doi.org/10.36721/PJPS.2020.33.1.SUP.333-342.1

A flavonoid driven phyto-pharmacological effects of *Capparis decidua Edgew.* in rodents

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Abstract: This study elicits the underlying mechanism(s) of *Capparis decidua* when used for different gut disorders. HPLC chromatogram of *C. decidua* extract (CD.Cr) and its respective fractions showed a variety of phytochemicals of which, kaempferol being in a high proportion. In mice, CD.Cr at doses of 70 and 150 mg/kg enhanced the wet feces output to 33 and 44% respectively as compared to carbachol (47.6%), while doses of 500 and 700 mg/kg, presented 41 and 70% safety against castor oil-driven diarrhea, respectively. Its flavonoid constituent, kaempferol at doses of (50 and 100 mg/kg) produced 51.7 and 82% safety when compared to nifedipine which provided 95% safety at dose of 40 mg/kg against castor oil-driven diarrhea like loperamide. In isolated jejunum preparations, *C. decidua* extract and its respective fractions (except pet-ether) produced atropine-sensitive inhibitory effects, whereas kaempferol and nifedipine showed atropine insensitive effects. Against high K^+-induced contractions, *C. decidua's* fractions and kaempferol both exhibited a concentration-related non-specific inhibition while displacing the Ca^{++} -CRCs to right-ward with suppression in maximal response like nifedipine. In isolated rat ileal preparations, CD.Cr and respective fractions elicited atropine-sensitive gut excitatory responses. In summary, this article reports *C. decidua's* laxative effect through cholinergic receptor activation as well as its antidiarrheal effects, where its flavonoid constituent kaempferol produces Ca^{++} antagonist like activity, thus justifying *C. decidua* folk use in constipation and diarrhea.

Keywords: *Capparis decidua*, laxative, cholinergic receptor agonist, antidiarrheal, Ca^{++} antagonist,

INTRODUCTION

Capparis decidua Edgew. (Family Capparidaceae) is a perennial, richly branched, glabrous, spiny, almost leafless woody shrub reaching to a height of 4-5m. It is commonly referred as Karir in Urdu and Caper berry in English. The plant is known for its berry-shaped fruits which is called "Della" in Punjab. The plant is abundantly found in dry, arid and sun exposed areas of Sind, Balochistan, Egypt, Socotra, Iran, Arabia, dry tropical Africa, Rajputana, Deccan Peninsula, Punjab and Gujarat (Zereen *et al*., 2015; Nazar *et al*., 2018).

C. decidua has been claimed to cure numerous diseases including hemorrhoids, diabetes, ulcers, asthma, gout, vomiting, boils, piles, inflammation, various pain causing conditions, pyrexia, malaria, cardiac and renal disorders, psychiatric problems, gastrointestinal disease conditions including constipation, diarrhea, dysentery, indigestion and rheumatism (Singh and Singh, 2011, Haq *et al*., 2011, Mann *et al*., 2013). The stem or bark of *C. decidua* has an acrid, sharp hot taste and has been used to treat pile, asthma, ulcer, diarrhea, dysentery, vomiting and inflammation. It is also effective as a laxative, purgative, analgesic and anthelmintic agent (Sharma 2003; Singh *et al*., 2011; Nazar *et al*., 2018).

Different phytochemicals have been found in *C. decidua* are spermidine, n-triacontanol, isocodonocarpine, capparisinine, capparidisine, 15- N- acetyl capparisine, capparisesterpenolide, decidua terpenolide A,B,C,D and E, 14-N- acetylcodonocarpine, cadabicine, stachydrine, capparisine, codonocarpine, capparine, cappariline, capparinine, glucocapparin, (sec-butyl, benzyl, isopropyl and methyl isothiocyanates as volatile oil), simiarenol, lupeol, taraxerol, N-pentacosane, stigmasterol, sitosterol, campesterol, avenasterol, (α-tocopherol, γ-tocopherol and δ-tocopherol *i.e* isoforms of tocopherols as vitamin E and ascorbic acid, vitamin C), carotenoids (lutein and β-carotene), flavonoid glycosides as rutin, isorhamnetin and kaempferol, (β-amyrin, octadecanol, tetracosanol, hexadecanol, gramisterol, cycloartanol and citrostadienol as aliphatic and triterpenic alcohol), phthalic acid, fatty acid (linoleic acid and oleic acid), carbohydrates, amino acids and different minerals like zinc, iron, potassium, calcium, phosphorus and manganese (Tlili *et al*., 2011;

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Singh *et al*., 2011; Anjum *et al*., 2018; Nazar *et al*., 2018). Kaempferol, a yellow colored flavonoid, commonly found in vegetative foods has also been found in this plant. Kaempferol has been reported for its antioxidant, antimicrobial, anti-inflammatory, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, hypotensive, anxiolytic, analgesic, antiallergic and hepatoprotective activities (Bigoniya *et al*., 2013). However its role in diarrhea and dysentery as well as the underlying mechanism for this antidiarrheal activity yet remains to be validated.

C. decidua showed anti-arthritis, anti-fungal and anti-bacterial (Sharma and Kumar, 2008; Anjum *et al*., 2018), parasitical (Abdalrahman *et al*., 2016), anti-viral, analgesic and anti-inflammatory (Singh and Singh, 2011; Mohammed *et al*., 2012), hypotensive (Shah and Gilani, 2011), anti-oxidant and anti-diabetic (sharma *et al*., 2010), anti-aging and hepato-protective (Aghel *et al*., 2010), anti-neoplastic (Luecha *et al*., 2009), anti-seizure and anti-fertility (Revathi *et al*., 2010), anti-platelet and hypo-lipidemic (Purohit and Vyas, 2006) activities. Keeping in view, the folk use of *C. decidua* in gastro-intestinal tract disorders like constipation and diarrhea (Sharma 2003; Singh *et al*., 2011; Nazar *et al*., 2018) and to further study its simultaneously present laxative and antidiarrheal effects, the objective of this study is to explore the scientific basis of these effects by using standard pharmacological protocols. This study has also been extended to elaborate the proportion and efficacy of gut stimulant and relaxant constituents in polarity-based fractions of *C. decidua*.

