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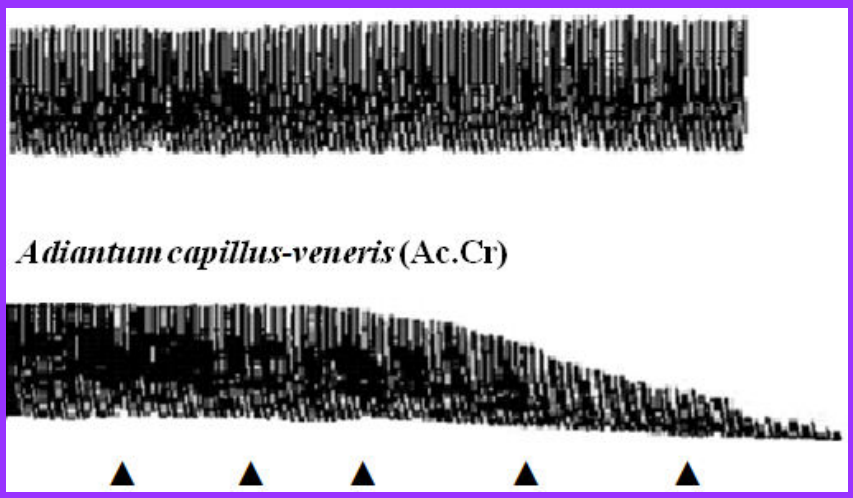

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Antidiarrheal and antispasmodic activities of *Adiantum capillus-veneris* are predominantly mediated through ATP-dependent K^+ channels activation

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Abstract

The study was tempted to explore the scientific basis for the medicinal use of *Adiantum capillus-veneris* L. in diarrhea using the *in vivo* and *in vitro* assays. The crude extract of dried leaves of *A. capillus-veneris* exhibited antidiarrheal effect against castor oil-induced diarrhea in mice at 300 and 500 mg/kg, similar to the effect loperamide. It was also found safe up to administered dose of 7 g/kg in mice. In isolated rabbit jejunum, extract of *A. capillus-veneris* showed a concentration-dependent relaxation of spontaneous and low K^+ (25 mM)-induced contractions and had weak inhibitory effect on high K^+ (80 mM), similar to the activity pattern of cromakalim, an ATP-dependent K^+ channel opener. Interestingly, its inhibitory effect on spontaneous contractions was potentiated in the presence of atropine. These data demonstrates that *A. capillus-veneris* possesses antidiarrheal and antispasmodic properties mediated possibly through ATP-dependent K^+ channels activation, thus providing scienti-fic basis to its folk use in abdominal colic and diarrhea.

Introduction

Adiantum capillus-veneris Linn. (Pteridaceae) is among the most commonly and widely distributed species. It is medicinally important in Ayurvedic system of medicine, being known as "Hansraj". The pteridophytes containing ferns and ferns allies have been used in folk medicine for more than 2000 years (Chopra et al., 1958). It is an ornamental plant growing to 0.3 m at slow rate. The plant grows in shades in moist but well drained neutral as well as alkaline soils and seeds ripen from May to September (Hakim et al., 1996). *A. capillus-veneris* is traditionally used to treat abdominal colic, diarrhea asthma, cough, diabetes and constipation (Duke et al., 2002). Its leaves have also been applied as anti-inflammatory, demulcent, expectorant, diuretics and tonic (Usmanghani et al., 1997).

There are multiple studies available in the literature demonstrating its various health benefits such as, antioxidant (Nilforoushzadeh et al., 2014), antibacterial and antifungal (Ishaq et al., 2014; Singh et al., 2008), antialopecia (Noubarani et al., 2014) antiurolithiasic (Ahmed et al., 2013), anti-inflammatory (Yuan et al., 2013), antinociceptive (Haider et al., 2013) and hypoglycemic (Ibraheim et al., 2011).

