academicJournals

Vol. 7(31), pp. 2293-2299, 17 August, 2013
DOI: 10.5897/JMPR12.1165
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Aerial parts of *Solanum agrarium* Sendtn. (Solanaceae) present the flavonoid myricetin 3,7,3' trimethyl ether and antispasmodic effect on guinea-pig ileum by blockade of voltage-gated calcium channels

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Accepted 26 July, 2013

Solanum agrarium **Sendtn. is popularly known in Northeastern Brazil as "gogóia". Previous work has verified the presence of the flavonoid kaempferol and showed the crude ethanolic extract from aerial parts of *S. agrarium* (SA-EtOH) to have non-selective antispasmodic effect on guinea-pig ileum. The aim was to isolate and identify the special metabolites and to investigate antispasmodic mechanism of SA-EtOH on guinea-pig ileum. From their aerial parts was isolated the flavonoid myricetin-3,7,3'-trimethyl ether. SA-EtOH shifted to the right cumulative concentration-response curves to carbachol or histamine in a reversible and non-competitive manner. SA-EtOH relaxed the ileum pre-contracted with 40 mM KCl (EC_{50}=17.4±3.5 µg/ml), 10^{-6} M carbachol (EC_{50}=119.4±24.3 µg/ml) or 10^{-6} M histamine (EC_{50}=18.7±4.6 µg/ml). This is most likely due to the inhibition of calcium influx through voltage-gated calcium channels (Ca_V). It was confirmed by SA-EtOH antagonizing $CaCl_2$-induced contractions in depolarizing medium nominally without Ca^{2+}, with non-parallel shift of the concentration-response curves to the right and reduction of E_{max}. The uncovering of SA-EtOH (EC_{50}=187.2±27.3 µg/ml) inhibiting tonic contractions induced by S-(-)-BayK8644 suggests that Ca^{2+} channel subtype involved is Ca_V-L. In conclusion, SA-EtOH shows antispasmodic effect on guinea-pig ileum due, in part, to blockade of calcium influx through Ca_V channels.**

Key words: *Solanum agrarium*, flavonoid, antispasmodic effect, guinea-pig ileum, Ca_V-L.

INTRODUCTION

Solanum L. is the largest and most complex genus of the Solanaceae family, with about 1400 species (Bohs, 2005) and showing tropical and subtropical distribution around the world. According to Child and Lester (2001), the subgenus *Leptostemonum* (Dunal) Bitter comprises 33 sections and about 450 species. These species can be recognized by a set of characters, among which: presence of thorns, anthers attenuated to the apex and indumentum consisting of a wide diversity of stellate trichomes. Its main centers of diversity and distribution are South America, Africa and Australia (Whalen, 1984).

In Brazil, some species of *Solanum* are used in folk medicine, mainly in the Northeast, where they are used for treatment of various diseases, among which are liver diseases, inflammation and external ulcers (Agra et al., 2008). Some species show spasmolytic, such as *Solanum stipulaceum* (Ribeiro et al., 2002), *Solanum megalonyx* Sendtn., *Solanum asterophorum* Mart. (Oliveira et al., 2006a,b) and *Solanum jabrense* Agra & Nee (Cavalcante et al., 2012); and antidiarrheal activity as *S. asterophorum* (Silva et al., 2012).

Solanum agrarium Sendtn. is a subshrub belonging to section *Acanthophora*, with distribution restricted to South America (Brazil and Venezuela), popularly known in Northeastern Brazil as "gogóia" and used in folk medicine for mycosis, diarrhea and gonorrhea (Ribeiro et al., 1989) and root decoction is used for treatment in prostatic inflammation and as abortive (Agra et al., 2008). In previous studies, absence of toxicity of crude ethanol extract from *S. agrarium* against *Biomphalaria glabrata* mollusk was observed (Silva et al., 2006). In addition, this extract showed non-selective antispasmodic effect on guinea-pig ileum and rat uterus (Santos et al., 2003). As an extension of our previous studies on *Solanum* species from Brazil (Silva et al., 2006; Oliveira et al., 2006a, b; Santos et al., 2003; Silva et al., 2003; Silva et al., 2002a, b; Cavalcante et al., 2013; Silva et al., 2012), we now present a study on *S. agrarium*, of the subgenus *Leptostemonum*, which looked into the isolation and structural identification of special metabolites and investigated the antispasmodic mechanism of the crude ethanolic extract from the aerial parts of *S. agrarium* (SA-EtOH) on guinea-pig ileum.

