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Ethnopharmacological basis for antispasmodic, antidiarrheal and antiemetic activities of *Ceratonia siliqua* pods

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Abstract

The study was conducted to provide the ethnopharmacological bases of the crude extract of seed pods of *Ceratonia siliqua* in the gastrointestinal spasm, diarrhea and emesis. In segregated rabbit jejunum, it showed dose-dependent (0.01-10 mg/mL) relaxation of spontaneous as well as carbachol (1 µM)-induced contraction. Pre-treatment of segregated rat ileum with *C. siliqua*, significantly ($p<0.0001$) suppressed the carbachol (1 µM)-induced contraction similar to atropine (1 µM). These results indicated that *C. siliqua* possesses spasmolytic activity through possible blockage of muscarinic receptor in jejunum preparations. Furthermore, the crude extract inhibited the castor oil-induced diarrhea, charcoal meal propulsion in mice and copper sulfate-induced retches in chicks in a dose-dependent manner (100, 200, 300 mg/kg). These *in vitro* and *in vivo* results indicate that *C. siliqua* possesses the spasmolytic and antidiarrheal activities mediated possibly through blockage of muscarinic receptors. Thus, this study provides a rationale for its folkloric use.

Introduction

Ceratonia siliqua L. (Fabaceae) known by its vernacular name Kharnub/Caroob (part used: Pods). Pods of C. siliqua are used in the food industry as food supplement and food additives. It has pharmaceutical as well as nutraceutical importance. As a drug carrier, it is used in the formulation of different dosage forms (Karim and Azlan, 2012). Pods are the good source of carbohydrate, dietary fibre, fat and protein. It also contains polyphenols, gallic acid, proanthocyanin, gallo-tannins, catechin, epicatechin gallate, quercetin glycosides, flavonoids, fructose, glucose and sucrose (Nasar-Abbas et al., 2016). It is a rich source of amino acids such as aspartic acid, alanine, glutamic acid and valine as well as minerals, potassium and calcium (Ayaz et al., 2007).

Multiple pharmacological activities have been reported on pods of *C. siliqua* including antidiabetic (Rtibi et al., 2016a), antibacterial (Meziani et al., 2015), antifungal and cytotoxic (Dhaouadi et al., 2014), anti-oxidant (Kumazawa et al., 2002; Rached et al., 2016), anti-proliferative (Roseiro et al., 2013), neuroprotective (Ahmed, 2010), human neutrophil myeloperoxidase inhibitor, scavenger of reactive oxygen species (Rtibi et al., 2015a), gastroprotective (Rtibi et al., 2015b), hepato-protective (Souli et al., 2015; Temiz et al., 2015) and anti-atherosclerotic effects (Valero-Muñoz et al., 2014).

Pods of *C. siliqua* are traditionally used in the treatment of diarrhea (Khare, 2008), vomiting, asthma, cough, hyperperistalsis, cardiovascular diseases, high cholesterol, diabetes, obesity, pain and inflammation (Duke, 2002).

Several *in vitro* and *in vivo* studies as well as clinical trials had been conducted on the pods of *C. siliqua* in recent years. However, the mechanistic basis for the ethnomedicinal use of pods for diarrhea and gastro-intestinal spasm need to be elucidated. The present

study was done to establish its possible mechanism in the multiple gastrointestinal disorders including the hyperactive gut, diarrhea and emesis.

Materials and Methods

Collection, identification and extraction of pods

C. siliqua pods were collected in May 2014 from the Manshera, Pakistan and recognized by Dr. Abdul Rehman Niazi, a taxonomist from the Department of Botany, The University of Punjab. The specimen of plant pod was deposited in the same Department with voucher No. 252015. The unripe pods were dried under the shed and grinded into coarse powder by an electric grinder. The powder material (1.6 kg) was soaked in 70% methanol at 25°C for 7 days with occasional shaking. This material was filtered stepwise through a muslin cloth and Whatman filter paper No. 1. The same practice was done in triplicate and obtained filtrates were combined. The filtrate was dried in the form of semisolid paste by evaporating the solvent on a rotary evaporator (M: 9230 Buchi, Switzerland). The approximate yield of crude extract of *C. siliqua* pods was 12.9%

Fractionation was performed by dissolving 10 g of the crude extract of *C. siliqua* pods in 100 mL distilled water. The solution was poured in separating funnel and an equal volume of dichloromethane was mixed in it with the manual shaking. The organic dichloromethane layer was removed and this procedure was repeated thrice. The obtained fractions were dried with the rotary evaporator and labeled as dichloromethane fraction and aqueous fraction.

