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Extract and chloroform fraction from *Syzygium cumini* leaves with vasorelaxant effect mediated by inhibition of calcium channels

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Hypertension is associated with endothelial dysfunction characterized by decreased vasorelaxation. Our research group recently demonstrated that hydroalcoholic extract of *Syzygium cumini* leaves (HESc) reduces the blood pressure in spontaneously hypertensive rats (SHR). This study evaluated the ability of HESc and chloroform fraction (CF) in promoting vasorelaxation on resistance arteries rings. Endothelium-deprived superior mesenteric artery rings were suspended in organ baths containing warm perfusion medium that was continuously bubbled with carbogen and then the vasorelaxant ability of HESc and CF were assayed. The cumulative additions of HESc (0.1 to 10 mg/mL) caused a concentration-dependent relaxant response, in precontracted preparations by NE or KCl. CF (0.1 to 1.0 mg/mL) exhibited remarkable vasorelaxant activity in preparations endothelium-denuded pre-contracted with NE, in a concentration-dependent manner. The pretreatment of TEA did not decrease significantly in relaxation. The incubation of CF (0.25 and 0.5 mg/ml) reduced in a concentration-dependent way, the E_{max} induced by NE in mesenteric artery, however, did not altered the pD_2 of the NE. Additionally, CF promoted concentration-dependent manner, maximal effect inhibition and also led to a significant rightward shift in the concentration-response curve for Ca^{2+} in endothelium-denuded rings. This finding indicates that *S. cumini* acts as a vasorelaxant agent and interfere with the responsiveness of vascular smooth muscle cell, probably acting on the regulation of intracellular Ca^{2+} levels through voltage-operated calcium channels.

Key words: *S. cumini,* calcium channels, vasorelaxant, medicinal plant, antihypertensive.

INTRODUCTION

Globally cardiovascular disease accounts for approximately 17 million deaths a year. Of these, complications of hypertension account for 9.4 million deaths worldwide every year. Hypertension is responsible for at least 45% of deaths due to ischemic heart disease and 51% of deaths due to stroke (WHO, 2013).

The objective of antihypertensive treatment is to achieve optimal blood pressure levels during therapy to reduce hypertension-related complications. The research literature indicates that secondary metabolites of herbs and spices exhibit antihypertensive effects contributing in reducing blood pressure levels and minimizing its complications (Al Disi et al., 2016).

Syzygium cumini (L.) Skeels, belongs to Myrtaceae family, is popularly known as jambolan, being is a medicinal plant known for its therapeutic properties for the treatment of different diseases such as inflammation, diabetes and hypertension (Morton, 1963; Pepato et al., 2005; Migliato et al., 2006; Dieckel et al., 2007; Abbas and Mushtaq, 2008). Its leaves are rich in phenolic compounds (Sanches et al., 2016) with great ability to act biologically in cardiometabolic disorders (Chagas et al., 2015).

According to Silva et al. (2012), hydroalcoholic extract of *S. cumini* leaves (HESc) exhibits no chronic toxicity. Additionally, the ability of HESc to reduce blood pressure in SHR suggesting antihypertensive property were demonstrated (Ribeiro et al., 2014).

In this context, this present study investigated the vasodilator effect of *S. cumini* leaves. This work assess the potential of HESc and and its active fraction in promoting vasorelaxation in resistance artery rings for the first time thereby, contributing to clarify the mechanism of its antihypertensive action.

MATERIALS AND METHODS

Plant material

Leaves of *S. cumini* were collected from the campus of the Federal University of Maranhão (2°33'11.7"S 44°18'22.7"W), São Luís, Brazil, in October 2013. A voucher specimen was identified and deposited in the herbarium of “Prof. Dr. Berta Lange de Morretes” Medicinal Plant Garden, UFMA (No. 1069).

