

## Myorelaxant and antispasmodic effect of an aqueous extract of *Artemisia campestris* L. via calcium channel blocking and anticholinergic pathways

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### Abstract

Intestinal spasms are violent contractions that occur in the intestine, which cause discomfort to people who have them. Medicinal plants are widely used in traditional Moroccan medicine to treat these problems, among these being *Artemisia campestris* L. This study aims to evaluate the relaxant and antispasmodic effects of an aqueous extract of this plant (ACAE). It was performed *in vitro* on isolated segments of both isolated rat and rabbit jejunum mounted in an organ bath and tension recordings made via an isotonic transducer. ACAE caused a myorelaxant effect on baseline rabbit jejunum contractions in a dose-dependent and reversible manner with an IC<sub>50</sub> of 1.52 ± 0.12 mg/ml. This extract would not act via adrenergic receptors pathway. On the other hand, the extract caused a dose-dependent relaxation of the jejunum tone in rat jejunum segments pre-contracted with either Carbachol (CCh; 10<sup>-6</sup> M) or high K<sup>+</sup> (KCl 75 mM) with an IC<sub>50</sub> = 0.49 ± 0.02 mg/ml and 0.36 ± 0.02 mg/ml respectively. In the presence of different doses of the extract, the maximum response to CCh and CaCl<sub>2</sub> was significantly reduced. This demonstrates that ACAE acts on both muscarinic receptors and voltage-dependent calcium channels. Thus, the plant extract acted on both muscarinic and nicotinic receptors and acts on the guanylate cyclase pathway, but not the nitric oxide pathway. These results indicate the mechanism by which *Artemisia campestris* L. acts as an effective antispasmodic agent in traditional Moroccan medicine.

**Key words:** anticholinergic, antispasmodic, *Artemisia campestris* L., calcium channel blocking, jejunum

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## Introduction

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Currently, traditional medicines are a very important therapeutic source, especially in developing countries. WHO estimates that 80% of their population use plant cures (1, 2). The Moroccan traditional pharmacopeia is full of a multitude of herbal recipes to prevent, cure, relieve or improve human well-being (3). In Morocco, *Artemisia campestris* L. is a plant used traditionally for the treatment of diseases such as diabetes, obesity (4), cancer (5), hypertension (6), allergy, asthma, pathology of the digestive system (7), gastric ulcer (8), diarrhea (9) and also as an antispasmodic (10). This wide use is due to the diversity of phytoconstituents and several active ingredients (11). *Artemisia campestris* L. is rich in flavonoids, phenolic acids, coumarins, and fatty acids (12). Different pharmacological studies show that *Artemisia campestris* L. has antibacterial (13), antitumor (14), anti-inflammatory (15), antiplatelet, antihypertensive, and vasorelaxant effects (16, 17).

According to Fakchich and Elachouri (7), an *Artemisia campestris* aqueous extract (ACAE) has a high ICF (Informant Consensus Factor) value of 0.92, which means that this herb was traditionally strongly used to treat gastrointestinal problems. As there has been no previous study in this pharmacological effect of *Artemisia campestris*, we have chosen to study the antispasmodic and myorelaxant activity of ACAE on isolated segments of the rat and rabbit jejunum to elucidate the mechanism of action of ACAE in gastrointestinal disorders.

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## Material and Methods

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We followed the previously described method used by Aziz et al. (18) and Makrane et al. (19).

### ***Plant material***

The aerial part of *Artemisia campestris* L. was collected from a desert area situated between Tendirra and Figui in Morocco. The plant was identified by Professor Elachouri Mostafa from the Department of Biology. The voucher specimen HUMPOM-151 is kept in the herbarium of the Faculty of Sciences, Mohamed the First University, Oujda (Morocco).

### ***Extract preparation***

According to traditional usage, the ACAE was prepared by infusion of 30 g of the aerial part in 300 ml distilled water for 30 min. The ACAE was obtained after filtration and evaporation to dryness *in vacuo* (yield: 19%). The extract was stored at  $-20^{\circ}\text{C}$  until use.

### ***Pharmacological drugs***

The following drugs were used: Carbamylcholine chloride (Carbachol, CCh), propranolol, yohimbine, prazosin, L-NAME, calcium chloride ( $\text{CaCl}_2$ ), and hexamethonium were purchased from Sigma Chemical Co. (Sigma-Aldrich, USA). Atropine was supplied by Research Biochemical Incorporated, USA and Verapamil by Tocris, USA. All chemicals used were of the analytical grade available and solubilized in distilled water.

### ***Animals***

The experimental animals used were male and female 6–8 week old Wistar rats (200–300 g) and 4 month old New Zealand rabbits (1.5–2 kg). All animals were kept in the animal house of the Faculty of Sciences (Oujda, Morocco) in an air-conditioned room with controlled lighting (12 h:12 h light-darkness cycle) and free access to food and water. Food was withdrawn 24 h before the experiment. All animals were cared for in

compliance with the internationally accepted Guide for the care and use of laboratory animals, published by the US National Institutes of Health (20).

### ***Isolated tissue experiments***

Animals were anesthetized with light ethyl ether inhalation, the abdominal cavity opened and 2 cm jejunum segments removed and maintained during the tests in aerated normal Krebs-Henseleit buffer (KHB) solution with the following composition (in mM): NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 10. The KHB solution was maintained at a temperature of 37 °C and a pH of 7.4 maintained with continuous bubbling with a mixture of 95% O<sub>2</sub>, 5% CO<sub>2</sub> for 1 h to maintain the physiological conditions of the animal. Each piece of jejunum was mounted in an isolated organ bath (10 ml). The physiological fluid was changed every 15 min to equilibrate the organ before adding the plant extracts or other drugs. For all experiments, the effects of each dose were recorded for at least 7 to 8 min. The graph tracing related to the contractile response of the intestine was recorded using the PROTOWIN Panlab software and an isotonic transducer (TRO015 / Panlab) connected to a force amplifier (ISO510A / Panlab).