Phytochemical investigations revealed alkaloids, cardiac glycosides, steroids, reducing sugars, tannins (Ishaq et al., 2014), triterpenoids such as, 30-normethyl fernen-22-one (capillirone, 1), hopan-3β-ol (capillirol B, 2), 4-α-hydroxyfilican-3-one, 3-β,4-α-dihydroxyfilicane (Haider et al., 2013), isoadiantone, isoadiantol-B, 3-methoxy-4-hydroxyfilicane and 3,4-dihydroxyfilicane, flavonoids such as, quercetin, quercetin-3-O-glucoside

and quercetin-3-O-rutinoside (Ibraheim et al., 2011), oleanane compounds such as, olean-18-en-3-one and olean-12-en-3-one (Nakane et al., 2002), beta-sitosterol, stigmasterol and capesterol (Marino et al., 1989) as plant constituents.

A. capillus-veneris is a native plant that is used traditionally in the treatment of a number of ailments including diarrhea. To the best of our knowledge, there is no evidence available in the literature for its medicinal use in diarrhea, this imperative study has been designed to provide pharmacological basis to its medicinal use in hyperactive gut disorders using *in vivo* assays and isolated tissue experiments.

Materials and Methods

Preparation of the crude extract

The leaves of *A. capillus-veneris* were purchased from herbal store in Multan, rendered them free of adulterated material through manual picking and identified by an expert taxonomist (Prof. Mumtaz Hussain Bukhari), Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan. A specimen voucher # AC-L-06-09-120, was preserved in the herbarium of the Natural Product Research Division, Department of Biological and Biomedical Sciences, the Aga Khan University, Karachi. The plant leaves were ground into coarse powder through electrically driven device. The powdered material was soaked in 80% aqueous-methanol (v/v) for 8 days in amber colored glass bottles with gentle shaking on alternate days. The soaked materials was passed through muslin cloth to remove vegetative debris followed by pressing of marks. The obtained fluid was subjected to filtration through Whatman grade No. 1 filter paper and evaporated to get thick pastes using rotary evaporator under reduced pressure at 37°C followed by freeze drying of thick paste material into dry form. The practical yield of *A. capillus-veneris* extract was 1.9% and it was found soluble in DMSO (1%).

Drugs

Acetylcholine, atropine sulfate, potassium chloride and loperamide hydrochloride were purchased from Sigma Chemicals Co., USA. Cromakalim and glibenclamide were purchased from Tocris, Ellisville, MO and RBI Chemicals Co, USA respectively. All chemicals used were of the analytical grade available and solubilized in distill water/saline except cromakalim and glibenclamide, which were dissolved in DMSO (1%). The vehicle used for solubilization was found inert on isolated tissue preparations in control experiments. Stock solutions of all chemicals were made fresh in normal saline on the day of the experiment.

Animals

BALB/c mice (weighing 20–25 g, n=60) and locally bred rabbits (weighing 1–1.5 kg, n=8) of both sexes, were housed at the Animal House of Aga Khan University under controlled environmental conditions (23–25°C). The animals were fasted for 16-18 hours before the experiment, whereas they were given tap water and standard diet routinely. Experiments were performed with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996). This study (77/2007-Pharm/BZU) was the part of the M. Phil thesis of Mr. Waseem Hassan which was approved by the Board of Studies, Bahauddin Zakariya University, Multan.

In vivo assays

Antidiarrheal activity

Mice (20-25 g, n=20) of either sex were fasted for 16-18 hours before the experiment. The animals were housed in individual cages and divided in 4 groups, for each n=5. The first group received saline along with solubilizing vehicle (10 mL/kg, orally), acted as negative control. The second group received loperamide (10 mg/kg) orally, serving as positive control. The remaining two groups received 300 and 500 mg/kg doses of the crude extract of *A. capillus-veneris*, respectively. After 1 hour of the respec-tive treatments, each animal received 10 mL/kg castor oil orally through a feeding needle. After 5 hours, the cages were inspected for the presence of typical diarrheal droppings; the absence was regarded as a positive result, indicating protection from diarrhea (Janbaz et al., 2014).

Acute toxicity testing

A total of 40 BALB/c mice (20–25 g) of either sex were equally divided into four groups. The test was performed using increasing doses of the crude extract of *A. capillus-veneris* (1, 3 and 7 g/kg) given orally in 10 mL/kg control vehicle to different animals serving as the test groups. Another group of mice was administered control vehicle (10 mL/kg) orally as the negative control. The animals were allowed food and water *ad libitum* and kept under regular observation for 6 hours to observe their piloerection, changes in exploratory behavior and blindness, while lethality was monitored up to 24 hours.