MATERIALS AND METHODS

Collection, identification and preparation of plant extracts

The stem part of the plant was obtained from the vicinity of Mochiwala Jhang, Faisalabad Road, District Jhang, Punjab, Pakistan. This was authenticated by the expert taxonomist, Associate Professor. Dr. Mansoor Hameed, University of Agriculture Faisalabad (UAF), Pakistan. The collected specimen of *C. decidua* was kept at the University of Agriculture, Faisalabad for future reference vide voucher No.1129-2017. A total of 5 kg of the plant was shade dried, cleaned thoroughly and powdered coarsely by using electrically driven cutters. The powdered material was transferred to amber color glass jar and added with 5 L of aqueous- methanol (30:70). This material was retained for three days. The liquid was separated by using muslin cloth and further purified by passing through the filter paper. This practice of soaking and then filtration was executed thrice to get final combined filtrate. The combined macerate was transferred to the flask and attached to rotary evaporator to evaporate the liquid and finally get dark brown paste of *C. decidua* extract (yielding 8.4 % w/w).

For further liquid-led fractionation of the *C. decidua* extract, 30 gm of the extract was measured and transferred to separating funnel to dissolve completely in distilled water. Next, petroleum ether of same volume was also added to the funnel and shuddered for five minutes. On separation of layers after few minutes/hours, the upper layer i.e. petroleum ether was removed and this process of fractionation was repeated again. Petroleum ether was replaced by chloroform and then ethyl acetate to obtain the chloroform ($CHCl_3$.CD), ethyl acetate (Et.Ac.CD) and aqueous fractions (Aq.CD). Obtained fractions were evaporated on rota-vapor under reduce pressure and the aqueous fraction was freeze dried (Williamson *et al*., 1996).

Initial phytochemical trials

The *C. decidua* extract was tested by following the standard methods as described by Victor and Chidi (2009) for alkaloid, tannin, flavonoid, saponin, glycoside and phenol.

HPLC fingerprint assay

The HPLC of *C. decidua* extract (CD.Cr) and its fractions (Pet.CD, $CHCl_3$.CD, Et.Ac.CD and Aq.CD) was studied by using a Shimadzu (Japan) Prominence 10-AT equipped with a pump (LC-10AT), PDA detector (SPD-10AV) and column [Shim-Pack CLC-ODS (C-18), 25cm x 4.6mm, 5 µm]. Mobile-phase with 1 mg of sample per mL was prepared. Acetonitrile, acetic acid and methanol constitute the mobile-phase and supplied at flow rate of 1mL/ min at 25 ^{o}C, while detection was done at 280 nm wavelength. Kaempferol, quercetin, benzoic acid and rutin were used as qualitative standards.

Chemicals

Carbachol (CCh), kaempferol, acetylcholine (ACh), loperamide, atropine sulphate, nifedipine and Tween 80 were obtained from Sigma-Aldrich (St. Louis, Missouri, United States). Castor oil, thiopental sodium injection and isoflurane were obtained from Care Pharmacy (Faisalabd, Punjab, Pakistan). These chemicals were of the analytical grade. Other than water soluble chemicals were solubilized by using Tween 80.

Experimental animals

Bulb/c mice (20-25g), SD rats (200–250g) and locally born and raised rabbits (1-1.5kg) of both genders were utilized to perform this study. Animals were retained at animal house, at 22±3°C temperature and 55±5% humidity. The rats and mice were provided with tap water and standard diet *ad labitum* while rabbits were given the green fodder. These studies were executed as per verdicts of Institute of laboratory Animal Resources Commission on Life Sciences (1996), NRC, which also fulfill the IUCN Policy Statement on Research Involving Species at Risk of Extinction. The Institutional Review Board, Government College University Faisalabad, Punjab,

Pakistan also approved this study vide letter No.IRB No.583 dated 26-06-2018.

In-vivo studies

Acute toxicity trials

Bulb /c mice (n= 40) were likewise divided into four groups and resided in special cages with water and food *ad labitum*. First three groups received 3, 5 and 10 g/kg of *C. decidua* extract, respectively while the last group received saline (10 mL/kg) orally. The animals were monitored for any sign of behavioral changes, pilo-erection /or loco-motor activity for 6 h, however mortality was observed until 24 h.

Determination of laxative activity

Mice (n=30) consisting of both genders were starved for a night and divided into five groups. They were kept individually in special transparent plastic cages. The first group received saline (10 ml/kg) orally and the second one was injected with the carbachol (1 mg/kg) intra-peritonieally, while third, fourth and fifth groups were administered 70, 150 and 300 mg/kg respectively of *C. decidua* extract orally. After 4 h, the total figure of pellets (total figure of wet pellets plus total figure of dry pellets) production was computed and the proportion of wet pellets out of total pellets reflected the laxative activity (Mehmood *et al*., 2014).

Castor oil-driven diarrhea

Mice (n= 42) of either genders were kept fasted for 16 h and divided into seven groups. They were kept into special cages individually. One group was given saline (10mL/kg) and other group acting as the positive control, was administered with loperamide (10 mg/kg) orally. The next two groups received crude extract at doses of 500 and 700 mg/kg respectively. The fifth and sixth group received orally 50 and 100 mg/kg dose of kaempferol while the seventh group received nifedipine (40 mg/kg) orally. 1 h later, all groups were administered castor oil orally by using special gastric needle. After 3 h, the cages were monitored for total figure of pellets (total figure of wet pellets plus total figure of dry pellets) and percentage safety from diarrhea was determined as = 100 – [total figure of wet pellets / total pellets ×100] (Ghayur and Gilani, 2005).

