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Antispasmodic and relaxant effects of the hidroalcoholic extract of *Pimpinella anisum* (Apiaceae) on rat anococcygeus smooth muscle

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Abstract

The present work describes the mechanisms involved in the muscle relaxant effect of ethanol:water (40:60, 60:40 and 80:20) aerial parts extracts of *Pimpinella anisum*. Three hidroalcoholic extracts in which the proportion of ethanol was 40% ($HA_{40\%}$), 60% ($HA_{60\%}$) or 80% ($HA_{80\%}$) were tested for activity in the rat anococcygeus smooth muscle. The three extracts (50 μg/mL) inhibited acetylcholine-induced contraction. The extract $HA_{60\%}$ (5–50 μg/mL) concentration dependently relaxed acetylcholine-pre-contracted tissues (31.55 ± 3.56%). Conversely, $HA_{40\%}$ and $HA_{80\%}$ did not exert relaxant action. Pre-incubation of the preparations with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 3 μM) and oxyhemoglobin (10 μM) reduced the relaxation induced by $HA_{60\%}$ (percentage of relaxation: 6.81 ± 1.86%, 13.13 ± 5.87% and 2.12 ± 1.46%, respectively). Neither indomethacin (10 μM) nor tetraethylammonium (1 mM) affected the relaxation induced by $HA_{60\%}$. Incubation of the tissues with L-NAME significantly enhanced the maximal contraction induced by acetylcholine, indicating an inhibitory role for NO in the modulation of the contractile response of anococcygeus smooth muscle to acetylcholine. However, simultaneous addition of L-NAME and $HA_{60\%}$ resulted in an effect similar to that observed with L-NAME alone, further confirming the observation that *Pimpinella anisum* acts by realizing NO. Additionally, $HA_{60\%}$ did not alter $CaCl_2$-induced contraction. Collectively, our results provide functional evidence that the effects elicited by the hidroalcoholic extract of *Pimpinella anisum* involve the participation of NO and subsequent activation of the NO-cGMP pathway. The relaxant action displayed by *Pimpinella anisum* justifies its use in the folk medicine as an antispasmodic agent.

Keywords: *Pimpinella anisum*; Apiaceae; Relaxation; Nitric oxide; Anococcygeus smooth muscle

1. Introduction

Plant extracts are considered nowadays as potential bioactive agents that can interfere and alter different cellular processes. *Pimpinella anisum* L. (*Pimpinella anisum*) is a grassy plant with white flowers and small green to yellow seeds, which grows in many warm regions in the world including Brazil. *Pimpinella anisum* is a plant rich in volatile oils, which are employed in the folk medicine. A concoction of seeds in hot water is used as a carminative, antiseptic, diuretic, digestive and a folk remedy to insomnia and constipation (Bisset, 1994). Furthermore, several therapeutic effects including those on digestive disorders, gynecologic, and also anticonvulsant, anti-asthma and dyspnea have been described for the seeds of *Pimpinella anisum* in ancient medical books (Aboabrahim, 1970).

Many biological actions of *Pimpinella anisum* have previously been reported. The essential oil of *Pimpinella anisum* has

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been described to exert fungicidal activity in a concentration-dependent manner (Soliman and Badeaa, 2002). Singh et al. (2002) provided evidence that the essential oil extracted from the seeds of *Pimpinella anisum* has strong antibacterial activity against eight human pathogenic bacteria. In addition, the fruit essential oil of *Pimpinella anisum* possesses anticonvulsant activity in the mouse (Pourgholami et al., 1999). More recently, it has been found that *Pimpinella anisum* oil increases glucose absorption and reduces urine output in the rat (Kreydiyyeh et al., 2003). Moreover, antioxidant and antimicrobial activities of water and ethanol extracts of *Pimpinella anisum* seed were described by Gülçin et al. (2003). Biological assays have shown that *Pimpinella anisum* also exerts relaxant actions. In this line, Reiter and Brandt (1985) described that the volatile oil from this plant displays relaxant effects on the isolated tracheal muscles of guinea pig. The aqueous and ethanol extracts as well as the essential oil of *Pimpinella anisum* relaxed isolated tracheal chains of the guinea pig pre-contracted with methacholine (Boskabady and Ramazani-Assari, 2001). These authors have also described that the relaxant response induced by *Pimpinella anisum* is due to its inhibitory effects on muscarinic receptors.