MATERIALS AND METHODS

Chemical studies

General experimental procedures

Infrared (IR) spectra were obtained on FT-IR 1750 Perkin-Elmer spectrometer in KBr pellets and UV-Vis measures were used on VANKEL 50 UV-Vis Varian (Autralia). Nuclear magnetic resonance (NMR) spectra were recorded on Mercury-Varian 200 operating at both 200 MHz for ^{1}H and 50 MHz for ^{13}C (LTF/UFPB). Silica gel 60 (0063-0.2 mm/70-230 mesh, Merck) and Sephadex LH-20 (Amersham Biosciences-Sweden) were used for column chromatography (CC) separations. Chromatosheets of silica gel 60 F_{254} (Merck) were used for the analysis of thin-layer chromatography (TLC) separations.

Plant material

Aerial parts of *S. agrarium* were collected in the semi-arid (Caatinga) of Paraiba, Northeastern Brazil, and identified by Maria de Fátima Agra Ph D., LTF/UFPB. A voucher specimen (Agra 6523) is deposited in the Herbarium Prof. Lauro Pires Xavier (JPB) of Universidade Federal da Paraíba (UFPB).

Extraction and isolation

Dried and finely powdered aerial parts of *S. agrarium* (179.0 g) were extracted with ethanol (EtOH) through exhaustive maceration at room temperature. The extract was concentrated under reduced pressure in a rotaevaporator. The crude residue (50.0 g) was dissolved in acetic acid solution and fractioned with hexane:ether (1:1) and ethyl ethanoate (EtOAc). Solvents were evaporated to dryness to obtain hexane:ether (18.1 g) and EtOAc (6.0 g) fractions. The resulting flavonoid fractions were analyzed on commercial TLC aluminum sheets (Merck silica gel 60 F_{254}), and the spot visualization was made under UV light (366 nm) after spraying with diphenylboryloxyethylamine (NP) solution in MeOH. The EtOAc extract from *S. agrarium* showed the presence of flavonoids, and was subsequently submitted to column chromatography with Sephadex LH-20 and $CHCl_3$:MeOH as elution system to obtain 6.1 mg of flavonoid. Myricetin-3,7,3'-trimethyl ether (Figure 1): RMN of ^{1}H (200 MHz, DMSO-D_6) δ_H (mult*; J* in Hz, H): 3.88 (s; 3H-3), 3.90 (s; 3H-3'), 3.91 (s; 3H-7), 6.30 (*d*; 2.2; H-6), 6.67 (*d*; 2.2; H-8), 7.40 (s; 2H-2',6'), 12.74 (s; OH-5), RMN of ^{13}C (50 MHz, DMSO-D_6), δ_C: 56.40 (OCH_3-7), 56.63 (OCH_3-3'), 60.16 (OCH_3-3), 98.42 (C-6), 92.85 (C-8), 105.14 (C-2'), 106.48 (C-10), 110.62 (C-6'), 121.67 (C-1'), 138.34 (C-4'), 138.34 (C-3), 146.26 (C-5'), 149.17 (C-3'), 157.02 (C-2), 162.74 (C-5), 165.17 (C-7), 179.54 (C-4).

Pharmacological studies

Animals

Adult guinea-pigs (*Cavia porcellus*) of both sex, weighing 300-500 g, from Biotério Prof. Thomas George of the UFPB were used. The animals had free access to food and water and were kept in rooms at 21 ± 1°C submitted to a 12-h light-dark cycle and fasted for 18 h before the experiments. All experimental procedures were performed in accordance with the Animal Research Ethic Committee of LTF/UFPB guidelines (protocol CEPA/LTF: 0605/09).

Drugs and salts

Calcium chloride dehydrate ($CaCl_2.2H_2O$), potassium chloride (KCl) and sodium bicarbonate ($NaHCO_3$) were purchased from Vetec – Brazil. Monosodium phosphate 1-hydrate ($NaH_2PO_4.H_2O$), glucose ($C_6H_{12}O_6$), magnesium sulfate monohydrate ($MgSO_4.H_2O$) and hydrochloric acid (HCl) were purchased from Nuclear – Brazil.

