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Studies on Prokinetic, Laxative, Antidiarrheal and Gut Modulatory Activities of the Aqueous-methanol Extract of *Celtis africana* and Underlying Mechanisms

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Abstract: This study describes the prokinetic, laxative and antidiarrheal activities of the aqueous-ethanol extract of *Celtis africana* (Ca.Cr) in mice, together with gut stimulatory and inhibitory activities using isolated gut preparations in an attempt to explore possible mechanisms of action. Ca.Cr, showed atropine-sensitive prokinetic and laxative activities in mice at low doses (30 and 100 mg kg^{-1}), followed by antidiarrheal effect at next higher doses (300 and 1000 mg kg^{-1}). In spontaneously contracting rabbit jejunum, Ca.Cr, showed a dose-dependent (0.03-3 mg mL^{-1}) spasmogenic effect followed by spasmolytic effect at higher concentrations (5-10 mg mL^{-1}). Activity-directed fractionation revealed that the atropine-sensitive spasmogenic component was concentrated in the aqueous fraction, while the spasmolytic component was separated in the organic fraction. When studied against the high K^+ (80 mM)-induced contractions, both Ca.Cr and Ca.Cl caused dose-dependent (0.01-5.0 mg mL^{-1}) inhibition, later being more potent, while both shifted the Ca^{++} concentration response curves to the right, similar to verapamil. These data showed that the crude extract of *C. africana* possesses prokinetic, laxative and spasmodic activities mediated through muscarinic receptor activation concentrated in the aqueous fraction while, antidiarrheal and spasmolytic activities via Ca^{++} antagonist activity, separated in the chloroform fraction.

Key words: *Celtis africana*, prokinetic, laxative, antidiarrheal, spasmogenic, spasmolytic, cholinergic, Ca^{++} antagonist

INTRODUCTION

The *Celtis Africana* Burm.f. (family Ulmaceae) is one of 70 species of genus Celtis, which is considered a medicinal plant and is used to treat indigestion, eye infection and edema Koduru *et al.* (2007). In South Africa, its bark is used as a remedy for cancer, fever and headaches (Krief *et al.*, 2005).

The plant has been shown to possess antifungal activity specifically against *Cryptococcus* neoforms (Mokoka *et al.*, 2010). A recent study showed that it contains *C*-glycosyl flavonoids with antioxidant and urease inhibitory activities (Perveen *et al.*, 2011). More recently, different bioactive amides have been isolated with antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities (Al-Taweel *et al.*, 2012).

However, there is no report in the literature showing its effectiveness in gastrointestinal disorders. In this investigation, we showed first time that the crude extract of *C. africana* possesses combination of prokinetic, laxative, antidiarrheal, spasmodic and spasmolytic activities with possible mode of action explored. Activity-directed fractionation revealed that the gut stimulatory effect (cholinergic) is concentrated in the aqueous fraction, while the constituent(s) with inhibitory effect separated in the chloroform fraction.

MATERIALS AND METHODS

Plant material: The aerial parts of *C. africana* (2.5 kg) was collected from Riyadh (Saudi Arabia) and air-dried. The identity of the plant was verified by Dr. M. Atiqur Rahman, Plant Taxonomist, College of Pharmacy, King

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Saud University, Riyadh. A voucher specimen (No. 44) was deposited in the herbarium of Department of Pharmacognosy, King Saud University.

Preparation of the crude extract: The aerial part of *C. africana* (2.5 kg) was shade-dried, ground and extracted at room temperature with EtOH:H_2O (8:2, thrice). A part of ethanol extract (100 g) was divided into *n*-hexane (30 g), $CHCl_3$ (20 g), *n*-BuOH (30 g) and water (20 g) soluble sub-fractions.

Drugs: The following reference chemicals were obtained from the sources specified: acetylcholine chloride, loperamide hydrochloride, verapamil hydrochloride, potassium chloride (Sigma Chemical Company, St. Louis, MO, U.S.A.) and castor oil (Karachi Chemical Industries, Karachi, Pakistan). Chemicals used for making physiological salt solutions including potassium chloride, calcium chloride, glucose, magnesium chloride, magnesium sulfate, potassium dihydrogen phosphate, sodium bicarbonate, sodium dihydrogen phosphate and sodium chloride were obtained from Merck (Darmstadt, Germany). All chemicals used were of the highest purity grade. Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh in normal saline on the day of experiment.