In-vitro studies

Preparation of isolated rabbit-jejunum segments and spasmolytic activity

At terminal day rabbits free from gender restrictions were kept fasted for 14 h. They were injected with thiopental sodium (60-90 mg/kg). Afterwards the cervical dislocation was completed by a solid rod blow to the posterior portion of skull. An incision was performed to abdomen for the isolation of jejunum, then immersed into Tyrode's solution in petri-dish to divide into 2-3 cm of pieces. These pieces were suspended in double jacket 10 mL organ bath full of Tyrode's solution, maintained at 37°C and carbagen (95% O_2 and 5% CO_2) was bubbled through it. The test material poured into the bath and its effects were recorded by means of isometric force transducer fixed with the Power Lab Data Acquisition System (AD Instruments, Sydney, Australia). The jejunum preparation was first equilibrate and then stabilized by addition of acetylcholine (Ach, 0.3 µM) repeatedly. In such circumstances, rabbit jejunum showed pendular movements of contractions and the relaxations and the responses of the test material were studied as average change in these movements of jejunum (Mehmood *et al*., 2011).

For validation of supposed mechanism(s) involved in spasmolytic activity, sustained contractions were induced in the isolated jejunum preparations by addition of high K^+. This is an established fact that high K^+ (80 mM) to organ bath leads to opening of L-type calcium channels and subsequently influx of calcium, which results in the contractile response (Bolton, 1979). Thus those agents which inhibit these high K^+-driven contractions is recognized as a blocker of Ca^{++} influx through blockade of VDCCs (Godfraind, 2017). To confirm the CCBs (calcium channel blocker) like activity, a control calcium CRCs (Ca^{++}-concentration response curve) was drawn in absence and presence of test material in K^+-rich and calcium free medium (Ghayur and Gilani, 2005).

Isolated rat-ileum preparations and spasmogenic activity

Rats were starved for 24 h and then euthanized by using isoflurane (2-5 % v/w). The abdomen was cut by using sharp edge blades and ileum was separated. The ileum was cut into 2-3 cm long pieces and suspended into 10 mL organ bath full of Tyrode's solution, maintained at 37°C and carbagen bubbled through it. Each tissue response was recorded on power lab acquisition data setup using isometric force transducers. Each preparation was stabilized by acetylcholine as for rabbit jejunum.

To characterized the underlying stimulatory mechanism, the plant extract and respective fractions were tested on rat ileal pieces with and without atropine (0.1µM), a cholinergic antagonist, pyrilamine, a histaminegic receptor antagonist (1µM), and methysergide, a serotonergic antagonist (1µM) (Mehmood *et al*., 2014). Complete or partial blockade of the stimulatory effects of plant extract and its fractions in the presence of any of aforementioned antagonist(s) illicit the involvement of respective pathway(s) in the excitatory effect of the test material.

STATISTICAL ANALYSIS

Data is shared in mean ± Standard Error of Mean (S.E.M) where "n" showed number of tests or animals used. EC50 showed median effective concentration with 95% (CIs)

confidence intervals. One-way analysis of variance (ANOVA) followed by Dunnet's test was applied to laxative and antidiarrheal activities. P & lt; 0.05 was considered as significant difference. Concentration-response curves (CRCs) were analyzed by non-linear regression. Two-way ANOVA followed by Bonferroni's post-test correction was used for comparison of CRCs with control. Graphs were prepared on Graph Pad program (San Diego, Calf. USA).

RESULTS

Initial phytochemical trials

These procedures unveiled the alkaloid, saponin, tannin, flavonoid, glycoside and phenol as phyto-constituents of *C. decidua*.

HPLC fingerprints assays

HPLC trials of CD.Cr and respective fractions were completed as defined in earlier section of methodology. The HPLC fingerprints are shown in fig. 1

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In-vivo Activity

Acute toxicity trials

In mice CD. Cr was proven safe to the highest tried dose of 10g/kg as it was devoid of any sign of salivation, diarrhea, lethargy, convulsions, tremors, sleep and coma up to 6 h of the study and no death was observed until 24 h in all groups.

Laxative activity

C. decidua extract at 70 and 150 mg/kg of dose exhibited 33 and 44% of wet feces in mice; carbachol (1mg/kg) produced 47.6 % while saline treated group showed 9.52 % of wet feces. While at 300 mg/kg, the next higher dose, it exhibited a fall in wet (11.11 %) and total fecal output. This weak fecal production executed the existence of both laxative (at lower-doses) and antidiarrheal (at higher-doses) effects of *C. decidua* extract (table 1).

Castor oil-driven diarrhea

C. decidua extract and kaempferol produced dose-related antidiarrheal effects in mice. The animals treated with the crude extracts of *C. decidua* at 500 and 700 mg/kg of dose exhibited 45.7 and 76.7 % safety, while kaempferol at 50 and 100 mg/kg of dose showed 51.7 and 82 % safety, respectively while nifedipine (40 mg/kg) produced 95 % safety from castor oil-driven diarrhea. The positive control of loperamide treated animals showed 100% safety, while the negative control (saline treated animals) exhibited only 22 % safety as seen in table 2.