Isolation of alkaloids like component from the dichloromethane fraction of pod extract

Dichloromethane fraction was extracted with acidified water (pH 2-3 diluted with hydrochloric acid). The organic layer was discarded. The aqueous acidic layer was made alkaline with sodium bicarbonate to adjust the pH 10 and then back extracted with dichloromethane. The dichloromethane phase was separated, evaporated and labeled as alkaloid free base fraction of *C. siliqua*.

Animals (♂/♀) and housing environment

Local bread rabbits (1.2-1.7 kg), white albino mice (28-35 g) and poultry chicks (40-50 g) were obtained from the local animal market and placed in controlled temperature (25 ± 2°C) with light-dark cycle in the animal house of the Faculty of Pharmacy, Bahauddin Zakariya University, Multan with the open access to standard diet (Hi-Tech Feeds Pvt. Ltd) and water *ad libitum*. All the animals were kept on fasting 16 hours before the start of experiment with the free access to water.

Chemicals and drugs

Acetylcholine chloride, carbachol, atropine sulfate, ethylene tetraacetic acid, magnesium chloride and potassium chloride were acquired from the Sigma Chemical Company (USA). Calcium chloride, dichloromethane, glucose, magnesium sulfate, potassium dihydrogen phosphate, sodium dihydrogen phosphate, sodium bicarbonate and methanol were purchased from the Merck Dermstadt (Germany). Loperamide was kindly gifted by the Saffron Pharmaceuticals Pvt. Ltd. (Pakistan). Chlorpromazine was obtained from the Highnoon Laboratories Limited (Pakistan). Copper sulfate was obtained from the Scharlau Chemie (Spain). Ammonium hydroxide, sodium chloride and sodium hydroxide were purchased from the BDH Laboratories (England).

Phytochemical analysis

The extract of *C. siliqua* pods was investigated for the presence of secondary metabolites including alkaloids, flavonoids, glycosides, phenols, saponons and tannins (Saeed et al., 2012). The dichloromethane fraction was evaluated for the presence of alkaloids, fats, resins and waxes (Jones and Kinghorn, 2005).

In vitro experiments

Segregated rabbit jejunum and rat ileum preparations

The antispasmodic effect of *C. siliqua* pods was investigated by using segregated rabbit jejunum and rat ileum by slight modification according to the method described previously (Bashir et al., 2006). Respective segments of 2-3 cm were suspended in 15 mL tissue bath (radnoti®) containing Tyroid's solution maintained at 37°C, oxygenated with carbogen gas. The isotonic transducer (MLT0015) was used to record the tissue response through Power Lab® data acquisition system (Australia). Each tissue was equilibrated for 30 min before the addition of any test substance. Rabbit jejunum exhibited spontaneous rhythmic contraction and was used to test the antispasmodic activity directly without the use of agonist. Under the same experimental conditions, rat ileum acted as a quiescent smooth preparation and was considered more helpful for perusing the contractile response of agonists like carbachol.

Calcium channel blocking and potassium channel opening activities

To access the antispasmodic effect, whether it was through the calcium channel blocking activity, K^+ (80 mM) was added to the tissue bath to attain sustained contraction of the jejunum tissue of rabbit. The test substance was then added in a cumulative manner to observe the concentration-dependent inhibitory response. In addition to calcium channel blocking activity,

potassium channel activation experiment was also performed to determine the mechanism of antispasmodic activity. To confirm whether it was through potassium channel opening effect, K^+ (25 mM) was added in tissue bath to attain sustained contraction of the rabbit jejunum tissue. The test material was then added in a cumulative manner to observe concentration -dependent inhibitory response (Janbaz et al., 2016; Rafique et al., 2016).

Muscarinic receptor blocking activity

To confirm the antispasmodic effect, rabbit jejunum was treated with 1 µM carbachol to induce contraction and *C. siliqua* extract was added in a cumulative fashion (1-10 mg/kg) to attain concentration-dependent spasmolytic response. Furthermore, for the assessment of antispasmodic activity, whether it was through anti-muscarinic effect, rat ileum was used and treated with 1 µM carbachol to attain three successive peaks of carbachol. The tissue preparations were subsequently washed and incubated with increasing doses of test material/standard anti-muscarinic drug (atropine) for 30 min and the response of 1 µM carbachol was achieved to note any effect of test material or atropine on the control peaks of carbachol. For further confirmation, the concentration response curves (CRCs) of carbachol for the dichloro-methane extract and atropine were constructed on the rat ileum (Gilani and Aftab, 1992).