Animals: BALB/c mice (weighing 20-25 g), guinea-pigs (weighing 400-600 g) and local breed rabbits (weighing 1-1.5 kg) of either sex, were housed at the animal house of the Aga Khan University under a controlled environment (23-25°C). The animals were kept in plastic cages (47×34×18) with sawdust (changed at every 48 h) and were fasted for 24 h before starting the experiment. In routine, they were given tap water *ad libitum* and a standard diet consisting of (g kg^{-1}): flour 380, fiber 380, molasses 12, NaCl 5.8, nutrivet L 2.5, potassium metabisulfate 1.2, vegetable oil 38, fish meal 170 and powdered milk 150. The experiments were performed with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, NRC (1996).

***In vivo* experiments:** Charcoal meal GI transit test. The method of Mascolo *et al.* (1994) was used with slight modifications as described earlier (Najeeb-ur-Rehman *et al.*, 2012). Mice fasted for 18 h were divided into different groups (n = 6). Two of the groups were treated orally with increasing doses of Ca.Cr 30 and 100 (mg kg^{-1}), acting as the test groups. One group, serving as normal control, was given saline (10 mL kg^{-1}). The next group was administered CCh (1 mg kg^{-1}) as the positive control. After 15 min, the animals were given 0.3 mL of charcoal meal of distilled water suspension containing 10% gum acacia, 10% vegetable charcoal and 20% starch. The animals were sacrificed after 30 min and the abdomen was opened to excise the whole small intestine. The length of the small intestine and the distance between the pylorus region and the front of the charcoal meal was measured to obtain the charcoal transport ratio or percentage. In order to assess the involvement of acetylcholine (ACh)-like prokinetic effect of the extract and CCh, further groups of mice were pretreated with atropine (10 mg kg^{-1} i.p.) 15 min prior the administration of the extract or CCh.

Laxative activity test: Mice fasted for 6 h before the experiment were placed individually in cages lined with clean filter paper. The animals were divided into seven groups (n = 6); the first group acting as the negative control and administered saline (10 mL kg^{-1}, p.o.), while the next group received CCh (1 mg kg^{-1}, i.p.), which served as the positive control. The third and fourth groups received orally, 30 and 100 mg kg^{-1} of Ca.Cr, respectively. To determine the mechanism underlying its laxative effect, separate sets of mice (group # 5, 6 and 7) were pretreated with atropine (10 mg kg^{-1}, i.p.) one hour before administration of the extract or CCh. After 18 h, the feces production (total number of feces and total number of wet feces per group) in all animals was counted and the percentage increase in wet feces relative to that of total fecal output was recorded, which was considered as the laxative effect (Najeeb-ur-Rehman *et al.*, 2012).

Antidiarrheal activity: The antidiarrhoeal activity was studied in mice as described previously (Shah *et al.*, 2011). Mice (20-25 g) of either sex were fasted for 24 h before the experiment. The animals were housed in individual cages and divided in 7 equal groups, for each n = 5. The first group received saline along with normal saline (10 mL kg^{-1}, p.o.), acted negative control. The second and third groups received Ca.Cr, 300 and 1000 mg kg^{-1} respectively. Fourth group received loperamide (10 mg kg^{-1}), as positive control. Afterwards, the cages were inspected for the presence and absence of typical diarrheal droppings; the absence was noted as a positive result, indicating protection from diarrhea.

***In vitro* experiments:** The spasmolytic/spasmogenic activities were studied on isolated rabbit jejunum and guinea-pig ileum preparations as described previously (Najeeb-ur-Rehman *et al.*, 2012). Approximately 2 cm long segments of jejunum or ileum were suspended in tissue baths containing Tyrode's solution maintained at 37°C and aerated with carbogen (95% O_2 and 5% CO_2).

