating that the EEtOH-Zr/leaves can interfere with the contractile response to phenylephrine (Figure 4).

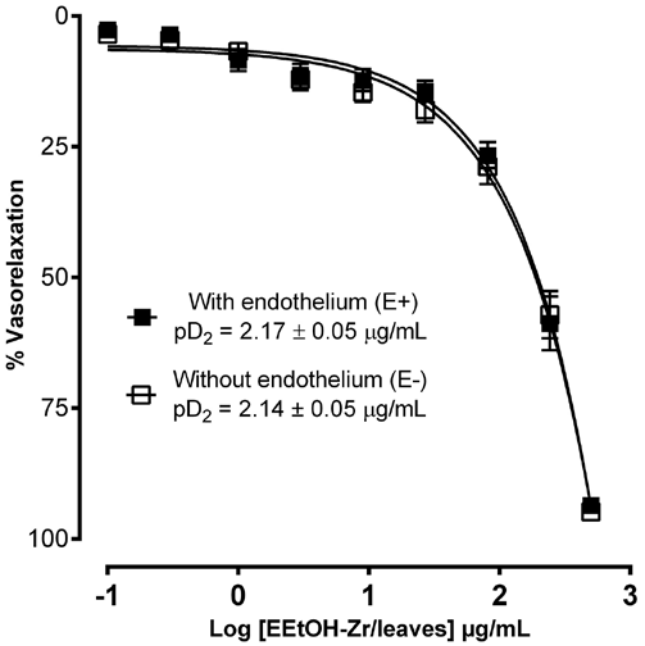

FIGURE 2 - Concentration-response curves of the vasorelaxant effect of EEtOH-Zr/leaves on upper mesenteric artery rings isolated from normotensive mice in the E+(■) and non-E (□) vascular endothelium rats. Values were expressed as mean ± SEM of 5 experiments.

DISCUSSION

In general, acute toxicity tests are intended to define the scope of the lethal dose of a drug administered in a single dose or in a short-time interleaved dose. These tests are part of the initial pharmacological screening, which is observed during the action of the drug on important parameters and functions. Studies with EEtOH-Zr/leaves showed that the oral dose of the extract did not cause behavioral changes such as stimulant or depressant action, and death within 30 to 240 minutes, 24 hours and 14 days after the administration. No alterations were observed in the biochemical parameters nalyzed (Figure 5).