In vivo experiments

Castor oil-induced diarrhea in albino mice

White albino mice were divided into five groups with five mice in each group. Group I received 10 mL/kg normal saline (0.9%) by the oral route using the gavage method and served as negative control group. Group II was treated with 10 mg/kg loperamide by the oral route and considered as positive control. Treatment Group III, IV and V were administered with the oral dose of crude extracts (100, 200 and 300 mg/kg) respectively. After 1 hour, all the animals were administered with 10 mL/kg castor oil and placed in individual cages with bloating paper on the base of each cage. Six hours later, each cage was inspected to count the number of wet feces. The total number of wet feces in the treatment groups and positive control group were compared with the negative control group to note the anti-diarrheal activity of *C. siliqua* pods (Akindele et al., 2014).

Charcoal meal gastrointestinal transit time in albino mice

Five groups of mice, each containing 5 mice, after overnight fasting were labeled as Group I (negative control), Group II (positive control), Group III, IV and V (treatment group). Group I was orally administered with 10 mL/kg normal saline, Group II was intraperitonially treated with 10 mg/kg atropine and Group III, IV and V were orally administered with the crude extract of *C. siliqua* at the dose of 100, 200, 300 mg/kg respectively. After 20 min, all animals were fed with 0.5 mL charcoal meal. Charcoal meal consisted of 5% deactivated charcoal suspension containing 5% methylcellulose. Mice of all groups were dissected 30 min post-charcoal meal treatment to remove the small intestine. Mice were killed by cervical dislocation after anesthetizing with chloroform prior to dissection. The total length of the small intestine and charcoal meal transport from the pylorus sphincter to the front of charcoal was measured. The charcoal transport in the intestine was expressed in percentage. The percentage inhibition was calculated as:

Percentage inhibition = negative control – test group/ negative control x 100

Antiemetic activity in chicks

To access the antiemetic activity of the extract of *C. siliqua* pod, the chick emetic model was used as described previously (Aleem and Janbaz, 2017). Chicks were sub-divided into five groups with five chicks in each group. Group I (negative control) was administered with 10 mL/kg normal saline orally. Group II (positive control) was treated with 150 mg/kg chlorpromazine orally. Group III, IV and V were treated orally with 100, 200, 300 mg/kg crude extract of *C. siliqua* respectively. After 15 min, all chicks were administered with 50 mg/kg copper sulfate to induce retches. The number of retches was recorded for 10 min in each chick of all the groups.

Statistical analysis

The median effective concentration (EC_{50}) value with confidence interval (CI) 95% was measured to analyze the response of segregated rabbit jejunum preparation in spontaneous and carbachol-induced contractions. One-way analysis of variance (ANOVA) followed by Dunnet's test was used for the rat ileum preparations, antidiarrheal, charcoal meal propulsion and antiemetic activities. The p value of <0.05 was considered as statistically significant. Non-linear regression was used to analyze the concentration response curves of carbachol in rat ileum.

Results

Phytochemical screening of aqueous methanolic extract of *C. siliqua* pods indicated the presence of some important secondary metabolites like alkaloids, flavonoids, phenols, saponins and tannins. Alkaloids, resins and waxes were present in the dichloromethane fraction of the *C. siliqua*.

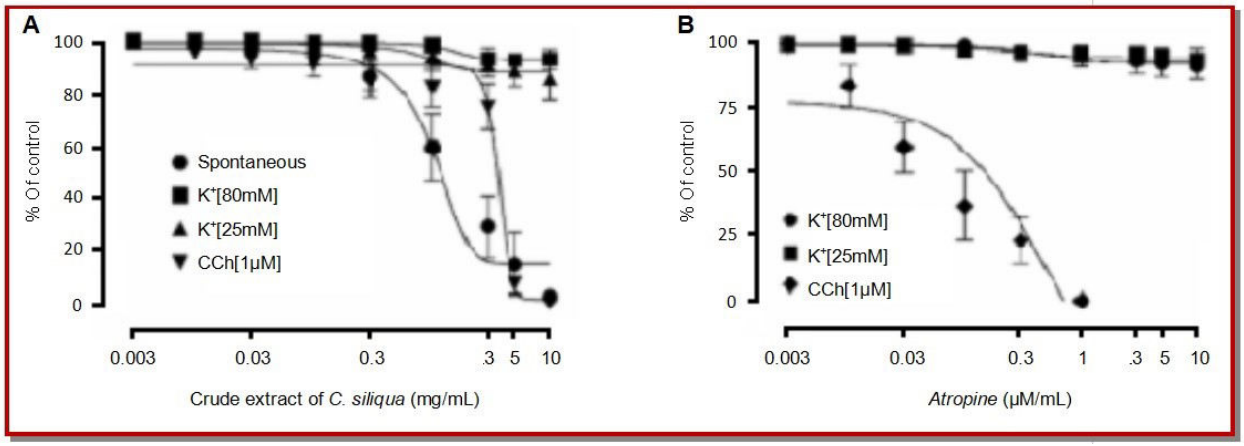

Figure 1: (A) Inhibitory effect of crude extract of *C. siliqua* on spontaneous, high-K, low-K, carbachol (1 µM)-induced contraction in segregated rabbit jejunum. (B) Effect of atropine on high-K, low-K and carbachol (1 µM)-induced contraction in segregated rabbit jejunum. n=5