### ***Myorelaxant activity in isolated rabbit jejunum segments***

After stabilizing the baseline contractions (7 min) of rabbit jejunal smooth muscle segments, cumulative doses (0.1–3 mg/ml) of ACAE were added to the isolated organ chamber. We also tested the effect of this extract in the presence of three adrenergic receptor antagonists (Prazosin, propranolol and yohimbine) at a concentration of ( $5.0 \times 10^{-5}$  M) for each of them in the chamber.

### ***Antispasmodic effect of Artemisia campestris L. extract on rat jejunum segments via calcium channel blocking***

To evaluate the antispasmodic activity, we pre-contracted jejunal smooth muscle segments with KCl 75 mM after stabilization, and then added cumulative doses of ACAE to the isolated organ bath for final concentrations of between 0.1 to 1 mg/ml. To confirm the effect of ACAE on calcium channels, without stopping the recording, normal KHB was replaced by calcium-free Krebs solution with the following composition (in mM): (NaCl 121.7, KCl 4.7, CaCl<sub>2</sub> 0, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 10 and EDTA (0.1 mM) to remove calcium from the tissues for 10 min, then replaced with KHB rich in potassium and without calcium (in mM); NaCl 48, KCl 75, CaCl<sub>2</sub> 0, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub>, 1.2 and glucose 10. After stabilization, increasing and cumulative doses of CaCl<sub>2</sub> from 0.1 to 10 mM were added to the control. We followed the same protocol for the rest of the tests except that the ACAE were added before CaCl<sub>2</sub>.

### ***Anti-cholinergic effect of Artemisia campestris L. extract on rat jejunum segments***

To study anticholinergic activity, we pre-contracted jejunal smooth muscle segments with CCh ( $10^{-6}$  M). After stabilization, cumulative doses of ACAE were added to the isolated organ bath for final concentrations of between 0.1 and 1 mg/ml. To confirm the effect of our plant on cholinergic receptors, we added in the organ bath cumulative, increasing doses of CCh ( $3.10^{-8}$  M -  $3.10^{-5}$  M) in both the absence and presence of ACAE. To better understand the mechanism of action of the extract, tissues were pre-incubated for 20 min in either atropine ( $10^{-6}$  M) or hexamethonium ( $10^{-4}$  M), then the tissues were contracted, and the concentration of the extract that induced the maximum relaxation (1 mg/ml) was added.

### ***The relaxant effect of Artemisia campestris L. extract on the NO/cGMP pathway***

To analyze the effect of the plant extract on the nitric oxide (NO) and guanylate cyclase pathway (GC), we have pre-incubated the jejunum segments for 20 min in either L-NAME ( $10^{-4}$  M) or methylene blue ( $10^{-5}$  M). Following contraction of the tissues with a KCl rich medium, the concentration of the extract that induced the maximum effect (1 mg/ml) was added.

### ***Statistical analysis of the results***

The results were expressed as the mean  $\pm$  S.E.M. Moreover, the difference between the groups was calculated with a one-way analysis of variance (ANOVA) using GraphPad Prism 5 for windows, followed by a post hoc Tukey test. The difference was considered to be significant when  $P$  is less than 5%.

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## **Results**

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### ***Myorelaxant activity on isolated segments of rabbit jejunum***

ACAЕ has inhibited basal contractions of rabbit jejunum segments in a dose-dependent manner with an  $IC_{50} = 1.52 \pm 0.12$  mg/ml. The difference between the control and the 3 mg/ml dose is statistically extremely significant ( $P \leq 0.001$ ) (Fig. 1B). When rinsing with normal KHB was performed, contractions have been found to resume normally after a few minutes, showing that ACAЕ has a reversible effect (Fig. 1A).

In the presence of a combination of three adrenergic blocking agents, Propranolol ( $5.10^{-5}$  M), Prazosin ( $5.10^{-5}$  M), and Yohimbine ( $5.10^{-5}$  M), ACAЕ (3 mg/ml) caused an inhibition of the contraction of the smooth muscle of the rabbit jejunum segments. This inhibition is comparable to that obtained with the extract alone without inhibitors.

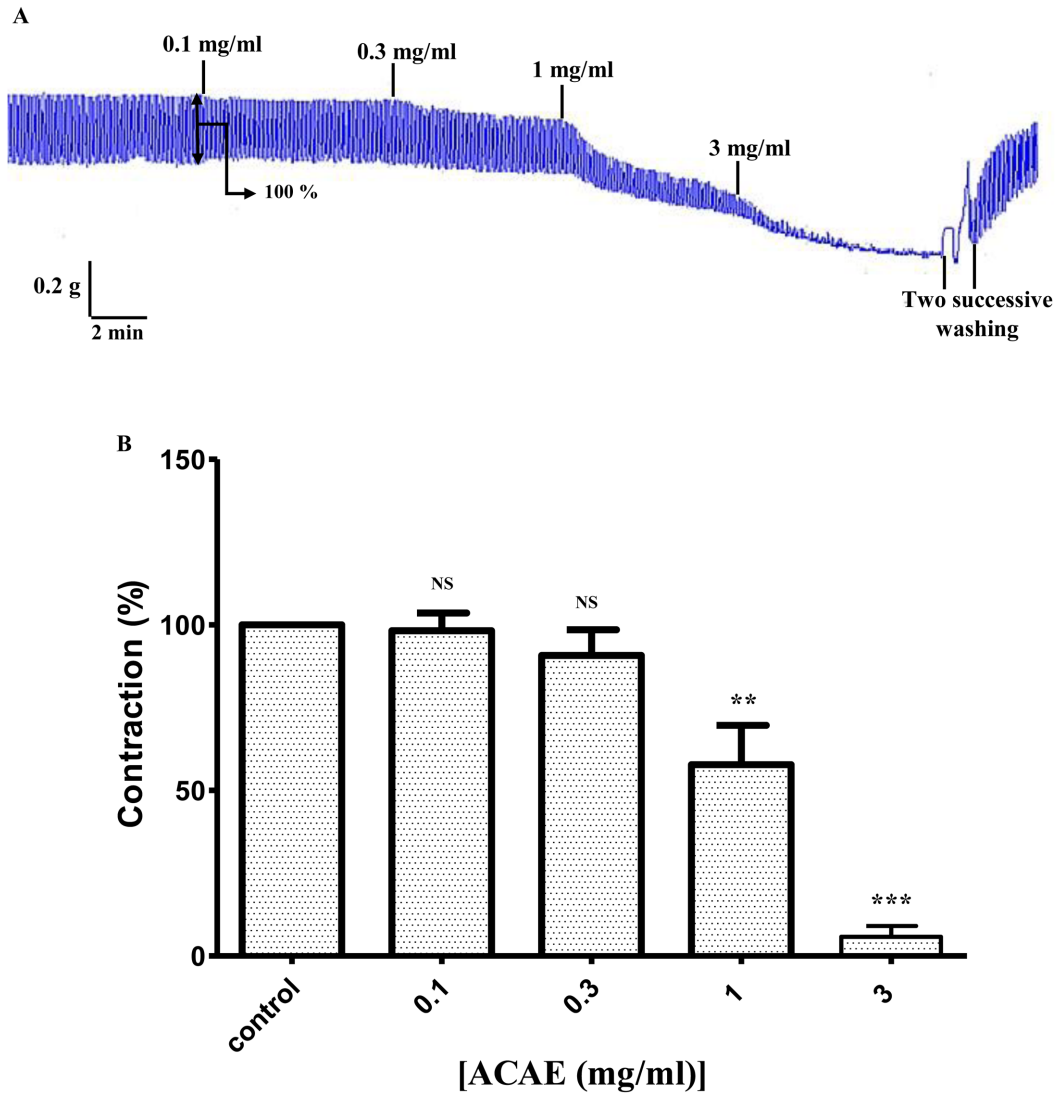
### ***Antispasmodic effect of Artemisia campestris L. extract on rat jejunum segments via calcium channel blocking***

