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Spasmolytic Action of *Centaurium erythraea* on Rabbit Jejunum is through Calcium Channel Blockade and NO Release

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Authors' contributions

This work was carried out in collaboration between all authors. Authors A Chda and MEK have performed experiments on isolated rabbit jejunum and wrote the protocol. Authors A Chokri and AT managed the analyses of study and the literature searches. Author KEA realized statistical analysis and author RBC designed the study and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The aim of the present study was to assess the antispasmodic effect of the aqueous extract from aerial parts of *Centaurium erythraea* (AECE) on isolated rabbit jejunum.
Methods: Myorelaxant and spasmolytic effects of AECE (0.3-10 mg/ml) were tested directly on spontaneous contractions and after spasm induction. To evaluate whether the effect of AECE involves a Ca^{2+} channel blockade, the tissues were placed in Ca^{2+}-free Tyrode's solution, then calcium was added in the presence of AECE. In addition, to investigate the involvement of the NO /cGMP pathway in the spasmolytic effect of AECE, the concentration-effect curve was achieved in the presence of L-NAME as a nitric oxide synthesis inhibitor, or ODQ as a specific cGMP inhibitor.
Results: Aqueous extract of *Centaurium erythraea* significantly reduced the jejunum's

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spontaneous contractions ($p<0.05$). When the jejunum was incubated in Ca^{2+}-free Tyrode's solution or in high K^+-Ca^{2+}-free Tyrode's solution, AECE significantly inhibited the recovery of spontaneous contraction as well as those induced by high K^+ during Ca^{2+} supplementation. Also, AECE shifted to the right the concentration responses curve of high K^+ induced jejunum contraction similarly to the effect produced by verapamil, a known calcium channel blocker, suggesting a presence of calcium antagonistic constituent(s) in AECE. By contrast, pretreatment with L-NAME or ODQ significantly reduced the antispasmodic effect, and shifted to the right the response curves of AECE, demonstrating the involvement of the NO pathway in this effect.

Conclusion: The main finding of our work suggests that AECE contains spasmolytic constituents mediating their effect at least through Ca^{2+} influx blockade and NO-cGMP pathway activation.

Keywords: Centarium erythraea; spasmolytic; NO/cGMP; calcium influx restriction; rabbit jejunum.

1. INTRODUCTION

Centaurium erythraea L. (CE) of the Gentianaceae family, known in Morocco under its vernacular name "Gosset El Haya", is used in traditional medicine as related by some ethnopharmacological surveys for the treatment of digestive disorders, kidney diseases, as an antipyretic and to treat diabetes [1- 4].

The literature also shows that *in vitro*, aqueous extract of *Centaurium erythraea* possesses anti-inflammatory activity [5], anti-hyperglycemic activity [6,7] a diuretic effect [8] and gastroprotective effect [9]. *Centaurium erythraea* chloroform extract has been found to have an inhibitory action on digestive enzymes and on angiotensin converting enzyme (ACE) [10].

Previous phytochemical investigations on CE yielded a variety of plant secondary metabolites including centauroside, flavonoids, gentiopicrin, isocumarin and phenolic acids [11,12]. Many CE compounds are reported to exhibit important biological activities such as secoiridoid glycoside and gentiopicroside, which have been assessed for antibacterial and free radical scavenging activities [13,14], phenolic compounds isolated from the flowers of CE for antioxidant activity [15], and xanthone derivatives for antimutagenic properties [16].

By contrast, contraction of the smooth muscle, including that of rabbit jejunum, is dependent on an increase in the cytosolic free Ca^{2+} levels [17,18], which is due to either influx via voltage-dependent Ca^{2+} channels or release from intracellular stores [19-21]. It is well documented that high K^+ elicits membrane depolarization and thus opens the voltage-dependent Ca^{2+} channels to cause an influx of Ca^{2+} and ultimately induce muscle contraction [22-24]. It is also well known that Ca^{2+} channel blockers such as verapamil inhibit the Ca^{2+} influx via voltage-dependent channels into smooth muscle cells [25,26].

In addition to calcium channel blockers, nitric oxide (NO) is an important agent that can affect gastrointestinal motility. Nitric oxide formation is known to occur in many cell types, including vascular endothelial cells, platelets, and epithelial cells, and NO is an important mediator in numerous physiological processes. Nitric oxide induced relaxation via a cyclic GMP-dependent protein kinase (PKG) system has been reported in a wide range of types of gastrointestinal smooth muscle, including rat ileum and duodenum [27,28], dog duodenum [29], and human jejunum and oesophagus [30,31], which was related to the decrease of the intracellular calcium concentration. Since, this decrease of $[Ca^{2+}]$ i (restriction of influx or/and of the release from reticulum sarcoplasmic) has been often proposed as the origin of the myorelaxant and spasmolytic effects of many medicinal plants [32-37]. The present work was designed to test whether the spasmolytic effect of AECE on isolated rabbit jejunum is due to a possible decrease of $[Ca^{2+}]$ i through voltage calcium channel blockade and/or NO release. Also, because of the high cost of the drugs used in the treatment of intestinal spasms, this study has been undertaken to provide scientific proof to justify the popular medicinal use of AECE as a spasmolytic remedy by people who do not have access to modern medicine. To our knowledge, this is the first report on the spasmolytic effect of AECE.