Preparation of the hydroalcoholic extract of *S. cumini*

Leaves were dried at room temperature and pulverized. The crude extract was prepared by maceration of the leaf powder (300 g) in 70% ethanol (1:3 w/v), and concentration in a rotary evaporator under reduced pressure at a temperature below 60°C and lyophilized. The extract thus obtained was called the hydroalcoholic extract of *S. cumini* leaves (HESc) with a dry weight of 49.8 g and yield 16.6% (Ribeiro et al., 2014).

HESc aliquots were kept at 4ºC, protect from light, until further experimental use, when powdered HESc was resuspended in water at desired concentrations. HESc was partitioned by sequential extraction using hexane, chloroform (CF), ethyl acetate, and n-butanol. Based on previous results demonstrating that the CF was the most potent in inducing vascular relaxation *in vitro* (Ribeiro, 2007), we evaluated the effects of this fraction. The chloroform (CF) fraction were evaporated, with a yield 5.2% and tested to evaluate the vasorelaxant effect. In this study, phytochemical screening by CF revealed the presence of phenols.

Animals

Male 12-week-old spontaneously hypertensive rats (SHR) or normotensive (Wistar), *Rattus norvegicus*, weighing 250 to 300 g, obtained from the animal house of UFMA were used. The animals were housed under controlled conditions of temperature (21 ± 2°C) under a 12 h light-dark cycle, with ration and water available *ad libitum*. All The experimental protocols were reviewed and approved by the Animal Research Ethics Committee of UEMA, Brazil (Number 17/2012) and all the methods in this study were carried out in accordance with the approved guidelines.

Drugs

Norepinephrine hydrochloride, Acetylcholine and Tetraethylammonium chloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of high analytical grade purity from Merck Darmstadt.

Tissue preparation

Preparations of the mesenteric artery were obtained as described by Borges et al. (1999), Abreu et al. (2003) and Amaechina and Omogbai (2007) and ring segments (3 to 5 mm) of the superior mesenteric artery were placed between stainless steel wires (50 μm in diameter) and immersed in an organ bath chamber (5 mL) containing Krebs nutritive solution (118 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 15.5 mM $NaHCO_3$, 2 mM $CaCl_2$, and 11mM glucose, pH 7.4) at 37°C, equilibrated with 5% CO_2/ 95% O_2. The preparations were first equilibrated under a tension of 1.0 g and washed at intervals of 10 min, for 60 min. Changes in the isometric tension of the preparations were measured with an isometric force transducer (PowerLab, ADInstruments Pty. Ltd., Sydney, Australia).

Due to the endothelial dysfunction and the inefficient production of vasodilators by the epithelial cells present in hypertensive syndrome, we chose to perform all experiments on endothelium-denuded mesenteric arteries, to demonstrate the vascular relaxing property of the extract and fractions without the interference of endothelium-derived factors. Vascular endothelium removal was confirmed by the absence of a relaxation response by Acetylcholine (10 μM) to induce more than 70% inhibition of vessels precontract with norepinephrine (NE 10 μM).

Effect of HESc on contraction induced by NE or KCl

After the stabilization period, endothelium- denuded mesenteric artery rings, obtained of SHRs, were pre-contracted with NE (10 μM) or KCl (80 mM) and, on the tonic phase, different concentrations of HESc (0.1, 0.25, 0.5, 5 and 10 mg/ml) were added cumulatively to organ bath. The relaxant effect was expressed as the percentage of NE or KCl induced contraction.

