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Spasmolytic activity of *Hyptis macrostachys* Benth. (Lamiaceae)

Iara Leão Luna de Souza[1], Gislaine Alves de Oliveira[1], Rafael de Almeida Travassos[1], Luiz Henrique César Vasconcelos[1], Ana Carolina de Carvalho Correia[1], Italo Rossi Roseno Martins[1], Massilon da Silva Moreira dos Santos Júnior[1], Vicente Carlos de Oliveira Costa[1], Josean Fechine Tavares[1,2], Marcelo Sobral da Silva[1,2] and Bagnólia Araújo da Silva[1,2]*

[1]Centro de Ciências da Saúde (CCS), Universidade Federal da Paraíba (UFPB), Campus I, 58.051-970, PO Box 5009, João Pessoa, Paraíba, Brazil.
[2]Departamento de Ciências Farmacêuticas (DCF), Universidade Federal da Paraíba, Campus I, 58.051-970, João Pessoa, Paraíba, Brazil.

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***Hyptis* is a worldwide genus cited by its medicinal uses. *Hyptis macrostachys* Benth., a species popularly known in Brazil as "alfavaca-brava" and "hortelã-do-mato", is used in folk medicine to relief asthma, cough and bronchitis symptoms. Thus, this study investigated a possible spasmolytic effect of the aerial parts ethanol extract from *H. macrostachys* Benth. (HM-EtOH$_{PA}$) on several smooth muscle models. On the rat aorta, the uterus was observed and guinea pig trachea HM-EtOH$_{PA}$ did not show relevant spasmolytic action in all tested concentrations (243 to 729 µg/ml). However, on guinea pig ileum, HM-EtOH$_{PA}$ (9 to 729 µg/ml) significantly ($p < 0.001$) inhibited in a concentration-dependent manner the contractions induced by carbachol (CCh) 10^{-6} M and histamine 10^{-6} M as well as relaxed this organ in an equipotent and concentration-dependent (1 to 729 µg/ml) manner when pre-contracted with CCh 10^{-6} M, histamine 10^{-6} M and KCl 40 mM, suggesting its actions in the voltage-gated calcium channels (Ca_V) blockade. Since HM-EtOH$_{PA}$ (9 to 729 µg/ml) inhibited Ca^{2+}-induced contractions in Ca^{2+}-free depolarizing medium and relaxed ileum pre-contracted with S-(-)-Bay K8644 (3×10^{-7} M) in a concentration-dependent manner, the hypothesis of Ca_v was confirmed. Thus, *H. macrostachys* Benth. showed a selective spasmolytic action on guinea pig ileum by blocking Ca^{2+} entry through Ca_V.**

Key words: Lamiaceae, *Hyptis macrostachys* Benth., relaxant, antispasmodic, calcium channel.

INTRODUCTION

Plants are a known source of chemical constituents with spasmolytic activity that relieve colicky pain, which constitute a very important symptom of gastrointestinal motility disorders such as dyspepsia (indigestion), intestinal spasms, peptic and duodenal ulceration, nausea and vomiting, constipation and irritable bowel syndrome (Williamson et al., 1996; Sadraei et al., 2003).

Lamiaceae members have been used for centuries in folk medicine (Deo et al., 2011), being characterized as a flowering family and also called of mint family (Harley et al., 2004). The *Hyptis* genus includes around 400 species distributed in American, West Africa, Fiji Island (Oceania) and Western India (Raja, 2012). In Brazil, *Hyptis* species are found in the northern region and have high economic

*Corresponding author. E-mail: bagnoliasilva@yahoo.com or bagnolia@pq.cnpq.br. Tel: +55-83-32167126. Fax: +55-83-3216-7502.

importance since they are source of aromatic essential oils (Falcão and Menezes, 2003).

Some *Hyptis* spp. presented interesting biological activities such as anti-hyperglycemic (Mishra et al., 2011), antiulcerogenic (Caldas et al., 2011), hypotensive (Santos et al., 2007), anti-inflammatory and antinociceptive activities (Raymundo et al., 2011). *Hyptis* spp. have shown spasmolytic effect, such as *Hyptis capitata,* which is used in folk medicine for asthma treatment (Almtorp et al., 1991) and *Hyptis suaveolens* that is used to treat dysmenorrhoea (Agra et al., 2007).

Hyptis macrostachys Benth. is a species found in the Semi-Arid region of Brazil popularly known as “alfavaca-brava” and “hortelã-do-mato”. In folk medicine, tea and syrup of its aerial parts are used orally to relief symptoms in asthma, cough and bronchitis (Agra et al., 2008). Based on the ethnopharmacological uses and spasmolytic activity related for *Hyptis* genus and *H. macrostachys* Benth., this study aimed to investigate a possible spasmolytic action of this species on guinea pig ileum and trachea, rat uterus and male rat aorta and so contribute to Lamiaceae pharmacological studies.

MATERIALS AND METHODS

Plant

H. macrostachys Benth. was collected in Matureia municipality, Paraíba, in March 2009 and identified by Maria de Fátima Agra (PhD) of the Department of Botany of Universidade Federal da Paraíba (UFPB). A voucher specimen was deposited in the Herbarium by Prof. Lauro Pires Xavier (JPB) under the identification code of AGRA 6947.