In vitro experiments

Spasmolytic activity

The spasmolytic activity of the test material was studied by using isolated rabbit jejunum preparations (Mehmood and Gilani, 2010; Khan et al., 2013). Respective segments of 2 cm in length were suspended

individually in 10 mL tissue baths containing Tyrode's solution, aerated with a mixture of 95% oxygen and 5% carbon dioxide (carbogen) and maintained at 37°C using thermocirculator. The composition of the Tyrode's solution in mM was: KCl 2.68, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.55 (pH 7.4). Intestinal responses were recorded isotonically using Bioscience Transducers coupled with PowerLab data acquisition system (ADInstruments, Sydney, Australia). Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug and then stabilized with a sub-maximal concentration of acetylcholine (ACh, 0.3 µM) and the bath fluid was subsequently replaced with normal Tyrode's solution before starting the experiment.

The myorelaxant effect of the crude extract of *A. capillus-veneris* was assessed on spontaneously contracting isolated rabbit jejunum. For elucidation of the possible mechanism of spasmolytic effect, low K^+ (25 mM) and high K^+ (80 mM) were used to depolarize the isolated tissues which in turn produced sustained contractions, which are considered useful for determining the different inhibitory mechanisms like, K^+ channels activation (Gilani et al., 2008; Khan et al., 2011) or Ca^{++} channel blockade (Mehmood et al., 2011; Janbaz et al., 2014), respectively. The test material was then added in a cumulative fashion to obtain concentration-dependent inhibitory response curves. The relaxation of isolated tissue preparations was expressed as percent of the control response mediated by added low K^+ or high K^+-induced concentrations. To characterize the nature of K^+ channels involved, the spasmolytic effect of the test material was studied on low K^+-induced contractions in the absence and presence of glibenclamide (GB), an ATP-dependent K^+-channel blocker (Frank et al., 1994).

Statistical analysis

All the data expressed are mean ± standard error of mean (S.E.M, n = number of experiments) and the median effective concentrations (EC_{50} values) with 95% confidence intervals (CI). The concentration-response curves were analyzed by non-linear regression (Graph-PAD program, Graph-PAD, San Diego, CA, USA). The Chi-square test was applied to differentiate the results of antidiarrheal activity assay. Probability of less than 0.05 was considered significantly different.

Results

Oral administration of the crude extract of *A. capillus-veneris* exhibited dose-dependent inhibitory effect against castor oil-induced diarrhea in mice by producing 40 and 60% protection at respective doses of 300 and 500 mg/kg. The positive control of loperamide caused 100% protection at the dose of 10 mg/kg, while the group administered only saline and castor oil showed no protection against diarrhea as shown in Table I.

Table I

Antidiarrheal effect of *A. capillus-veneris* against castor oil-induced diarrhea in mice

Treatment	n	Diarrhea after 5 hours	% Protection
Saline (10 mL/kg) plus Castor oil (10 mL/kg)	5	5	0
Loperamide (10 mg/kg) plus castor oil (10 mL/kg)	5	0[b]	100
Extract (300 mg/kg) plus castor oil (10 mL/kg)	5	3[a]	40
Extract (500 mg/kg) plus castor oil (10 mL/kg)	5	2[a]	60

[a]$p<0.05$ and [b]$p<0.01$ versus Group No. 1 (Chi-square test)

The plant extract was well tolerated by the animals up to the tested oral dose of as high as 7 g/kg. No sign of acute toxicity like restlessness, seizures and piloerection was noticed over the period of observation (6 hours) and there was no death recorded up to 24 hours.

In order to investigate the presence of gut relaxant constitutes in plant extract, which might be mediating its antidiarrheal activity, isolated rabbit jejunum was selected for further studies.