Effect on segregated rabbit jejunum and rat ileum

Aqueous methanolic extract of *C. siliqua* pods relaxed the spontaneous rhythmic contraction of segregated rabbit jejunum preparations in a concentration-dependent manner from the dose range of 0.01-10 mg/mL, with EC_{50} value of 1.9 mg/mL (1.3 to 2.7: CI, 95%). It did not relax the high K+ (80 mM and 25 mM)-induced contraction in the rabbit jejunum. The crude extract relaxed the carbachol (1 µM)-induced contraction in the rabbit jejunum at a higher dose of 10 mg/mL with EC_{50} value of 13.1 mg/mL (0.5 to 1.6: CI, 95%) like atropine. Atropine did not relaxed high K+(80 mM)-induced contraction but it relaxed the carbachol (1 µM)-induced contraction in the rabbit jejunum with EC_{50} value of 0.04 mg/mL (0.02 to 0.1: CI, 95%) (Figure 1A, B).

Pre-treatment of rat ileum with the crude extract (0.003-3 mg/mL) caused a dose-dependent suppression of carbachol (1 µM) peaks like atropine (1 µM). Carbachol (1 µM): Mean ± SEM 100 ± 0, atropine (1 µM), 0.63 ± 0.31, and crude extract at different doses (0.003-3 mg/mL) [(0.003): 67.1 ± 2.2, (0.01): 36.2 ± 1.8, (0.03): 35.1 ± 1.6, (0.1): 30.3 ± 2.1, (0.3): 28.6 ± 2.2, (1): 4.9 ± 0.6, (3): 2.0 ± 0.8] respectively (Figure 2A).

Pre-treatment of rat ileum with dichloromethane fraction also caused a dose-dependent suppression of carbachol (1 µM) peaks at different doses (0.01-3) like atropine (1 µM). Control peaks of carbachol (1 µM) with the mean ± SEM: 100 ± 0, atropine (1 µM): 0.6 ± 0.3 and dichloromethane fraction at the dose range (0.01-3 mg/mL) showed mean ± SEM values [(0.01): 49.7 ± 3.3, (0.03): 45.9 ± 4.3, (0.1): 38.6 ± 4.4, (0.3): 36.8 ± 3.7, (1): 27.3 ± 3.6, (3): 1.6 ± 0.9] respectively (Figure 2B).

Similarly, when the alkaloid-free base fraction of *C. siliqua* pods was tested on the rat ileum, it suppressed the carbachol (1 µM) peaks at a lower dose than the crude extract and its dichloromethane fraction like

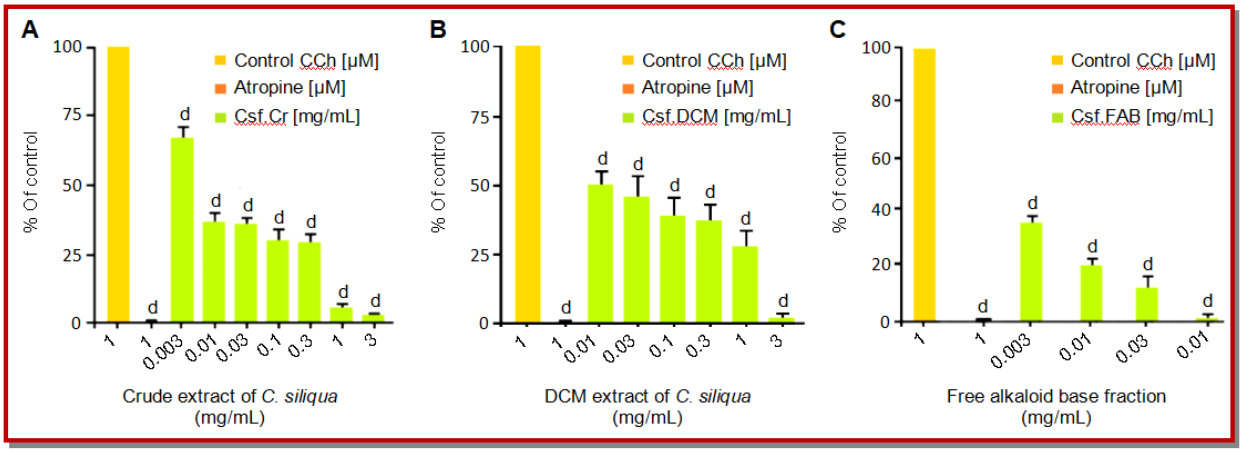

Figure 2: Inhibitory effect of (A) Crude extract of *C. siliqua,* (B) Diclorometh ane (DCM) fraction of *C. siliqua*, (C) Free alkaloid base fraction of DCM fraction of *C. siliqua* on carbachol (1 uM)-induced contraction in segregated rabbit jejunum. n=5. a ($p< 0.05$), b ($p< 0.01$), c ($p< 0.001$), d ($p< 0.0001$) were considered statistically significant followed by One-Way-Analysis of variance (ANOVA). n=5