Extraction

The collected plant material was dried in a stove with circulating air at 40°C temperature and then was chopped by a mechanical mill. The fine powdered from aerial parts (3.0 kg) was macerated with 95% ethanol (5 L) during 72 h. The extraction solution was concentrated under reduced pressure using a rotary evaporator to give 200 g of ethanol extract of the aerial parts from *H. macrostachys* Benth. (HM-EtOH$_{PA}$).

Drugs and salts

Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), calcium chloride bihydrate ($CaCl_2.2H_2O$), potassium chloride (KCl) and magnesium chloride ($MgCl_2$) were purchased from Vetec Química Fina Ltd (Duque de Caxias, RJ, Brazil). Monosodium phosphate-1-hydrate ($NaH_2PO_4.H_2O$) and monopotassium phosphate (KH_2PO_4) were purchased from Nuclear (São Paulo, Brazil). Glucose and sodium bicarbonate ($NaHCO_3$) were purchased from Dinâmica (São Paulo, Brazil). Sodium chloride (NaCl) was purchased from Fmaia (São Paulo, Brazil).

Histamine dihydrochloride, Cremophor EL®, diethylstilbestrol, oxytocin, acetylcholine (ACh), arachidonic acid (AA) and phenylephrine hydrochloride (Phe) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Ethanol PA was purchased from Reagen Ltd (Colombo, PR, Brazil). Carbamylcholine hydrochloride (CCh) was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA). All substances were dissolved in distilled water except for HM-EtOH$_{PA}$, which was solubilized in Cremophor EL® (3%), dissolved in distilled water to the concentration of 10 µg/ml, and re-diluted in distilled water as required for each experimental protocol. The final Cremophor EL® concentration in the organ-bath never exceeded 0.01% (v/v).

Animal and organ manipulation

Experiments were performed with trachea rings and ileum segments of guinea pigs (*Cavia porcelus,* 300 to 500 g), rat uterus (150 to 250 g) and male rat aorta (250 to 350 g) (*Rattus norvegicus*) from the bioterium of Prof. Thomas George of the Centro de Biotecnologia (CBiotec)/UFPB. The animals had full access to food and water, were kept in rooms at 21 ± 1°C and submitted to a 12 h light-dark cycle. Adult guinea pigs of both sexes were fasted for 18 h before the experiments (only water available). Adult female rats were treated with diethylstilbestrol (1.0 mg/kg subcutaneously (s.c.)) 24 h before the experiments for estrus induction. All experimental procedures were performed in accordance with guidelines approved by Animal Research Ethic Committee of UFPB (protocol: 0506/05).

Animals were euthanized by cervical dislocation. Organs were immediately removed, cleaned of adhering fat and connective tissue and suspended under organ baths containing the following physiological solutions: modified Krebs for ileum, Locke-Ringer for uterus and Krebs for trachea and aorta, kept under appropriate temperature (37°C for ileum, trachea and aorta, and 32°C for uterus) and continuously aerated with a mixture of 95% O_2 and 5% CO_2. The solutions composition (mM) were Krebs: NaCl (118), KCl (4.55), $MgSO_4$ (5.7), KH_2PO_4 (1.1), $CaCl_2$ (2.52), $NaHCO_3$ (25), glucose (11); modified Krebs: NaCl (117), KCl (4.7), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25), glucose (11); Locke Ringer: NaCl (154), KCl (5.63), $MgCl_2$ (2.1), $CaCl_2$ (2.16), $NaHCO_3$ (5,95), glucose (5.55). The pH was adjusted to 7.4.

All organs were suspended in 5 ml organ baths under resting load of 1.0 g. To register isometric contractions, organs were suspended in steel rods, connected to a force transducer (FORT-10), attached to an amplifier (TMB4M, World Precision Instruments, Sarasota, FL, USA) and connected to an A/D converter into a PC running Biomed® software (BioData, Brazil). To isotonic contractions, organs were suspended by cotton yarn and recorded on smoked drum through levers coupled to kymographs.

Effect of HM-EtOH$_{PA}$ on phenylephrine-induced tonic contractions in rat aorta in both absence and presence of endothelium

After a 60 min resting period, the aortic rings were contracted with phenylephrine (3×10^{-7} M) and the isometric tension was recorded. When a stable contraction was attained (15 to 20 min), acetylcholine (10^{-6} M) was added to the organ bath to confirm the presence of functional endothelium by the presence of acetylcholine-induced relaxation above 50% of maximal tension (Furchgott and Zawadzki, 1980). In some aortic rings, the luminal surface was low rubbed with Krebs-wet cotton to remove the endothelial layer. The absence of functional endothelium was confirmed by the absence of acetylcholine-induced relaxation or when the relaxation was inferior to 10% of maximal tension. During the tonic phase of a second response to phenylephrine, HM-EtOH$_{PA}$ (243 and 729 µg/ml) was cumulatively added as an attempt to obtain concentration-relaxation curves in both absence and presence of functional endothelium. The relaxant effect induced by HM-EtOH$_{PA}$ was expressed as the reverse percentage of the initial contraction force elicited by phenylephrine.