2. MATERIALS AND METHODS

2.1 Plant Materials

Centaurium erythraea aerial part (leaves and flowers) was collected locally from northern

Morocco, Taounate (Lat: 34.52, Long: -5.06) between May and June (2014). The fresh herb was then pooled and stored at room temperature in a dry place prior to use. The plant was identified and registered as specimen number (MA-FSTF 14) at our institution (Department of Biology, FST, USMBA, Fès, Morocco). 20 g of the air-dried aerial parts of this plant were boiled in distilled water (200 ml). The mixture was then filtered through whatman filter paper, and thereafter the water was removed under vacuum in a rotary evaporator until dry. The percentage yields based on the dried starting material was 15% for dried aqueous extract. The extract was stored at -20°C until the pharmacological investigations were performed.

2.2 Animals and Tissue Preparation

Rabbits of both sexes (1.8-2.5 kg) were kept in a standard environmental condition in terms of humidity, temperature and light. The animals had free access to water and food until the experiment. However, food was withdrawn 24 hours prior to the experiment. The present study was performed according to international and institutional rules regarding animal experiments (NIH Publication No. 85-23, revised 1996). The rabbits were slightly anesthetized with ether and then stunned by a blow to the head and exsanguinated. Segments of jejunum of about 2-3 cm were quickly isolated and mounted in an organ bath containing Tyrode solution (50 ml) between two stainless steel hooks under 1g initial tension. The lower hook was fixed at the bottom of the organ bath and upper one was connected to an isotonic transducer (Harvard transducer, UK) connected to a Harvard Universal Oscillograph (UK). The Tyrode solution composition (in mM): 136 NaCl, 2.7 KCl, 1.4 $CaCl_2$ $2H_2O$, 0.5 $MgCl_2$ $6H_2O$, 11.9 $NaHCO_3$, 0.42 NaH_2PO_4 and 5.56 Glucose (bubbled continuously with 95% O_2, 5% CO_2, pH 7.4 at 37°C) [34]. Each piece of jejunum was allowed to equilibrate and stabilize for at least 30 min with washout every 10 min.

2.3 Effect of AECE on Spontaneous Contractions

After a stabilization period (30 min), the eventual myorelaxant effect of AECE (0.3-10 mg/ml) was tested directly on spontaneous rabbit jejunum contractions. Segments of jejunum that did not show spontaneous contraction were discarded from the experimental protocol.

2.4 Effect of AECE on the KCl and ACh Induced Contractions

In order to assess the spasmolytic effect of our extract, the spasm was induced by Tyrode containing high KCl (60 mM) or with acetylcholine (ACh, 10^{-5} M). Once the plateau of contraction elicited by spasmogen was achieved, AECE was added to the organ bath cumulatively (0.3, 1, 3, 10 mg/ml).

2.5 AECE Effect on Calcium Induced Contraction

To evaluate whether the effect of AECE involves a Ca^{2+} channel blockade, the tissue samples were first allowed to stabilize in normal Tyrode's solution, which was subsequently replaced with Ca^{2+}-free Tyrode's solution containing EDTA (2 mM). The jejunum was kept in this solution for 10 minutes. This method was adopted from [36,38] where EDTA was used, so as to remove any calcium that might have been released from the tissue. Ca^{2+}-free Tyrode's solution was then replaced by high K^+ (60mM)-Ca^{2+}-free solution. A control concentration response curve was obtained by adding $CaCl_2$ (0.5 to 10 mM) to the bath. The study was repeated once in the presence of the submaximal concentration of AECE (3 mg/ml) and twice in the presence of verapamil (5.10^{-6} M), which acted as a positive control.

2.6 AECE Effect in the Presence of L-NAME and ODQ

To investigate the involvement of the NO /cGMP pathway in the spasmolytic effect of AECE, the concentration-effect curve was achieved after 20 minutes' tissue incubation with 10^{-4} M of L-NAME (NG-nitro-L-arginine methyl ester) as a nitric oxide synthesis inhibitor or 10^{-5} M of ODQ (1H [1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one) as a specific cGMP inhibitor. Each jejunum preparation was used only for one of the spasmogens and antagonists.