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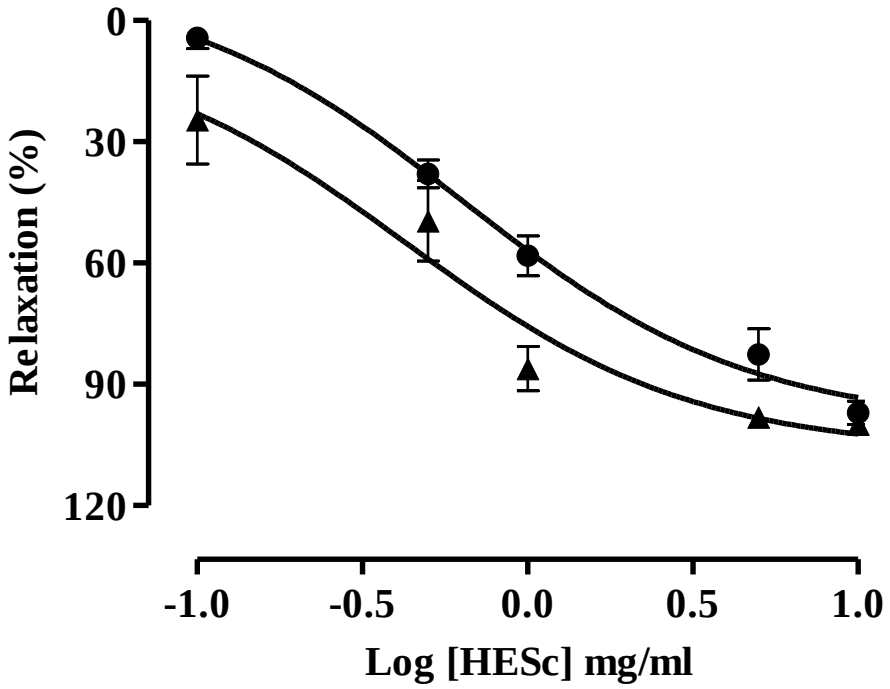

Figure 1. Concentration-relaxation curves for HESc (0.1 - 10 mg/ml). Endothelium-deprived mesenteric arteries precontracted by NE (●) or KCl (▲). Values are expressed as mean ± SEM (n = 5).

CF on contraction induced by NE in presence or absences of K^+- blocker

After the stabilization period, two successive contractions of similar magnitude were induced with NE (10 µM) in endothelium- denuded rings, obtained of SHR. During the tonic phase of the third contract, different concentrations of CF (0.01, 0.05, 0.10, 0.50 and 1.0 mg/mL) were added cumulatively to the organ bath. To examine the role of K^+ channels in the HESc or CF induced relaxation, arteries rings pre-contract with NE (10 µM) were constructed in absence or presence of TEA (0.3 mM). The results were expressed as the percentage of NE induced contraction.

CF effect on arterial smooth muscle contraction induced by NE or Ca^{2+}

The preparations were first equilibrated under a tension of 1.0 g and washed at intervals of 10 min. After 60 min of successive washes, cumulative dose-response curves to NE (10^{-9} to 10^{-4} M) were constructed in the absence or presence of CF (0.25 and 0.5 mg/ml). To evaluate the antagonistic action of CF against Ca^{2+}, vascular tissue was stabilized with normal Krebs solution. After 30 min, the fluid of the preparation was replaced with Ca^{2+}-free Krebs solution (60 mM K^+, nominally Ca^{2+} free).

Also, after 30 min of successive washes, the basal tone was recovered, permitting to obtain cumulative concentration-response curves to $CaCl_2$ (10^{-6} to10^{-2} M) in the absence or presence of CF (0.25 and 0.5 mg/ml). The concentration necessary to elicit 50% of the maximum response (EC_{50}) was determined using nonlinear regression analysis. The negative logarithms of the EC_{50} values (pD_2) were used for statistical analysis. In the experiments involving high extracellular K^+, Krebs solution containing 60 mM KCl was prepared by replacing an equimolar concentration of NaCl with KCl.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-test was used for, multiple comparisons and Student t-test for comparison of unpaired data. A p-value < 0.05 was considered significant and the statistical analysis was performed using the GraphPad Prism® 5.0 program.

RESULTS

In endothelium-denuded rings HESc (0.1 to 10 mg/ml) inhibited the sustained tonic contraction induced by NE 10 µM (Figure 1) in a concentration-dependent manner (E_{max} values= 97.20 ± 2.90%; EC_{50} values= 2.82 mg/ml). Additionally, HESc also promoved a prominent vasorelaxant effect in arteries rings contracted with KCl (Figure 1), with a maximum relaxation of 100.0 ± 0.0% (EC_{50} 1.11 mg/ml). The relaxant effect of HESc was reversible as the tissue regained its spontaneous activity at least within one hour after repeated washout.