Intestinal responses were recorded isotonically using Bioscience transducers attached to Powerlab Data Acquisition System (AD Instruments, Sydney, Australia) linked to a computer installed with Labchart software (version 6). The tissues were allowed to equilibrate for 30 min prior to addition of any chemical substance. The tissues were stabilized following repeated exposure to 0.3 μM acetylcholine (3-5 times) after washing with the Tyrode's solution until the sub-maximal responses of uniform amplitude were obtained. The observed modulation of spontaneous rhythmic contractions was used to test spasmolytic or spasmogenic activity in isolated rabbit jejunum preparation, whereas, induction of contraction with test or control drugs above that of the basal tone was used to measure spasmogenic activity in guinea-pig ileum.

Statistical analysis: The data expressed are Mean±standard error of mean (SEM, n = number of experiments) and the median effective concentrations (EC_{50} values) with 95% Confidence Intervals (CI). One way Analysis of Variance (ANOVA) followed by Dunnett's test or unpaired t-test was used to assess the laxative activity, while one-way ANOVA followed by Tukey's test was employed for the effect of plant extract in charcoal meal transit. The Concentration-response Curves (CRCs) were analyzed by non-linear regression. All the graphs, calculations and statistical analysis were performed using GraphPad Prism 4 for windows (GraphPad Software, San Diego, California, USA).

RESULTS

In vivo findings

Effect of Ca.Cr on charcoal meal: The crude extract of *C. africana* dose-dependently propelled charcoal meal through the small intestine of mice (Fig. 1). The distance travelled by the saline treated group was 62.0±3.4% of total length of small intestine, while the positive control group receiving CCh (1 mg kg^{-1}) significantly enhanced the movement ($p<0.01$ versus saline) of charcoal meal to 93.5±6.48%. The plant extract at the dose of 30 and 100 mg kg^{-1}, moved charcoal meal to the level of 85.9±4.72% ($p<0.05$) and 92.9±4.63% ($p<0.01$), respectively, when compared with the saline treated group. In mice pretreated with atropine, all the excitatory effects were markedly inhibited as evident in Fig. 1.

Laxative activity: The oral administration of Ca.Cr produced 67±2.86 and 74±3.56% (Mean±SEM, n = 6) wet feces in mice at 30 and 100 mg kg^{-1}, respectively. The positive control receiving CCh (1 mg kg^{-1}), produced

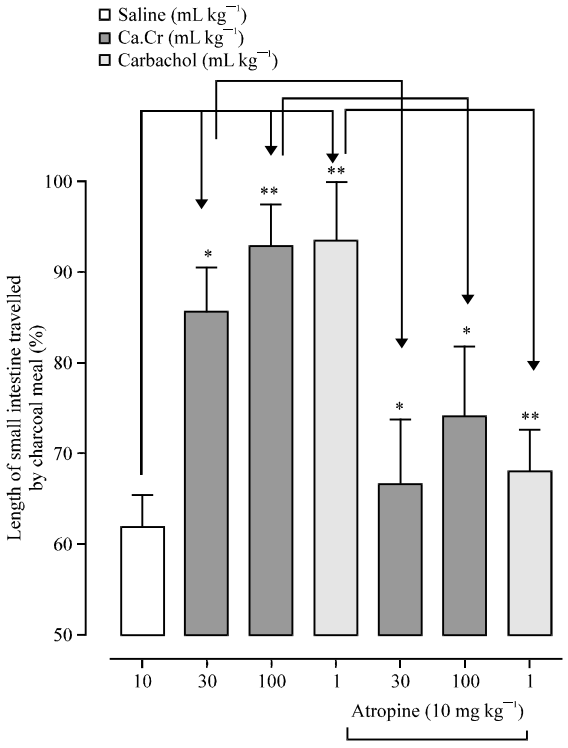

Fig. 1: Bar diagram showing the dose-dependent effect of *C. africana* (Ca.Cr) on the travel of charcoal meal through small intestine of mice, in the absence and presence of atropine. *p<0.05, **p<0.01, One-way ANOVA followed by Tukey's test (n = 6)

80±3.5% wet feces, while the saline treated group did not form any wet feces. When animals were pretreated with atropine (10 mg kg^{-1}), the laxative effect of Ca.Cr at 30 and 100 mg kg^{-1} declined to 25±2.02% and 13.5±4.8%, respectively; further details are shown in Table 1.