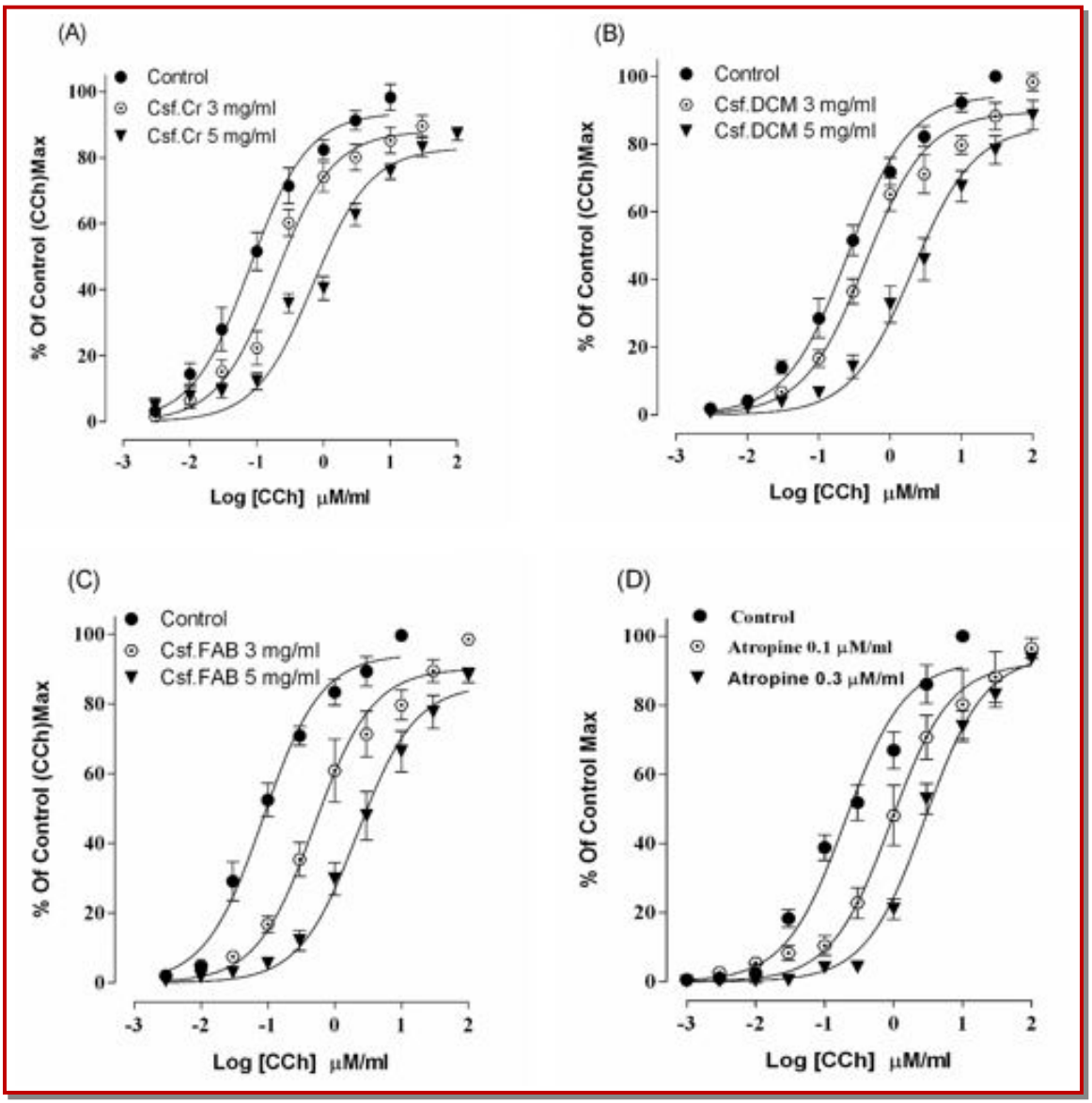

Figure 3: Concentration response curves in rat ileum of carbachol in the presence of different concentrations of (A) *C. siliqua* (Csf.Cr), (B) Dichloromethane fraction (Csf.DCM), (C) Free alkaloid base fraction of dichloromethane fraction (Csf.DCM), (D) Standard anti-muscarinic agent, atropine

atropine. Control of carbachol (1 µM) peaks with mean ± SEM values 100 ± 0, atropine (1 µM): 0.6 ± 0.3 and at different doses of free alkaloid base fractions (0.003-0.1) showed mean ± SEM values [(0.003): 35.5 ± 1.7, (0.01): 19.3 ± 2.1, (0.03): 11.9 ± 2.5, (0.1): 0.9 ± 0.5] respectively (Figure 2C).