Effect of HM-EtOH$_{PA}$ on carbachol-induced tonic contractions in guinea pig trachea in both absence and presence of epithelium

After a 60 min resting period, the tracheal rings were contracted with CCh (10^{-6} M) and the isometric tension was recorded. When a stable contraction was attained (15 to 20 min), arachidonic acid (10^{-4} M) was added to the organ bath to confirm the presence of epithelium by the presence of arachidonic acid-induced relaxation above 50% of maximal tension (Tschirhart et al., 1987). In some tracheal rings, the luminal surface was gently rubbed with Krebs-wet cotton to remove the epithelial layer. The absence of epithelium was confirmed when arachidonic acid-induced relaxation was absent or when the relaxation was inferior to 10% of maximal tension. During the tonic phase of a second response to CCh, HM-EtOH$_{PA}$ (243 and 729 µg/ml) was cumulatively added as an attempt to obtain concentration-relaxation curves in both absence and presence of epithelium. The relaxant effect induced by HM-EtOH$_{PA}$ was expressed as the reverse percentage of the initial contraction force elicited by CCh.

Effect of HM-EtOH$_{PA}$ on oxytocin and carbachol-induced phasic contractions in rat uterus

After a 40 min stabilization period, two similar dose-response curves were obtained with oxytocin (10^{-2} IU/ml) and CCh (10^{-5} M). The contraction process was repeated until a stable response to oxytocin or CCh was obtained. HM-EtOH$_{PA}$ (243 and 729 µg/ml) effects were then determined by pre-incubating the uterine strips for 15 min with a single concentration of the extract in independent experiments before adding oxytocin or CCh (Revuelta et al., 2000).

Effect of HM-EtOH$_{PA}$ on histamine and carbachol-induced phasic contractions in guinea pig ileum

After a 30 min stabilization period, two similar dose-response curves were obtained with a submaximal concentration to CCh (10^{-6} M) and histamine (10^{-6} M). The contraction process was repeated until a stable response to CCh or histamine was obtained. HM-EtOH$_{PA}$ (9 to 729 µg/ml) effects were then determined by pre-incubating the ileum strips for 15 min with a single concentration of the extract in independent experiments before adding CCh or histamine (Daniel et al., 2001).

Effect of HM-EtOH$_{PA}$ on KCl, histamine and carbachol-induced tonic contractions in guinea pig ileum

After the stabilization period, a contraction was induced with KCl (40 mM), CCh (10^{-6} M) or histamine (10^{-6} M). During the tonic phase, HM-EtOH$_{PA}$ (1 to 729 µg/ml) was cumulatively added as an attempt to obtain concentration-relaxation curves in different preparations. The relaxant effect induced by HM-EtOH$_{PA}$ was expressed as the reverse percentage of the initial contraction force elicited by CCh.

Effect of HM-EtOH$_{PA}$ on $CaCl_2$-induced contractions in a depolarizing nominally Ca^{2+}-free medium in guinea pig ileum

After the appropriated stabilization period, the modified Krebs solution was replaced by a depolarizing Ca^{2+}-free modified Krebs solution (KCl, 4 mM was increased to 40 mM with equimolar exchange for NaCl). After 45 min, two concentration-response curves to $CaCl_2$ (10^{-6} to 10^{-1} M) were constructed in absence of HM-EtOH$_{PA}$. After washing, the tissues were pre-incubated with different concentrations of HM-EtOH$_{PA}$ (9 to 729 µg/ml) for 15 min and a new $CaCl_2$ concentration-response curve was constructed. The maximal contraction obtained with $CaCl_2$ concentration-response curve was considered to be 100% (control), and all contractions were assessed referring to it. Each preparation was exposed to a single concentration of HM-EtOH$_{PA}$ (Van Rossum, 1963).

Effect of HM-EtOH$_{PA}$ on S-(-)-Bay K8644-induced tonic contractions in guinea pig ileum

After the stabilization period, the ileum was partially depolarized by adding KCl (15 mM) for 10 min (Usowicz et al., 1995) and a contraction was induced with S-(-)-Bay K8644 (3×10^{-7} M), a selective voltage-dependent calcium channel (Ca_v) agonist to L-type or Ca_V1 (Ferrante et al., 1989). In the sustained tonic phase of the contraction, HM-EtOH$_{PA}$ (9 to 729 µg/ml) was added cumulatively in different preparations, in order to obtain a relaxation curve. The relaxation was expressed as the reversal percentage of initial contraction elicited by contractile agents.

Statistical analysis

Results were statistically analyzed using Student's test and one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc when appropriate. Differences between values were considered significant when a calculated $p < 0.05$.

IC_{50} (Molar concentration of an antagonist which produces 50% of its maximum inhibitory response) and EC_{50} (molar concentration of an agonist which produces 50% of its maximum effective response) were calculated with nonlinear regression and were used to express spasmolytic potency and maximum effect (E_{max}) was used to express spasmolytic efficacy. Values were presented as mean and standard error of mean (SEM) in all experiments.
All data were analyzed with GraphPad Prism software version 5.01 (GraphPad Software Inc., San Diego CA, USA).