2.7 AECE Effect in Presence of Yohimbine, Prasosin and Propranolol

To verify if the adrenergic receptors mediate the spasmolytic effect of AECE, the preparation was incubated simultaneously in the presence of adrenergic inhibitors (5.10^{-5} M) (prasosin propranolol and yohimbine) 20 minutes before KCl-induced contraction. Then, cumulative

concentration-effect curves of AECE (0.3, 1,3,10 mg/ml) were recorded.

2.8 Drugs

ACh, L-NAME, ODQ, prazosin, yohimbine, propanolol and verapamil were purchased from Sigma Chemicals Co (St Louis, MO, the United States). All the drugs were dissolved in distilled water, except ODQ, which was dissolved in DMSO. The drugs were stored at -20°C until use in the pharmacological experiment.

2.9 Statistical Analysis

The results were expressed as Mean ± SEM. The comparison between the control and the treated samples was analyzed using student's t-test (significance at $p < 0.05$). The plateau of the contraction caused by each spasmogen (KCl and ACh) in the absence of the extract or applied antagonists was considered as the 100% contraction. Comparisons between groups were performed by an analysis of variance (ANOVA) test for repeated measurements, followed by the Bonferroni t test, and a difference was considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Effect of AECE Extract on Spontaneous Contractions of Rabbit Jejunum

AECE (0.3-10 mg/ml) was tested on isolated rabbit jejunum to determine its myorelaxant effect. As shown in Figs. 1 and 2, the cumulative concentrations of AECE altered the spontaneous contractions of the isolated rabbit jejunum in a concentration-dependent manner since the percentage of contraction decreased to 76, 52±3, 4 ($p<0.01$) for the lowest concentration and was almost totally inhibited at the highest concentration to 2±2.01 ($p<0.001$).

To investigate an eventual interference of AECE extract with Ca^{2+} influx, the spontaneous contraction of the jejunum was examined in Ca^{2+}-free Tyrode in the absence and in the presence of the extract. In Ca^{2+}-free Tyrode, spontaneous contraction was markedly reduced. The addition of the Ca^{2+} rapidly restores the jejunum activity (Fig. 3a), indicating the importance of Ca^{2+} influx in spontaneous contraction. As clearly shown in Fig. 3b, the presence of AECE inhibited the recovery of spontaneous contraction during Ca^{2+} supplementation.

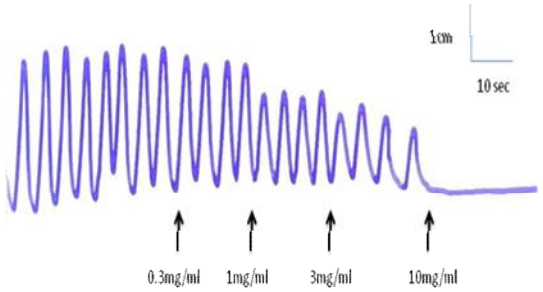

Fig. 1. Original tracing showing the spasmolytic effect of AECE on the spontaneous contraction of isolated rabbit jejunum

3.2 Effect of AECE Extract on KCl and ACh Induced Contractions of Rabbit Jejunum

When the spasm was induced by KCl (60 mM) or ACh (10 µM), as shown in Fig. 4, AECE reduced the jejunum's contraction in a concentration-dependent manner, with total relaxation at the highest concentration (10 mg/ml) for both spasmogens. Indeed the percentage of K^+-induced contraction deceased to 9.75±2.44 ; 27.75±1,09 and 38.75±3,06 as well as that induced by ACh to 14.75±2.83, 28.50±4.83 and 61.80±6.57 respectively for AECA concentrations of 0.3, 1 and 3 mg/ml.

In order to determine whether the spasmolytic effect of AECE is due to a blockade of voltage-dependent Ca^{2+} channels, the extract was assayed on high K^+ (60 mM)-Ca^{2+}-free Tyrode solution. As shown in Fig. 5, verapamil used as a positive control, and a submaximal concentration of AECE inhibited the recovery of the high K^+ induced contraction during calcium supplementation and shifted to the right in a similar way to the Ca^{2+} concentration response curves, suggesting that the spasmolytic effect of AECE is through voltage-dependent Ca^{2+} channel blockade.

