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Pharmacological evaluation and validation for the folkloric use of *Oligochaeta ramosa* in constipation and diarrhea

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Abstract

Crude extract of *Oligochaeta ramosa* and its fractions were studied to rationalize its traditional use in GIT disturbance. In spontaneous contracting jejunum preparation, *O. ramosa* (0.01-1.0 mg/mL) caused a transient spasmogenic effect followed by the spasmolytic effect at higher doses (3.0-10.0 mg/mL). In atropinized jejunum preparation, *O. ramosa* inhibit the spontaneous and K⁺ (80 mM)-induced contraction at the similar doses (0.01-1.0 mg/mL), suggesting calcium channel blocking effect. The calcium channel blocking effect was confirmed when pretreatment of tissue with *O. ramosa* produced a dose-dependent shift in Ca²⁺ dose- response curve to the right, similar to that produced by the verapamil. Activity-directed fractionation revealed that the spasmolytic effect is concentrated in the dichloromethane fraction while, aqueous fraction contains both spasmogenic and spasmolytic constituents. This study validate the presence of both spasmogenic and spasmolytic components mediated through muscarinic receptor activation and calcium channel blockade respectively, which may explain its traditional uses in constipation and diarrhea.

Introduction

Oligochaeta ramosa (Roxb.) (Family; *Asteraceae*) known by the Synonym of *O. albispina*; is a straggling herb (Qureshi and Bhatti, 2008). It's flowering and fruiting time of is from January to March. Leaves are obovate whereas heads are ovoid (pale-purple in color). The achenes are angled and grooved while base is narrow and dull-brown (Vardhana, 2008).

Phytochemical investigations revealed the presence of flavone (jaceosidine), sesquiterpene lactone (cynaropicrine, amberin, ambersins A, ambersins B, apigenin, crysoeriol, jaseocidine, ramosine, tricontane), long-chain esters, cycloartane-triterpenoids and glycoside (5,7,4'-trihydroxy-3,8-dimethoxyflavone 5-O-β-D-glucopyranoside) (Forgacs et al., 1981; Khan et al., 2004; Khan et al., 2005a; Khan et al., 2005b; Ibrahim et al., 2010; Ibrahim et al., 2012).

O. ramosa has folkloric reputation as laxative, anti-pyretic, antiemetic, antimicrobial, purgative, astringent, antidote and resolvent (Bhattacharjee, 2005; Khare, 2007) and is traditionally used to cure cough, wounds, skin irritation, external swelling, and diseases of liver (Kiritkar and Basu, 1987; Vardhana, 2008). For the blood purification juice of the fresh plant is used with black pepper (Qureshi and Bhatti, 2008). Beside a number of traditional uses, this herb was not previously pharmacologically evaluated for the possible mode(s) of action. In this study, we provide the evidence that the plant exhibit anti-diarrheal and antispasmodic activities, mediated possibly through the dual blockade of muscarinic receptors and Ca²⁺ channels. Moreover, the activity-guided fractionation of the crude extract was carried out, showing that the biologically active constituents were widely distributed in the organic and aqueous fractions.



Material and Method

Collection and extraction of plant material: *O. ramosa* (collected from the local punsar in Multan) was authenticated by an expert Taxonomist Prof. Altaf Dasti of Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan. Adulterants free plant material was crushed into coarse powder and subjected to triple maceration for extraction (Hussain et al., 2013). Coarse powdered material (1.5 kg) was macerated with 70% methanol in air tight amber glass bottles (25°C), with occasional shaking thrice a day for three days. After maceration, it was filtered through muslin cloth (double layered), followed by filtration through a Whatman-1 filter paper. The filtrate was stored in amber glass air-tight container. Same procedure was subsequently repeated twice after each two days and filtrates of these three macerations were combined and evaporated at 40-50°C in rotary evaporator (Rotavapor, BUCHI labrotechnik AG, Model 9230, Switzerland) attached with a vacuum pump and a recirculation chiller. The dark green crude extract was lyophilized to remove moisture contents and the approximate yield was 30%. Methanol crude extract of *O. ramosa* was stored in amber glass container at -20°C.