ACAЕ caused a dose-dependent relaxation of the jejunum segments of the rat pre-contracted by KCl 75 mM with an  $IC_{50} = 0.36 \pm 0.02$  mg/ml and a total relaxation at 1 mg/ml, which was highly significant compared to the control (Fig. 2). In the presence of different doses of the extract (0.1, 0.3, 1 mg/ml), the maximum response to the increasing cumulative amount of  $CaCl_2$  was significantly reduced while shifting the dose-response curves of contraction to the right and down (Fig. 3). Verapamil ( $10^{-6}$  M), which is an antagonist of L-type calcium channel blocker, had a comparable effect to that of 1 mg/ml of ACAЕ (data not shown).

### ***Anti-cholinergic effect of Artemisia campestris L. on rat jejunum segments***

The concentration of 0.3 mg/ml of ACAЕ caused a significant decrease ( $P < 0.01$ ) in the tone of rat jejunum segments induced by carbachol  $10^{-6}$  M, while the addition of 1 mg/ml caused a total decrease of tone with an  $IC_{50} = 0.48 \pm 0.02$  mg/ml (Fig. 4). In the presence of different doses of the extract, the contractile response to CCh significantly reduced and shifted the dose-response curves of contraction to the right and down. At a dose of 1 mg/ml, the ACAЕ showed total inhibition of the contraction of the jejunum segments (Fig. 5).

The relaxant effect of ACAЕ on a KCl-induced contraction in the presence of muscarinic (atropine) and nicotinic (hexamethonium) inhibitors was significantly reduced by 51.16% and 78.88% respectively, compared to the effect of the extract in the absence of these inhibitors (Fig. 6). Atropine and hexamethonium did not have a significant inhibitory effect on KCl-induced contraction.



**Fig. 1.** Original tracing (A) and histogram (B) showing the effect of increasing doses of ACAE on the amplitude of spontaneous contractions of segments of the rabbit jejunum. The reduction of tone is not significant (NS) until 1 mg/ml (\*\* $P \leq 0.01$ ) and 3 mg/ml (\*\*\*) ( $P \leq 0.001$ ) (mean  $\pm$  S.E.M.  $n=5$ ).

### The relaxant effect *Artemisia campestris* L. extract on the NO/cGMP pathway

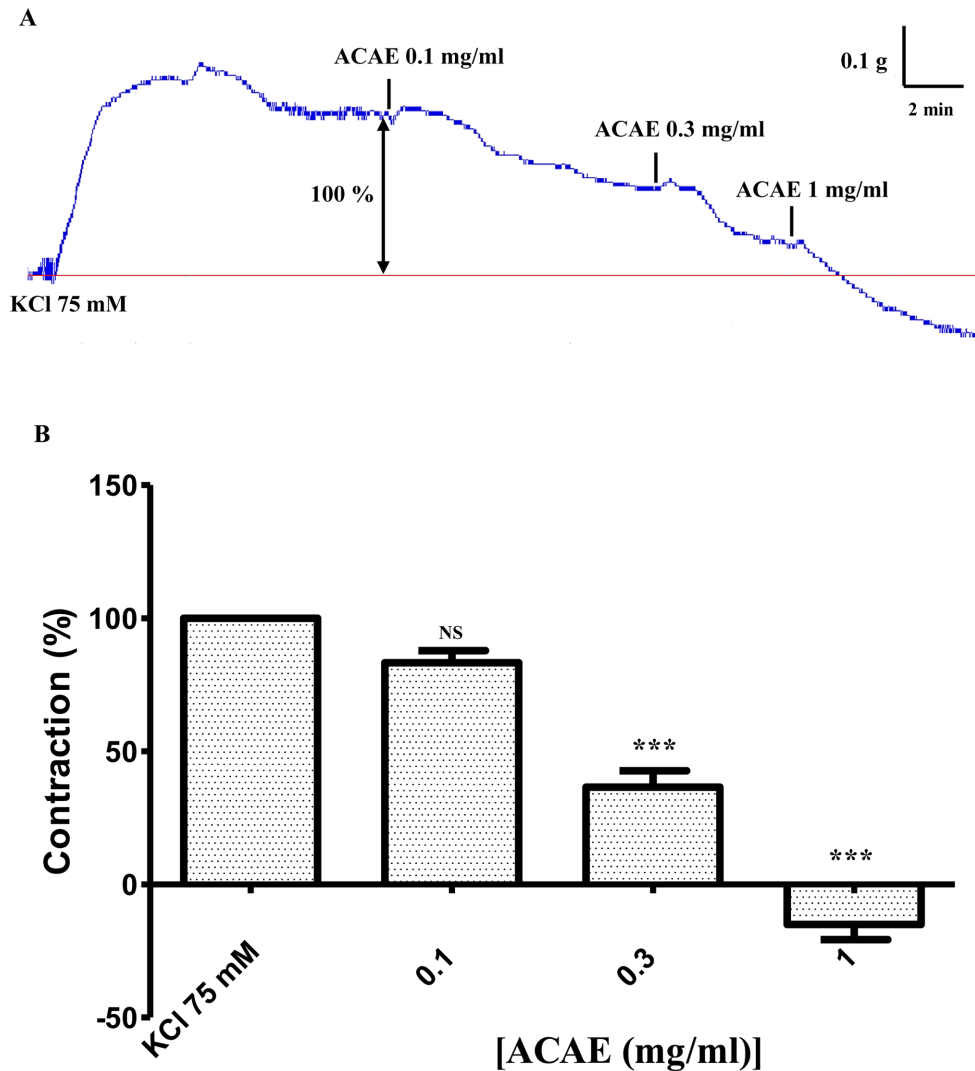
The effect of ACAE on rat jejunum segments pre-incubated with L-NAME and contracted with CCh was weakly reduced by 14.65% (not significant) compared to the control. When the intestine was pre-incubated with methylene blue, the effect of the extract was reduced significantly by 28.82% (Fig. 7). L-NAME did not affect the CCh-induced contraction of rat jejunum segments, but methylene blue inhibited the contraction induced by CCh by 50% (data not shown).