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Sodium chloride (NaCl) was purchased from Dinâmica – Brazil. Carbamilcholine chloride was purchased from Merck – Brazil. Histamine dihydrochloride, S-(-)-Bay K8644 and Cremophor® were purchased from Sigma-Aldrich – USA.

Preparation of extracts for pharmacological testing

A crude ethanolic extract from aerial parts of *S. agrarium* (SA-EtOH) was solubilized in cremophor (3%, v/v) and diluted in distilled water to obtain the stock solution (10 mg/mL), which was stored at 0°C, and the working solutions were freshly prepared daily. Cremophor concentration in organ baths never exceeded 0.01% (v/v) throughout the experiment, being ineffective on both contraction and relaxation of the studied organ.

Tissue preparation

Guinea-pigs were euthanized through cervical dislocation and exsanguination, the ileum being immediately removed. The modified Krebs solution (mM): NaCl (117.0), KCl (4.7), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), glucose (11.0) and $NaHCO_3$ (25.0); and high-K^+ isosmotic solution for 70 mM KCl: NaCl (51.7), KCl (70.0), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), glucose (11.0) and $NaHCO_3$ (25.0); solutions adjusted to pH 7.4, continuously bubbled with carbogen mixture (95% O_2, 5% CO_2). The tissue was suspended in 5 mL organ baths under resting load of 1.0 g at 37°C. The isotonic contractions were recorded using isotonic levers coupled to kymographs and smoked drums (DTF, Brazil). For the record of isometrics contractions was used an isometric transducer (FORT-10) connected to an amplifier (TMB4M), both from World Precision Instruments (EUA), connected to an analog/digital converter board (Biodata – Brazil) installed on computer with BioMed© version RV2 software. Tissues were allowed to stabilize for 30 min.

Characteristics of carbachol or histamine induced contraction blockade

After stabilization, two similar carbachol or histamine cumulative concentrations-response curves were induced and SA-EtOH was incubated in absence of carbachol or histamine for 15 min in different concentrations and independent experiments. After that, a new carbachol or histamine cumulative curve was obtained in the presence of SA-EtOH. The average amplitude of concentration-response curves to carbachol or histamine was considered to be 100%, and all contractions were assessed. Each preparation was exposed to only one concentration of SA-EtOH. The antagonism exerted by SA-EtOH was evaluated based on the analysis of concentration values of a substance that produces 50% of its maximal effect (EC_{50}) and the maximum effect (E_{max}) values of carbachol or histamine, assessed through concentration-response curves in absence (control) and presence of SA-EtOH.

SA-EtOH effect on tonic contractions induced by KCl, carbachol or histamine

After the stabilization period, an isometric contraction was elicited with carbachol (10^{-6} M), histamine (10^{-6} M) or KCl (40 mM). Contractile agents remained in contact with the preparation until a plateau was reached, after which the tissue was washed out. The plateau characterizes the tonic component of the contraction. After 30 min. the process was repeated and, on the contraction plateau, SA-EtOH was added cumulatively. Subsequent concentrations were added only after the response to the previous was stabilized.

Relaxation was expressed as the reversal percentage of initial contraction elicited by contractile agents. EC_{50} values were expressed as mean ± S.E.M of EC_{50} individual values assessed through nonlinear regression.

SA-EtOH effect on $CaCl_2$-induced contractions in depolarizing nominally Ca^{2+}-free medium

After the stabilization period, modified Krebs solution was replaced with depolarizing nominally Ca^{2+}-free solution for 45 min. Two similar $CaCl_2$ cumulative concentrations-response curves were induced, and SA-EtOH was incubated in the absence of $CaCl_2$ for 15 min and a third $CaCl_2$ cumulative curve was obtained in the presence of SA-EtOH. The maximal contraction obtained with the first $CaCl_2$ concentration-response curve was considered to be 100%, and all contractions were assessed referring to it. Each preparation was exposed to a single SA-EtOH concentration.