Extracts solution preparation and fractionation: Methanol crude extract of *O. ramosa* (0.3 g) was dissolved in 0.1 mL of 100% dimethyl sulphoxide (biologically inactive) and volume was made up to 1 mL with distill water to prepare stock solutions (300 mg/mL) (Hussain et al., 2014a). The stock solutions were subjected to series of dilutions to make 30, 3 mg/mL on the day of experiment. For the activity-directed fractionation, crude extract of *O. ramosa* (20 g) was dissolved in sufficient volume of distilled water and an equal volume of the dichloromethane was added into it and was shaken vigorously in a separating funnel (Williamson et al., 1963) and after overnight hanging on the stand, extract was separated into two layers; upper aqueous layer and lower organic solvent layer (DCM). Organic solvent layer was evaporated on rotary evaporators, whereas aqueous layer was lyophilized to remove moisture contents. For experimentation (*in vitro*), DCM was dissolved in 10% dimethyl sulfoxide, while aqueous extract was dissolved in distilled water/saline. The vehicles used for solubility were found to be inactive in all experiments.

Animals and housing conditions: Animals (male/female) used in this study were local strain rabbits (1.0-1.8 kg) and mice (30-40 g), which were kept at animal house (23-25°C) of Faculty of Pharmacy, Bahauddin Zakariya University, Multan. All rabbits had free access to food and tap water *ad libitum* but food was withdrawn atleast 24 hours prior the commencement of experiments. The rabbits were sacrificed following a blow on back of thehead and dissected to remove jejunum for *in*

vitro isolated tissue experiments. All the experiments were performed by following ruling of Institute of Laboratory Animal Resources, Commission on Life Sciences (National Research Council, 1996), approved by the Ethical Committee of Bahauddin Zakariya University, Multan, with having reference number EC/02/2011 dated, 14 February.

Drugs and chemicals: Acetylcholine chloride, atropine sulfate, carbachol, dicyclomine, cyproheptadine, pyrilamine, potassium chloride, verapamil hydrochloride and magnesium chloride, ethylene tetra-acetic acid (EDTA) were purchased from Sigma Chemicals Co. St Louis, MO, USA. Calcium chloride, glucose, magnesium sulfate, potassium dihydrogen phosphate, sodium bicarbonate, sodium dihydrogen phosphate, and methanol were obtained from Merck, Darmstadt, Germany. Ammonium hydroxide, sodium chloride, and sodium hydroxide were purchased from BDH Laboratory supplies, Poole, England. The vehicles used for the drugs solubilization, do not possess any effect on tissue functioning and contractility in control experiments.

Phytochemical screening: Methanol extract was subjected to phytochemical screening for the detection of alkaloids, carbohydrates, tannins, saponins, anthraquinones, steroids and flavonoids as possible important constituents of the plant, according to standard method (Evans, 2006). Appearance of yellowish brown coloration on mixing of Dragendorff's reagent with HCl treated aqueous plant extract solution, conform the presence of alkaloids in extract. Molisch's, benedict's and Fehling's tests were performed for the detection of carbohydrates. Formation of froth on vigorous shaking of the aqueous extract solution, conform the presence of saponin. Development of blue green or dark green coloration on mixing of aqueous FeCl₃ with extract solution indicated presence of phenols and tannins. The appearance of pink, violet or red coloration on exposure to NH₄OH of the mixture of benzene with aqueous solution of plant extract already acidified with 1% HCl was taken as presence of anthraquinones among the plant constituents. The plant material was deemed positive for flavonoids when it gave a yellow color with AlCl₃ reagent.

Acute toxicity test: For the acute toxicity of the plant, mice were used which were fasted 24 hours prior to test but had free access to water. Sixteen mice were divided into four groups (each 4). Group 1 (control group) was given normal saline (0.9% NaCl) orally whereas group 2, 3 and 4 were orally administered with 3, 5 and 7 g/kg of *O. ramosa* respectively. All 4 groups were observed for possible lethargy and mortality for 24 hours.