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## Discussion

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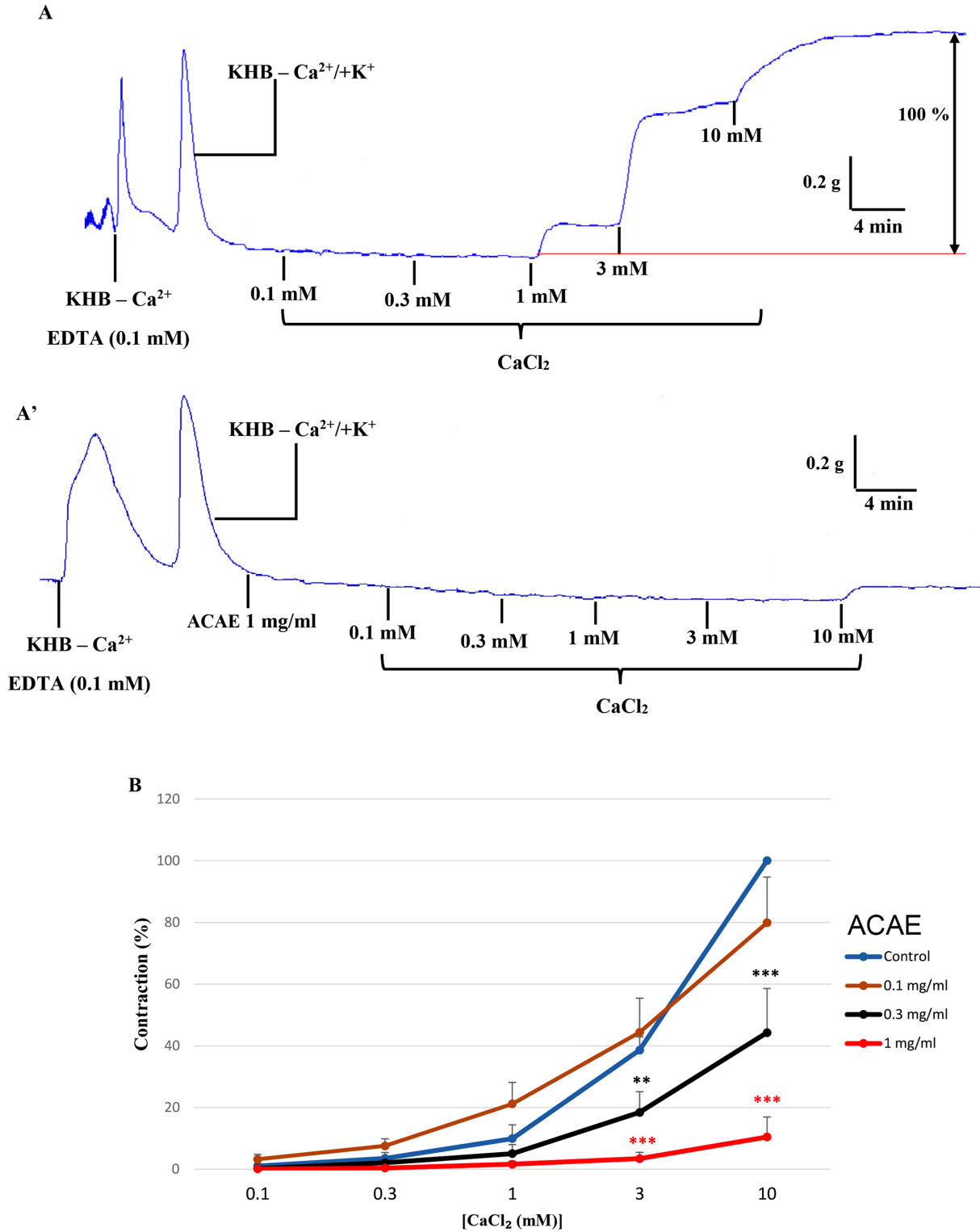
Spontaneous phasic contractions of the longitudinal and circular muscle layers of the intestine result from a cyclical depolarization/repolarization cycle, known as electrical slow waves, which result from the intrinsic pacemaker activity of Interstitial cells of Cajal (ICC) which are electrically coupled to smooth muscle cells. These waves of depolarization activate voltage-dependent calcium channels in the smooth muscle cells and



**Fig. 2.** Original tracing (A) and histogram (B) showing the effect of ACAE on the contractions of segments of the rat jejunum induced by 75 mM KCl. The difference is statistically significant to the control (KCl 75 mM) at 0.3 and 1 mg/ml ( $***P \leq 0.001$ ; mean  $\pm$  S.E.M.  $n=6$ ). NS: Not Significant.

rhythmical mechanical contractions are generated (21). The basic spontaneous contractions of the rabbit jejunum are larger and easier to evaluate than those of the rat jejunum. For this reason, we chose to test the effect of ACAE on this jejunum. ACAE caused a decrease in the amplitude and tone of spontaneous contractions of the smooth muscle in segments of the rabbit jejunum. The dose of 3 mg/ml gave a total inhibition of this contraction. After a double wash with a physiological solution, the tone and amplitude of these basic contractions was restored. Therefore, addition of ACAE had a myorelaxant effect on base contractions of segments of the rabbit jejunum in a dose-dependent and reversible manner. It could be acting either directly on smooth muscle cells and/or on ICC.