3.3 Does the AECE Effect Involve NO Release?

To investigate the involvement of NO and/or cGMP in the spasmolytic effect of AECE, the preparation was incubated with L-NAME (10^{-4} M), a specific inhibitor of the NO synthase (NOS), or

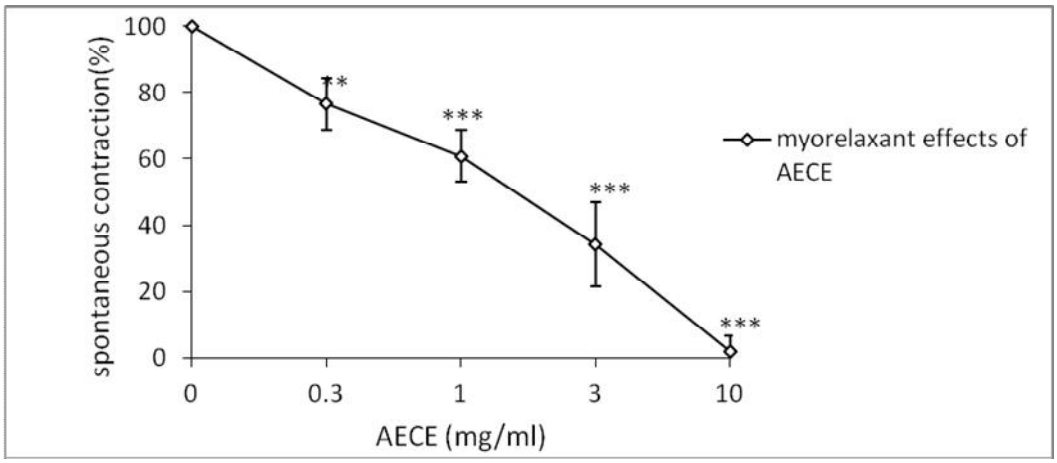

Fig. 2. Myorelaxant effects of cumulative concentrations of AECE (mg/ml) on the spontaneous contraction of isolated rabbit jejunum. Results are expressed as means ± SEM (n = 6); values are compared to the control. ** P</0.01 and *** P<0.001

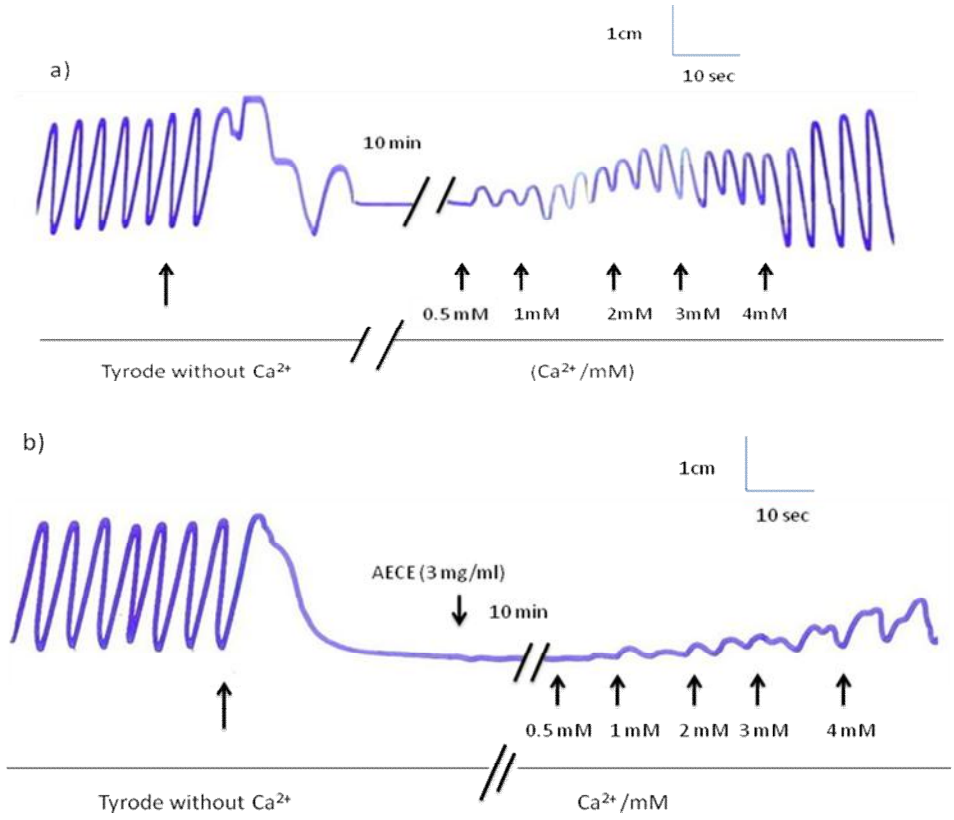

Fig. 3. Typical tracing of contractile activity of rabbit jejunum in Tyrode Ca^{2+} free without (a) and with (b) a sub maximal concentration of AECE (3 mg/ml)

ODQ, a selective inhibitor of guanylyl cyclase. As shown in Fig. 6, L-NAME and ODQ enhanced significantly ($p<0.05$) the basal tone of spontaneous contraction, and they significantly altered the spasmolytic effect of AECE on the Ach-induced contraction. Indeed, the highest concentration of AECE (10 mg/ml), which completely inhibited the Ach-induced contraction

(Fig. 4) achieved only 48.34±4.25% ($p<0.01$) (Fig. 6a) and 47.95±4.64% ($p<0.01$) (Fig. 6b) of relaxation respectively in the presence of L-NAME and ODQ.