RESULTS

Effect of HM-EtOH$_{PA}$ on phenylephrine-induced tonic contractions in rat aorta in both absence and presence of endothelium

HM-EtOH$_{PA}$ (243 and 729 µg/ml) relaxed aorta rings pre-contracted with Phe (3×10^{-7} M) in both absence (E_{max} = 1.8 ± 0.9 and 97.4 ± 2.6%, respectively, n = 3) and presence (E_{max} = 1.7 ± 1.0 and 99.1 ± 0.9%, respectively, n = 3) of functional endothelium (Figure 1). HM-EtOH$_{PA}$ relaxant effect was reversed 60 min after its removal from organ baths (data not shown).

Effect of HM-EtOH$_{PA}$ on carbachol-induced tonic contractions in guinea pig trachea in both absence and presence of epithelium

HM-EtOH$_{PA}$ (243 and 729 µg/ml) relaxed tracheal rings pre-contracted with CCh (10^{-6} M) in both absence (E_{max} = 4.6 ± 2.9 and 12.8 ± 5.6%, respectively, n = 3) and presence (E_{max} = 17.8 ± 6.4 and 43.2 ± 5.8%, respectively, n = 3) of functional epithelium (Figure 2). HM-EtOH$_{PA}$

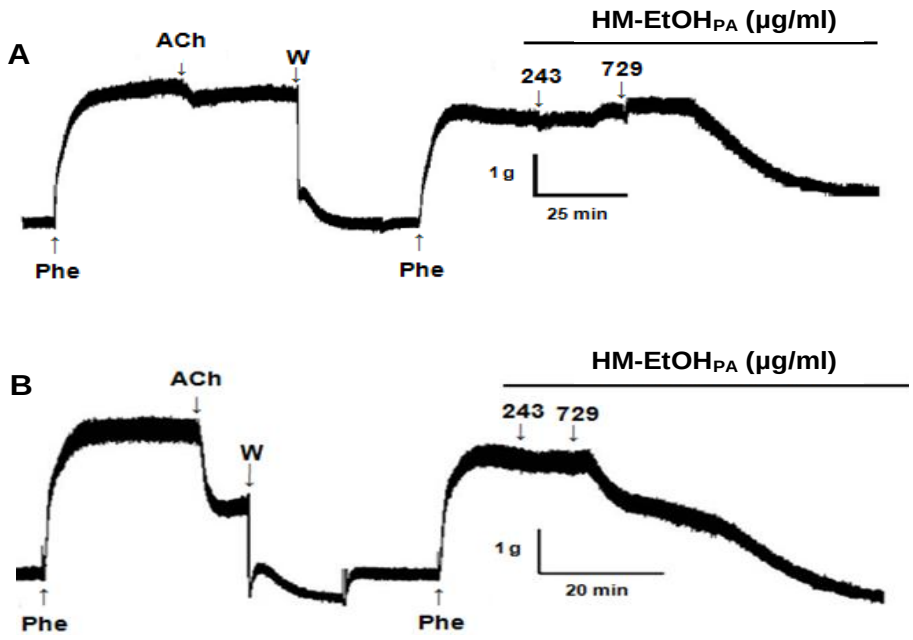

Figure 1. Representative register of HM-EtOH$_{PA}$ relaxant effect (243 and 729 µg/ml) on rat aorta pre-contracted with Phe (3×10^{-7} M) in functional endothelium absence (A) and presence (B) (n = 3).