The plant extract inhibited the spontaneous contractions in rabbit jejunum with EC_{50} value of 1.50 mg/mL (1.11-2.30, 95% CI, n=5) as shown in Figure 1A. When studied in the tissue pretreated with atropine, the observed inhibitory effect of the extract was potentiated with resultant EC_{50} value of 0.43 mg/mL (0.38-0.67, n=4) as shown in Figure 2.

When tested against induced contractions, the crude extract of *A. capillus-veneris* inhibited potently low K^+ (25 mM)-induced contractions with an EC_{50} value of 0.49 mg/mL (0.27-0.65, n=5) compared to its effect on high K^+ (80 mM)-induced contractions, where it produced only 37 ± 15.7% (n=4) relaxation as maximum at highest tested concentration of 5 mg/mL (Figure 1B and Figure 3A). Similarly, cromakalim showed strong inhibitory effect against low K^+-induced contractions with EC_{50} value of 0.29 mg/mL (0.25-0.34, n=4), while it had very weak inhibitory effect (20 ± 9.50%, n=6) on high K^+-induced contractions. When the inhibitory effect of the plant extract was reproduced on low K^+ in the presence of glibenclamide (GB, 10 µM), it was significantly reduced with resultant EC_{50} value of 1.45 mg/mL (1.25-2.30, n=5) vs. 0.49 mg/mL (0.27-0.65, n=5), similar to the effect of cromakalim when reproduced in the presence of glibenclamide as shown in Figure 3B.