In-vitro activity

Response on rabbit jejunum

The plant extract partially inhibited the pendular movement of the spontaneously contracting rabbit-jejunum, while after the addition of atropine (0.1 µM) to the organ bath, the response of the *C. decidua* extract was extremely potentiated and it completely inhibited the rabbit-jejunum with IC_{50} value of 3.86 mg/mL (2.64 - 5.64, 95% CI, n = 4-6) in a concentration-related manner. The crude extract of *C. decidua* also inhibited the high K^+ (80 mM)-created contractions. Kaempferol also produced the same effect and inhibited the spontaneously contracting jejunum and high K^+-created contraction in rabbit-jejunum with IC_{50} value of 2.43 (1.53 - 3.93) in concentration-related manner, these effects of CD.Cr and kaempferol are similar to nifedipine [0.12 (0.12 -0.43)] in rabbit-jejunum as seen in fig. 2a.

The chloroform ($CHCl_3$.CD), ethyl acetate (Et.Ac.CD) and aqueous (Aq.CD) fractions inhibited the pendular movements of rabbit-jejunum with IC_{50} value of 2.53 (1.33 - 4.82), 4.72 (2.50 - 6.89) and 4.75 (3.24 - 7.04), respectively and their effects were potentiated in atropinized tissue with respective IC_{50} value of 0.94 (0.67 - 1.31), 3.40 (1.81 - 6.40) and 1.95 (1.27 - 3.01). However, the inhibitory effect of pet-ether (Pet.CD) fraction [EC_{50} value = 0.68 (0.39 - 1.19)] was insensitive to atropine (fig. 2).

When evaluated for CCBs like activity, CD.Cr incubation tissues produced a rightward non-parallel move in Ca^{++} concentration-response curves with significant inhibition to the control response of Ca^{++} at 1 mg/mL (55±2.88%, n=4-6) and 3mg/mL (79 ± 2.08%, n=4-6), while kaempferol inhibit the maximum response of Ca^{++} at 3 µM (33.3 ± 3.33) and 10 µM (71.5 ± 6.09) like nifedipine which inhibit the effect of Ca^{++} at 0.03 µM (31. 6 ± 1.66) and 0.1 µM (65 ± 2.88) as seen in fig. 3. The addition of Pet.CD (0.1-0.3 mg/mL), $CHCl_3$.CD (0.3-1 mg/mL) and Et.Ac.CD (1-3 mg/mL) to organ bath also executed a rightward non-parallel move in the CRCs of Ca^{++} with significant inhibition to the control response of Ca^{++} to (25 ± 5 - 66.6 ± 4.45), (35 ± 2.90 - 62.4 ± 1.45) and (38.3 ± 1.66 - 75 ± 2.78) respectively as seen in fig. 3, except its aqueous fraction which lacking these effects (data not shown).

Effect on rat ileum

The crude extract of *C. decidua* exhibited excitatory effects on rat-ileum in varying dose of 0.01-3 mg/mL, getting its highest effect of 43.3 ± 1.66% (mean ± SEM, n = 4-6) to acetylcholine (0.3 µM) effects. To characterize the excitatory effect of CD.Cr, the rat-ileum preparations were first atropinized (0.1 µM). In atropinized ileum the excitatory effects of both CD.Cr and Ach were blocked (fig. 4). Kaempferol and nifedipine exhibited no effect on rat-ileum as seen in fig. 4.

Among the respective fractions, $CHCl_3$.CD, Et.Ac.CD and Aq.CD fractions exhibited excitatory effects at concentration range of 0.3, 0.1-1 and 0.03-3 mg/mL, getting their highest of 8.36 ± 1.65, 36.6 ± 1.67 and 55 ± 2.88%, respectively, however the aqueous fraction

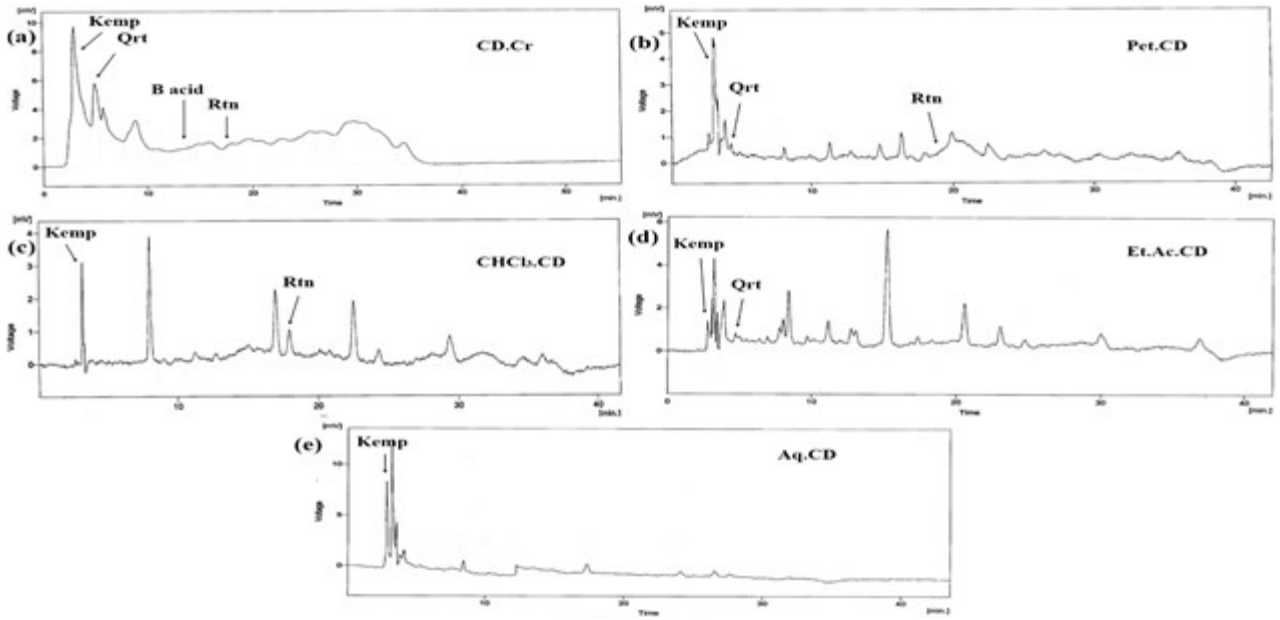

Fig. 1: The HPLC chromatogram of the crude extract of the stem part of *C. decidua* (CD.Cr) (a) and its petroleum ether (Pet.CD) (b), chloroform ($CHCl_3$.CD) (c), ethyl acetate (Et.Ac.CD) (d) and aqueous (Aq.CD) (e) shows the peaks of kaempferol (kaemp), quercetin (Qrt), benzoic acid (B.acid) and rutin (Rtn) along with other unidentified compounds.