SA-EtOH effect on tonic contractions induced by S-(-)-Bay K8644

After the stabilization period, the ileum was partially depolarized by adding of 15 mM KCl for 10 min (Conte-Camerino et al., 1987; Usowicz et al., 1995), which induced a 0.3 µM S-(-)-Bay K8644 contraction, a selective Ca_V agonist to L-type (Ferrante et al., 1989). During stabilization of the contraction tonic phase, SA-EtOH was added cumulatively in order to obtain a concentration-inhibition curve. Relaxation was expressed as described.

Statistical analysis

Values were expressed as mean ± S.E.M. Statistical analysis was performed using GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA). Differences between means were compared using t-test (non-paired) and/or one-way ANOVA followed by Bonferroni's test as appropriate and p values < 0.05 were considered indicative of significance.

RESULTS AND DISCUSSION

In the present study, it was demonstrated for the first time the isolation and identification of the flavonoid myricetin-3,7,3'-trimethyl ether from aerial parts of *S. agrarium* Sendtn. The most important finding was that the crude ethanolic extract from aerial parts of *S. agrarium* (SA-EtOH) shows antispasmodic effect on guinea-pig ileum by blockade of voltage-gated calcium channels (Ca_V).

Chromatographic fractionation of extract from aerial parts of *S. agrarium* on Sephadex LH-20 led to the isolation of the flavonoid myricetin-3,7,3'-trimethyl ether (Figure 1). Data from ^1H and ^{13}C NMR were compared to those on literature (Kumari et al., 1984), validating this structure. This flavonoid was previously isolated from *Solanum pubescens* (Kumari et al., 1984), other species of subgenus *Leptostemonum*, to which belongs *S. agrarium*.

The presence of secretory structures that accumulate flavonoid aglycones in *Solanum* subg. *Leptostemonum*

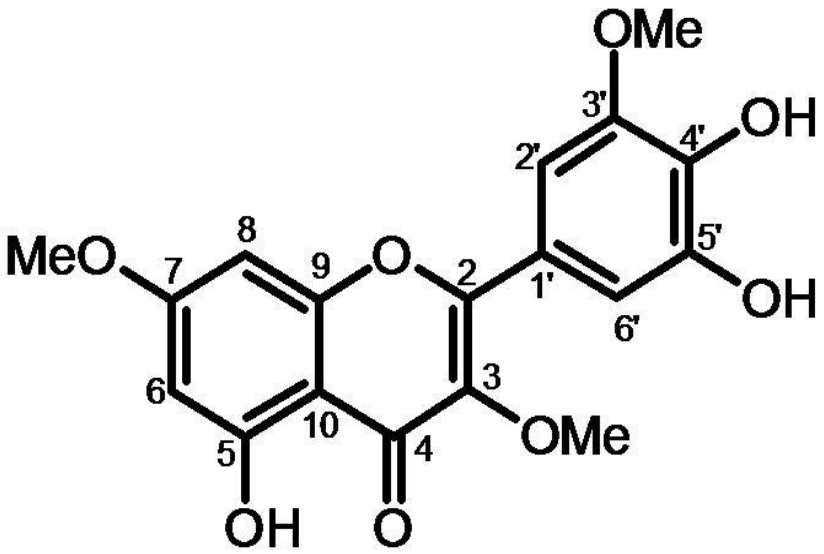

Figure 1. Chemical structure of flavonoid myricetin-3,7,3'-trimethyl ether.

species was recently reported by Wollenweber et al. (2005), Silva et al. (2002a,b) and Silva et al. (2003) for three species of section *Erythrotrichum* Child (*S. jabrense* Agra, *S. paludosum* Moric. and *S. rhytidoandrum* Sendtn.), which revealed the same chemical profile, the presence of the same flavonoid aglycones. In addition to the phytochemistry profile, species of *Erythrotrichum* show common morphological characters, among which are the presence of ferruginous indumentum with stellate-globular trichomes and fruit with glandular-pubescent epicarp. Aerial parts of *S. agrarium* was previously studied (Silva et al., 2003) by high performance liquid chromatography (HPLC) verifying the presence of the flavonoid kaempferol and other phenolic substances.