Effect on Castor oil-induced diarrhea in mice: In our experimental settings, Ca.Cr showed a dose-dependent antidiarrheal effect in terms of % protection against castor oil-induced diarrhea in mice. All animals in castor oil-treated group showed diarrhea, while animal pretreated with Ca.Cr before castor oil administration showed 20 and 60% protection from diarrhea at respective doses of 300 and 1000 mg kg^{-1} vs. castor oil untreated group. Loperamide (10 mg kg^{-1}) pre-treated group exhibited complete protection. Further details are given in Table 2.

In vitro findings:

Effects on rabbit jejunum and guinea-pig ileum: When tested in spontaneously contracting rabbit jejunum, Ca.Cr exhibited a concentration-dependent (0.1-3 mg mL^{-1}) mild stimulatory effect followed by inhibitory effect at the next

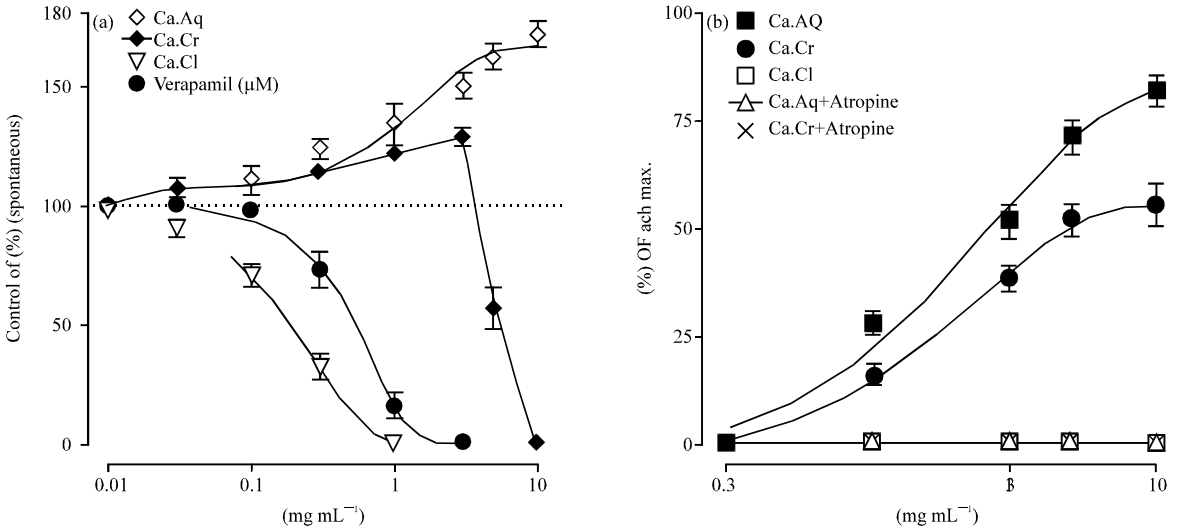

Fig. 2(a-b): Concentration-response curves showing effect of (a) Ca.Cr, Ca.Aq, Ca.Cl and verapamil on spontaneously contracting rabbit jejunum preparations and (b) Ca.Aq and Ca.Cr alone and in presence of atropine on guinea-pig ileum (B). The values shown are mean SEM. from 4 to 5 determinations

Table 1: Effect of atropine on the laxative activity of the crude extract of *C. africana* (Ca.Cr) in mice

Group No.	Treatment	Dose (mg kg^{-1})	Mean defecation/group	Mean number of wet feces/group	Mean% of wet feces
1	Saline (p.o., mL kg^{-1})	10	3.1±0.6	0	0
2	Carbachol (p.o.)	1	11.6±1.05***	9.5±1.08***	80.0±3.50
3	Ca.Cr (p.o.)	30	9.1±0.79**	6.16±0.54*	67.0±2.86
4		100	12.5±0.99**	9.33±0.84**	74.0±3.56
5	Carbachol (p.o.)+Atropine (i.p.)	1+10	3.83±0.3***	0.33±0.21***	8.83±5.83
6	Ca.Cr (p.o.)+Atropine (i.p.)	30+10	5.3±0.88**	1.5±0.22***	25.0±2.02
7		100+10	5.5±0.71**	0.83±0.3***	13.5±4.80