Table 1: The laxative properties of the *C. decidua* extract (CD.Cr) and carbachol (CCh) in mice

S. No.	Medication	Quantity (mg/kg)	Mean pellets / group (numbers)	Mean wet pellets / group (numbers)	% age safety from diarrhea
1	Saline + castor oil (10 mL/kg)	10	8.16 ± 0.60	6.33 ± 0.49	22
2	Loperamide + castor oil	10	2.5 ± 0.42 **	0 ± 0.00 **	100
3	CD.Cr (p.o) + castor oil	500	5.83 ± 0.79 *	3.16 ± 1.44 *	45.7
4	CD.Cr (p.o) + castor oil	700	4.33 ± 0.55 **	1.0 ± 0.36 **	76.7
5	Kaempferol (p.o) + castor oil	50	4.83 ± 0.60 *	2.33 ± 0.84 *	51.7
6	Kaempferol (p.o) + castor oil	100	3.84 ± 0.47 **	0.66 ± 0.42 **	82
7	Nifedipine (p.o) + castor oil	40	3.16 ± 0.47 **	0.16 ± 0.16 **	95

Data is shown as mean ± Standard Error of Mean (SEM), (n) = 6 animals / group. * $p < 0.05$ and ** $p < 0.01$ expresses an evaluation of group 2–5 vs group 1, (One-way analysis of variance (ANOVA) followed by Dunnet's test)

Table 2: The anti-diarrheal properties of the crude extract of *Capparis decidua* against castor oil-driven diarrhea in mice

S. No	Medication	Quantity (mg/Kg)	Mean pellets / group (numbers)	Mean wet pellets / group (numbers)	% laxation
1	Saline (10 mL/kg)	10	3.5 ± 0.76	0.33 ± 0.11	9.52
2	CCh	1	10.83 ± 0.74 **	5.16 ± 0.70 **	47.6
3	CD.Cr (p.o)	70	7.53 ± 0.76 *	2.55 ± 0.50 *	33
4	CD.Cr (p.o)	150	8.33 ± 0.66 **	3.66 ± 0.55 **	44
5	CD.Cr (p.o)	300	3.00 ± 0.68	0.34 ± 0.22	11.11

Values shown are mean ± SEM, n= six animals/group. * $p < 0.05$, ** $p < 0.01$ expresses an evaluation of group 2 -7 versus group 1 (One way ANOVA followed by Dunnet's test)

exhibited the more strong effects. In atropinized tissue these respective effects of $CHCl_3$.CD, Et.Ac.CD and Aq.CD fractions were completely blocked. Pet-ether fraction did not show any excitatory effect (fig. 5).

The crude extract were reassessed for excitatory effects in tissues pretreated with pyrilamine and methysergide, no change was observed in the resultant response compared to control responses in rat-ileum preparations, hence data was not shown.