Species of *Acanthophora* Dunal are considered phylogenetically more derived than those of *Erythrotrichum*. Among the characters used to define the group are retention of juvenile leaves (repanda and spines acicular) and indument with simple and stellate trichomes throughout the whole plant (Nee, 1991). According to literature review, only one other species of *Acanthophora* was studied for the presence of flavonoids, *Solanum mammosum* L., which showed methoxylated aglycones (Wollenweber et al., 2005). Comparing the studies from the three species of *Erythrotrichum* (Silva et al., 2002a,b; Silva et al., 2003) to *S. agrarium*, of *Acanthophora*, it is possible to observe that the flavonoid myricetin-3,7,3'-trimethyl ether also show chemical characteristics in the most derived subgenus *Leptostemonum*.

A recent phylogenetic study of subgenus *Leptostemonum*, based on molecular biology, suggested the displacement of *S. agrarium* from *Acanthophora* to *Erythrotrichum* (Levin et al., 2005). Since the crude ethanolic extract from aerial parts of *S. agrarium* (SA-EtOH) showed antispasmodic effect on guinea-pig ileum (Santos et al., 2003), it was decided to investigate the SA-EtOH action mechanism on guinea-pig ileum. Initially, it was investigated to verify if SA-EtOH acted directly at receptor level or through a signaling pathway after receptor activation. It was established that, carbachol cumulative concentration-response curves were non-parallelly shifted to the right and E_{max} reduced from 100% (control) to 89.5 ± 4.5; 69.3 ± 4.6; 49.6 ± 6.0; 30.7 ± 5.2 and 12.6 ± 5.8%, in the presence of 27, 81, 243, 500 and 750 µg/mL of SA-EtOH, respectively (Figure 2A). EC_{50} values for carbachol increased from $0.3 \pm 0.01 \times 10^{-7}$ M (control) to $0.8 \pm 0.1 \times 10^{-7}$; $1.5 \pm 0.2 \times 10^{-7}$; $2.4 \pm 0.9 \times 10^{-7}$; $4.0 \pm 1.3 \times 10^{-7}$ and $9.3 \pm 1.2 \times 10^{-7}$ M in the presence of 27, 81, 243, 500 and 750 µg/mL of SA-EtOH, respectively. To confirm non-participation of receptors, the same experiment was performed using histamine agonist. The effects were evaluated with cumulative histamine curves and SA-EtOH shifted the cumulative concentration-response curves of histamine to the right in a non-parallel and E_{max} reduced from 100% (control) to 92.5 ± 4.7; 80.4 ± 4.5; 58.4 ± 5.2; 28.0 ± 4.2 and 6.0 ± 2.5% in the presence of 9, 27, 81, 243 and 500 µg/mL of SA-EtOH, respectively (Figure 2B).

EC_{50} histamine values increased from $1.9 \pm 0.3 \times 10^{-7}$ M (control) to $3.2 \pm 0.6 \times 10^{-7}$; $3.9 \pm 0.7 \times 10^{-7}$; $2.8 \pm 0.5 \times 10^{-7}$; $3.7 \pm 0.9 \times 10^{-7}$ and $8.7 \pm 3.6 \times 10^{-7}$ M in presence of 9, 27, 81, 243 and 500 µg/mL of SA-EtOH, respectively. This suggests non-competitive antagonism and that antispasmodic effects induced by SA-EtOH do not involve muscarinic or histaminic receptors. Thus, it is more likely that SA-EtOH acts on other step of the events

