Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
20(5): 762-766, Out./Nov. 2010
Received 6 Jul 2009; Accepted 4 Dec 2009; Available online 19 Nov 2010.

Article

Vasorelaxant effect of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, dichloromethane extract on rat mesenteric artery

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RESUMO: "Efeito vasorelaxante do extrato diclorometano de *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, em artéria mesentérica de ratos". O efeito vasorelaxante do extrato diclorometano de *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae (HFDE), em anéis isolados de artéria mesentérica de ratos foi avaliado nesse estudo. Em anéis intactos, pré-contraídos com fenilefrina (10 μM), HFDE (0,1-3000 μg/mL) induziu vasorelaxamento de maneira dependente de concentração (E_{max} = 119±14%; n = 6), o qual não foi afetado após remoção do endotélio (E_{max} = 116±6%; n = 6), após KCl 20 mM (E_{max} = 135±9%; n = 6) ou em anéis pré-contraídos com KCl 80 mM (E_{max} = 125±4%; n = 6). Em anéis sem endotélio, HFDE (300 ou 1000 μg/mL) inibiu as contrações induzidas por $CaCl_2$ (inibição máxima = 25±7% e 95±1%, respectivamente). Além disso, HFDE promoveu um vasorelaxamento adicional (15±3%; n = 7) sobre o relaxamento máximo de 10 μM de nifedipina (78±3%, n = 7). Em conclusão, HFDE induz efeito vasorelaxante através de uma via independente de endotélio, possivelmente devido à inibição do influxo de Ca^{2+} através de canais de Ca^{2+} operados por voltagem.

Unitermos: *Hyptis fruticosa,* extrato diclorometano, efeito vasorelaxante, artéria mesentérica, ratos.

ABSTRACT: Vasorelaxant effect of *Hyptis fruticosa* dichloromethane extract (HFDE) on isolated rings of rat mesenteric artery was evaluated in this study. In intact rings, HFDE (0.1-3000 μg/mL) induced concentration-dependent vasorelaxations (E_{max} = 119±14%; n = 6) of phenylephrine tonus that were not modified after endothelium removal (E_{max} = 116±6%; n = 6), after KCl 20 mM (E_{max} = 135±9%; n = 6) or in rings pre-contracted with KCl 80 mM (E_{max} = 125±4%; n = 6). In endothelium denuded rings, HFDE (300 or 1000 μg/mL) inhibited contractions induced by $CaCl_2$ (maximal inhibition = 25±7% and 95±1%; respectively). Furthermore, HFDE promoted an additional vasorelaxation (15±3%; n = 7) after maximal response of 10 μM nifedipine (78±3%; n = 7). In conclusion, HFDE induces vasorelaxant effect through an endothelium-independent pathway, which mostly seems to occur due inhibition of the Ca^{2+} influx through voltage-operated Ca^{2+} channels.

Keywords: *Hyptis fruticosa,* dichloromethane extract, vasorelaxant effect, mesenteric artery, rats.

INTRODUCTION

The use of medicinal plants for the treatment of human diseases has increased considerably worldwide. Evaluation of the effects of these plants on organs and systems has contributed to the development of the scientific basis for their therapeutic application, and also has enriched considerably the therapeutic arsenal for the treatment of a number of diseases (Elizabetsky, 1986).

Hyptis genus, Lamiaceae, is composed by four hundred species distributed at all American Continent. In Brazil, this species is mainly distributed at the central region (Harley, 1988).Various species of this genus are used in the folk medicine because of its antiinflammatory, antinociceptive, anticonvulsant and antiulcerogenic actions (Barbosa & Ramos, 1992; Akah & Nwambie, 1993; Kuhnt et al., 1995; Bispo et al., 2001).

Hyptis fruticosa Salzm. ex Benth., Lamiaceae, popularly known in Brazil as "alecrim-do-campo" or "alecrim-do-vaqueiro", is an aromatic sub-bush plant which

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ISSN 0102-695X
DOI: 10.1590/S0102-695X2010005000003

grows up to 1.5 m found on the Brazilian northeastern coast. Phytochemical studies performed in our laboratory have demonstrated that leaves of this plant present tanins, terpenes, steroids and alkaloids, and absence of saponins (unpublished data). Previous pharmacological studies have demonstrated that *H. fruticosa* presented analgesic (Silva et al., 2006; Cândido, 2006; Menezes et al., 2007), larvicidal (Silva et al., 2008) and hypotensive activities (Santos et al., 2007). Thus, the aim of this work was to evaluate the vasorelaxant effect of *Hyptis fruticosa* dichloromethane extract (HFDE) and its action mechanism in rats.