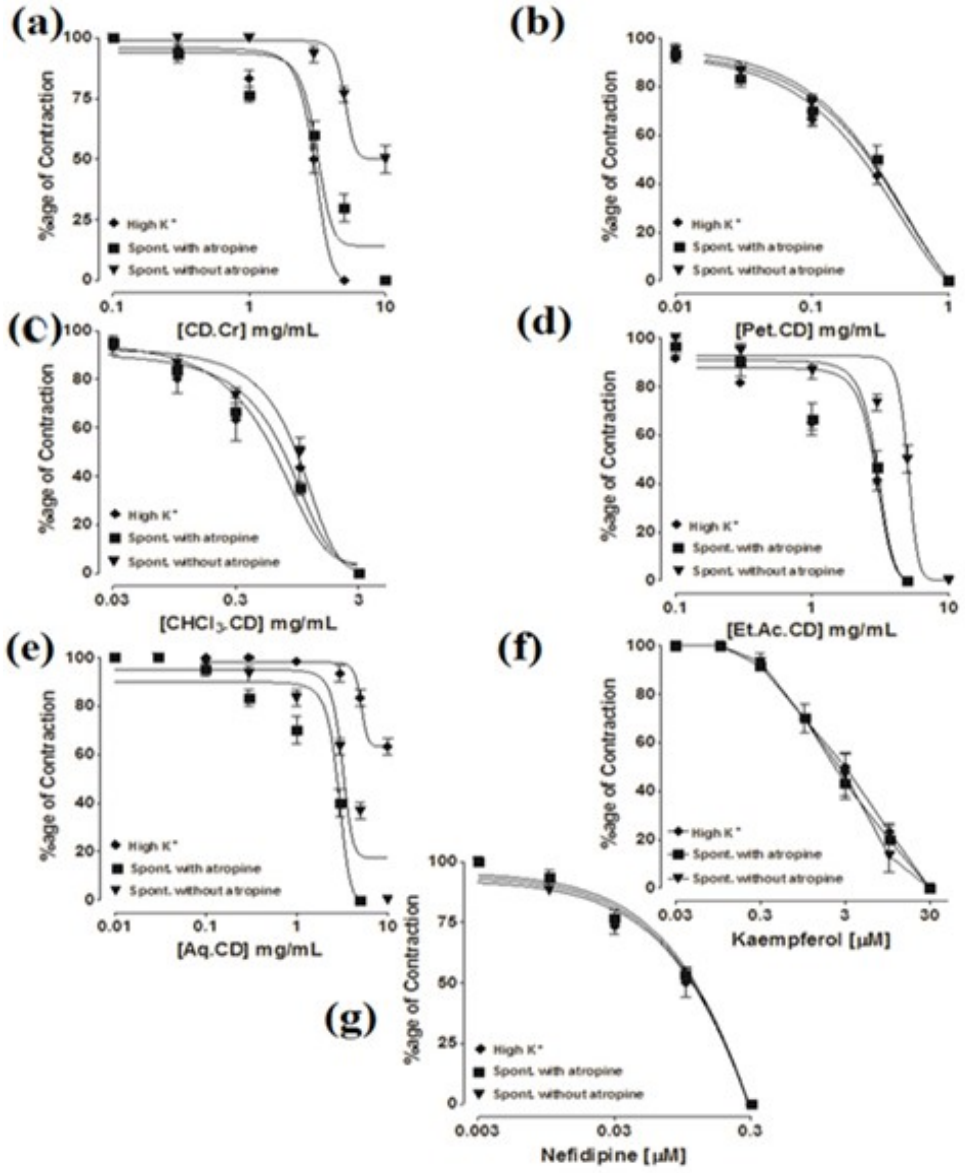

Fig. 2: The concentration-related inhibitory effect of the *C. decidua* stem extract (CD.Cr) (a) and its petroleum ether (Pet.CD) (b), chloroform ($CHCl_3$.CD) (c), ethyl acetate (Et.Ac.CD) (d), aqueous (Aq.CD) fractions (e) kaempferol (f) and nifedipine (g) against spontaneous (with and without atropine) and high K^+ (80 mM)-created contractions on rabbit-jejunum. Values are stated as mean ± S.E.M, n = 4-6.

DISCUSSION

C. decidua has been established safe up to the highest tried dose of 10 g/kg. Its vernacular usage in constipation was endorsed when at lesser doses of 70 and 150 mg/kg, it enhanced the total fecal output along with the wet feces in mice. Constipation results from the slow bowel movements, which leads to infrequent and/or hard movement of stool. On the other hand laxatives increase the water contents of feces and the frequency of defecation (Chatoor and Emmanuel, 2009). Carbachol, a cholinomimetic drug (Brown and Taylor, 2006), increased the gut motility through cholinergic activation (Dijken & Wied, 1961). Carbachol (positive control) also enhanced the total fecal output, its water contents and frequency of defecation. On the other hand CD.Cr at dose of 300 mg/kg showed very weak laxative effects. *C. decidua* is also famous for antidiarrheal effect in traditional system of medicine, which was validated when in castor oil-driven diarrhea, the *C. decidua* extract (500 and 700 mg/kg), kaempferol (50 and 100 mg/kg) and nifedipne (40 mg/kg) showed prominent antidiarrheal activity like loperamide in mice. Castor oil is a triglyceride, which produces ricinoleic acid in intestinal lumen by lipase

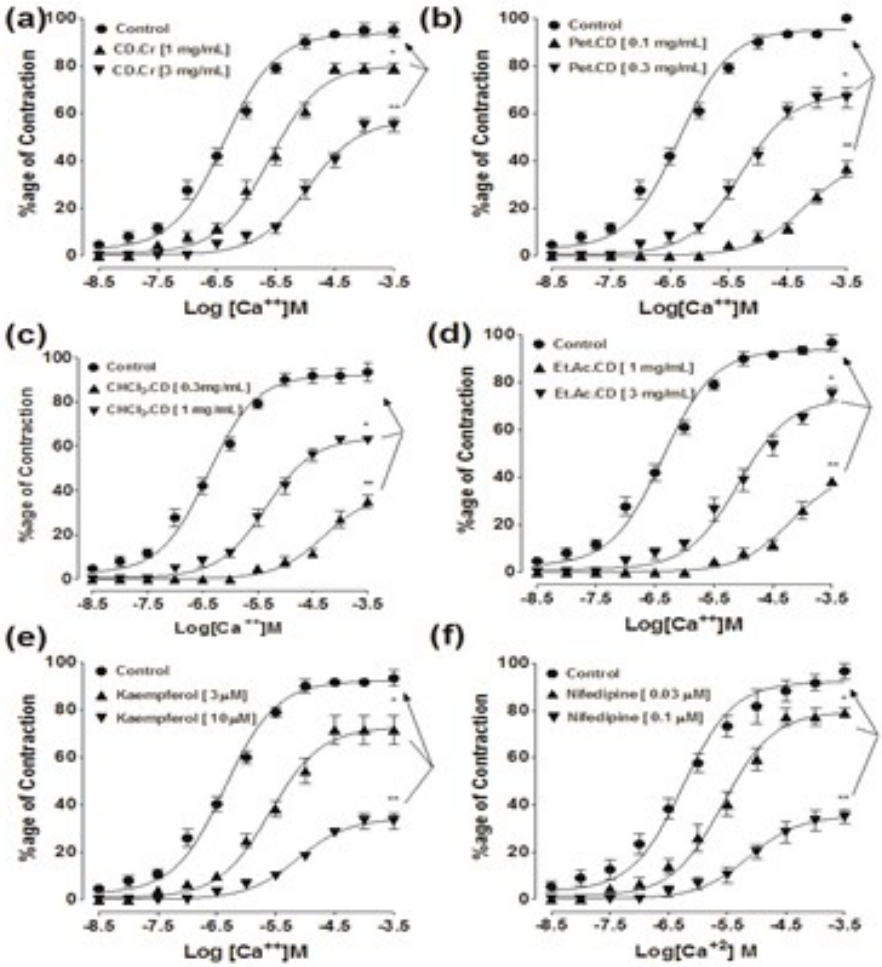

Fig. 3: The concentration–response curves of Ca^{++} with and without different concentrations of the crude extract of stem of *C. decidua* (a), respective fractions, petroleum ether (Pet.CD) (b), chloroform ($CHCl_3$.CD) (c), ethyl acetate (Et.Ac.CD) (d), kaempferol (e) and nifedipine (f). The values are stated as mean ± SEM from 4–6 determinations. *p < 0.05, **p<0.01 (two-way ANOVA followed by Bonferroni's post-test correction).