Values are Mean±SEM, n = 6, *p<0.05, **p<0.01 and ***p<0.001 show a comparison of group No. 2, 3 and 4 vs. group No. 1 (One-way ANOVA followed by Dunnett's test), group No. 5 vs. group No. 2, group No. 6 vs. group No. 3 and group No. 7 vs. group No. 4 (unpaired t-test)

Table 2: Antidiarrheal activity of *C. africana* crude extract (Ca.Cr) in mice, on castor oil (10 mL kg^{-1})-induced diarrhea

Treatment (p.o.), dose (mg kg^{-1})	No. of mice out of five with diarrhea	Protection (%)
Saline (10 mL kg^{-1})+castor oil	5/5	0
Ca.Cr+Castor oil		
300+10	4/5	20
1000+10	2*/5	60
Loperamide+castor oil	0**/5	100

*p<0.05 and **p<0.01 vs. Saline+Castor oil treated group (χ^2-test)

higher concentration of 5 and 10 mg mL^{-1}. Similar to parent crude extract, the aqueous fraction (Ca.Aq), also showed dose-dependent (0.1-10 mg mL^{-1}) spasmogenic effect, with higher potency than that of the crude extract, whereas, the chloroform fraction (Ca.Cl) was devoid of any stimulant effect; instead, it showed only inhibitory effect on the spontaneously contractions of jejunum with EC_{50} value of 0.19 mg mL^{-1} (0.15-0.23 CI). Verapamil also inhibited dose-dependently (0.03-3 μM) the spontaneous contractions of jejunum with EC_{50} value of 0.47 μM (0.41-0.54 CI), as shown in Fig. 2a.

In order to further investigate the spasmogenic action of test materials, isolated guinea-pig ileum, a quiescent preparation considered useful for spasmodic activity was used. Both the crude extract and its aqueous fraction exhibited atropine-sensitive spasmogenic effects, reaching their maximum 51.5±5 and 81.5±3.4%, respectively compared to the ACh maximum, whereas, the chloroform fraction was found devoid of stimulant effect (Fig. 2b).

The rabbit jejunum was used to study Ca^{++} antagonist activity initially constructing inhibitory dose-response curves against high K^+-induced contractions and then constructing concentration-response curves (CRCs) of Ca^{++} in the absence and presence of plant material (Shah *et al*., 2011). When tested against high K^+ (80 mM)-induced contractions, the crude extract and its chloroform fraction inhibited dose-dependently the high K^+-induced contractions with respective EC_{50} values of 2.47 (1.98-3.07 CI) and 0.77 (0.61-0.97 CI) mg mL^{-1}, whereas, the aqueous fraction did not show any inhibitory effect on high K^+-induced contraction (Fig. 3a). Verapamil, also inhibited high K^+-induced contraction as expected with EC_{50} value of 0.17 (0.15-0.19) as shown in Fig. 3a.