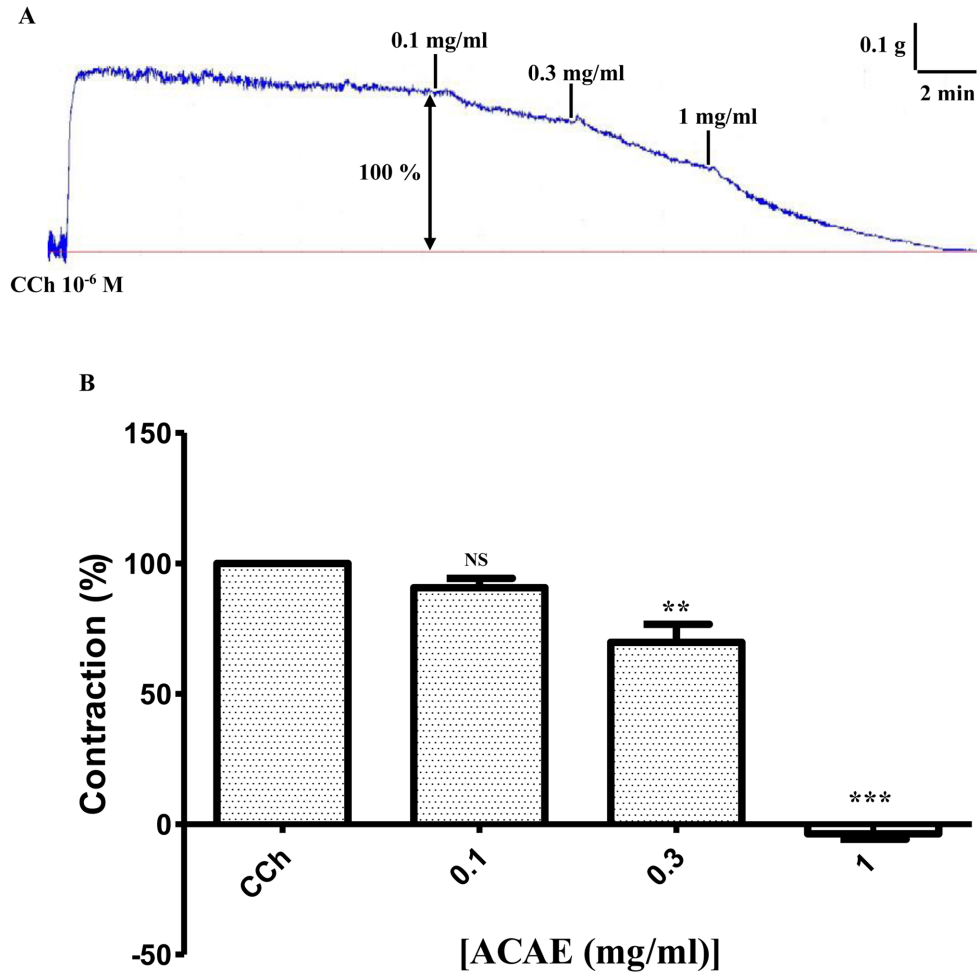
The autonomic nervous system acts via the release of norepinephrine and adrenaline to inhibit intestinal contractions (22–24) by the attachment of these neurotransmitters to the adrenergic receptors, all coupled to trimeric G proteins (25). We wanted to determine if ACAE acts via  $\alpha$  and  $\beta$  adrenergic receptors. For that purpose, we added three adrenergic inhibitors simultaneously to the organ bath: yohimbine, prazosin, and propranolol, antagonists of  $\alpha 1$ ,  $\alpha 2$ , and  $\beta$  adrenergic receptors respectively. In the presence of these inhibitors, adrenaline did not affect basal contractions, and the relaxant effect of ACAE was identical to that obtained in



**Fig. 3.** Original tracings (A, A') and (B) the dose-response curves to CaCl<sub>2</sub> in the presence (A') and absence (A) of ACAE on segments of the rat jejunum. The difference is statistically significant to the control (in the absence of ACAE) at higher concentrations of ACAE and Ca<sup>2+</sup> (\*\**P*≤0.01; \*\*\**P*≤0.001; mean ± S.E.M. *n*=5).

their absence. This suggests that the effect of the plant extract did not operate through the adrenergic receptor pathway.