enzyme with a strong laxative effect by changing the transport of electrolytes, water and intestinal motility (Mathias *et al*., 1978). Thus, the agents which show the safety against castor oil-driven diarrhea are understood to have the antidiarrheal activity.

The isolated rat ileum is a semi-quiescent preparation and considered to be a better to characterize the stimulant effects of test substance (Unno *et al*., 2005; Najeeb-ur-Rehman *et al*., 2012). The crude extract of *C. decidua* exhibited excitatory effects in rat-ileum through cholinergic pathway involvement. It was confirmed when its excitatory effects were completely abolished after the addition of atropine, a well-known anticholinergic drug (Brown and Taylor, 2006), to tissue organ bath. This effect showed the presence of acetylcholine like spasmogenic components of *C. decidua* in addition to its spasmolytic constituents. Noteworthily Kaempferol was found devoid of any stimulatory effects.

In rabbit jejunum the *C. decidua* extract and kaempferol both showed dose dependent inhibitory effects. Isolated rabbit jejunum produces a regular pendular movement, a steady state of contraction and relaxation (Mehmood et al., 2011), which are govern through the rise in cytosolic Ca^{++} concentrations ultimately forming the Ca^{++} - calmodulin complex by depolarization (Bolton, 1979). The complete inhibition of rhythmic contractions of jejunum via test substance indicated its interference with cytosolic Ca^{++} influx.

To further test the underlying mechanism of these observed inhibitory effects, the *C. decidua* extract and kaempferol was studied against the high K^+-created contraction in rabbit jejunum. High K^+ (80 mM) involves the calcium influx through VDCCs (voltage dependent Ca^{++} channels) in the cell. The plant extract and kaempferol produced the dose-related relaxation of high K^+-created contractions similar to nifedipine, a known

Ca^{++} channel blocker (Godfraind, 2017), hence this effect confirms the interference of extract with calcium entry *via* antagonizing the calcium channels. The calcium channel blockers (CCBs) like activity was confirmed when CD. Cr and kaempferol at slightly higher concentrations moved the Ca^{++} CRCs to right by suppressing the highest response which indicates the Ca^{++} antagonist like activity of these agents like nifedipine. It is a well-known fact that the calcium channel blocker produced spasmolytic and antidiarrheal activity (Ghayur and Gilani, 2005; Taqvi *et al*., 2009), hence, the Ca^{++} antagonist like activity of *C. decidua* and kaempferol confirms their antidiarrheal effect.

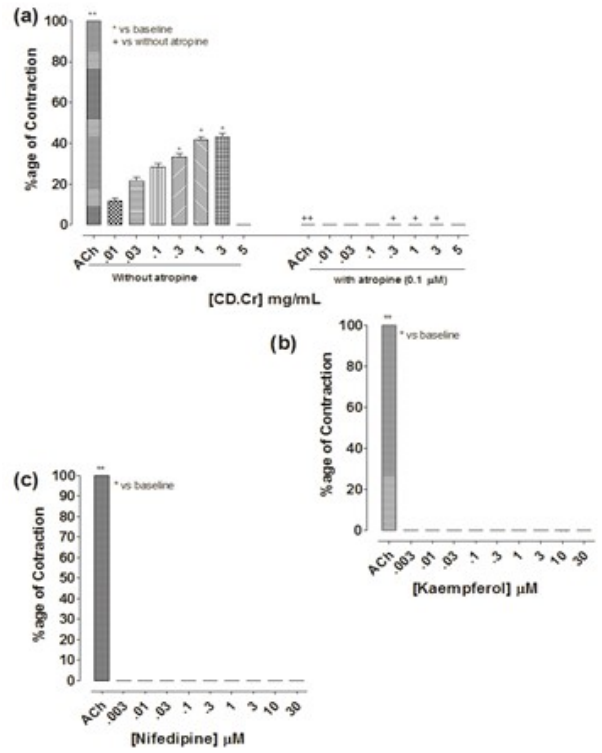

Fig. 4: The concentration-related excitatory responses of C. decidua stem extract (a), kaempferol (b) and nifedipine (c) without and with atropine (0.1µM) on isolated rat-ileum. Values are stated as mean ±S.E.M, n=4-6 {*/$^{+}$P<0.05 and **/$^{++}$P<0.01} vs. baseline and without atropine responses (One-way ANOVA followed by Dunnet's test).