In Figure 2A was observed CF (0.1 to 1 mg/ml) also exhibited vasorelaxant activity in preparations endothelium-denuded pre-contracted with NE (10µM), in a concentration-dependent manner (Table 1). The relaxant effect of CF also was reversible after repeated washout. The vasorelaxant effect induced by HESc or CF was available in preparations with TEA (1mM). The Figure 2B showed that 30 min of TEA-pretreatment in

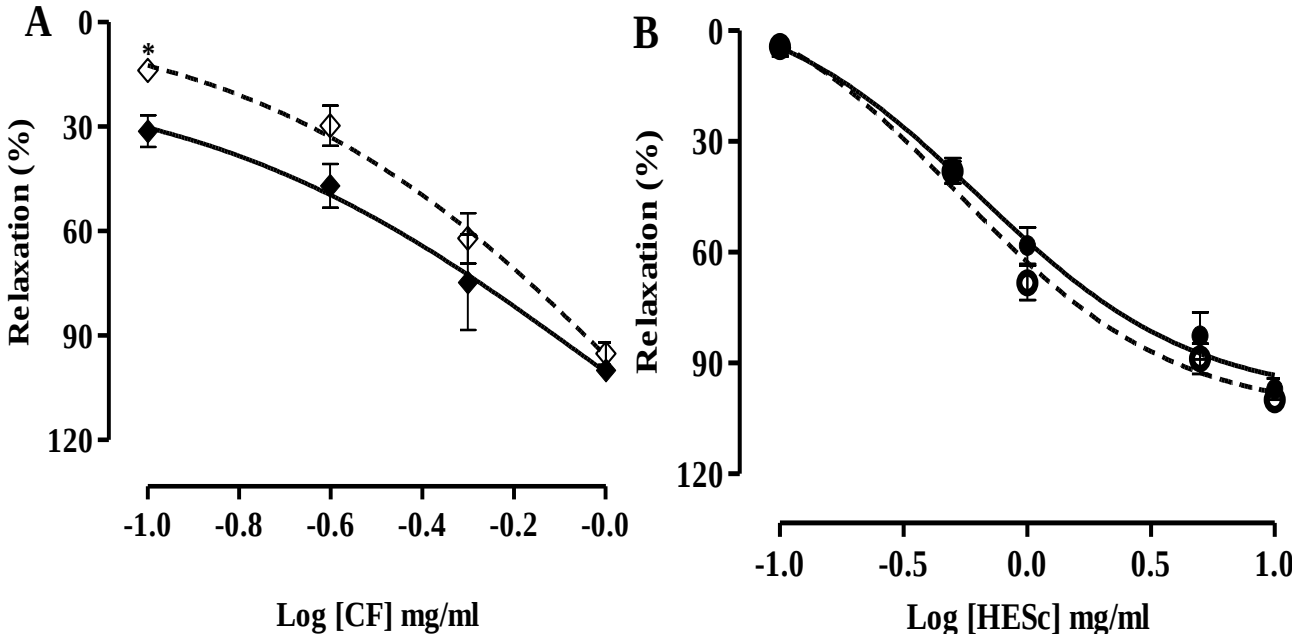

Figure 2. Concentration-relaxation curves for HESc (0.1 - 10 mg/ml) or CF (0.1-1.0 mg/ml) in presence or absence TEA (1mM). Endothelium-deprived mesenteric arteries precontracted by NE in presence (◊) or absence TEA (♦) for CF (**A**) or in presence (●) or absence TEA (○) for HESc (**B**). Data are expressed as mean ± S.E.M. (n = 5). p <0.05 vs CF without TEA.

Table 1. Parameters of concentration-relaxation curves for chloroform fraction (CF) from *Syzygium cumini* leaves in presence or absence of TEA.