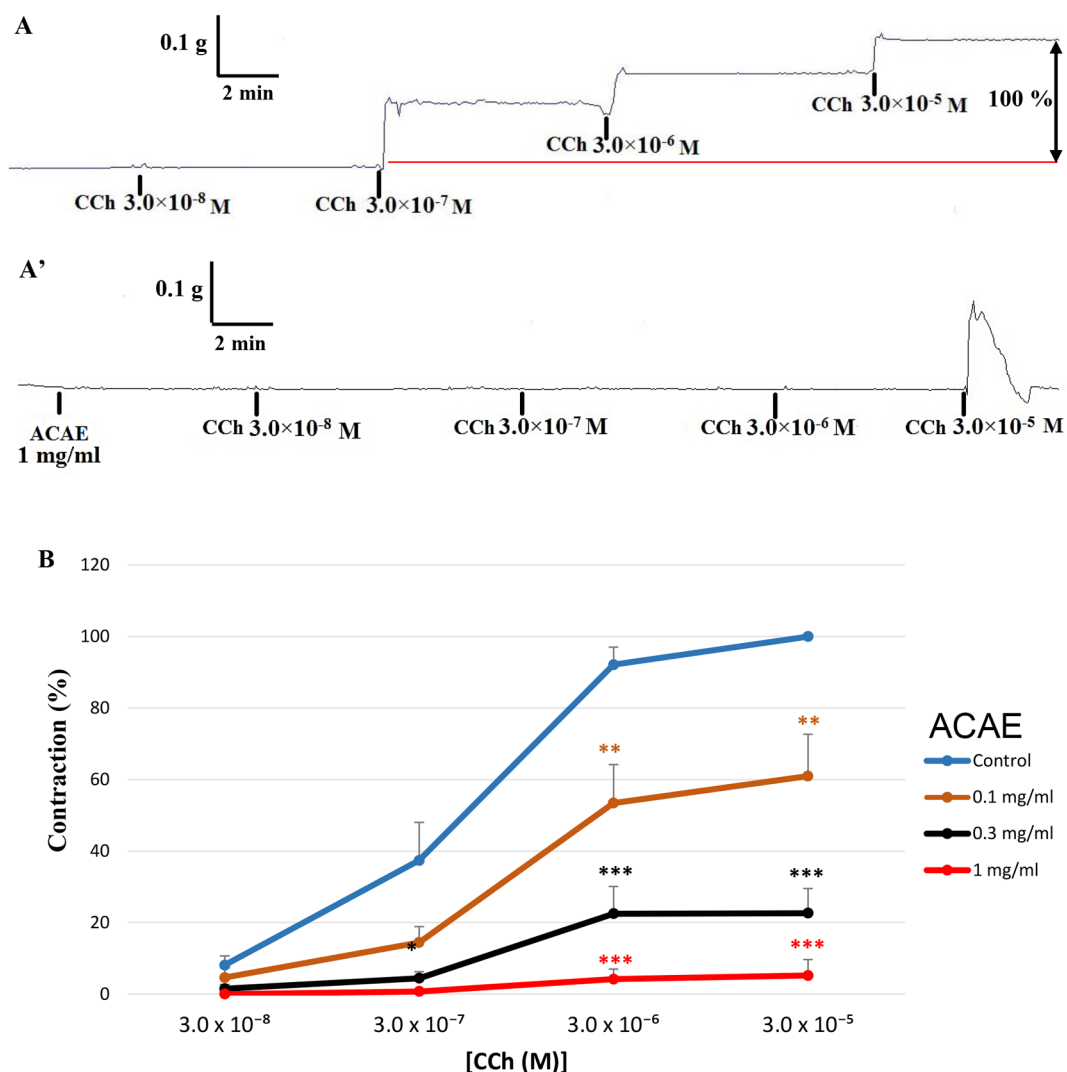
Muscarinic antagonists inhibit the contractions of the gastrointestinal tract induced by acetylcholine and



**Fig. 4.** Original tracing (A) and histogram (B) showing the effect of ACAE on the contractions of segments of rat jejunum induced by CCh  $10^{-6}$  M. The difference is statistically significant to the control at 0.3 and 1 mg/ml (\*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ; mean  $\pm$  S.E.M.  $n=6$ ). NS: Not Significant.

other muscarinic agonists (such as CCh) mediated mainly via M2 and M3 receptors (26). These last are coupled to G-proteins, but the signal transduction varies. M3 receptors are predominant in the Interstitial cells of Cajal and are the primary mediator of contraction, while the contribution of the M2 receptors that are predominant in intestinal smooth muscle cells is less clear. The latter couple to pertussis toxin-sensitive G<sub>i/o</sub> proteins and modulate contraction, at least in part by inhibiting cyclic AMP (cAMP)-dependent relaxation and by regulating smooth muscle ion channel activity. M3 receptors couple preferentially to G<sub>q/11</sub> proteins which induce stimulation of phospholipase C (PLC) hydrolysis of phosphoinositides resulting in the formation of inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) and diacylglycerol (DAG). In turn, InsP<sub>3</sub> releases Ca<sup>2+</sup> from the sarcoplasmic reticulum and DAG activates protein kinase C (PKC), leading to the phosphorylation of various proteins (27). Carbachol is a cholinergic agonist, which is a structural analog of acetylcholine and is not degraded by acetylcholinesterase. It causes an increase in tone by acting on M2 and M3 muscarinic receptors (28). ACAE inhibited the tone induced by CCh in a dose-dependent manner, and this inhibition was reduced in the presence of atropine, which is a specific inhibitor of muscarinic receptors. The extract could act on the pathways of these receptors. We suggest that the plant extract may contain components that have a cholinergic receptor blocking effect. This hypothesis was confirmed when the extract shifted the carbachol dose-response curve to the right and down, inhibiting the contractile response of intestinal smooth muscle at increasing doses of carbachol.