The rat anococcygeus smooth muscle, first described by Gillespie (1972), is a paired smooth muscle with dense sympathetic innervations, which represents most of its total innervations in addition to a variety of innervations including cholinergic, serotoninergic, purinergic, and non-adrenergic, non-cholinergic (NANC) ones. Then, because of its diversity of receptors, anococcygeus muscle is a widely used smooth muscle preparation for the study of the effects of new drugs and their possible mechanisms on adrenergic, cholinergic and nitrergic transmission.

Because *Pimpinella anisum* is highly used in the folk medicine, a study of its physiological properties was deemed necessary and informative. At present, the mechanisms involved in the relaxant action induced by the extract of *Pimpinella anisum* are poorly understood. This work aimed to investigate the possible mechanism(s) underlying the relaxant action of the hidroalcoholic extract of *Pimpinella anisum* on rat anococcygeus smooth muscle.

2. Materials and methods

2.1. Plant material

The aerial parts of *Pimpinella anisum* were purchased from Companhia das Ervas – Ribeirão Preto, São Paulo, Brazil.

2.2. Preparation of the hidroalcoholic crude extracts

Dried, powdered aerial parts (50 g) of *Pimpinella anisum* were extracted with 300 mL of three hidroalcoholic solutions, in which the proportion of ethanol was 40% ($HA_{40\%}$), 60% ($HA_{60\%}$) or 80% ($HA_{80\%}$) for 10 min, using a shaker at temperature a of 50 °C. After filtration on paper filter, the extracts were concentrated in a rotary evaporator to eliminate the ethanol and then lyophilized to give 2.3 g, 2.4 g and 2.2 g of $HA_{40\%}$, $HA_{60\%}$ and $HA_{80\%}$, respectively.

2.3. Analysis of the crude extracts

The $HA_{40\%}$, $HA_{60\%}$ and $HA_{80\%}$ HPLC analyses were carried out in a Shimadzu LC 10Avp system, diode array detector (UV-Vis-DAD) SPD-M10Avp and analytic column (Shimadzu, 4.6 mm × 250 mm; 5 μm; 100 Å), operating with the software CLASS-VP version 5.02. For HPLC runs, a gradient of acidified H_2O (2% HOAc) (solvent A) and MeOH (2% HOAc) (solvent B) at a flow rate 0.95 mL min^{-1} was used. The gradient was as follows: 0 min, 5% B; 5 min, 30% B; 8 min, 34% B; 11 min, 55% B; 17 min, 5%B; 23 min, 5% B. UV detection was made at 200–400 nm (scan) using DAD and then set to 280 and 320 nm. The chromatographic analysis of the extracts indicated aromatic compounds as the main constituents.

2.4. Pharmacological assays

2.4.1. Tissue preparations

Male Wistar rats weighing between 190 and 210 g (50–60 days old) were decapitated and the anococcygeus muscle removed as previously described by Gillespie (1972). All procedures are in accordance with standards and policies of the University of Sao Paulo's Animal Care and Use Committee. Tissues were placed in a 5 mL organ chamber containing physiological salt solution (PSS) as follows (mM): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.1 D-(+)-glucose. The PSS was gassed with 5% CO_2 and 95% O_2 and maintained at 37 °C, pH 7.4, with periodical checking. Isotonic transducers were used to measure changes in contraction of the tissues, which were displayed on a Harvard Universal Student Oscillograph (Mass., USA) at a optimal basal tension of 1.0 g, which was determined by length-tension relationship experiments. Isolated muscles were allowed to equilibrate for 30–45 min before making experimental observations. The organ bath PSS was repeatedly replaced with fresh PSS every 15 min. After the equilibration period, tissues were stimulated with KCl (90 mM) to check their responsiveness.

2.4.2. Determination of the period of incubation

The incubation period was determined using the EC_{50} value of acetylcholine. For this purpose, the preparations were stimulated before and after incubation with the extract ($HA_{80\%}$ at 50 μg/mL) for 15, 30 and 60 min. Since no difference in the magnitude of contraction induced by acetylcholine was found after 30 and 60 min of incubation, the first was accepted as the ideal period of incubation (data not shown).