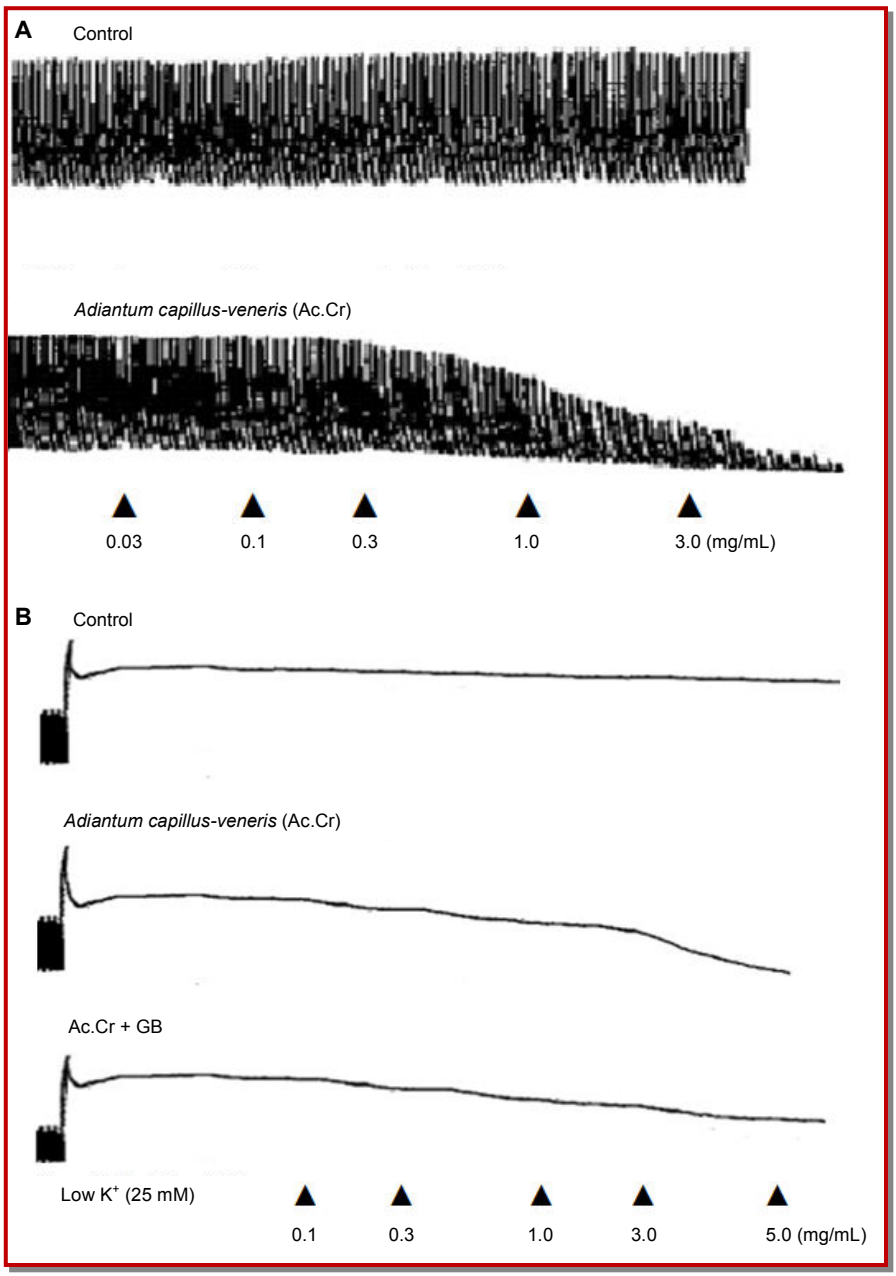

Figure 1: Tracing showing the concentration-dependent inhibitory effect of the crude extract of *A. capillus-veneris* (Ac.Cr) on (A) spontaneous and (B) low K^+ (25 mM)-induced contractions without and with glibenclamide (GB, 10 µM) in isolated rabbit jejunum preparations

Discussion

Keeping in view the medicinal use of *A. capillus-veneris* in hyperactive gut disorders, like diarrhea and abdominal colic (Duke et al., 2002), this study was planned to evaluate antidiarrheal and antispasmodic activities of the plant extract using an animal model and isolated tissue experiments. Antidiarrheal and acute toxicity studies were conducted in mice, while to determine the insight into mechanism(s), isolated rabbit jejunum preparations were employed.

In castor oil-stimulated diarrheal mice, the plant extract showed significant protection at tested doses of 300 and 500 mg/kg, similar to the effects of loperamide, a standard antidiarrheal agent (Reynolds et al., 1984). Castor oil is known to cause increased intestinal fluid contents and promotes diarrhea indirectly through the effect of its active constituent, ricinoleic acid formed by the hydrolysis of oil (Iwao and Terada, 1962), which changes the electrolytes and water transport (Gaginella and Phillips, 1975) and generates massive contractions in transverse and distal colon (Croci et al., 1997). A

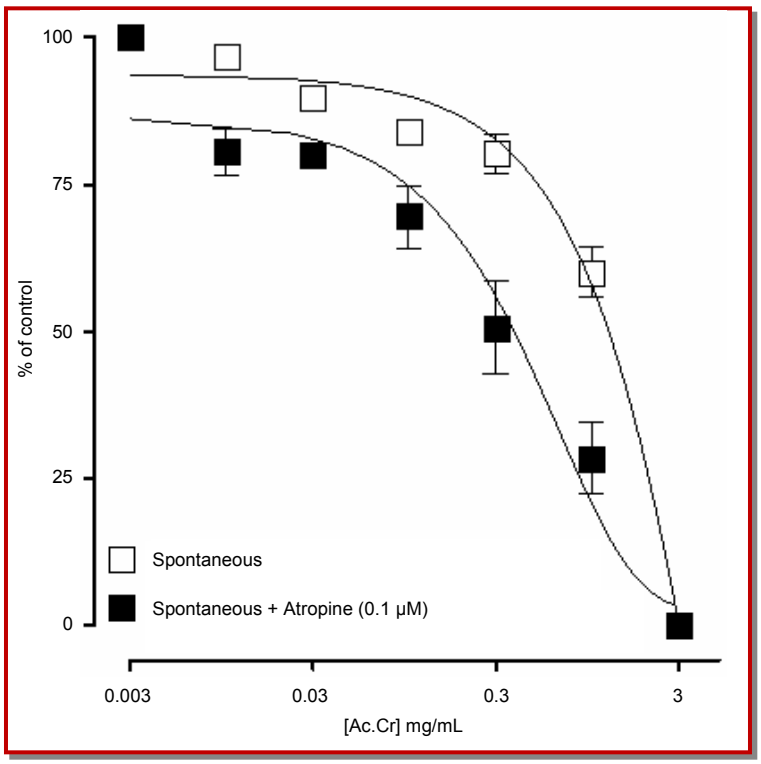

Figure 2: The concentration-dependent inhibitory effect of the crude extract of *A. capillus-veneris* (Ac.Cr) in the absence and presence of atropine (0.1 µM) on spontaneous contractions in isolated rabbit jejunum preparations. Values are expressed as mean ± S.E.M; n = 4-5

substance which inhibits castor oil-induced diarrhea is considered to possess strong antidiarrheal activity. There were no sign of acute toxicity or death when the plant extract was administered to mice up to the highest tested dose of 7 g/kg.