Fig. 7 shows that the selective inhibitors of the NO/cGMP pathway significantly altered the spasmolytic effect of AECE and shifted the relaxation curves to the right, since the submaximal response to AECE (3 mg/ml) was reduced from 55, 5±5, 63% to only15.64±2.82% in the presence of L-NAME (10^{-4} M) (n=4, $p<0.01$) and to only 13.84±1.14% in the presence of ODQ (10^{-5} M).

3.4 Does the AECE Effect Involve Adrenergic Receptors?

To verify if the adrenergic receptors mediate the spasmolytic effect of AECE, the inhibitory activity of the AECE (0.3-10 mg/ml) on KCl-induced contractions was tested in the presence of the α1, α2 and β adrenergic receptors antagonists: prasosin (5.10^{-5} M), propranolol (5.10^{-5} M) and yohimbine (5.10^{-5} M), respectively. Fig. 8 shows no change in the AECE responses during incubation with those antagonists.

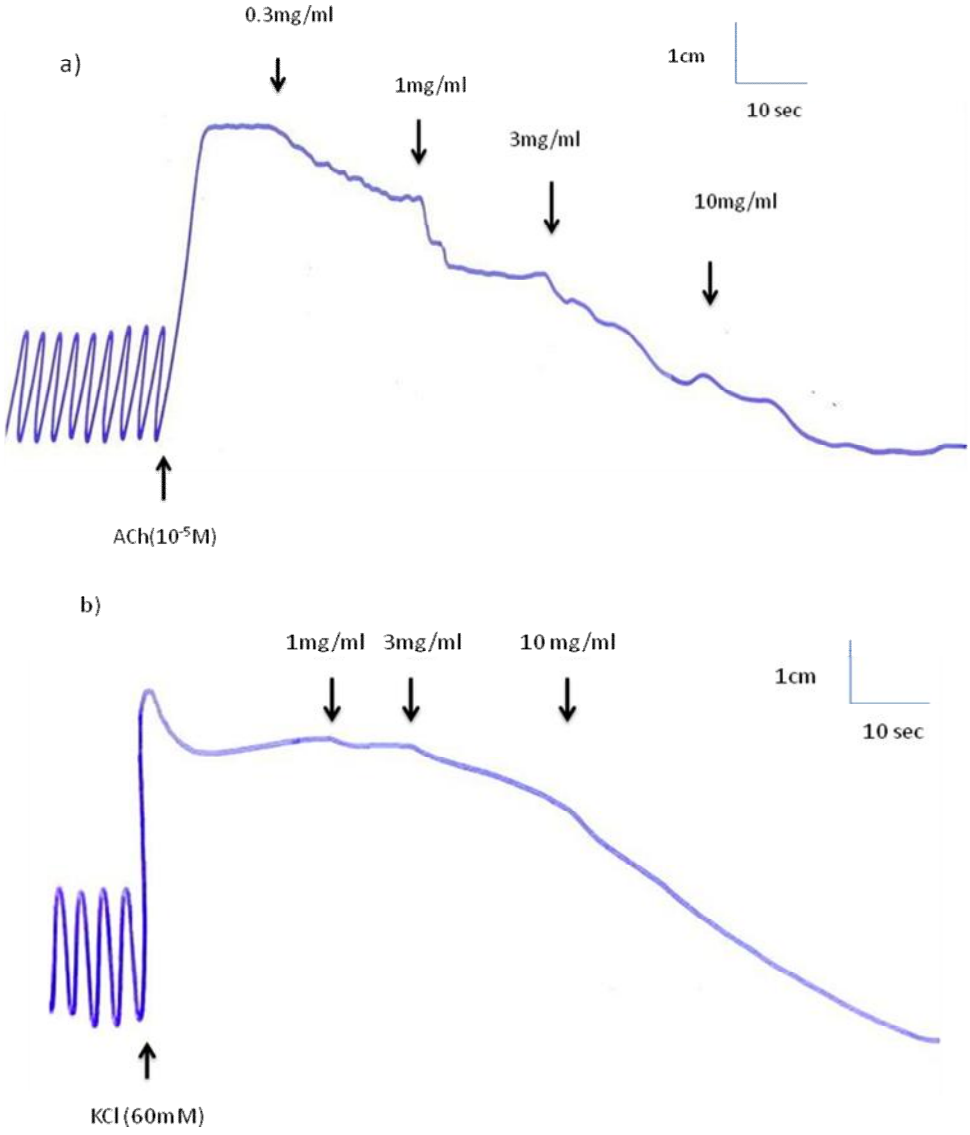

Fig. 4. Typical tracing showing the effect of AECE on KCl (60 mM) (a) and Ach (10^{-5}M) (b) induced contraction of rabbit jejunum