2.4.3. Effect of the hidroalcoholic extracts of Pimpinella anisum on contractions induced by acetylcholine

After equilibration, cumulative concentration–response curves for acetylcholine (10 nM–100 μM) were determined. The curves were obtained by a stepwise increase in the concentration of acetylcholine. Additions were made as soon as a steady response was obtained from the preceding concentration. The curves for acetylcholine were determined in the absence (control group) or after a 30 min incubation period with one of the three hidroalcoholic extracts ($HA_{40\%}$, $HA_{60\%}$ and $HA_{800\%}$)

of *Pimpinella anisum*. The preparations were incubated with 50 μg/mL of each extract.

2.4.4. Effect of the hidroalcoholic extracts of Pimpinella anisum on rat anococcygeus smooth muscle pre-contracted with acetylcholine

In another set of experiments, steady tension was evoked by acetylcholine (5 μM) and when the contraction reached a plateau, $HA_{40\%}$, $HA_{60\%}$ and $HA_{80\%}$ were added cumulatively (5–50 μg/mL). For comparison, the effect of sodium nitroprusside (10^{-8}–10^{-6} M), a donor of nitric oxide (NO) was also evaluated against the contractions induced by acetylcholine.

To investigate the possible mechanism(s) underlying the relaxant effect of $HA_{60\%}$, the preparations were pre-contracted with acetylcholine (5 μM) 30 min after being incubated with one of the following drugs: N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 3 μM), indomethacin (10 μM), oxyhemoglobin (10 μM) or tetraethylammonium (1 mM). Relaxation was expressed as percentage change from the acetylcholine-contracted levels.

2.4.5. Effect of L-NAME on contractions induced by acetylcholine

To examine the contribution of NO in modulating the response to acetylcholine, complete cumulative concentration–response curves for acetylcholine (10–100 μM) were obtained in the absence or in the presence of the NOS inhibitor L-NAME (100 μM). In another set of experiments, cumulative concentration–response curves for acetylcholine were performed in the presence of both L-NAME and $HA_{60\%}$.

2.4.6. Effect of $HA_{60\%}$ on $CaCl_2$-induced contractions

To assess the effects of $HA_{60\%}$ on $CaCl_2$-induced contractions, the tissues were first contracted with phenylephrine (0.1 μM) to deplete the intracellular Ca^{2+} stores in Ca^{2+}-free solution (approximately 40 min) containing EGTA (1 mM) and then rinsed in Ca^{2+}-free solution (without EGTA) containing acetylcholine (1 μM). The cumulative concentration–response curves for $CaCl_2$ (0.05–2 mM) were obtained in the absence (of control group) or after a 30 min incubation period with $HA_{60\%}$ (50 μM).

2.5. Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, ODQ (Sigma, St. Louis, MO, USA), potassium chloride, calcium chloride (Synth, São Paulo, Brazil), L-NAME, tetraethylammonium (Sigma/RBI, Natick, MA) and indomethacin (Calbiochem). Indomethacin was prepared as stock solutions in ethanol. ODQ was prepared as stock solution in dimethyl sulfoxide (DMSO). The other drugs were dissolved in distilled water. The bath concentration of ethanol or DMSO did not exceed 0.5%, which was shown to have no effect *per se* on the basal tonus of the preparations or on the agonist-mediated contraction or relaxation.

2.6. Data analysis

Contractions were recorded as changes in the displacement (mm) from baseline and expressed as a percentage of the maximum response induced by KCl (90 mM). Agonists concentration–response curves were fitted using a non-linear interactive fitting program (GraphPad Prism 2.00, Graph Pad Software Incorporated, Calig., USA). To study the effect of the hidroalcoholic extract on attenuating contraction or inducing relaxation, two pharmacological parameters were analysed: the E_{max} (maximal effect generated by the agonist) and pD_2 ($-\log EC_{50}$). Results were expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis of the E_{max} and pD_2 values was performed using one-way analysis of variance (ANOVA) or Student's "*t*"-test. Post-hoc comparisons were performed after ANOVA analysis using Bonferroni or Dunnet tests as indicated in the text and tables. The significance level considered in all tests was 0.05.