Moreover, log carbachol curves of crude extract, dichloromethane fraction and alkaloid-free base fraction of *C. siliqua* were constructed on rat ileum in comparison to atropine to confirm the anti-muscarinic effect. All the test materials shifted the carbachol curves towards the right in a parallel manner like atropine (Figure 3).

Castor oil-induced diarrhea in albino mice

The aqueous methanolic extract of *C. siliqua* pods exhibited anti-diarrheal activity at doses of 100, 200, 300 mg/kg respectively against the castor oil-induced diarrhea in white albino mice. The negative control (Group I) administered with saline showed the maximum diarrheal dropping after the administration of castor oil with percentage inhibition of diarrhea (0%). The positive control (Group II)-treated with loperamide showed maximum inhibition of diarrhea (89.2%). The treatment Group III, IV and V showed dose-dependent inhibition of diarrhea (20, 43.0, 67.6%) respectively (Figure 4A).

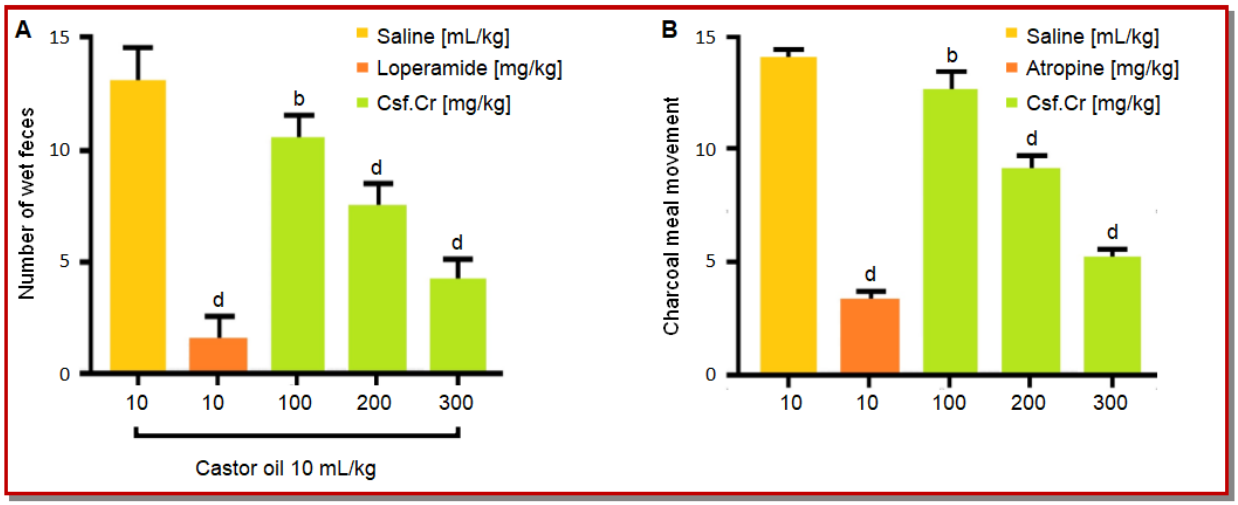

Figure 4: Dose dependent inhibitory effect of crude extract of *C. siliqua* on (A) castor oil-induced diarrhea in mice n=5, (B) Charcoal meal propulsion in mice n=5. a ($p< 0.05$), b ($p< 0.01$), c ($p< 0.001$), d ($p< 0.0001$) were considered statistically significant followed by One-Way-Analysis of variance (ANOVA)