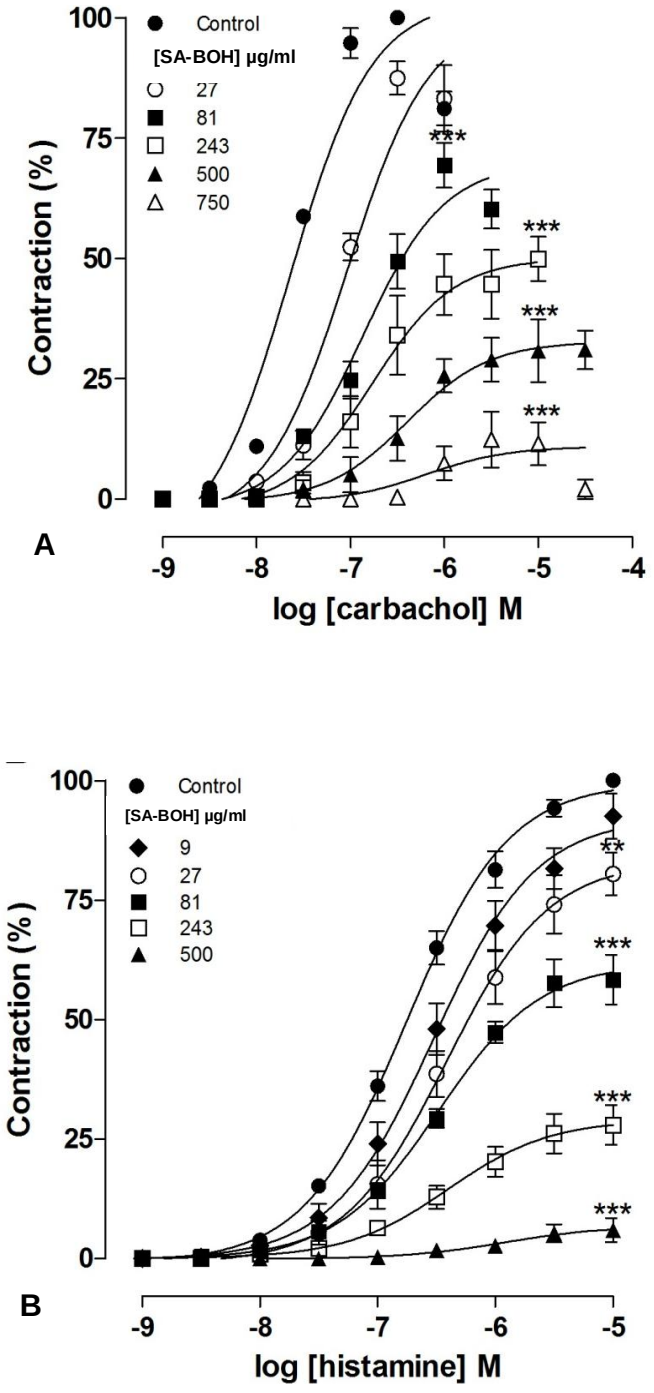

Figure 2. Curves concentracion-response cumulatives to carbachol (A) or histamine (B) on guinea-pig ileum (n = 5). The symbols and verticals bars represent the mean ± S.E.M., respectivety. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$ (control *vs*. SA-EtOH).

cascade that leads to smooth muscle contraction.

Since ileum is an organ totally dependent on membrane potential variation, the removal of extracellular Ca^{2+} blocks contractile response using both depolarizing agents (KCl) (electromechanical coupling) and agonists of mixed coupling (pharmaco and electromechanical) such as serotonin, carbachol and histamine, in seconds, suggest that the sources of intracellular Ca^{2+} do not contribute significantly to the tension level (Nouailhetas et al., 1985). However, the influence of extracellular Ca^{2+} is relatively higher in tonic contractile responses when compared to phasic ones (Triggle et al., 1979). Since tonic contraction is maintained almost exclusively by Ca^{2+} influx through Ca_V (Bolton, 1979; Bolton, 2006). In order to verify if SA-EtOH action mechanism on guinea-pig ileum involve blockage of Ca^{2+} influx through these channels, we evaluated the effects of SA-EtOH on the tonic component of KCl, carbachol or histamine-induced contractile response. SA-EtOH relaxed in a significant and dependent-concentration manner the ileum pre-contracted with 40 mM KCl (EC_{50} = 17.4 ± 3.5 µg/mL), 10^{-6} M carbachol (EC_{50} = 119.4 ± 24.3 µg/mL) or histamine (EC_{50} = 18.7 ± 4.6 µg/mL) (Figure 3). This suggests that SA-EtOH may inhibit the Ca^{2+} influx through Ca_V, producing non-selective antispasmodic effects.