3. Results

3.1. Effect of the hidroalcoholic extracts of Pimpinella anisum on contractions induced by acetylcholine

The effects of the three hidroalcoholic extracts of *Pimpinella anisum* (50 μg/mL) on the cumulative concentration–response curves for acethylcholine on isolated rat anococcygeus smooth muscle are shown in Fig. 1. The E_{max} values for acetylcholine were depressed in the presence of $HA_{40\%}$, $HA_{60\%}$ or $HA_{80\%}$. However, the extracts did not alter the pD_2 values for acetylcholine (Table 1).

3.2. Effect of the hidroalcoholic extracts of Pimpinella anisum on rat anococcygeus smooth muscle pre-contracted with acetylcholine

The hidroalcoholic extract of *Pimpinella anisum* ($HA_{60\%}$) at concentrations ranging from 5 to 50 μg/mL induced relaxation

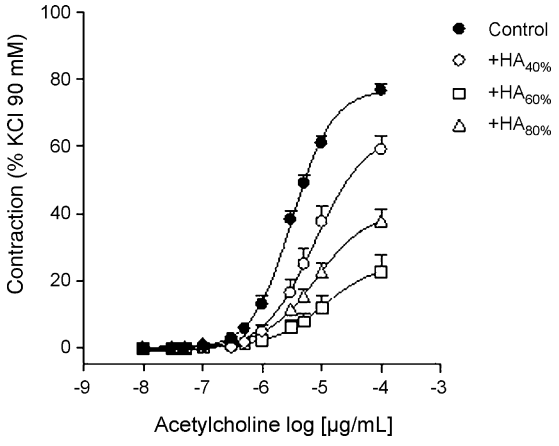

Fig. 1. Effect of the hidroalcoholic extracts of *Pimpinella anisum* ($HA_{40\%}$, $HA_{60\%}$ and $HA_{80\%}$) on acetylcholine-induced contractile response in rat anococcygeus smooth muscle. Concentration–response curves for acetylcholine were determined in the absence (of control group) or after a 30 min period of incubation with the extracts (50 μg/mL).

Table 1
Effect of the three hidroalcoholic extracts of *Pimpinella anisum* $HA_{40\%}$ (50:2.3 g/mL), $HA_{60\%}$ (50:2.4 g/mL) and $HA_{80\%}$ (50:2.2 g/mL) on acetylcholine-induced contraction of rat anococcygeus smooth muscle

Groups	E_{max} (%contraction)	pD_2	n
Control	79.58 ± 2.13	5.49 ± 0.04	5
$HA_{40\%}$ (50 μg/mL)	59.17 ± 3.90[a]	5.07 ± 0.03[a]	5
$HA_{60\%}$ (50 μg/mL)	22.59 ± 5.23[a]	4.97 ± 0.05[a]	4
$HA_{80\%}$ (50 μg/mL)	37.84 ± 3.51[a]	5.04 ± 0.04[a]	4

Values are mean ± S.E.M. of n experiments.
[a] Compared to control group (ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$).

on the sustained tonic contraction induced by acetylcholine in a concentration-dependent manner (E_{max}: 31.55 ± 3.56%, pD_2: 1.27 ± 0.05; $n = 9$). Surprisingly, neither $HA_{40\%}$ nor $HA_{80\%}$ induced relaxation (Fig. 2A). It can be seen in Fig. 2B that sodium nitroprusside induced relaxation of the tissues pre-contracted with acetylcholine. The relaxation induced by sodium nitroprusside (E_{max}: 93.98 ± 4.00%, $n = 5$) on acetylcholine-pre-contracted tissues was significantly different from those found for $HA_{60\%}$. Moreover, the pD_2 values (pD_2: 7.33 ± 0.21) found for sodium nitroprusside were higher than that found for $HA_{60\%}$.