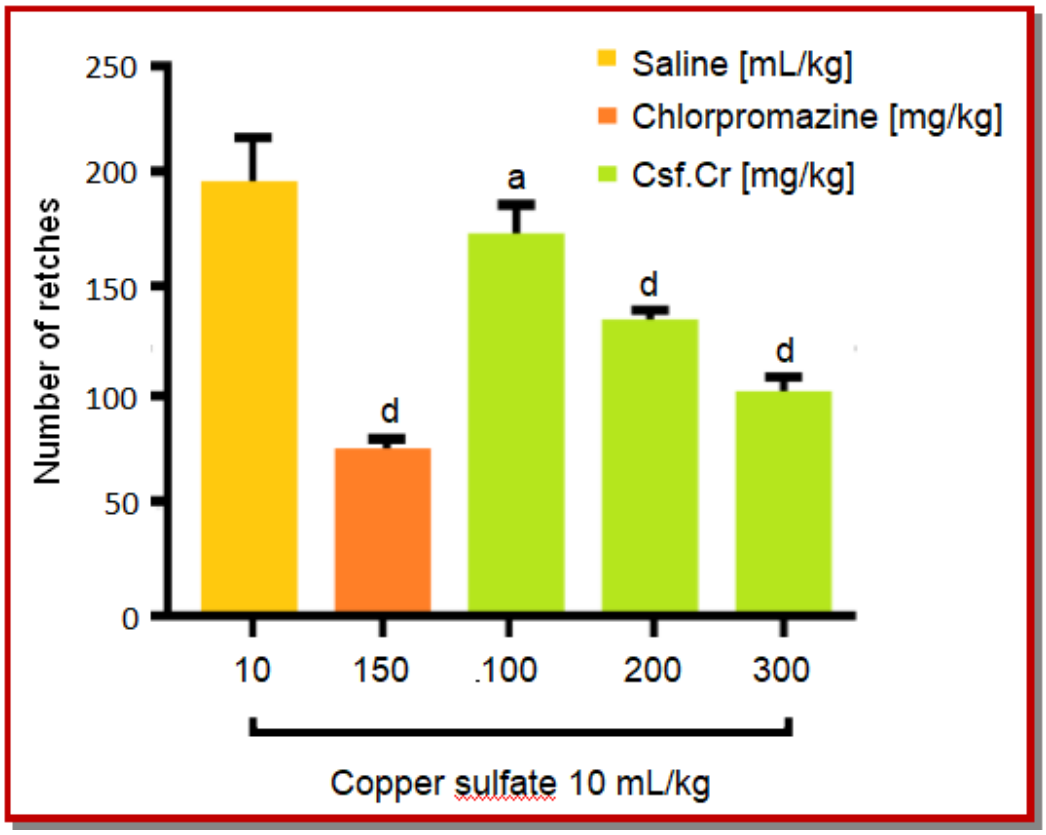

Figure 5: Dose dependent inhibitory effect of Crude extract of *C.siliqua Linn* on copper sulfate induced retches in chicks in comparison of chlorpromazine. a ($p<0.05$), d ($p< 0.0001$) were considered statistically significant followed by One Way Analysis of Variance (ANOVA). n=5

Charcoal meal gastrointestinal movement activity

The crude extract of *C. siliqua* pods inhibited the charcoal meal transport in the gastrointestinal tract of mice in a dose-dependent manner at the dose of 100, 200, 300 mg/kg respectively. Group I administered with normal saline showed the distance travelled by charcoal meal in the percentage inhibition (0%). Group II treated with atropine sulfate 10 mg/kg exhibited percentage inhibition of distance traveled by charcoal meal in gastrointestinal tract of mice (76.7%). Treatment group-III, IV and V at dose of 100, 200 and 300 showed percentage inhibition of distance traveled by charcoal meal (10.6, 35.3, 63.2%) respectively (Figure 4B).

Antiemetic effect in chicks

The extract of *C. siliqua* pods exhibited antiemetic activity. The control Group I showed the maximum number of retches in chicks (196.8 ± 9.3). Group II treated with chlorpromazine exhibited the percentage inhibition of retches (62.5%) while the treatment Group III, IV and V orally administered with crude extract 100, 200, 300 mg/kg showed %inhibition of the number of retches in chicks (13.1, 32.7, 49.1%) respectively. All the groups were compared with the Group I (negative control group) (Figure 5).

Discussion

In the present study, *in vitro* and *in vivo* studies were conducted to confirm the ethnopharmacological basis for the possible mechanism of action of aqueous methanolic crude extract of *C.siliqua* pods for the management of gastrointestinal disorder including spasms, diarrhea and emesis.

C. siliqua pods are used in various regions of the world including Pakistan for the treatment of diarrhea and constipation by traditional medicine practitioner. Recently a scientific *in vivo* study was conducted on immature and mature carob pods which claimed that phytochemical constituent(s) variations in immature and mature carob pods play a role in the management of diarrhea and constipation (Rtibi et al., 2016b).