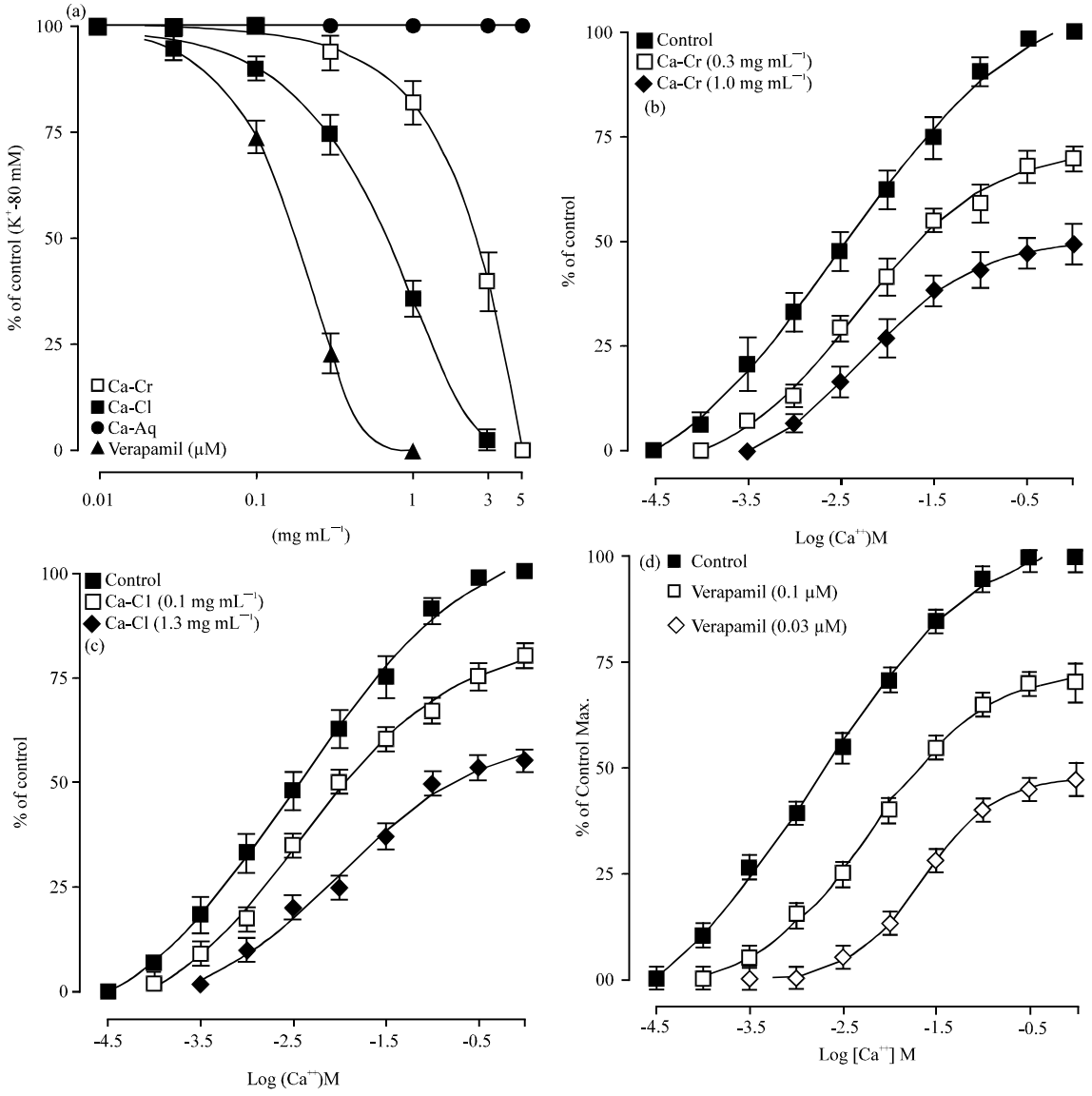

Fig. 3(a-d): (a) Shows dose-dependent inhibitory effects of the crude extract of the *C. africana* (Ca.Cr) and its chloroform (Ca.Cl) and aqueous (Ca.Aq) fractions and verapamil against high K^+-induced contraction in isolated rabbit jejunum preparations, while and (b-d) Show Ca^{++} concentration response curves (CRCs) of Ca.Cr, Ca.Cl and verapamil, respectively. Values shown are Mean±SEM from 4 to 5 determinations

Pretreatment of tissues with crude extract (0.3 and 1 mg mL^{-1}) and chloroform fraction (0.1 and 0.3 mg mL^{-1}), caused a concentration-dependent rightward shift in the Ca^{++} CRCs with suppression of the maximum response, like that caused by verapamil (Fig. 3a-d).

DISCUSSION

The crude extract of *Celtis africana* was tested in mice, where it propelled charcoal meal through the small intestine and increased the production of wet feces at lower doses (30 and 100 mg kg^{-1}), hence, showing prokinetic and laxative activities, similar to the effect of carbachol, a standard cholinergic agonist and accelerator of intestinal contents (Brown and Taylor, 2006). These gut stimulatory action of the extract was found sensitive to atropine, a muscarinic receptor blocker (Gilani *et al*., 1997), indicating the presence of some ACh-like component(s) in gut stimulant action. ACh is a neurotransmitter of the parasympathetic nervous system and is known to cause gut stimulation through the activation of M_3 muscarinic receptors subtype (Brown and Taylor, 2006); hence, the presence of ACh-like constituents explains its medicinal use as digestive aid.