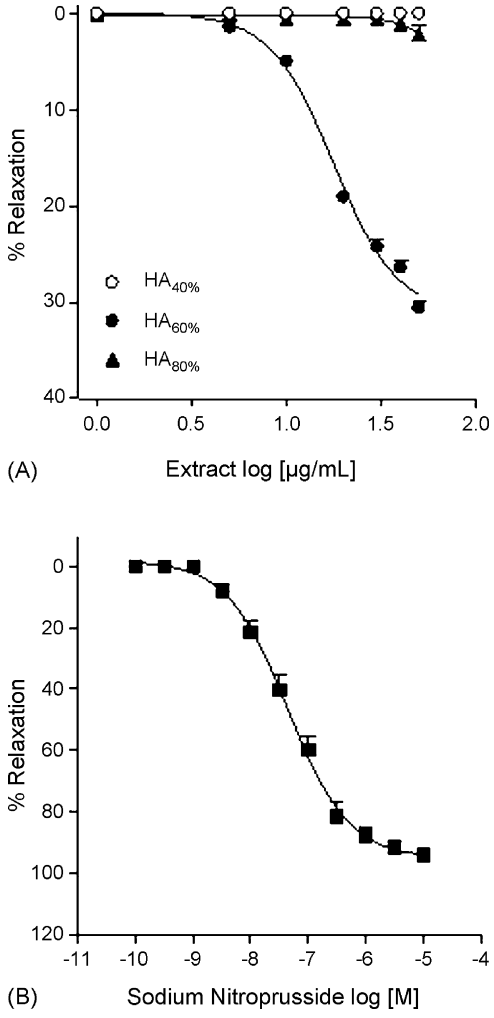

Fig. 2. Relaxation responses induced by the three hidroalcoholic extracts ($HA_{40\%}$, $HA_{60\%}$ or $HA_{80\%}$) of *Pimpinella anisum* (A) or sodium nitroprusside (B) on acetylcholine-pre-contracted rat anococcygeus smooth muscle. The relaxation was studied on anococcygeus smooth muscle submaximally pre-contracted with acetylcholine. Steady tension was evoked by acetylcholine and then the extracts (5–50 μg/mL) or sodium nitroprusside (0.1–10 nM) were added cumulatively.

3.3. Mechanisms underlying $HA_{60\%}$-induced relaxation on rat anococcygeus smooth muscle pre-contracted with acetylcholine

The experiments designed to investigate the mechanisms responsible for the relaxation induced by $HA_{60\%}$ showed that the non-selective ciclooxygenase inhibitor indomethacin did not have significant effect on the relaxation induced by $HA_{60\%}$. Pre-incubation of the tissues with tetraethylammonium, a non selective blocker of K^+ channels did not alter the relaxant effect of $HA_{60\%}$ (Fig. 3A, Table 2). On the other hand, the NO synthase inhibitor, L-NAME, reduced the relaxation and produced a rightward displacement of the concentration–response curve for the extract. The relaxation induced by the extract was strongly reduced in the presence of the NO scavenger, oxyhemoglobin. Similarly, ODQ, a guanylyl ciclase inhibitor, reduced the relaxation induced by $HA_{60\%}$ (Fig. 3B, Table 2).

3.4. Effect of L-NAME on contractions induced by acetylcholine

Incubation with L-NAME significantly enhanced the maximal contraction induced by acetylcholine when compared to the responses obtained in the absence of the inhibitor. $HA_{60\%}$ attenuated acetylcholine-induced contraction. However, the association of L-NAME and $HA_{60\%}$ resulted in an effect similar to that observed with L-NAME alone (Fig. 4, Table 3).

3.5. Effect of $HA_{60\%}$ on contractions induced by $CaCl_2$

As it can be seen in Fig. 5, $HA_{60\%}$ did not alter the contraction induced by $CaCl_2$. The E_{max} or pD_2 values for $CaCl_2$ (E_{max} = 75.23 ± 3.35%; pD_2 = 3.06 ± 0.05; $n = 4$) were not altered after incubation with $HA_{60\%}$ (E_{max} = 85.80 ± 5.20%; pD_2 = 3.12 ± 0.05; $n = 5$).

Table 2
Effect of indomethacin, tetraethylammonium, L-NAME, ODQ and oxyhemoglobin on the relaxant responses induced by $HA_{60\%}$ (50:2.4 g/mL) on rat anococcygeus muscle pre-contracted with acetylcholine

Groups	E_{max} (% relaxation)	pD_2	n
Control	31.55 ± 3.56	1.27 ± 0.05	9
Indomethacin (10 μM)	25.02 ± 2.63	1.34 ± 0.23	5
Tetraethylammonium (1 mM)	27.56 ± 4.64	1.21 ± 0.04	5
L-NAME (100 μM)	6.81 ± 1.86[a]	–	5
ODQ (3 μM)	13.13 ± 5.87[a]	–	6
Oxyhemoglobin (10 μM)	2.12 ± 1.46[a]	–	5

Values are mean ± S.E.M. of n experiments.
[a] Compared to control group (ANOVA followed by Dunnet's multiple comparison test, $P < 0.05$).