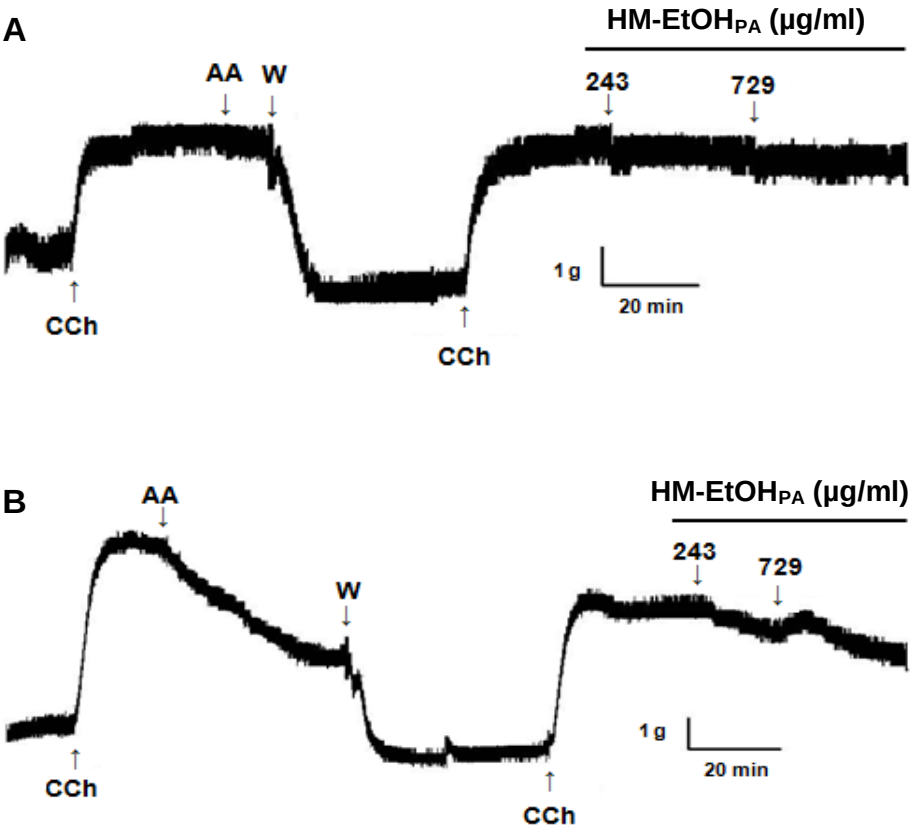

Figure 2. Representative register of HM-EtOH$_{PA}$ relaxant effect (243 and 729 µg/ml) on guinea pig trachea pre-contracted with CCh (10^{-6} M) in functional epithelium absence (A) and presence (B) (n = 3).

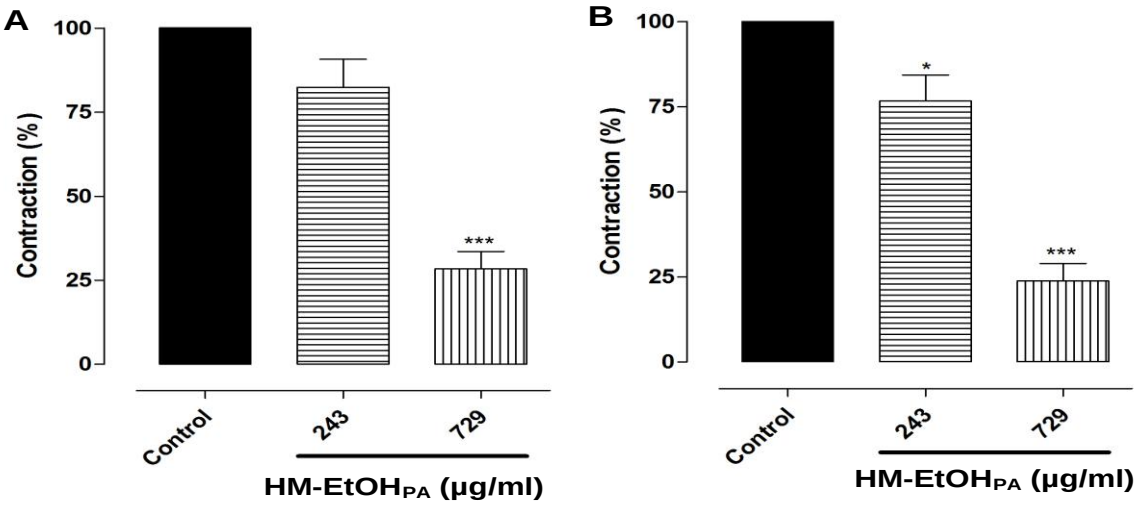

Figure 3. Effect of HM-EtOH$_{PA}$ on phasic contractions induced with CCh (10^{-5} M) (A) or oxytocin (10^{-2} UI/mL) (B) on rat uterus (n = 3). The columns and vertical bars represent mean and S.E.M. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by *p < 0.05 and ***p < 0.001 (Control vs. HM-EtOH$_{PA}$).

relaxant effect was reversed 60 min after its removal from organ baths (data not shown).

Effect of HM-EtOH$_{PA}$ on oxytocin and carbachol-induced phasic contractions in rat uterus

HM-EtOH$_{PA}$ (243 and 729 µg/ml) significantly ($p < 0.05$ and $p < 0.001$) antagonized the phasic contractions induced by CCh at 10^{-5} M (E_{max} = 17.6 ± 8.3 and 71.6 ± 5.2 µg/ml, respectively, n = 3) (Figure 3A) and oxytocin at 10^{-2} UI/ml (E_{max} = 23.3 ± 7.6 and 76.1 ± 5.1 µg/ml, respectively, n = 3) (Figure 3B) on rat uterus. HM-EtOH$_{PA}$ inhibitory effect was reversed 45 min after its removal from organ baths (data not shown).

Effect of HM-EtOH$_{PA}$ on histamine and carbachol-induced phasic contractions in guinea pig ileum

HM-EtOH$_{PA}$ (27 to 729 µg/ml) antagonized the phasic contractions induced by CCh at 10^{-6} M (IC_{50} = 164.7 ± 36.9 µg/ml, n = 5) (Figure 4A) and histamine at 10^{-6} M (IC_{50} = 93.2 ± 22.2 µg/ml, n = 5) (Figure 4B) on guinea pig ileum in a significant ($p < 0.001$) and concentration-dependent manner. HM-EtOH$_{PA}$ inhibitory effect was reversed 30 min after its removal from organ baths (data not shown).