MATERIAL AND METHODS

Drugs

The drugs used were: Acetylcholine chloride (Ach), L-phenylephrine chloride (Phe) and cremophor (a derivative of castor oil and ethylene oxide used to emulsify water-insoluble substances) (SIGMA). All compounds were dissolved in distilled water.

Extraction

Hyptis fruticosa Salzm. ex Benth., Lamiaceae, collected near São Cristóvão (S 10º 56` W 37º 11`), Brazilian State of Sergipe, was identified by Prof. Dr. Adauto Souza Ribeiro, Botanist in the Biology Department, Universidade Federal de Sergipe. A voucher specimen was deposited in the Herbarium of the Biology Departament, Universidade Federal de Sergipe (code n° ASE 01137). Aerial parts of *H. fruticosa* were dried at 40 ºC in an oven with air circulation and pulverized. The powder (500 g) was exhaustively extracted with methanol (1:5 p/v) by 8 dias to room temperature. After filtration, the solvent was removed under reduced pressure, yielding 78.5 g of the methanol extract. The HFDE was obtained from methanol extract by using the following solvents: dichloromethane, ethyl acetate and methanol yielding: 8.4 g (1.68%), 5.4 g (1.08%) and 29.5 g (5.90%), respectively.

Animals

Male Wistar rats (200-300 g) were used in all experiments. They were housed in conditions of controlled temperature (21±1 ºC) and exposed to a 12 h light-dark cycle with free access to food (Purina-Brazil) and tap water. All procedures described in the present work are in agreement with Animal Research Ethics Committee from Universidade Federal de Sergipe.

Solutions

The composition of the normal Tyrode's solution used was: NaCl 158.3, KCl 4.0, $CaCl_2 2H_2O$ 2.0, $NaHCO_3$ 10.0, $C_6H_{12}O_6$ 5.6, $MgCl_2.6H_2O$ 1.05 and $NaH_2PO_4H_2O$ 0.42 mM. K^+-depolarizing solutions (KCl 20, 60 and 80 mM) were prepared by replacing 20, 60 or 80 mM KCl in the Tyrode's solution with equimolar NaCl, respectively and nominally without Ca^{2+} solution was prepared by omitting $CaCl_2$.

Tissue preparation

Rats were euthanized by cervical dislocation and exsanguination. The superior mesenteric artery was removed, cleaned from connective tissue and fat, and sectioned in rings (1-2 mm), which were suspended in organ baths containing 10 mL of Tyrode´s solution, gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 ºC. Isometric tension was recorded under a resting tension of 0.75 g. During the stabilization period the solution was changed every 15 min (Altura & Altura, 1970). The isometric tension was recorded through a force transducer (Gould, Model GM2, USA) coupled to an amplifier-recorder (Gould, USA). Endothelium was removed by gently rubbing the intimal surface of the vessels. The presence of functional endothelium was assessed by the ability of acetylcholine (ACh) (10 µM) to induce more than 70% relaxation of pre-contracted vessels with phenylephrine (10 µM). The absence of the relaxation to ACh was taken as evidence that the vessel segments were functionally denuded of endothelium.

HFDE effect on phenylephrine (10 µM) induced tonus in isolated rat superior mesenteric artery rings with or without endothelium

After the stabilization period, two successive contractions of similar magnitude were induced with 10 µM Phe in rings with or without endothelium. During the tonic phase of the third contraction, different concentrations of HFDE (0.1; 0.3; 1; 3; 10; 30; 100; 300; 1000 and 3000 µg/mL) were added cumulatively to the organ bath. The relaxations were measured by comparing the developed tension before and after the addition of HFDE and expressed as percentage of relaxation from induced tonus. In other set of experiments, concentration-response curves were obtained in rings without endothelium before and after to the pre-incubated with 20 mM of KCl.

Effect of HFDE on contraction induced by KCl 80 mM in endothelium-denude rings

After the stabilization period, rings without endothelium were pre-contracted with K^+-depolarizing solutions (KCl 80 mM) and, on the tonic phase, different concentrations of HFDE (0.1; 0.3; 1; 3; 10; 30; 100; 300; 1000 and 3000 µg/mL) were added cumulatively to organ bath. The relaxations were measured as previously described.