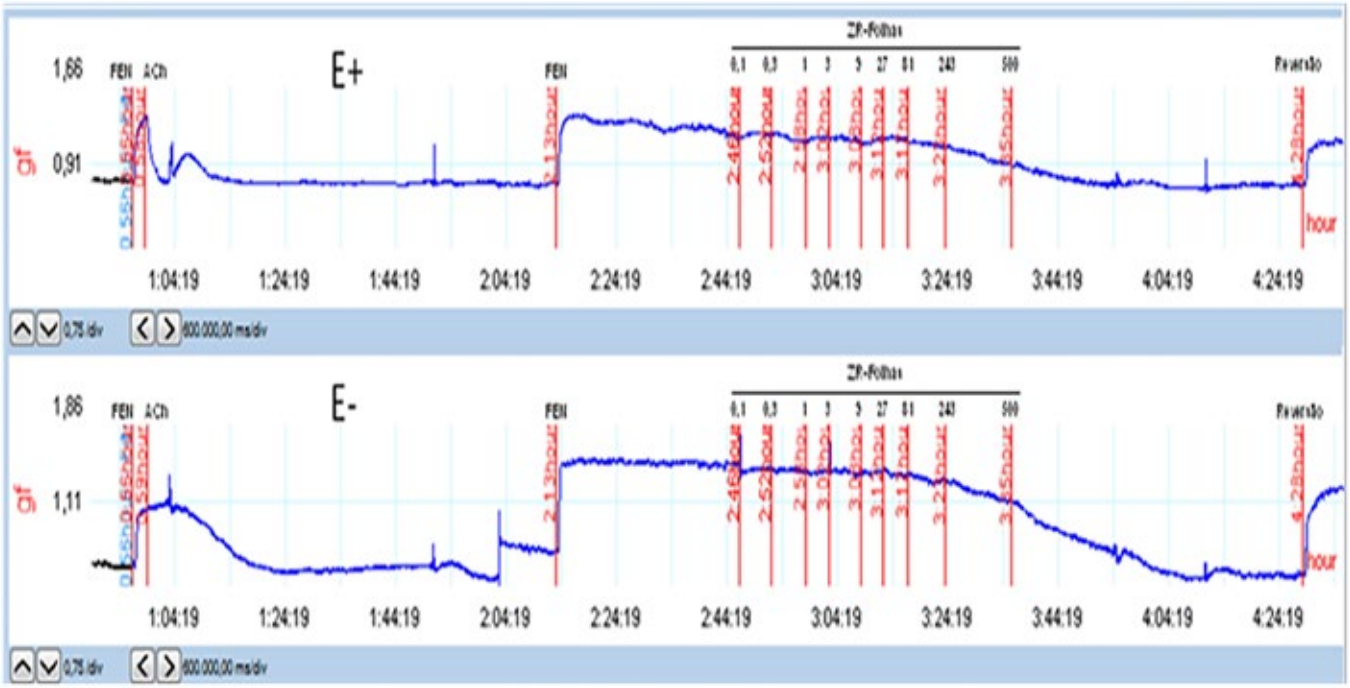

FIGURE 3 - Original tracing of the vasorelaxant effect of EEtOH-Zr/leaves on contractions induced by phenylephrine (10 µM) in presence (E+) or absence of vascular endothelium (E-).

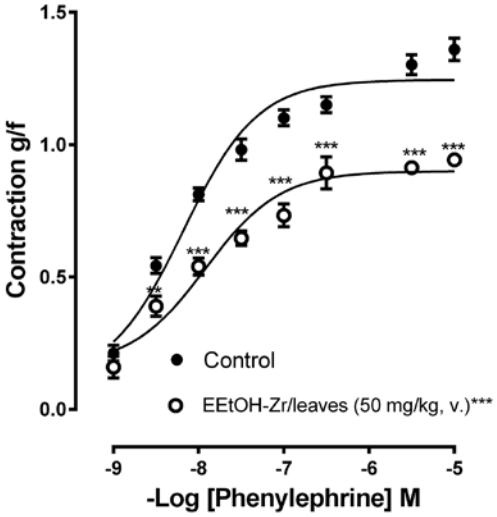

FIGURE 4 - Vascular Reactivity of EEtOH-Zr/sheets (50 mg/kg/7 days) on contractions induced by cumulative addition of phenylephrine (10^{-9}-10^{-5} M) in absence of the mesenteric vascular endothelium. Values were expressed as mean ± s.e.m. (●) control; (○) EEtOH-Zr (50 mg/kg/7 days); ** $p<0.05$, *** $p<0.001$ *vs*. control, n=12 rings.

In the evaluation of pharmacological activity on the vascular smooth muscle, the main observation is that EEtOH-Zr/leaves promoted a vasorelaxant effect dependent on the concentration and independent on the vascular endothelium in mesenteric artery rings pre-contracted with phenylephrine (Figure 3). The contraction and relaxation of the vascular smooth muscle can be regulated by extracellular Ca^{2+} influx via the receptor operative Ca^{2+} channel (ROCC) or the voltage-dependent Ca^{2+}-channel (CaV-L) without endothelial derived factors (Karaki *et al.*, 1997). The contractile activity of the smooth muscle of arteries and arteriole cells is the main determinant of the resistance to blood flow; it consequently influences the regulation of blood pressure (Jackson, 2000).

Natural products with biological activity have been reported in the literature as active in the regulation of the vascular tone. In order to verify the effect of the leaves of *Zanthoxylum rhoifolium* Lam on vascular smooth muscle, phenylephrine, an agonist of the α1-adrenergic receptors that is bound to the Gq/11 protein, was used. By