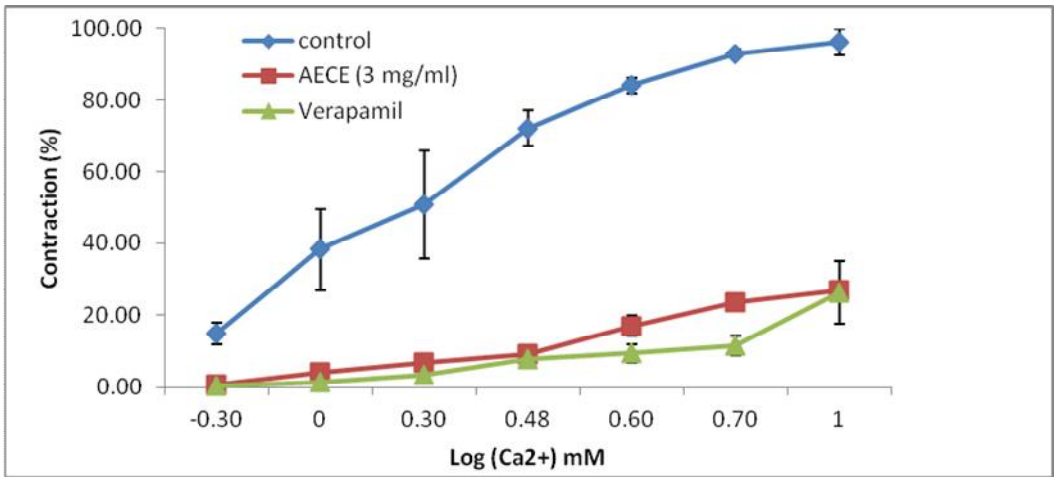

Fig. 5. Effect of exposure to verapamil (5.10^{-6} M, n=4) and to a sub maximal concentration of AECE (3 mg/ml, n=4) on KCl induced contraction of rabbit jejunum. Note the similar inhibition and similar right shifting of verapamil and AECE during Ca 2+ supplementation. Values are means ± SEM

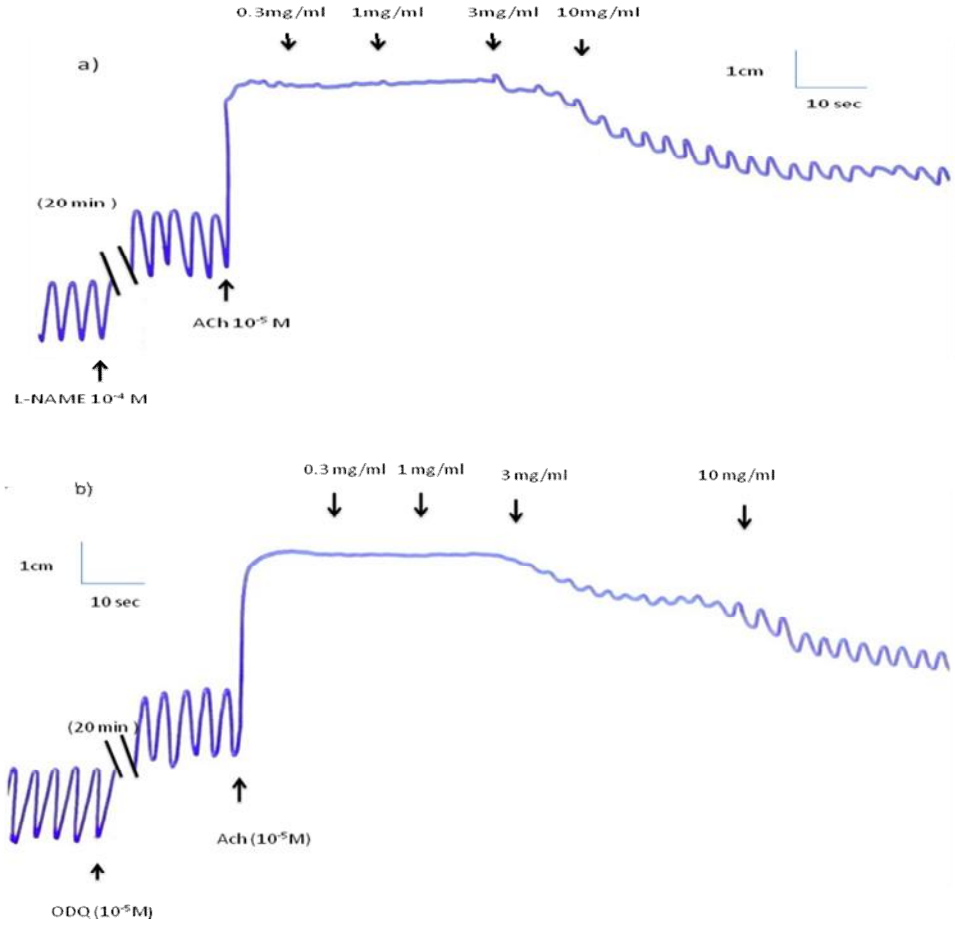

Fig. 6. Typical tracing showing that the incubation with L-NAME (10^{-4}M) (a) or ODQ (10^{-5}M) (b) altered the spasmolytic effect of AECE