Effect of HFDE on concentration-response curves to $CaCl_2$ in endothelium-denuded rings

After the stabilization period, the rings without endothelium were contracted with K^+-depolarizing solution (KCl 60 mM) and washed with normal Tyrode's solution until full recovery of initial tension. After this, they were incubated with nominally without Ca^{2+} solution for 15 min and afterwards exposed to nominally without Ca^{2+} solution with KCl to 60 mM for another 15 min (Goodfraind et al., 1986). Then, a first cumulative concentration-response curve to $CaCl_2$ (3 x 10^{-6}, 10^{-5}, 3 x 10^{-5}, 10^{-4}, 3 x 10^{-4}, 10^{-3}, 3 x 10^{-3}, 10^{-2} and 3 x 10^{-2} M) was obtained. In these same preparations, HFDE (300 or 1000 µg/mL) was individually pre-incubated for 15 min and a second cumulative concentration-response curve to $CaCl_2$ was obtained. This curve was compared with those obtained in the absence of HFDE and the results were expressed as percentages of the maximal response to $CaCl_2$ alone.

Effect of HFDE on maximal vasorelaxant response of nifedipine in endothelium denuded rings

Initially, a concentration-response curve to nifedipine was performed in order to determine the concentration of maximal vasorelaxant response in endothelium-denuded rings pre-contracted with 10 µM of Phe (data not shown). After this, others endothelium-denuded rings pre-contracted with 10 µM of Phe were incubated with 1000 µg/mL of HFDE or 10 µM of nifedipine, separately. In other set of experiments, endothelium-denuded rings pre-contracted with 10 µM of Phe were incubated with 10 µM of nifedipine and after obtainment of the maximal vasorelaxant response, HFDE (1000 µg/mL) was added in organ bath. The responses to each vasorelaxant agent were statistically compared.

Statistical analysis

Values were expressed as mean±SEM. When appropriate, one-way ANOVA or two-way ANOVA for repeated measures, both followed by Bonferroni post-test, was performed to evaluate the significance of the differences between means. Statistically different values were detected at a significance level of 0.05.

RESULTS AND DISCUSSION

In intact rings of rat isolated superior mesenteric artery, HFDE (0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 µg/mL, cumulatively) induced vasorelaxation in a concentration dependent manner of tonus induced by 10 µM phenylephrine (E_{max} = 119±14%; n = 6) (Figure 1).

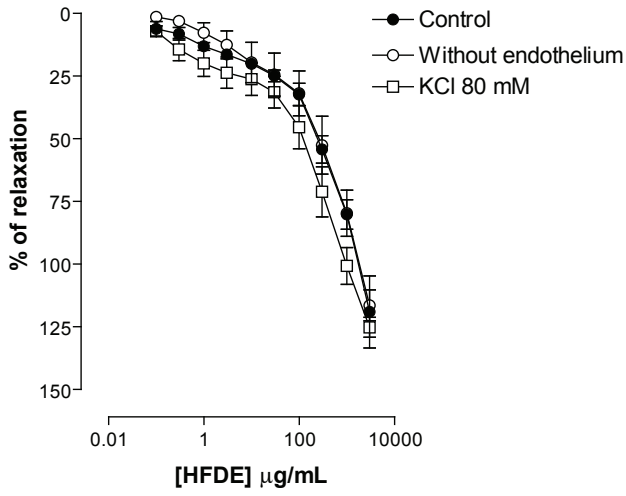

Figure 1. Concentration-response curves to HFDE (0.1; 0.3; 1; 3; 10; 30; 100; 300; 1000 and 3000 µg/mL) in rings of rat isolated superior mesenteric artery pre-contracted with 10 µM Phe (Control), without functional endothelium (Without endothelium) and rings without endothelium pre-contracted with K^+-depolarizing solutions (KCl 80 mM). Values are expressed as mean±SEM, n = 6. The data were analyzed with repeated measures two-way ANOVA followed by Bonferroni post-test.

It is well known that the endothelium is an important regulator of the vascular tone by releasing endothelium-derived relaxing factors (Moncada et al., 1991), mainly NO and COX-derived products, such as PGI2 (Moncada et al., 1991; Furchgott & Zawadzki, 1980). In order to investigate the participation of the endothelium in the vasorelaxant effect induced by HFDE, we performed experiments in the absence of functional endothelium. In these conditions, the vasorelaxant response induced by HFDE was not significantly changed (E_{max} = 116±6%; n = 6) (Figure 1). This suggests that the presence of endothelium is not essential for relaxant response expression and that an endothelium-independent pathway is probably implicated in this effect.