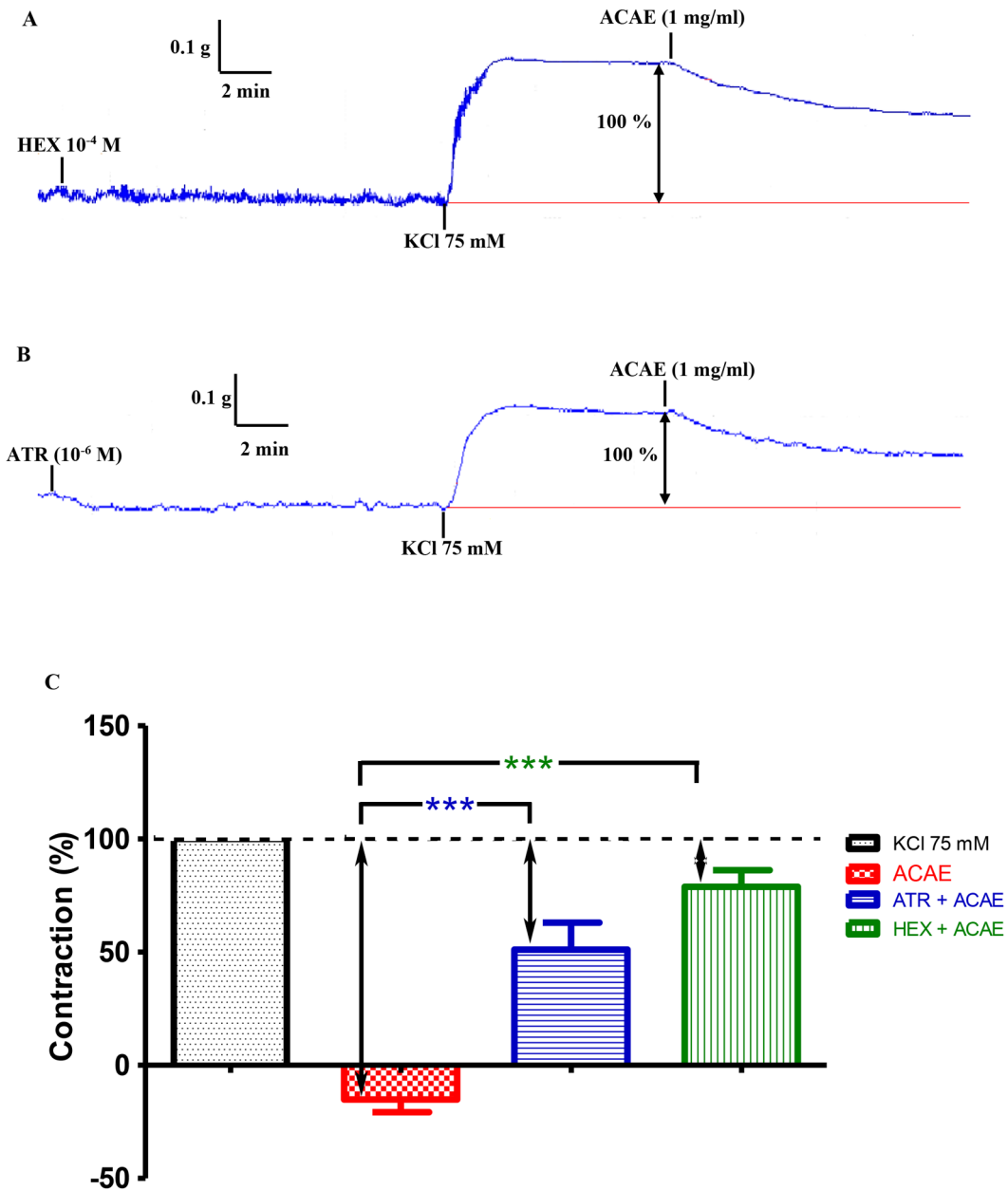


**Fig. 5.** Original tracings (A, A') and (B) the dose-response curves to CCh in the presence (A') and absence (A) of ACAE on segments of the rat jejunum. The difference is statistically significant to the control (in the absence of ACAE) at higher concentrations of CCh (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ; mean  $\pm$  S.E.M.  $n=6$ ).

Therefore, this inhibitory effect is similar to that of a non-competitive antagonist to the cholinergic receptors (19). The antagonist binds to the receptor at a site separate from the agonist-binding site (allosteric site) and results in conformational changes in the receptor with a decreased affinity of the receptor for its agonist.

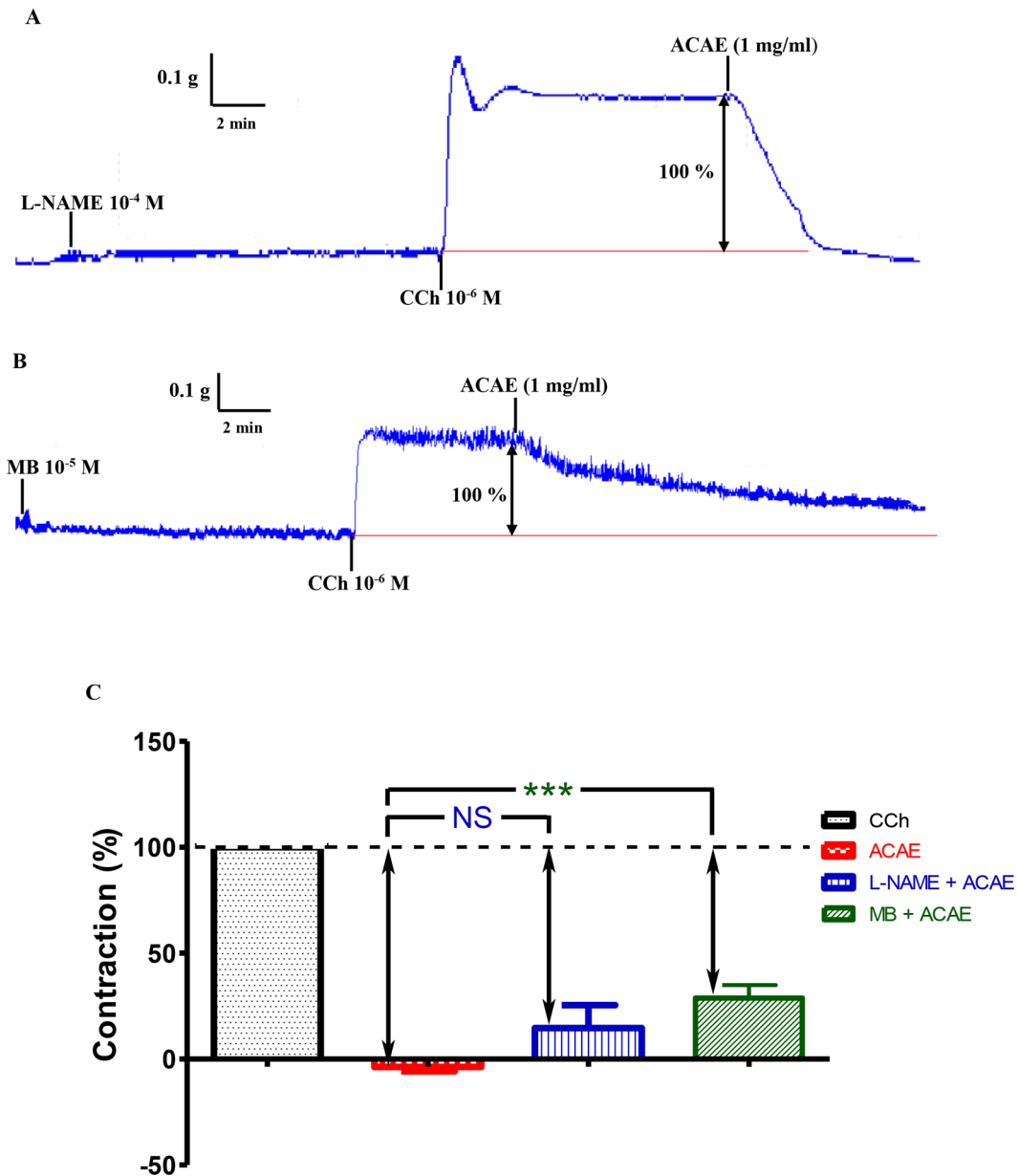
Extrinsic and enteric nervous systems (myenteric plexus and submucosal plexus) innervate the gastrointestinal tract. These systems contain nicotinic acetylcholine receptors (nAChRs) which are ligand-gated ion channels (29). To check the effect of our plant extract on these cholinergic pathways, we used hexamethonium, an antagonist of neuronal nicotinic receptors (30). Hexamethonium significantly altered the relaxing effect of ACAE by 78.88%, which indicates that the extract acts via nicotinic receptors. However, ACAE could act either directly on muscarinic and nicotinic receptors or by the release of acetylcholine from postganglionic neurons.