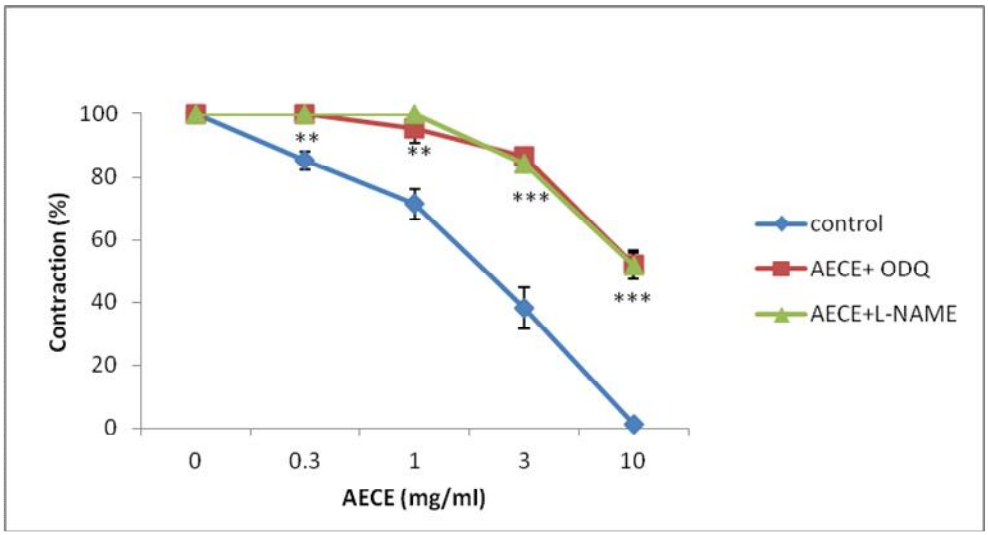

Fig. 7. The effect of AECE on the ACh induced contraction of jejunum in the absence and the presence of L-NAME (10^{-4}M) or ODQ (10^{-5}M). Values are shown as Mean ± SEM with n=4 **p<0.01, ***p<0.001) compared with the percentage of contraction in the presence of L-NAME or ODQ. Note the right shifting of relaxation in the presence of L-NAME or ODQ

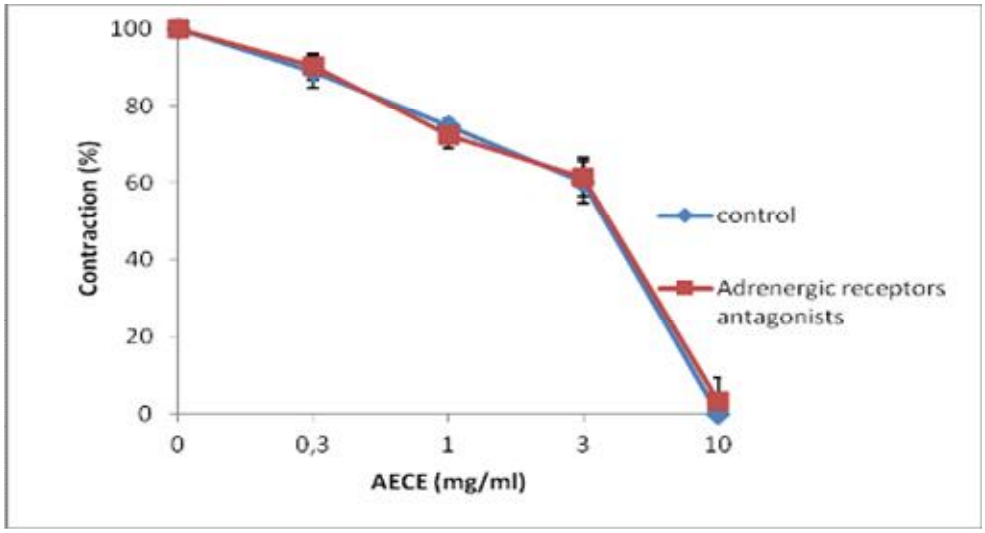

Fig. 8. Effect of AECE on the KCl (60mM) induced rabbit's jejunum contraction in the presence of adrenergic receptors antagonists (prazosin, yohimbine and propanolol). Values are shown as Mean ± SEM, n=4

4. DISCUSSION

The contractions of smooth muscle are dependent on an increase in cytoplasmic-free calcium through elevation of calcium influx and calcium release from reticulum sarcoplasmic [17,18]. This study showed that AECE is a potent spasmolytic agent, since it reduced both the spontaneous contractions and those produced by acetylcholine and high K^+ (spasmogens) in rabbit jejunum (Figs. 1 and 4).