CF (mg/mL)	E_{max} (%)		
	Without TEA	With TEA	p-values
0.1	32.40 ± 4.60	13.92 ± 2.35*	0.0164
0.25	47.10 ± 6.25	29.81 ± 5.80	0.0873
0.5	74.80 ± 13.70	62.20 ± 7.21	0.4764
1.0	100	95.30 ± 3.25	0.1658

Data are expressed as mean ± S.E.M. (n = 5). *p < 0.05 vs CF without TEA.

endothelium-denuded rings did not change the vasorelaxant response of HESc (E_{max} values= 99.75 ± 0.25%; EC_{50} values= 2.24 mg/ml). In addition, in relation to vasorelaxation promoted by CF, TEA reduced relaxative effect only in concentration of 0.1 mg/mL (Figure 2A and Table 1). It was also observed that in a lower concentration CF demonstrated a potential higher vasorelaxant HESc (Figure 3).

CF at concentrations of 0.25 and 0.5 mg/ml reduced the E_{max} value for NE in endothelium-denuded mesenteric artery (Figure 4 and Table 2). In addition, contractions induced by $CaCl_2$ in endothelium-deprived mesenteric arterial rings were reduced in a concentration-dependent manner after incubation with 0.25 and 0.5 mg/ml CF, (Figure 5 and Table 2). There was a displacement of the $CaCl_2$ curves to the right, changing pD2. These effects were reversed after washing with Krebs solution.

DISCUSSION

This study is the first to demonstrate the vasorelaxant activity of *S. cumini* leaves in mesenteric artery of SHR and possibly acting involving intracellular Ca^{2+} stores. These results support the elucidation of possible mechanisms involved in the antihypertensive effect of this plant species, a pharmacological property recently demonstrated by our group.

Structural and functional alterations occur in arterial hypertension and are important in the mechanisms that determine blood pressure and target of antihypertensive therapy (Oh et al., 2007; Oh et al., 2008). The sympathetic nervous system also participates in the development and maintenance of various forms of hypertension (Piascik et al., 1996). The magnitude of sympathetic over activity has been closely related to

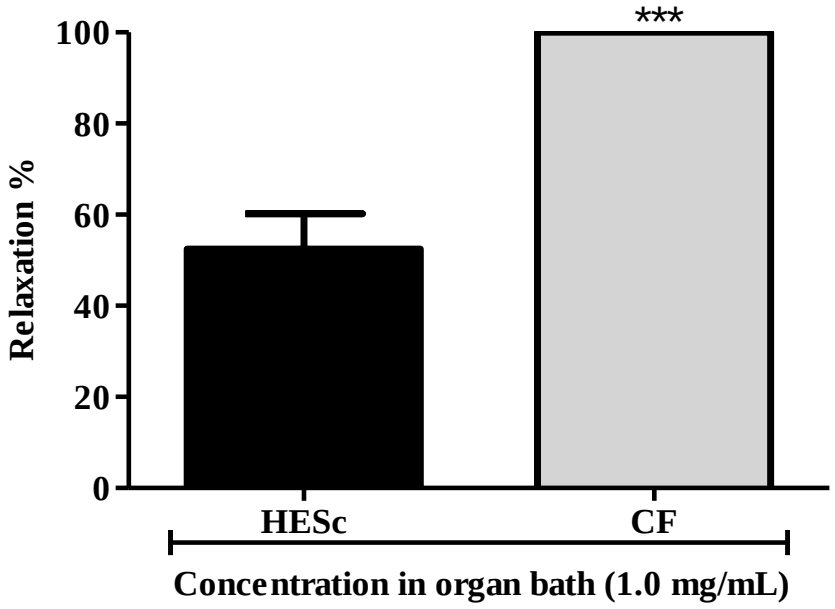

Figure 3. Comparative E_{max} (% values in terms of vasorelaxant effects) of HESc and CF at 1 mg/ml. Values are expressed as mean ± SEM of (*n* = 5) experiments. *** p < 0.00001 in comparison to HESc as reference.