Potassium channels importantly contribute to the determination and regulation of the vascular tone (Nelson & Quayle, 1995; Jackson, 2000). The electrochemical gradient for K^+ ions is such that the opening of K^+ channels results in the diffusion of this cation out of the cells with consequent hyperpolarization. This effect closes voltage-operated Ca^{2+} channels and leads to vasorelaxation (Jackson, 2000). In order to investigate the involvement of K^+ channels in the vasorelaxant effect of HFDE, we performed experiments in the presence of 20 mM of K^+. This procedure partially prevents the efflux of K^+ through the membrane and, therefore inhibits the relaxations mediated by the opening of K^+ channels (Campbell et al., 1996). Thus, In rings without endothelium pre-contracted with Phe and incubated with KCl 20 mM, the concentration-response curve to HFDE was not significantly changed

(Emax = 135±9%; n = 6) (Figure 2), suggesting that K^+ channels appears not to be involved in this response.

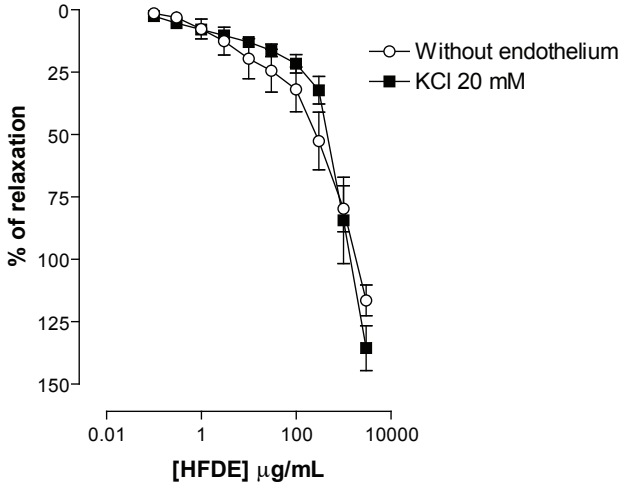

Figure 2. Concentration-response curves to HFDE (0.1; 0.3; 1; 3; 10; 30; 100; 300; 1000 and 3000 µg/mL) in rings of rat isolated superior mesenteric artery without functional endothelium pre-contracted with 10 µM Phe (Without endothelium), and rings without endothelium pre-contracted with Phe and incubated with K+-depolarizing solutions (KCl 20 mM). Values are expressed as mean±SEM, n = 6. The data were analyzed with repeated measures two-way ANOVA followed by Bonferroni post-test.

Calcium is the primary regulator of tension in vascular smooth muscle (Gurney, 1994). It is well known that the maintenance of smooth muscle contraction depends on Ca^{2+} influx from extracellular space through voltage- and/or receptor-operated calcium channels (VOCCs and/or ROCCs, respectively) (Karaki & Weiss, 1988). It is well reported that the increase of external K^+ concentration (KCl 80 mM) induces smooth muscle contraction through VOCCs activation and subsequent calcium release from the sarcoplasmic reticulum (Karaki & Weiss, 1988). The high K^+-induced contraction is inhibited by Ca^{2+} channel blockers or by removal of external Ca^{2+} and is, therefore, entirely dependent of Ca^{2+} influx (Karaki & Weiss, 1988). Thus, we evaluated the HFDE effect on endothelium-denuded rings pre-contracted with K^+-depolarizing solutions (KCl 80mM). This set of experiments revealed that HFDE induced vasorelaxations, which were not significantly different of those observed in rings pre-contracted with Phe (125±4%; n = 6) (Figure 1), suggesting that the HFDE inhibits Ca^{2+} influx through VOCCs.

In order to check the hypothesis above, we constructed a concentration-response curve to $CaCl_2$ (3 x 10^{-6}, 10^{-5}, 3 x 10^{-5}, 10^{-4}, 3 x 10^{-4}, 10^{-3}, 3 x 10^{-3}, 10^{-2} and 3 x 10^{-2} M) in presence of K^+-depolarizing solution (KCl 60 mM), before and after incubation with HFDE in doses of 300 and 1000 µg/mL. In these conditions, $CaCl_2$ induced contractions in endothelium-denuded rings of rat mesenteric artery in a concentration-dependent manner that were strongly inhibited after incubation with HFDE in concentrations of 300 and 1000 µg/mL (maximal inhibition = 25±7% and 95±1%; n = 6; respectively).