This hypothesis was confirmed by the observation that SA-EtOH inhibited Ca^{2+}-induced contractions in a depolarizing medium nominally without Ca^{2+} through non-competitive antagonism, shifting the concentration-response curve to the right in a non-parallel and concentration-dependent manner, reducing significantly E_{max} from 100% (control) to 86.7 ± 5.5; 68.3 ± 5.7; 65.1 ± 4.6; 57.1 ± 6.6; 30.1 ± 6.8 and 15.9 ± 1.9%, in the presence of 9, 27, 81, 243, 500 and 750 µg/mL of SA-EtOH, respectively (Figure 4). $CaCl_2$ EC_{50} values increased from 0.8 ± 0.1 x 10^{-3} M (control) to 1.0 ± 0.2x10^{-3}; 1.5 ± 0.5 x 10^{-3}; 1.2 ± 0.4 x 10^{-3}; 3.8 ± 1.5 x 10^{-3}; 5.9 ± 1.2 x 10^{-3} and 7.3 ± 1.5 x 10^{-3} M in the presence of 9, 27, 81, 243, 500 and 750 µg/mL of SA-EtOH, respectively.

In smooth muscles, Ca_V-L (dihydropyridine sensitive) are more characteristic and responsive to Ca^{2+} influx through Ca_V when the binding site is located in subunit α1, although there are four other unit complexes (2 α, 1 β e 1 γ) (Vogalis et al., 1991; Kuriyama et al., 1995; Knot et al., 1996). These channels are abundantly expressed in guinea-pig ileum and are currently known as CaV 1.2 (Caterral et al., 2005). To evaluate if the Ca_V channel involved in SA-EtOH response was Ca_V-L, SA-EtOH effects were investigated on S-(-)-Bay K8644 precontracted ileum, an agonist of Ca_V-L that acts by directly binding to the channel´s α1 subunit and not by depolarization (Spedding and Paoletti, 1992). SA-EtOH (EC_{50} = 187.2 ± 27.3 µg/ml) relaxed the ileum pre-contracted in a concentration-dependent manner, reached the E_{max} (Figure 5), suggesting that Ca_V-L channel is in fact involved. When comparing the relaxant potency of SA-EtOH on ileum pre-contracted with KCl it is possible to observe that SA-EtOH is 11 times more potent in relaxing ileum pre-contracted with KCl than with S-(-)-Bay K8644. This can be explained by the fact that

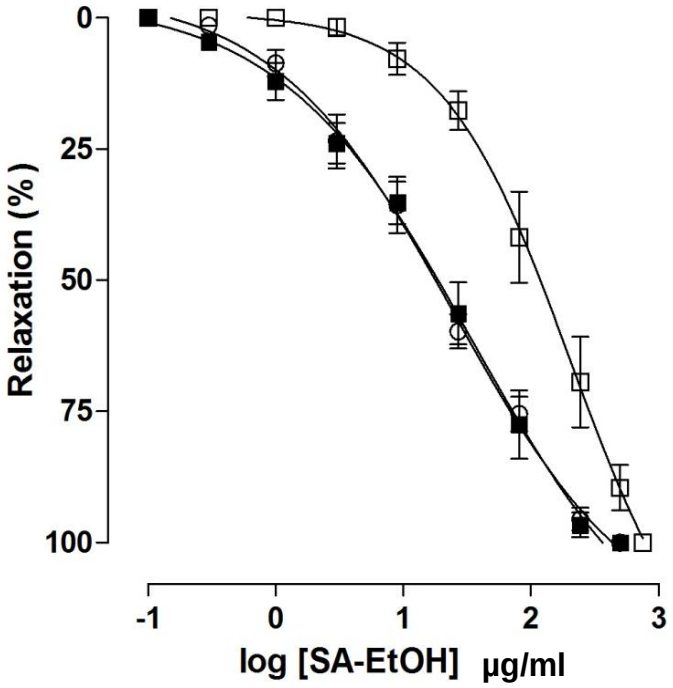

Figure 3. Effect of SA-EtOH on the tonic contractions induced by 40 mM KCl (○), 10^{-6} M carbachol (□) or histamine (■) on guinea-pig ileum (n = 5). Symbols and vertical bars represent mean ± S.E.M, respectivety.