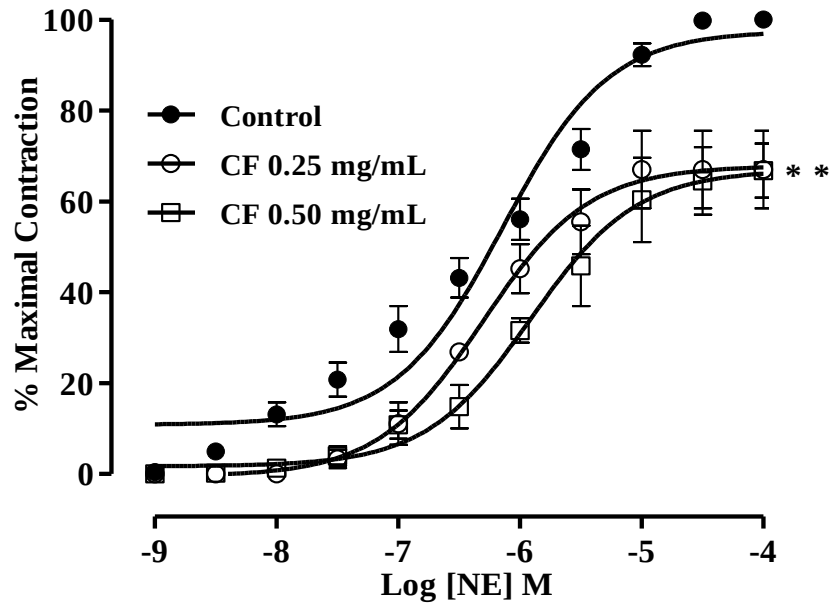

Figure 4. Cumulative dose-response curves to norepinephrine (NE) in isolated endothelium-deprived mesenteric arteries. Control (●), CF 0.25 mg/ml (○) and CF 0.5 mg/ml (□). Values are expressed as mean ± SEM (*n* = 5). *p < 0.05 vs NE Control.

hypertension-related end organ damage and predicts mortality and cardiovascular outcomes (Hering and Narkiewicz, 2013).

The contractile response of vascular smooth muscle cells (VSMCs) by NE is dependent on the Ca^{2+} influx from extracellular space through receptor-operated Ca^{2+} channels (ROCCs) (Karaki and Weiss, 1988; Nelson et al., 1990; Qayyum et al., 2016). In addition, potassium causes VSMCs contraction through activation of voltage dependent Ca^{2+} channels (VOCCs) (Thorneloe and

Table 2. Parameters of concentration-response curves for the effects of chloroform fraction (CF) from *Syzygium cumini* leaves on contraction induced by NE or Ca^{2+} in mesenteric arteries.

Concentration-response curve	E_{max} (%)	pD_2 (M)
NE control curve	100	-6.22 ± 0.15
NE + CF 0.25 mg/mL	67.02 ± 8.50**	-6.40 ± 0.14
NE + CF 0.5 mg/mL	66.80 ± 6.00**	-6.00 ± 0.13
Ca^{2+} control curve	100	-3.40 ± 0.13
Ca^{2+} + CF 0.25 mg/mL	79.30 ± 3.43**	-3.25 ± 0.05*
Ca^{2+} + CF 0.5 mg/mL	56.02 ± 8.7**,[a]	-2.40 ± 0.90**,[a]

The values indicate the mean ± SEM of the pD_2 and the E_{max} obtained from 5 experiments.*Significantly *vs* Control; [a]Significantly *vs* CF 0.25 mg/ml.