Effect of HM-EtOH$_{PA}$ on KCl, histamine and carbachol-induced tonic contractions in guinea pig ileum

HM-EtOH$_{PA}$ (3 to 729 µg/ml) showed an equipotent and concentration dependent spasmolytic effect in ileum pre-contracted with KCl at 40 mM (EC_{50} = 52.9 ± 4.7 µg/ml, n = 5), CCh at 10^{-6} M (EC_{50} = 54.8 ± 2.8 µg/ml, n = 5) or histamine at 10^{-6} M (EC_{50} = 38.9 ± 5.5 µg/ml, n = 5) (Figure 5). HM-EtOH$_{PA}$ relaxant effect was reversed 30 min after its removal from organ baths (data not shown).

Effect of HM-EtOH$_{PA}$ on $CaCl_2$-induced contractions in a depolarizing nominally Ca^{2+}-free medium in guinea pig ileum

HM-EtOH$_{PA}$ (27 to 729 µg/ml) concentration-dependently inhibited Ca^{2+}-induced contractions in Ca^{2+}-free depolarizing medium (Figure 6). $CaCl_2$ cumulative concentration-response curves were non-parallelly shifted to the right and E_{max} reduced from 100% (control) to 98.6 ± 0.8, 72.4 ± 1.6, 67.7 ± 2.3, 16.9 ± 2.5 and 2.3 ± 0.4% which indicates a possible Ca_v blockade.

Effect of HM-EtOH$_{PA}$ on S-(-)-Bay K8644-induced tonic contractions in guinea pig ileum

HM-EtOH$_{PA}$ (27 to 729 µg/ml) showed a concentration-dependent spasmolytic effect in ileum pre-contracted with S-(-)-Bay K8644 at 3 × 10^{-7} M (Figure 7). HM-EtOH$_{PA}$ was 8 folds more potent in relaxing the ileum pre-contracted with 40 mM KCl (EC_{50} = 444.4 ± 159.6 µg/ml, n = 5) than with S-(-)-Bay K8644 (EC_{50} = 52.4 ± 4.7 µg/ml, n = 5).

DISCUSSION

In the investigation of this study, ethanol extract of the

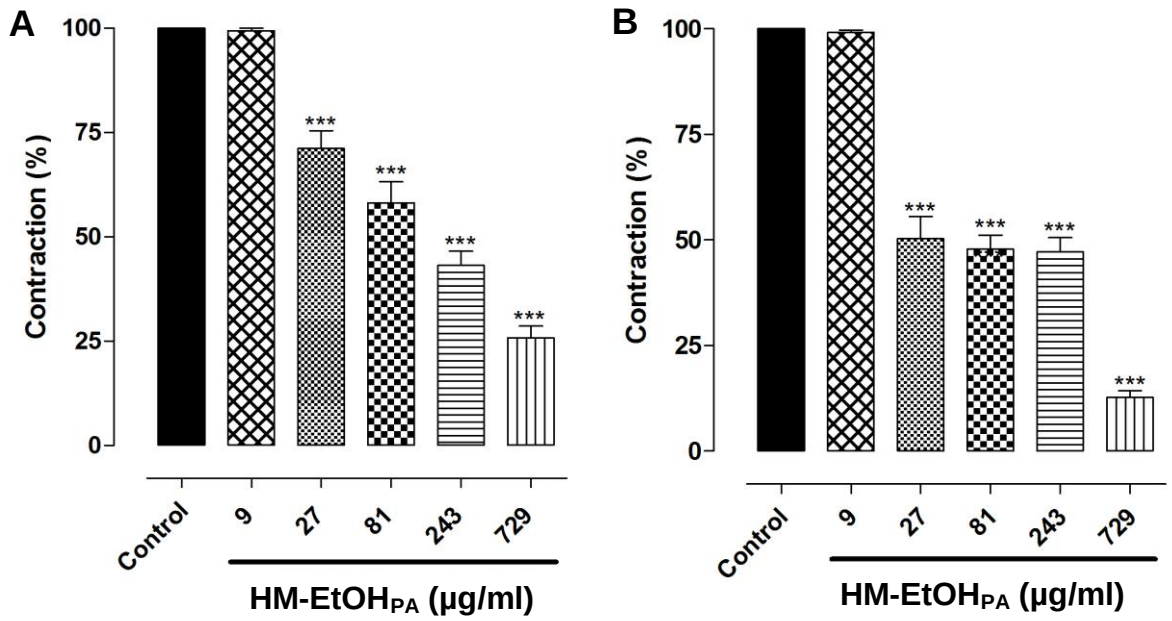

Figure 4. Effect of HM-EtOH$_{PA}$ on phasic contractions induced with CCh (10^{-6} M) (A) or histamine (10^{-6} M) (B) on guinea pig ileum (n = 5). The columns and vertical bars represent mean and SEM. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by ***p < 0.001 (Control vs. HM-EtOH$_{PA}$).