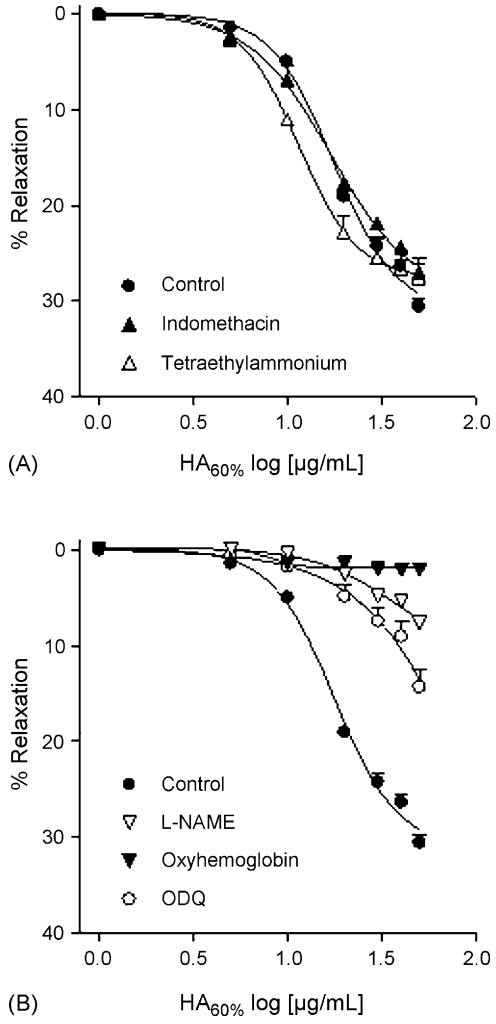

Fig. 3. Relaxation responses induced by the hidroalcoholic extract ($HA_{60\%}$) of *Pimpinella anisum* on rat anococcygeus smooth muscle pre-contracted with acetylcholine in the presence of tetraethylammonium (1 mM), indomethacin (10 μM) (A), L-NAME (100 μM), ODQ (3 μM) or oxyhemoglobin (10 μM) (B). The tissues were pre-incubated with the drugs for 30 min. Steady tension was evoked by acetylcholine, and then $HA_{60\%}$ (5–50 μg/mL) was added cumulatively.

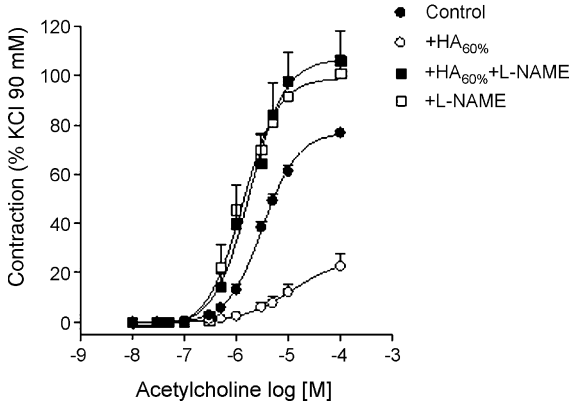

Fig. 4. Effect of L-NAME and the hidroalcoholic extract of *Pimpinella anisum* ($HA_{60\%}$) on acetylcholine-induced contractile response in rat anococcygeus smooth muscle. Concentration–response curves for acetylcholine were determined in the absence (control) or after a 30 min period of incubation with L-NAME (100 μM), $HA_{60\%}$ (50 μg/mL) or the association of L-NAME and $HA_{60\%}$.

Table 3
Effect of L-NAME, $HA_{60\%}$ (50:2.4 g/mL) or the association of both on acetylcholine-induced contraction of rat anococcygeus smooth muscle

Groups	E_{max} (% contraction)	pD_2	*n*
Control	79.58 ± 2.13	5.49 ± 0.04	5
L-NAME (100 μM)	100.90 ± 7.06[a]	5.89 ± 0.12[a]	5
$HA_{60\%}$ (50 μg/mL)	22.59 ± 5.23[a]	4.97 ± 0.05[a]	4
L-NAME + $HA_{60\%}$	105.60 ± 12.45[a]	5.74 ± 0.05[a]	4

Values are mean ± S.E.M. of *n* experiments.
[a] Compared to control group (ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$).