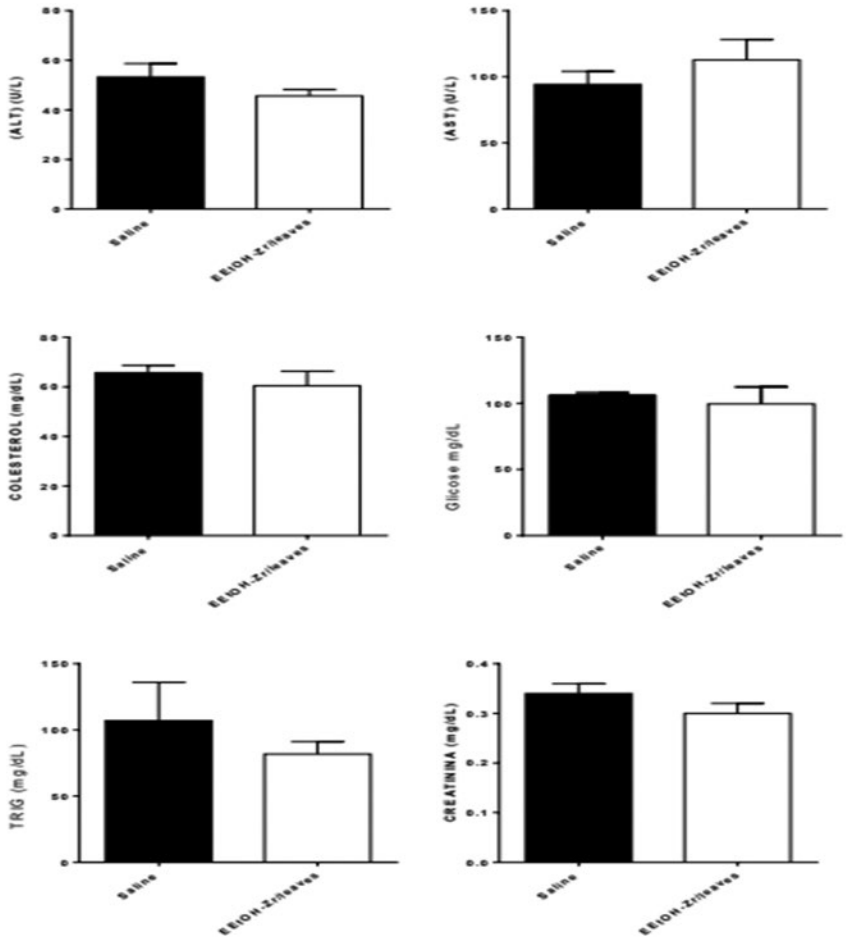

FIGURE 5 - Biochemistry analysis of the plasma of male rats treated with saline and EEtOH-Zr/leaves, orally, with a single dose of 2,000 mg/kg, in a volume of 10 mL/kg. Values were expressed as mean ± s.e.m. control; (■) and (□) EEtOH-Zr (14 days).

being activated, it induces the formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) through the hydrolysis of the phosphatidylinositol 4,5-biphosphate (PIP_2) of the plasmatic membrane. The IP_3 binds to its receptor in the sarcoplasmic reticulum (RIP_3), which induces the release of calcium, thus generating a process of vascular smooth muscle contraction (Zhang *et al.*, 2010). The contraction induced by phenylephrine is mediated by an increase in the Ca^{2+}- influx through the receptor-operated calcium channels and is also sensitive towards the voltage (Lee *et al.*, 2001). The agonists like phenylephrine cause an initial spike in Ca^{2+} followed by small sustained rise in Ca^{2+} above the basal levels, thus increasing the Ca^{2+} sensitivity of MLC phosphorylation and leading to increased contraction (Khalil, 2010).

In the present study, it was observed that *in vitro* administration of EEtOH-Zr/leaves induced concentration-dependent vasorelaxant effect, despite the presence of the vascular endothelium, which suggests non-involvement of derived relaxing factors from endothelium (EDFR) in that response (Figure 2). In experiments to verify the effect of oral treatment with extended EEtOH-Zr/leaves on the contractions induced by phenylephrine, it was observed that the extract was able to inhibit the contractions induced by phenylephrine with reduction of the maximal effect (Emax) (Figure 4) in endothelium-denuded mesenteric rings. These results suggest that EEtOH-Zr/leaves somehow influence the contractile responses induced by phenylephrine probably by acting on the release of calcium from intracellular stores or inhibiting the calcium influx through the membrane via the ROCC. In a previous study with the bark of the stem of *Z. rhoifollium*, we demonstrated a vasorelaxation response like that found with the leaves, what leads us to reflect on the bioactive molecules present in the two extracts.

CONCLUSION

The EEtOH-Zr/leaves showed no acute toxicity in the animals after 14 days of treatment and alter the contractile response induced by phenylephrine upper mesenteric artery rings isolated from rats. Vasorelaxant activity also showed concentration-dependent and independent on vascular endothelium in the preparations of arteries pre-contracted with phenylephrine.

ACKNOWLEDGMENTS

The authors thank CNPq, CAPES and FAPEPI for the scholarships and for the financial support, and Mr. Carlos Alberto de Deus for the animal care. The authors alone are responsible for the contents and writing of the article. They also thank teacher A. Borghi for the English review of the manuscript.

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Received for publication on 15th November 2017
Accepted for publication on 07th May 2018