Physiological mediators such as, acetylcholine, histamine, substance P, cholecystokinins, prostaglandins and 5-hydroxytryptamine (Pasricha, 2006) and some ion channels like, K^+ or Ca^{++} have their established role in regulatory function of gastrointestinal system (Pasricha, 2006).

To assess the presence of gut inhibitory component(s) in *A. capillus-veneris*, when it was tested on rabbit jejunum, it showed the presence of antispasmodic activity along with ACh-like gut stimulant effect, which was evident by its potentiated inhibitory effect when reproduced in the presence of atropine, a cholinergic antagonist (Brown and Taylor, 2006). The observed additional presence of gut stimulant component(s) might be a possible explanation for the documented medicinal use of *A. capillus-veneris* in constipation (Duke et al., 2002), however, further detailed studies are required justify its use in hypoactive gut disorders.

It has been observed that most of the medicinal plants or plant based remedies exhibit inhibitory effect on spontaneously contracting rabbit jejunum through K^+ channel activation (Gilani et al., 2008; Khan et al., 2011) and/or Ca^{++} channel blockade like mechanisms (Shah et al., 2011; Khan et al., 2013; Janbaz et al., 2014). To study the involvement of these inhibitory pathways, low K^+ (25 mM) and high K^+ (80 mM) are used to depolarize tissues which distinguish K^+ channel opening (KCO) and Ca^{++} antagonist like mechanisms (Gilani et al., 2008; Janbaz et al., 2014). K^+ channel openers (increase in K^+ efflux) and Ca^{++} antagonists (inhibition of Ca^{++} entry) are known to cause intestinal smooth muscle relaxation by decreasing intracellular free Ca^{++}, through respective mechanisms of membrane hyperpolarization, hence such agents are attested for their therapeutic potential in hyperactive gut disorders (Poggioli et al., 1995; Lee et al., 1997).

To test whether the inhibitory effect of *A. capillus-veneris* extract was also mediated via similar mechanisms, it was tested on low K^+ and high K^+-induced sustained contractions in rabbit jejunum. The plant extract caused complete relaxation of low K^+, while partial inhibition of high K^+-induced contractions, similar to the effect of cromakalim, a known ATP-dependent K^+-channel opener (Poggioli et al., 1995). These data indicate that the KCO-like spasmolytic constituent(s) in *A. capillus-veneris* are likely to be responsible for its observed antidiarrheal activity in mice. To characterize the nature of K^+ channels involved in antispasmodic effect of the