At higher doses (300 and 1000 mg mL^{-1}), the plant extract caused antidiarrheal effect. The co-existence of laxative and antidiarrheal constituents is common in herbal remedies, such as *Lavandula stoehas* (Jabeen *et al*., 2007), psyllium husk, *Phyllanthus emblica* (Mehmood *et al*., 2011; Mehmood *et al*., 2012), Ginger (Ghayur and Gilani, 2005), *Lepidium sativum* (Najeeb-ur-Rehman *et al*., 2011, 2012). It appears as the co-existence of antispasmodic and antidiarrheal constituent is probably meant by nature not to allow the gut stimulant effect to go beyond a certain limit, beyond which it could have been harmful, causing abdominal cramp, as in the case with chemical drugs used in constipation (Gilani *et al*., 2005a), which is in line with general perception that natural products possess "side-effect neutralizing" combinations (Gilani and Atta-ur-Rahman, 2005).

To study the possible mode of the observed prokinetic, laxative and antidiarrheal properties of the extract, we further used isolated rabbit jejunum and guinea-pig ileum preparations. In the spontaneously contracting rabbit jejunum preparation, the crude extract of *C. africana* showed spasmogenic effect followed by spasmolytic activity at higher doses; the aqueous fraction showed only spasmogenic effect, while the organic fraction showed only the spasmolytic action, suggesting that the spasmogenic and spasmolytic activities of the crude extract have been distributed in the aqueous and organic fractions, respectively.

To further study the possible mechanism of the spasmogenic activities observed in the crude extract and its aqueous fraction, we used guinea-pig ileum, a quiescent preparation considered useful for this purpose (Ghayur and Gilani, 2005), where both, the crude extract and its aqueous fraction, produced excitatory effect, like that of ACh. The aqueous fraction showed significantly higher efficacy for gut stimulatory effect than the crude extract, which may be partly due to the presence of the spasmolytic constituent(s) in the crude extract. Like in the *in vivo* studies, the spasmogenic effect in both gut preparations (rabbit jejunum and guinea-pig ileum), was atropine sensitive indicating that the gut stimulation is mediated via activation of muscarinic receptors.

As the plant extract and its organic fractions also showed inhibitory effects on the spontaneously contracting rabbit jejunum, we further extended the study to know the mode of action for its inhibitory effect. In our earlier studies, we observed that the inhibitory effect of the medicinal plants is usually mediated through blockade of Ca^{++} channels (Syed Taqvi *et al*., 2006; Gilani *et al*., 2000, 2005b). To investigate whether the spasmolytic effect of the plant and its subsequent fractions is also mediated via a similar mechanism(s), they were tested on high K^+-induced contractions. High K^+ (>30 mM) is known to cause smooth muscle contractions through opening of voltage-dependent L-type Ca^{++} channels, thus allowing influx of extracellular Ca^{++} causing a contractile effect (Bolton, 1979) and the substance causing inhibition of high K^+-induced contraction is considered an inhibitor of Ca^{++} influx (Godfraind *et al*., 1986). Both the parent crude extract and chloroform fraction inhibited the high K^+-induced contractions, like that caused by verapamil, a standard Ca^{++} antagonist (Fleckenstin, 1977), indicating CCB-like actions, however, the aqueous fraction was found devoid of any inhibitory effect. The Ca^{++} antagonist effect was further confirmed when Ca.Cr and Ca.Cl shifted the Ca^{++} CRCs to the right, like that caused by verapamil. Ca^{++} antagonists have been shown to be beneficial in gut disorders resulting from hyperactivity such as abdominal cramps and diarrhea (Pasricha, 2006). Activity-guided fractionation revealed that the spasmolytic component (s) of the crude extract of *C. africana* is distributed in the organic fraction, while the spasmogenic component is separated in the aqueous fraction as the aqueous fraction showed higher efficacy for the stimulant effects compared to the parent extract.

CONCLUSION

This study shows that the crude extract of *C. africana* possesses combination of prokinetic, laxative and antidiarrheal activities in mice. The *in vitro* studies showed similar patron of activity, atropine sensitive gut stimulant effect at lower doses followed by gut relaxant action via Ca^{++} antagonist action at high doses, which is perhaps meant by nature to offset the excessive gut stimulant effects usually seen with high doses of laxative drugs. Activity-directed fractionation revealed that the gut stimulant effect is concentrated in the aqueous fraction, while the constituents with inhibitory effect separated in the organic fraction.

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