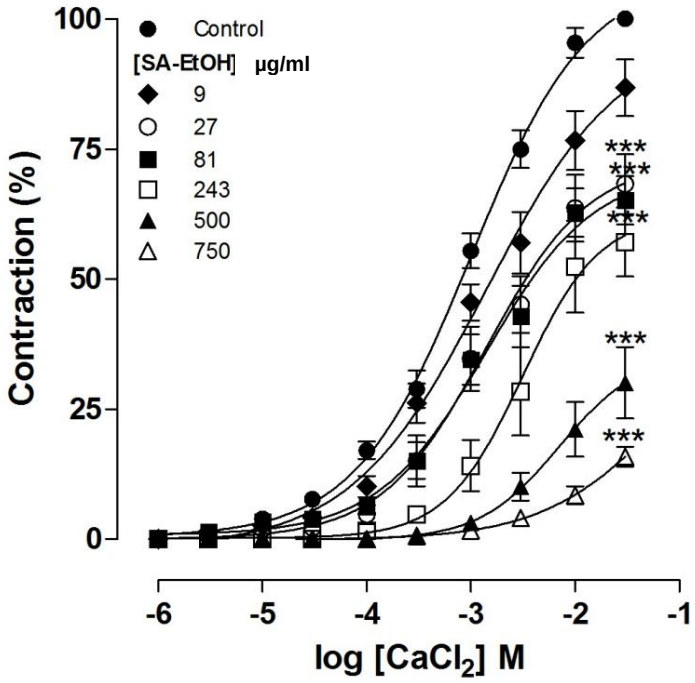

Figure 4. Curves concentracions-response cumulatives to $CaCl_2$ in depolarizing medium nominally without Ca^{2+} on guinea-pig ileum (n = 5). The symbols and vertical bars represent the mean ± S.E.M., respectivety. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by *** $p < 0.001$ (control *vs*. SA-EtOH).

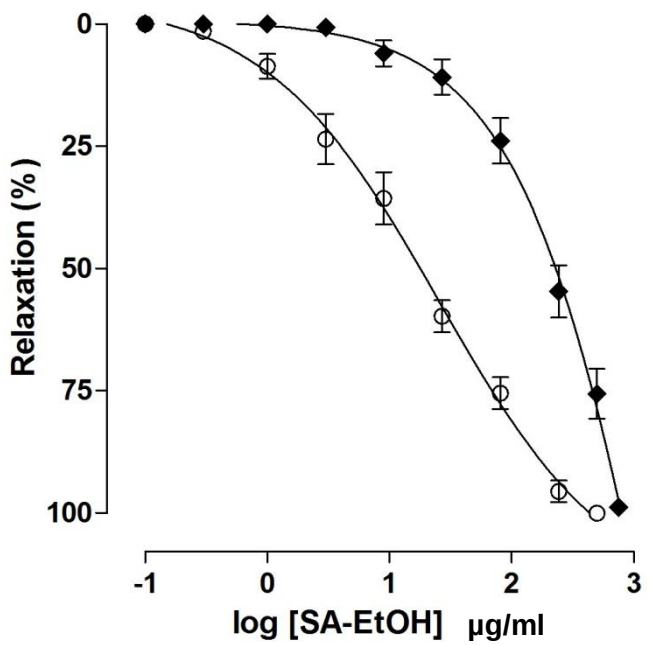

Figure 5. Effect of the SA-EtOH on the induced tonic contractions by 40 mM KCl (○) and 3 x 10^{-7} M S-(-)-Bay K8644 (◆) (n = 5). Symbols and vertical bars represent mean ± s.e.m., respectively.

KCl, in addition to inducing Ca_V-L 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 maintains contraction mainly by direct activation of Ca_V-L (Spedding and Paoletti, 1992).

In conclusion, our results show for the first time that from *Solanum agrarium* was isolated the flavonoid myricetin-3,7,3'-trimethyl, and that the mechanism of antispasmodic action of crude ethanolic extract from aerial parts of *S. agrarium* (SA-EtOH) on guinea-pig ileum is due, partially , to the inhibition of Ca^{2+} influx through Ca_V-L channels. However, we do not discard the role of other possible mechanisms that have not been studied yet.

ACKNOWLEDGEMENTS

The authors are grateful to José Crispim Duarte for technical assistance; and CNPq, CAPES, RENORBIO and FACEPE for financial support.

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