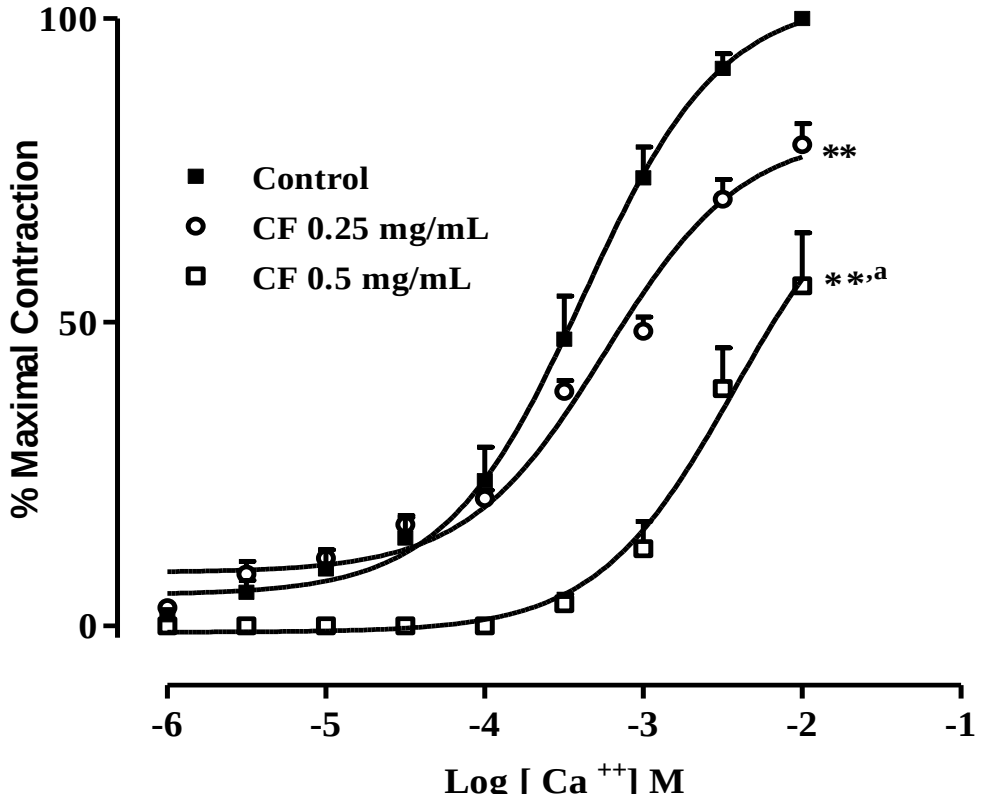

Figure 5. Cumulative dose-response curves to $CaCl_2$ in isolated endothelium-deprived and depolarized mesenteric arteries from spontaneously hypertensive rats. Control (■), CF 0.25 mg/ml (□) and CF 0.5 mg/ml (□). Values are expressed as mean ± SEM (n = 5). **p < 0.01 vs Ca^{2+} Control; [a]p < 0.01 vs CF 0.25 mg/ml.

Nelson, 2005; Ayele et al., 2010). The results presented in Figure 1 indicate that HESc promotes vasorelaxation of the pre-contracted mesenteric artery by NE and KCl, which would be suggestive of blocking Ca^{2+} influx through the plasma membrane. Recently, our laboratory hypothesized that HESc would prevent Ca^{2+} influx through VOCCs on isolated rat jejunum (Monteiro et al., 2018).

In VSMCs, K^+ channels play important roles in the regulation of vascular tone (Tanaka et al., 2004). Large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca} channels), which have been extensively studied in VSMCs, contribute to the control of vascular tone (Brenner et al., 2000) and have been therapeutic targets in the treatment of cardiovascular disease (Saponara et al., 2006).

To determine if the vasorelaxant effect induced by

HESc involves BK_{Ca} –channel activation, it was incubated in mesenteric artery without endothelium preparations with a selective inhibitor of this channel TEA (1mM) (Garcia and Kaczorowski, 1992; Campbell, 1993; Jackson, 2005; Eichhorn and Dobrev, 2007). Similar study was realized by Matsumoto et al. (2010) when assessing the vasodilator alkaloid isolated from *Cassia siamea* in mesenteric artery without endothelium preparations of rats. The results in Figure 2B showed that vasorelaxant effect of *S. cumini* leaves does not occur by direct activation of BK_{Ca} channels.

A particular feature of phytomedicines is their complex composition, that is, the 'phytocomplex', with different specific effects, however a wider array of effects and healing properties are guaranteed only by the phytocomplex (Medeiros et al., 2008). In order to further characterize this effect, HESc was submitted to liquid partition, as described under material and methods. To continue the study, we decided to work with CF, whose previous results suggest potential vasorelaxant effect in mesentric arteries of normotensive rats (Ribeiro, 2007).