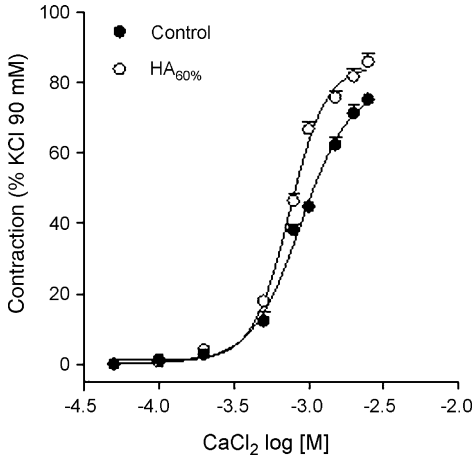

Fig. 5. Effect of the hidroalcoholic extract of *Pimpinella anisum* ($HA_{60\%}$) on $CaCl_2$-induced contractile response in rat anococcygeus smooth muscle. Concentration–response curves for $CaCl_2$ were determined in Ca^{2+}-free solution containing acetylcholine (1 μM). The curves were determined in the absence (control) or after a 30 min period of incubation with $HA_{60\%}$ (50 μg/mL).

4. Discussion

The present findings describing the relaxant action of the hidroalcoholic extract of *Pimpinella anisum* on the anococcygeus smooth muscle corroborate those of previous studies describing that *Pimpinella anisum* displays relaxant effects on tracheal (Boskabady and Ramazani-Assari, 2001) and smooth muscle preparations (Reiter and Brandt, 1985).

The anococcygeus muscle is a paired smooth muscle, arranged in parallel bundles to form a sheet allowing easy diffusion of drugs and ions, which makes it a unique and dynamic smooth muscle preparation for experimental pharmacology (Araujo and Bendhack, 2003). Both the sympathetic and parasympathetic divisions of the autonomic nervous system innervate the rat anococcygeus muscle (Gillespie and McGrath, 1973). Moreover, the rat anococcygeus responds to muscaric agonists. Muscarinic receptors of the M_3 subtype are present on smooth muscle where they induce contraction (Weiser et al., 1997). The current findings show acetylcholine-induced contraction of the anococcygeus muscle in a concentration-dependent fashion. However, the three hidroalcoholic extracts of *Pimpinella anisum* in which the proportion of ethanol was 40% ($HA_{40\%}$), 60% ($HA_{60\%}$) or 80% ($HA_{80\%}$) attenuated acetylcholine-induced contraction, further characterizing the antispasmodic action of *Pimpinella anisum*. Furthermore,

$HA_{60\%}$ concentration dependently relaxed acetylcholine-pre-contracted tissues. Interestingly, neither $HA_{40\%}$ nor $HA_{80\%}$ produced relaxation. The pD_2 values as well as the percentage of relaxation induced by sodium nitroprusside on anococcygeus smooth muscle pre-contracted with acetylcholine were higher when compared to those found for $HA_{60\%}$, indicating that sodium nitroprusside is more potent than the extract at inhibiting acetylcholine pre-contracted tissues.

Another aspect investigated in this study was the possible mechanisms underlying the relaxant effect induced by the extract. Indomethacin, a non-selective cyclooxygenase inhibitor, was not found to affect the relaxation caused by the $HA_{60\%}$. Therefore, it appears that cyclooxygenase pathways do not play a role in mediating the relaxant effects of *Pimpinella anisum*. The opening of K^+ channels in the cell membrane of smooth muscle cells increases K^+ efflux causing membrane potential hyperpolarization, which leads to relaxation (Nelson and Quayle, 1950). Tetraethylammonium, a non-selective blocker of K^+ channels, did not affect the relaxation induced by the extract. Thus, it seems unlikely that the relaxant response induced by the extract involves the opening of K^+ channels.