In *in vitro* experiments, *C. siliqua* pods extract inhibited the spontaneous rhythmic contraction of segregated rabbit jejunum preparation at dose 10 mg/mL. It indicates that extract exhibited spasmolytic activity. To identify which type of mechanism(s) are involved in the spasmolytic action, various experiments including a) calcium channel blocking activity, b) potassium channel opener activity and c) anti-muscarinic activity were performed. Majority of plants induce their spasmolytic effect through these mechanisms as reported in various previous plant extract studies (Gilani et al., 2008; Khan et al., 2011; Janbaz et al., 2014). The crude extract did not relax K+ (80 mM) and K+ (25 mM)-induced spastic contraction in segregated rabbit jejunum preparation. It rules out the calcium channel blocking and potassium channel opener activities. Crude extract relaxed the carbachol (1 µM)-induced sustained contraction in segregated rabbit jejunum preparation at dose 10 mg/mL. It speculated that the spasmolytic activity might be mediated through muscarinic receptor blockage effect. Gastrointestinal smooth muscles typically express both M_3 and M_2 receptors. Transient receptor potential channels are prominent nonselective cation channels in gastrointestinal smooth muscle. These nonselective cation channels are activated by stimulation of muscarinic receptors M_3 and M_2 involved in the regulation of gastrointestinal motility via different signalling pathways: a) activation of phospholipases, b) production of inositol 1,4,5-triphosphate and diacylglycerol, c) release of Ca^{++} from the sarcoplasmic reticulum, d) inhibition of potassium channel, e) activation of L-type Ca^{++} channels, f) inhibition of adenylate cyclise and g) activation of nonselective cation channels (Gerthoffer, 2005). For further confirmation, rat ileum was used to establish the base of possible mechanistic validation of *C. siliqua*. The crude extract, dichloromethane fraction and free alkaloid base fraction of the extract suppresses the peak of carbachol (1 µM)-induced contraction in rat ileum at a lower dose like atropine and found that free alkaloid fraction of *C. siliqua* being more potent. So, it might be possible that the antispasmodic effect of crude extract, dichloromethane fraction and free base alkaloid fraction of *C. siliqua* extract was possibly mediated through blockage of muscarinic receptor M_3. Anti-muscarinic agents are well proven for their use in gut ailments including gastrointestinal spasm, which provides a mechanistic basis for the folkloric use of the *C. siliqua* in spasm and diarrhea. This mechanistic discovery provides the basis for performing *in vivo* GIT charcoal meal propulsion and anti-diarrheal activities of the plant. In castor oil-induced diarrheal model of mice, *C. siliqua* decreased diarrheal droppings similar to the positive control group (loperamide), an antimotility agent. In the small intestine, castor oil is converted into an active ingredient recinolic acid which increases intestinal fluid contents. It causes diarrhea through changes in electrolyte and water transport and generates enormous contraction in the transverse and distal colon (Iwao and Terada, 1962). *C. siliqua* inhibits the charcoal transport and severe contraction in experimental animals. These *in vivo* experiments prove that the *C. siliqua* pods exhibit antidiarrheal activity that might be due to anti-muscarinic effects. When administered *C. siliqua* (100, 200 and 300 mg/kg) decreased the propulsive movement of the charcoal meal through GIT and increased the GI transit time, it supported the antidiarrheal activity of *C. siliqua* similar to that of atropine, a famous anti-muscarinic agent that decreases the movement of intestinal contents.

Copper sulfate induces emesis by acting on the motor system of the body as well as the somatic system and autonomic nerve flow (Wang and Borison, 1951). Various sensory pathways trigger the emetic reflex including gastrointestinal vagal afferents, area postrema for detection of circulating toxins, vestibular complex system and forebrain descending pathways (Horn, 2017). On the bases of folkloric use of *C. siliqua* in the treatment of vomiting, chick emetic model was adopted to screen its possible antiemetic activity. Vomiting center/chemoreceptor trigger zone (CTZ) present in the medulla oblongata. Emesis is caused by direct stimulation of motor pathway or indirect stimulation of chemoreceptor trigger zone. Antiemetic

activity of *C. siliqua* was comparable with chlorpromazine for copper sulfate induced emesis that might be mediated by inhibition of chemoreceptor trigger zone (Hussain et al., 2015).

It is previously reported that various phytochemical constituents such as alkaloids, phenols, tannins, saponins and flavonoids play an important role in spasmolytic, anti-diarrheal and antiemetic actions through various mechanisms including muscarinic receptor antagonist. The presence of alkaloids, saponins, tannins, flavonoids and phenols in *C. siliqua* might be responsible for spasmolytic, anti-diarrheal and antiemetic activities.

Conclusion

The aqueous methanolic extract of *C. siliqua* possesses antispasmodic and anti-diarrheal activities possibly through blockage of muscarinic receptors. It also exhibits the antiemetic activity. These anti-muscarinic and antiemetic effects might be mediated due to the presence of phytochemical constituents like alkaloids, phenols, saponins and tannins.

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Ethical Issue

All the experimental studies followed the rules of the Institute of Laboratory Animal Resources, Commission on life Sciences (National Research Council, 1996) and approved by the Animal Ethical committee of the University (reference no. EC/05PhDL/2013).

Conflict of Interest

Authors declare no conflict of interest.

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