In Figure 2A and 3 it was observed that, CF in the lower concentration exhibited remarkable vasorelaxant ability in a concentration-dependent manner, when compared to HESc at the same concentration, suggesting that CF contains the active compounds present in phytocomplex and the vasorelaxant response probably implicate an endothelium-independent pathway or inhibits Ca^{2+} influx or by hyperpolarization produced by K^+- channels (Karaki and Weiss, 1988; Eichhorn and Dobrev, 2007).

Figure 2B and Table 2 suggest that the presence of TEA did not alter the vasorelaxant response pattern of CF at higher concentrations. Additionally, reduced inhibitory effect of the CF at 0.1 mg/mL by TEA showed implication of BK_{Ca} –channel activation in the induced vasorelaxation at lower concentrations of fraction (Table 1).

Studies have shown that a number of plant products including polyphenols, flavonoids, and various plant extract exert antihypertensive effects that might be owing to vasorelaxant action (Curin and Andriantsitohaina, 2005; Wang et al., 2014; Van Rymenant et al., 2017). Chemical studies of *S. cumini* leaves were performed by Ruan et al. (2008) and showed that CF contains phenolic acids, the other complex phenolic compounds. The results of phytochemical screening showed that CF is rich in these compounds, which may be responsible for the vasorelaxant property of the plant.

Previously, we have shown that the maximal response induced by α_1-adrenoceptor agonist NE in SHRs, suggest that HESc contains components that interfere with the reactive response of the vascular musculature. In the present study, the incubation of CF (0.25 and 0.5 mg/ml) reduced in a concentration-dependent way the E_{max} induced by NE in mesenteric artery, however, did not altered the pD_2 of the NE, suggesting that CF contains components that interfere with the reactive response of the vascular musculature, possibly interfering with the mechanisms of Ca^{2+} homeostasis in VSMCs.

Contractions of smooth muscles induced by high K^+ have been widely used in understanding Ca^{2+} roles in biological systems. To check the effect of the CF in $[Ca^{2+}]_i$, a concentration-response curve to Ca^{2+} (10^{-6} and 10^{-2}M) in presence of K^+ - depolarizing solution (KCl 60 mM) was constructed, before and after incubation with CF 0.25 and 0.5 mg/ml, that induced concentration-dependent manner, maximal effect inhibition and also led to a significant rightward shift in the concentration-response curve for Ca^{2+} in endothelium-denuded rings (Figure 5 and Table 2). These findings support the notion that, the CF can block Ca- influx from the extracellular space and acts as a non-competitive Ca^{2+} antagonist (Figure 5). Clinically, Ca^{2+} antagonist are potentially used to treat hypertension (Tep-Areenan and Sawasdee, 2011).

Conclusion

The results demonstrate that *S. cumini* L. (Skeels) causes vasorelaxant effect and interfere with the responsiveness of vascular smooth muscle cell, probably as a result of the blockade of Ca^{2+} channels, as demonstrated in this study. These effects can be attributed to the presence of phenolic compounds detected by phytochemical screening.

The *S. cumini* leaves showed an excellent potential as a vasodilator agent for the treatment of hypertension. The findings may provide a possible candidate drug for clinical medical use to treat cardiovascular diseases in the future. However, further experiments are necessary to clearly elucidate this assumption.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

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ABBREVIATIONS

SHR, Spontaneously hypertensive rat; **HESc,** Hydroalcoholic extract; **CF,** Chloroform fraction; **NE,** Norepinephrine; **VSMC,** Vascular smooth muscle cell; **E_{max},** Maximum effect; **ROOC's,** Receptor-operated Ca^{2+} channels; **VOOC's,** Voltage-dependent Ca^{2+} channels; **BK_{Ca},** Large-conductance Ca^{2+} activated K^+ channels;

TEA, Tetraethylammonium.

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