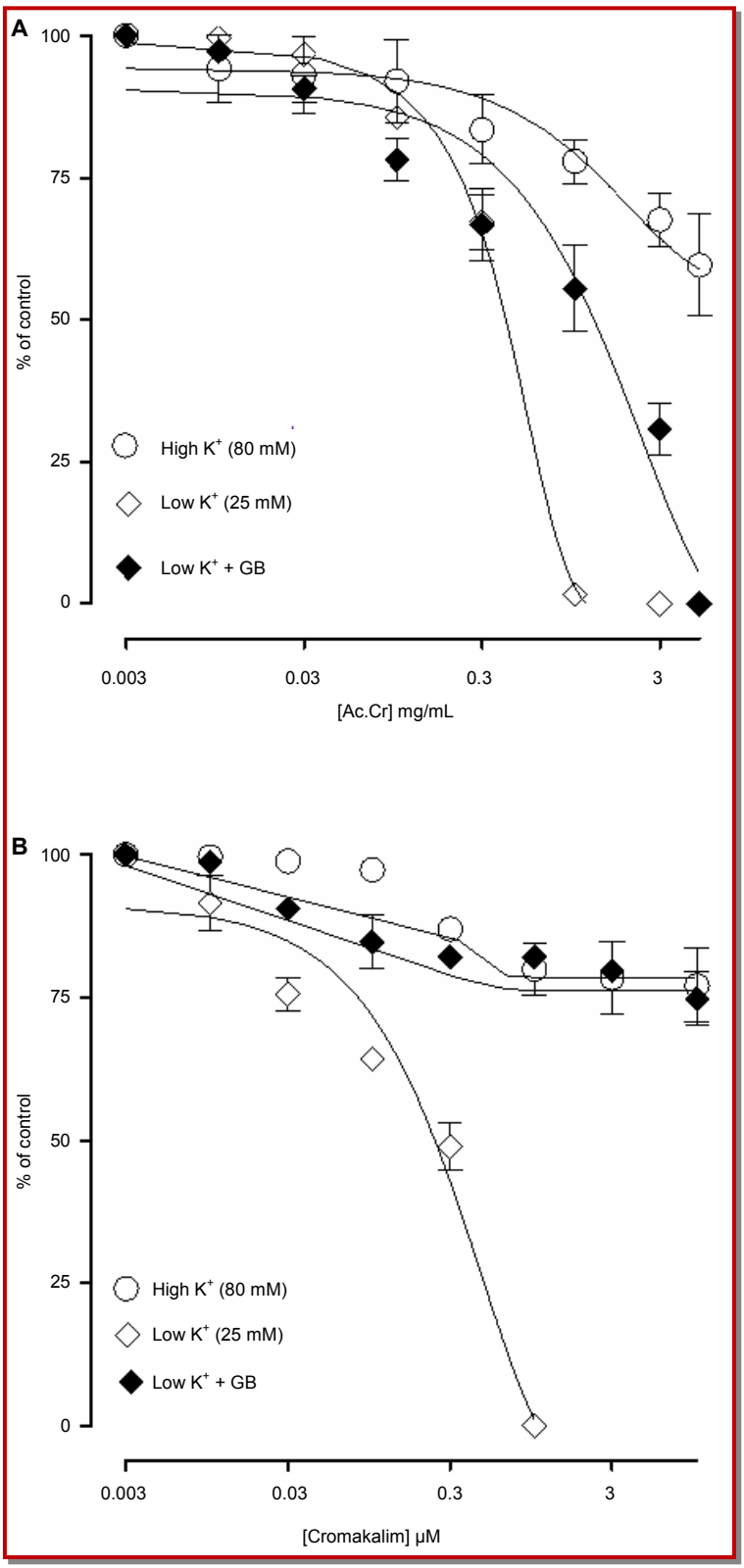

Figure 3. The concentration-dependent inhibitory effect of (A) the crude extract of *A capillus-veneris* (Ac.Cr) and (B) cromakalim against low K^+ (25 mM) in the absence and presence of glibenclamide (GB, 10 µM) and against high K^+ (80 mM)-induced contractions in isolated rabbit jejunum preparations. Values are expressed as mean ± S.E.M; n = 4-6

crude extract of *A. capillus-veneris*, the inhibitory CRCs of the plant extract against low K^+ were constructed in the absence and presence of GB. Interestingly, the inhibitory effect of the extract was significantly inhibited in the presence of GB, while the inhibitory effect of cromakalim was almost blocked in the presence of GB. This indicates that the observed spasmolytic activity of the plant extract mainly involves ATP-sensitive K^+ channels, however, the involvement of other inhibitory pathways cannot be ruled out without further detailed studies. Multiple types of K^+ channels are abundantly present in intestinal smooth muscles and are also known for their inhibitory influence in hypermotile gut (Vogalis, 2000). This study highlights the potential of this popular medicinal herb for the development of newer therapeutic options to treat hyperactive gut disorders such as, gut spasms and diarrhea.

In conclusion, these data suggests that *A. capillus-veneris* possesses antidiarrheal and antispasmodic activities mediated predominantly through ATP-dependent K^+ channels activation like pathway, thus, providing an evidence to its medicinal use in abdominal colic and diarrhea.

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