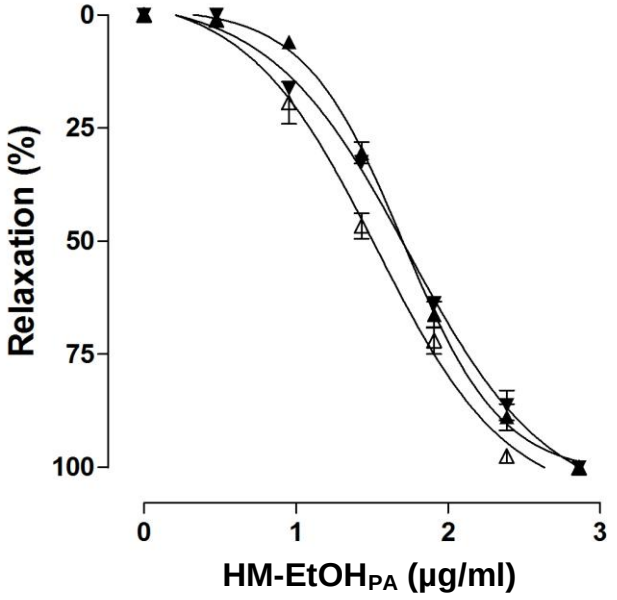

Figure 5. Effect of HM-EtOH$_{PA}$ on tonic contractions induced with KCl (40 mM) (▲), CCh (10^{-6} M) (▼) or histamine (10^{-6} M) (△) on guinea pig ileum (n = 5). The symbols and vertical bars represent mean and SEM, respectively. One-way ANOVA followed by Bonferroni's test.

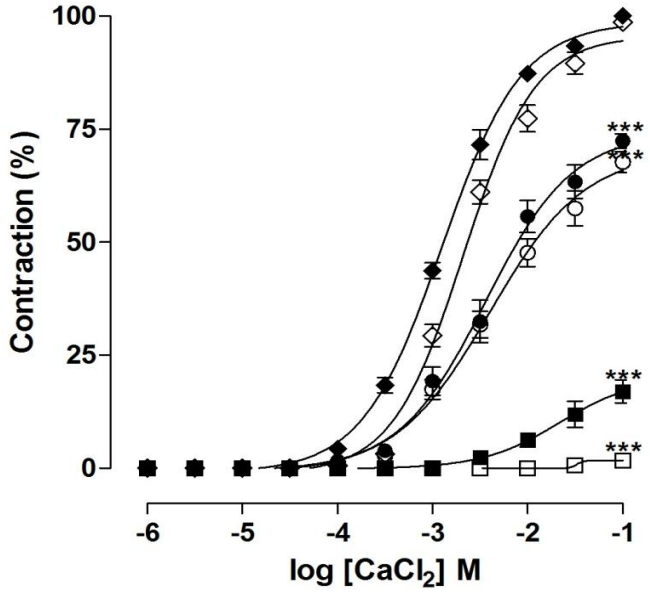

Figure 6. Cumulative concentration-response curves to $CaCl_2$ in depolarizing medium nominally without Ca^{2+} in the absence (◆) and presence of HM-EtOH$_{PA}$: 9 (◇), 27 (●), 81 (○), 243 (■) and 729 µg/mL (□) (n = 5). Symbols and vertical bars represent the mean and S.E.M. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by ***p < 0.001 (Control vs. HM-EtOH$_{PA}$).

aerial parts from *H. macrostachys* Benth. (HM-EtOH$_{PA}$) was studied on several smooth muscle models and showed a selective spasmolytic effect on guinea pig ileum and possibly the observed effect is due to blockade of the Ca_v that reduces cytosolic calcium concentration ($[Ca^{2+}]_c$) leading to smooth muscle relaxation.

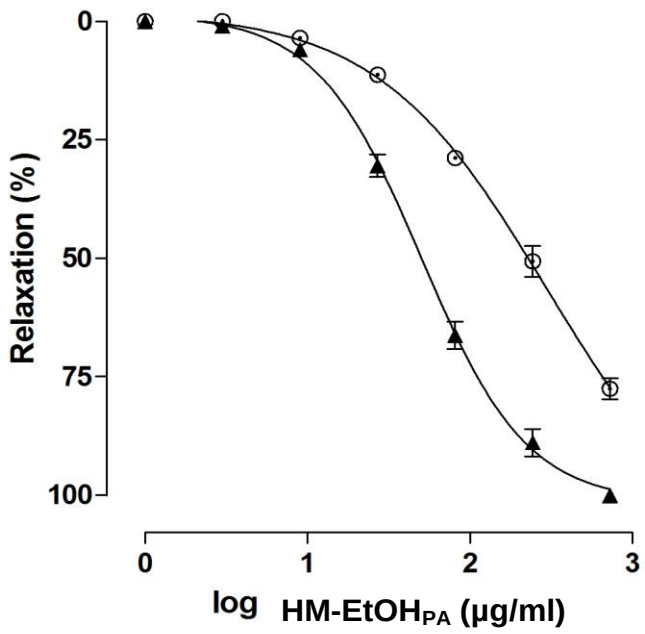

Figure 7. Effect of HM-EtOH$_{PA}$ on tonic contractions induced with KCl (40 mM) (▲) or S-(-)-Bay K8644 (3 x 10^{-7} M) (⊙) on guinea pig ileum (n = 5). The symbols and vertical bars represent mean and SEM, respectively.