To investigate whether AECE interacts with Ca^{2+} influx, rabbit jejunum was exposed to a Ca^{2+}-free Tyrode solution. Our results show that AECE inhibited the restored spontaneous contraction obtained when Ca^{2+} was added to the bath, suggesting that the plant extract probably acts at least as a Ca^{2+} channel blockade, since spontaneous contraction of smooth muscle mainly involves those channels. Our results are in agreement with many other studies [34-40] demonstrating that the spasmolytic effect of the

plant extract was mainly mediated through calcium antagonism.

However, KCl-induced contraction has long been known to be due to membrane depolarization causing Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCCs) [22,25]. Indeed, high K^+ is ineffective in the absence of external Ca^{2+} [41]. Furthermore, ACh activates M_2 muscarinic receptors to open non-selective cation channels and L-type (VDCCs) and elevates inositol triphosphate (IP3) production, which causes release of Ca^{2+} from sarcoplasmic reticulum [17,21]. According to Gordienko et al. [42] and Berridge [43], IP_3 Ca^{2+} release is even facilitated by Ca^{2+} influx through voltage-operated channels. Thus, it is possible to speculate that the AECE might also cause the spasmolytic effect through the inhibition of extracellular calcium influx.

In order to clarify the involvement of VDCCs blockade during the AECE spasmolytic effect, high K^+ Ca^{2+}-free Tyrode was used to depolarize tissue preparation. As mentioned in the results section (Fig. 5), applying cumulative concentrations of Ca^{2+} restores completely KCl induced contractions, which were significantly inhibited when the preparation was incubated with the submaximal concentration of AECE (3 mg/ml). Therefore, it seems that AECE has at least inhibited the Ca^{2+} influx through blockade of VDCCs, since substances that inhibit KCl-induced contraction of the smooth muscle are referred to as voltage-dependent calcium channel blockers [43].

On the other hand, it is well documented that NO has become one of the most important candidates for mediating nonadrenergic noncholinergic smooth muscle relaxation through the gastrointestinal tract [29,30,44]. It is now widely accepted that NO acts via an increase of cellular concentration of cGMP [30,28,45] which activates in it turn a protein kinase G (PKG) responsible for the decrease in the $[Ca^{2+}]i$ probably by increasing uptake of Ca^{2+} through activation of sarcoplasmic reticulum Ca^{2+} - ATPase (SERCA) [45]. Therefore, in our study we investigated if the antispasmodic activity of AECE was mediated by an eventual involvement of NO / cGMP pathways. The pretreatment with L-NAME or ODQ induced an increase of the jejunum basal tone as shown in Figs. 6a and 6b, thereby confirming the implication of the NO/cGMP pathway to maintain the basal tone of rabbit jejunum. Also, pretreatment with L-NAME or ODQ produced a similar inhibition and similar right shifting of the AECE relaxation curves (Fig. 7) suggesting the involvement of both NO and c GMP in the antispasmodic effect of AECE. Our findings correlate with those of the literature, which confirm the involvement of the NO-cGMP pathway in the spasmolytic action of many plant extracts, such as *Achillea millefolium* [28], *Pimpinella anisum* [46], *Lepechinia caulescens* [38] and *Cymbopogon citratus* [47].

The inhibitory effect of AECE remained unchanged during the simultaneous presence of prasosin, yohimibine and propanolol (α1, α2 and β adrenergic receptors blockers respectively), as shown in Fig. 8, suggesting a non-implication of the adrenergic pathway in the antispasmodic effect of *Centaurium erythraea*.

Previous phytochemical studies have reported that extract of *Centaurium erythraea* contains Gentiopicroside, xhantone and flavonoid derivatives such as quercetin and kaempferol [11,14,48]. Furthermore, *in vitro* studies have shown that these compounds inhibit the smooth muscle motility [49-52]. Therefore, it is possible to assume that the spasmolytic effect of aqueous extract of *Centaurium erythraea* may be due at least to these compounds.

5. CONCLUSION

Our study demonstrated that AECE had a potent spasmolytic effect on rabbit jejunum through at least a blockade of extracellular Ca^{2+} influx and an activation of the NO/cGMP pathway (activation of SERCA). Thus, the use of *Centaurium erythraea* in traditional medicine for the treatment of gastrointestinal disease is now supported and justified by the results described in this study. However, an additional investigation is necessary to determine the main bioactive molecules of AECE responsible of this spasmolytic effect.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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