Contraction of smooth muscle cells depends on intracellular calcium concentration, which is increased following the opening of the voltage-dependent calcium channels. Indeed, an extracellular  $\text{Ca}^{2+}$  flux passes to the intracellular medium (31). This causes the release of intracellular  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum.



**Fig. 6.** Original tracings (A, B) and histogram (C) showing the effects of ACAE (1 mg/ml) on contractions of segments of the rat jejunum pre-incubated with hexamethonium (HEX)  $10^{-4}$  M (A) and atropine (ATR)  $10^{-6}$  M (B) for 20 min and then pre-contracted by 75 mM KCl. In C, ACAE-induced relaxation was significantly inhibited by ATR or HEX ( $***P < 0.001$ ; mean  $\pm$  S.E.M.  $n=6$ ).

These channels open following depolarization of the membrane caused, in particular, by the increase of the concentration of  $K^+$  in the extracellular medium that occurs at the origin of a tonic contraction. To evaluate the effect of the ACAE on calcium channels, we pre-contracted the segments of rat jejunum with a medium rich in potassium (75 mM). ACAE reduced this contraction in a dose-dependent manner with the maximum relaxation at a dose of 1 mg/ml. This means that the ACAE has an effective blocking effect on calcium channels (32). According to Godfraind et al. (33), any substance that prevents these contractions (induced by the KCl rich medium) is considered to be an inhibitor of voltage-gated calcium channels. This hypothesis was reinforced by the fact that the extract at 0.1, 0.3, and 1 mg/ml shifted the dose-response curve of  $CaCl_2$  to the right and down by inhibiting the response to increasing doses of calcium, therefore, this inhibitory effect is similar to that of



**Fig. 7.** Original tracings (A, B) and histogram (C) showing the effects of ACAE (1 mg/ml) on contractions of segments of the rat jejunum pre-incubated with L-NAME  $10^{-4}$  M (A), methylene blue (MB)  $10^{-5}$  M (B), for 20 min. Then pre-contracted by CCh  $10^{-6}$  M. In C, ACAE-induced relaxation was significantly inhibited by MB but not by L-NAME (NS: Not Significant,  $***P < 0.001$ ; mean  $\pm$  S.E.M.  $n=6$ ).

a non-competitive antagonist to the voltage-dependent calcium channels (19). These findings are reinforced when we used verapamil, which is an antagonist of L-type voltage calcium channel blocker, which had an effect comparable to that of 1 mg/ml of ACAE.

NO-GC is expressed in several cell types in the gastrointestinal tract, such as smooth muscle cells and Cajal interstitial cells (34). NO diffuses into smooth muscle cells and will bind to guanylate cyclase to increase the intracellular concentration of cyclic guanosine monophosphate (cGMP). The cGMP will in turn activate a protein kinase G-I (PKG-I). PKG-mediated phosphorylation may cause activation of  $K^+$  channels and hyperpolarization; inhibition of L-type  $Ca^{2+}$  channels and  $Ca^{2+}$  influx; increased  $Ca^{2+}$  efflux through activation of the

Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; Ca<sup>2+</sup> sequestration through SERCA activation; reduction of Ca<sup>2+</sup> mobilization through the inhibition of the sarcoplasmic reticulum IP<sub>3</sub> receptor or the phospholipase C-dependent formation of IP<sub>3</sub>; or activation of the MLC phosphatase. The latter may be achieved directly via phosphorylation of the smooth muscle cells phosphatase or indirectly via inhibition of the inactivating RhoA pathway, ultimately resulting in dephosphorylation of the constricting smooth muscle cells (35, 36). The effect of ACAE on rat jejunum segments pre-incubated with L-NAME (NOS inhibitor) (37) and contracted with CCh was weakly reduced (not significantly) compared to the control. When the intestine was pre-incubated with methylene blue (a GC inhibitor) (38), the effect of the extract was reduced significantly. We can suggest that our extract had no effect on the formation of NO, but could act on the guanylate cyclase pathway by decreasing the intracellular calcium level of smooth muscle cells by interfering with one of the pathways of the effect of PKG cited above.

*Artemisia campestris* L. contains flavonols and phenol acids, as well as chlorogenic acid, 3,4-dicaffeoylquinic acid (chlorogenic acid A), 3, 5-dicaffeoylquinic acid (chlorogenic acid B), 4,5-dicaffeoylquinic acid (chlorogenic acid C) (12, 16). These molecules are the phytochemical basis for the spasmolytic activity (39). Therefore, it may be that one or more of these molecules found in this plant could act alone or synergistically on one of the pathways we have tested.

*Artemisia campestris* L. extract has been shown to cause myorelaxant and antispasmodic activities mediated predominantly through nicotinic, rather than muscarinic receptors. The extract could also act as an L-type voltage calcium channel blocker. It had less effect on the guanylate cyclase pathway. These results explain the pharmacological basis behind the use of this plant in Moroccan traditional medicine as an antispasmodic intestinal agent.

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## Conflict of Interest

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The authors declare no conflict of interest.

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