Polarity based solvent-led extraction of *C. decidua* extract showed presence of both spasmolytic and spasmogenic components in different potency. The crude extract, its aqueous, ethyl acetate and chloroform fraction were found to possess both spasmolytic and spasmogenic components with varying degrees. The petroleum fraction was found to possess only relaxant effect. On further distribution of spasmolytic effectiveness, the pet-ether and chloroform fractions were found to possess prominent spasmolytic effects relative to ethyl acetate fraction while the aqueous fraction was observed with lesser spasmolytic activity, thus, indicating that the nonpolar plant components, as alkaloids and flavonoids, which are more likely to be soluble in organic solvents having spasmolytic property. As reported that the alkaloids and flavonoids as ephedrine, morphine, peprine, kaempferol, rutin, kaempferol etc (Ajay *et al*., 2003; Qiu *et al*., 2014) have spasmolytic properties also support our study. In rat ileum, CD. Cr and its fractions produced excitatory effects mediated through the activation of cholinergic pathway with different strength. The aqueous fraction was observed with the strongest excitatory effects followed by ethyl acetate and chloroform fractions while pet-ether was devoid of any excitatory effect, thus revealing the polar-nature of spasmogenic constituents. It is reported that flavonoids and cardiac glycosides have stimulant effect on duodenum (Ehile *et al*., 1990). As saponin, tanins, glycosides, flavonoids are more likely to be soluble in water which is also in-line to our studies.

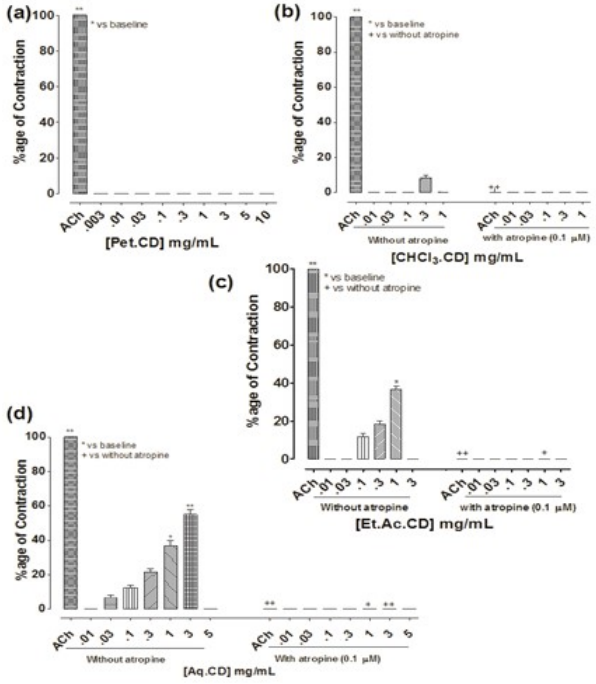

Fig. 5: The concentration-related excitatory effects of different fractions of C. decidua, petroleum ether (Pet. CD) (a), chloroform ($CHCl_3$.CD) (b), ethyl acetate (Et.Ac. CD) (c), aqueous (Aq.CD) (d) without and with atropine (0.1µM) on isolated rat-ileum. Values are stated as mean ± S.E.M, n=4-6 {*/$^{+}$P<0.05 and **/$^{++}$P<0.01} vs. baseline and without atropine responses (One-way ANOVA followed by Dunnet's test).

The data showed that excitatory effect of *C. decidua* is mediated through the muscarinic involvement, which increase the gut motility directly effecting on gut musculature, however the muscarinic agonist could not be directly used to treat the constipation due to their non-specificity in action and leads to undesirable effects as bradycardia, diarrhea, abdominal cramps, salivation,

convulsions, respiratory increase and increased urination (Pasricha, 2006). *C. decidua* possesses combination of gut stimulant and relaxant components by nature which are meant to neutralize their excessive effects when required. This dual nature of the plant is meant to overcome the harmful effects associated to the gut stimulant component as abdominal cramps and diarrhea mostly seen with other synthetic drugs used for the treatment of constipation etc. Therefore dual activity of *C. decidua* as excitatory (cholinergic agonist) and spasmolytic agent (Ca^{++} channel blockers) has the benefits to treat both the conditions of constipation and diarrhea. This dual nature of herbal remedy is commonly observed in other herbs as *Zingiber officinale* (Ghayur and Gilani, 2005), *Piper nigrum* (Mehmood and Gilani, 2010), Psyllium husk (Mehmood *et al*., 2011) and *Carissa carandas* (Mehmood *et al*., 2014). Thus these studies endorse the possible synergistic and /or adversative responses that overcome mixtures of same herbal/natural remedies.

Kaempferol, a flavonoid found in relatively high proportion in stem of this plant as analyzed in HPLC chromatogram was studied for its possible underlying spasmolytic mechanism, which is similar to nifedipine on high K^{+}-created contractions on rabbit-jejunum. In mice kaempferol also showed antidiarrheal effects against castor oil-driven diarrhea which clearly indicates that kaempferol partially collaborate for antidiarrheal activity of *C. decidua* in addition to other components as quercetin, β-sitosterol, and rutin, which are also documented to possess the spasmolytic activity (Ajay *et al*., 2003; Mehmood *et al*., 2014). The existence of these phytochemicals with spasmolytic activity supports its folk use in diarrhea and dysentery, while the presence of saponin, tanins, glycosides and flavonoids known to possess the spasmogenic activity (Ehile *et al*., 1990) supports its medicinal use in constipation.

CONCLUSION

These studies showed that *C. decidua* possess the laxative and antidiarrheal effects. The laxative effects are governed through cholinergic receptor activation while the antidiarrheal effects were intervened through Ca^{++} channels blockage. Further studies on fractions validated that the petroleum fraction produced its spasmolytic effects exclusively through involvement of calcium channels blockade pathway, aqueous fraction predominantly involve the cholinergic activation, while other chloroform and ethyl acetate fractions showed both spasmogenic and spasmolytic effects. This study also showed that kaempferol produced its antidiarrheal effects mediated through the blockage of calcium channels.

ACKNOWLEDGMENT

We would like to acknowledge the support of Department of Pharmacology, Faculty of Pharmaceutical Sciences, GCU, Faisalabad for lab facilities. We also acknowledge the support of animal house staff, Government College University Faisalabad, for assistance during the studies.

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