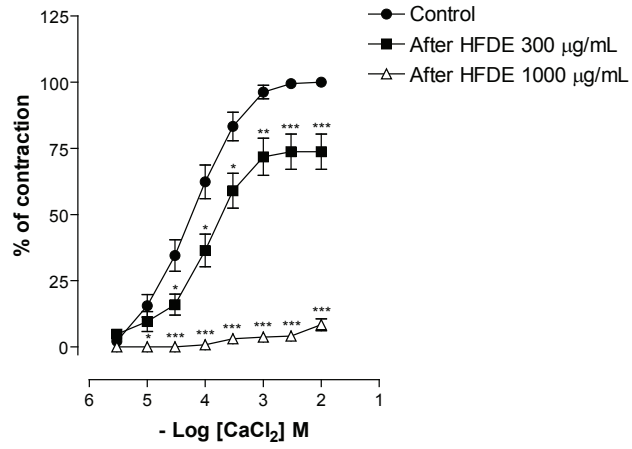

Figure 3. Concentration-response curves to $CaCl_2$ (3 x 10^{-6}, 10-5, 3 x 10^{-5}, 10^{-4}, 3 x 10^{-4}, 10^{-3}, 3 x 10^{-3}, 10^{-2} and 3 x 10^{-2} M) in rings of rat superior mesenteric artery, without endothelium before (control) and after pre-incubation with HFDE at concentrations of 300 and 1000 µg/mL, separately. Values are expressed as mean±SEM, n = 6. The data were analyzed with repeated measures two-way ANOVA followed by Bonferroni post-test. *p< 0.05, **p< 0.01 and ***p< 0.001 *vs* control.

As reported by Chan et al. (2000), nifedipine, a L-type voltage-operated Ca^{2+} channel selective blocker, also inhibited the concentration-response curve to $CaCl_2$, suggesting strongly that HFDE could be acting possibly as a calcium channel blocker.

Finally, we performed experiments in that were observed the vasorelaxation response of HFDE (1000 µg/mL) and 10 µM of nifedipine, separately, and the effect of HFDE (1000 µg/mL) after maximal vasorelaxant response induced by 10 µM of nifedipine. In this condition, HFDE (1000 µg/mL) or nifedipine (10 µM) were capable of inducing vasorelaxation of Phe tonus (E_{max} = 79±9 and 78±3%; n = 6, respectively), and HFDE (1000 µg/mL) induced a small but significant additional vasorelaxation effect on the maximal vasorelaxation of nifedipine (10 mM) (15±3%; n = 7), suggesting that HFDE appears to be acting in major part by the same pathway of the nifedipine.

However, the observation of an additional vasorelaxation after maximal response of nifedipine allow us to hypothesize that other pathway appears to be implicated in the HFDE-induced response, possibly involving intracellular calcium stores. However, further experiments are necessary to clearly elucidate this assumption.

In conclusion, these results demonstrate that the dichloromethane extract of *Hyptis fruticosa* Salzm. ex Benth., Lamiaceae, (HFDE) produces vasorelaxant effect in rat superior mesenteric artery through an endothelium-

independent pathway, which appears to be due in major part to inhibition of the Ca^{2+} influx through voltage-operated Ca^{2+} channels.

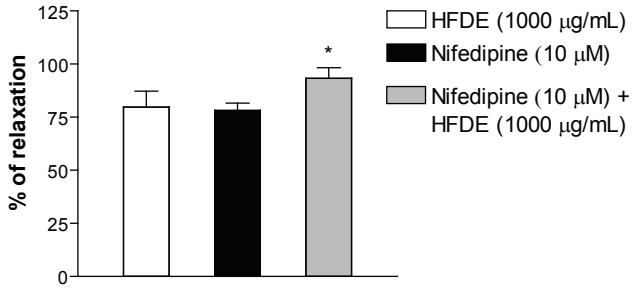

Figure 4. Vasorelaxant effect of nifedipine (10 μM) before and after administration of HFDE (1000 μg/mL) in rings of rat isolated superior mesenteric artery without functional endothelium pre-contracted with 10 μM Phe. Values are expressed as mean±SEM, n = 6. The data were analyzed with one-way ANOVA followed by Bonferroni post-test. *$p< 0.05$ *vs* Nifedipine.

ACKNOWLEDGEMENTS

We thank technical assistance of Mr. Osvaldo Andrade Santos. This work was supported by grants from CNPq, CAPES, FAPITEC-SE, Ministério da Saúde, SES/SE, Brazil.

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