Nitric oxide, enzymatically synthesized from the amino acid L-arginine, was first discovered in the vascular endothelium (Furchgott and Zawadzki, 1980; Moncada et al., 1989). In our study, pre-treatment of the tissues with L-NAME was associated with a rightward displacement of the curve and a reduction of the relaxation induced by the extract. This observation indicates that the activation of the enzyme NO synthase play a role in the relaxant response induced by *Pimpinella anisum*. It is well accepted that reaction with haemoglobin is the major mechanism for disarming NO bioactivity. Thus, a hallmark for establishing a role for NO in any biological system has been whether oxyhemoglobin scavenges its bioactivity (Palmer et al., 1987). We found that oxyhemoglobin powerfully inhibited the relaxation induced by the extract, further indicating the participation of NO in this response.

There is now consistent evidence that NO produces relaxant response in smooth muscle through cGMP-dependent mechanism (Moncada et al., 1989; Robertson et al., 1993). Thus, we sought to determine the possible requirement of cGMP pathway in the relaxant action of the extract. The selective inhibitor of guanylyl cyclase enzyme, ODQ (Garthwaite et al., 1995), inhibited the relaxant response induced by the extract. Such findings confirm the involvement of the NO-cGMP pathway in extract-mediated relaxant responses.

In addition to the adrenergic and cholinergic innervations, the ultra structure of the anococcygeus muscle also supports an inhibitory non-adrenergic, non-cholinergic (NANC) innervations that uses NO as the main neurotransmitter (Rand and Li, 1990). Based on this observation, a possible modulatory action of NO on acetylcholine-induced contraction was evaluated in the present study. Incubation of the tissues with L-NAME significantly enhanced the maximal contraction induced by acetylcholine, indicating an inhibitory role for NO in the modulation of the contractile response of anococcygeus smooth muscle to acetylcholine. As mentioned before, $HA_{60\%}$ attenuated acetylcholine-induced contraction. However, simultaneous addition of L-NAME and $HA_{60\%}$ resulted in an effect similar to that observed with L-NAME alone. The lack of the inhibitory effect of $HA_{60\%}$ in the presence of L-NAME, strengthens our findings that *Pimpinella anisum* acts by realising NO.

Another aspect investigated in this study was whether $HA_{60\%}$-induced relaxation was related to inhibition of Ca^{2+} influx from the extracellular medium. In many cases, the constrictor-induced contraction of rat anococcygeus smooth muscle is largely mediated by Ca^{2+} influx and inhibition of Ca^{2+} influx usually leads to relaxation (Araujo and Bendhack, 2003). The acetylcholine-induced contraction is dependent on the presence of extracellular Ca^{2+} and $HA_{60\%}$ relaxed the acetylcholine-pre-contracted tissues, hence raising the possibility that $HA_{60\%}$ may affect Ca^{2+} influx. However, we noted that $CaCl_2$-induced contraction in Ca^{2+} free medium containing acetylcholine was not altered by $HA_{60\%}$, further supporting the notion that blocked-off extracellular Ca^{2+} influx through Ca^{2+} channels presented in the smooth muscle cells does not play a role in the relaxant response induced by *Pimpinella anisum*.

Chemical studies demonstrated that the *Pimpinella anisum* seed contain anethole (Chandler and Hawkes, 1984; Fujita and Nagasawa, 1960), estragole (Zargari, 1989), eugenol (Monod and Dortan, 1950), pseudoisoeugenol (Reichling et al., 1995), methylchavicol and anisaldehyde (Wagner et al., 1984), coumarins, scopoletin, umbelliferon, estrols (Burkhardt et al., 1986), terpene hydrocarbons (Kartnig et al., 1975), polyenes and polyacetylenes (Schulte et al., 1970) as the major compounds. Among these compounds, eugenol, estragole (Boskabady and Ramazani-Assari, 2001) and anethole (Albuquerque et al., 1995) were described to exert relaxant effect. Further studies need to be carried out to determine whether the relaxant effect of the hidroalcoholic extract of *Pimpinella anisum*, described in the present study, could be attributed to the action displayed by these compounds.

In summary, the present findings demonstrate that the hidroalcoholic extract of *Pimpinella anisum* reduced the contractions induced by acetylcholine in the rat anococcygeus smooth muscle. Additionally to its antispasmodic effect, the extract also relaxed the preparations pre-contracted with acetylcholine. The action displayed by the extract is mainly dependent on the activation of the NO-cGMP pathway. The relaxant action displayed by *Pimpinella anisum*, described in the present study, justifies its use in the folk medicine as an antispasmodic agent.

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