Initially, a possible relaxant effect of HM-EtOH$_{PA}$ on rat aorta pre-contracted by Phe in both functional endothelium absence and presence were evaluated (Figure 1), but the relaxant effect was only observed in the highest concentration tested, although not in concentration-dependent manner.

In folk medicine, some species of *Hyptis* (e.g. *H. capitata*) (Almtorp et al., 1991) as well as *H. macrostachys* Benth., were used for asthma treatment (Agra et al., 2008). A possible activity of HM-EtOH$_{PA}$ on guinea pig trachea contracted by CCh in both functional epithelium absence and presence were evaluated. Figure 2 showed that HM-EtOH$_{PA}$ relaxant effect was significant ($p < 0.05$) but not concentration-dependent. These findings suggested that aerial parts of *H. macrostachys* did not presented spasmolytic effect on non-sensitized (healthy) guinea pig trachea, but an action in asthma of *H. macrostachys* is not discarded, being necessary studies using asthmatic animal model (e.g. sensitized guinea pigs) to confirm or refute this species usage in folk medicine.

Additionally, a possible antispasmodic action on rat uterus since other species of *Hyptis* are used for dysmenorrhoea treatment was evaluated (Agra et al., 2007) and an antagonism of phasic contractions induced by CCh or oxytocin was observed, but only in a higher concentration (Figure 3).

Interestingly, as shown in Figures 4 and 5, in guinea pig ileum, HM-EtOH$_{PA}$ inhibited both CCh and histamine-induced contractions and relaxed this organ pre-contracted by KCl, CCh and histamine in a concentration-dependent manner presenting a selective spasmolytic activity to guinea pig ileum. Furthermore, as HM-EtOH$_{PA}$ was equipotent in antagonize CCh or histamine-induced contractions and its pharmacological potency presented no difference in tonic contractions induced by KCl (electromechanical coupling), CCh or histamine (pharmacomechanical and electromechanical coupling), it can be indicated suggesting that HM-EtOH$_{PA}$ is not acting at receptor level, but possibly in a common step in the contraction pathway elicited by these contractile agents in guinea pig ileum.

Ileal smooth muscle presents biphasic contraction where muscle exhibits a fast and transient contraction in the first phase, followed by a long-lasting second phase characterized by maintained tonic contraction (Horie et al., 2005). Since both phasic and tonic contractions depend on extracellular Ca^{2+} as they are inhibited in its absence (Honda et al., 1996) and the main responsible for Ca^{2+} entry are Ca_v (Rembold, 1996). This led to the evaluation of the participation of these channels on HM-EtOH$_{PA}$ spasmolytic action.

Thus, $CaCl_2$ induced contractions in depolarizing nominally Ca^{2+}-free medium were used to assess the possible Ca_v blockade. This protocol is based on the fact that contraction will be obtained almost exclusively by Ca^{2+} from extracellular medium, since depolarization promoted by elevated extracellular potassium concentrations leads to Ca_v opening (Rembold, 1992). HM-EtOH$_{PA}$ inhibited significantly ($p < 0.001$) $CaCl_2$ induced contractions, shifting them to the right and reducing E_{max}, reinforcing the hypothesis of calcium influx blockade.

In smooth muscle, Ca_v1 are the main responsible to Ca^{2+} influx. These channels are subdivided as $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ are sensitive to dihydropyridine and high voltage (Alexander et al., 2008). Ca_v are composed by 4 subunits (2 α, 1 β and 1 γ) where α1 forms the pore that leads Ca^{2+} influx (Kuriyama et al., 1995). Thus, the next step was to confirm and identify the Ca_v subtype involved on HM-EtOH$_{PA}$ spasmolytic activity. Therefore, tonic contractions were obtained using S-(-)-Bay K8644, a specific dihydropyridine derivative agonist for Ca_v1 that binds directly with α1 subunit to open these channels, but not by depolarization (Spedding and Paoletti, 1992). HM-EtOH$_{PA}$ was more potent in relaxing the ileum pre-contracted with KCl than S-(-)-Bay K8644, which can be explained by the fact that KCl, in addition to induce Ca_V activation by depolarization, utilizes other mechanisms to sustain the tonic phase of smooth muscle contraction, such as Ca^{2+} sensitization involving translocation and activation of RhoA Kinase (Ratz et al., 2005). On the other hand, S-(-)-Bay K8644 keeps contraction mainly by direct activation of Ca_V (Spedding and Paoletti, 1992). Furthermore, as the more expressive Ca_v subtype in ileum is $Ca_v1.2$ (Catterall et al., 2005), it is suggested that the Ca^{2+} influx blockade through $Ca_v1.2$ is

implicated in the mechanism of HM-EtOH$_{PA}$ spasmolytic action on guinea pig ileum.

In conclusion, this study demonstrated that HM-EtOH$_{PA}$ did not have a relevant spasmolytic activity on rat uterus, rat aorta and guinea pig trachea, but showed a selective action on guinea pig ileum, probably, due to a Ca^{2+} influx blockade through $Ca_V1.2$.

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