***In vitro* experiment on rabbit jejunum:** Methanolic crude extracts of the plant were tested on isolated rabbit jejunum preparations for possible presence of spasm-

genic and/or spasmolytic activity, as described previously (Gilani et al., 2000). Isolated rabbit jejunum segments (2-3 cm length) were suspended in 10 mL isolated tissue baths containing Tyrode's solution (KCl 2.68, NaCl 136.9, MgCl₂ 1.05, NaHCO₃ 11.90, NaH₂PO₄ 0.42, CaCl₂ 1.8 and glucose 5.55 mM), bubbled with 95% O₂ and 5% CO₂ (carbogen), at normal body temperature (37°C). A preload of 1 g was applied and intestinal responses were recorded isotonicly using Bioscience transducers and Harvard Student Oscillograph. Prior to the addition of any drug, each tissues were allowed to equilibrate for at least 30 min. Isolated rabbit jejunum preparations exhibit spontaneous rhythmic contractions and allow testing of the spasmogenic and/or spasmolytic effect without application of an agonist (Gilani et al., 1994). The contractile effect of the plant material was assessed as the percent of the maximum effect produced by the control drug, acetylcholine (1.0 μM).

Determination of Ca²⁺ antagonist activity: To assess whether the spasmolytic activity of the test plant was through calcium channel blockade (CCB), K⁺ (80 mM), as KCl was added to depolarize the spontaneously contracting isolated jejunum preparation (Farre et al., 1991), which produce a sustained contraction.

The test materials were applied in a cumulative manner to the sustained contractions to achieve concentration-dependent inhibitory response (Van-Rossum, 1963). The observed relaxant effect of the test materials on K⁺ (80 mM)-induced contraction was expressed as percent of the control response mediated by K⁺.

Calcium channel blocking effect of the test substances were confirmed by the previously reported method (Gilani et al., 2005). The isolated rabbit jejunum preparations were allowed to stabilize in normal Tyrode's solution, which were subsequently replaced with Ca²⁺-free Tyrode's solution to which EDTA (0.1 mM) was added for 30 min, in order to remove calcium from the tissues. This bath solution was further replaced with K⁺-rich and Ca²⁺-free Tyrode's solution, having the following composition (mM): MgCl₂ (1.05), KCl (50), NaHCO₃ (11.90), NaCl (91.04), glucose (5.55), NaH₂PO₄ (0.42), and EDTA (0.1). Subsequent to an incubation period of 30 min., cumulative Ca²⁺ concentrations were applied to the tissue bath to obtain control calcium dose-response curves. On achievement of the superimposable control calcium dose-response curves (usually after two cycles), the tissues were then washed and incubated with the plant extract for 60 min. Then concentration response curves of Ca²⁺ were constructed and compared to the control curves. The concentration response curves for Ca²⁺ were developed in the presence of different concentrations of the plant to assess a possible Ca²⁺ channel blocking effect (Bolton, 1979).

Statistical analysis: The data is expressed as mean ±

standard error of mean and EC₅₀ (median effective concentration) values are given with 95% confidence intervals (95% CI) and the logarithmic dose response curves were plotted by using "Graphpad Prism" version 6, (Graph Pad Software, San Diego, CA, USA). Student t-test was applied for assessment of the observations. p<0.005 was believed to be statistically significant.

Results and Discussion

The crude extract of *O. ramosa* was found to contain alkaloids, glycosides, saponin, tannin, flavonoids, steroidal compounds, carbohydrates, ketones, pentose and soluble starch while anthraquinones and coumarins were absent.

In view of its traditional use in constipation, crude extract of *O. ramosa* was tested on spontaneously contracting isolated rabbit jejunum preparations which exhibited the dual effect (spasmogenic as well as spasmolytic) (Figure 1). Crude extract caused a dose-dependent spasmogenic effect at the dose range of 0.01-1.0 mg/mL, while at the next higher dose (3.0 mg/mL) spasmolytic effect is observed with EC₅₀ of 1.67 mg/mL (95% CI: 0.32-2.95, n=5). The observed contractile responses to crude extract were expressed as percentage of the maximal response to acetylcholine (0.3 μM), i.e., 17.9 ± 3.5, 17.7 ± 7.8, 20.2 ± 8.8, 21.8 ± 5.7, 38.2 ± 5.2 and 46.2 ± 5.2 (mean ± SEM; n=5), at tissue bath concentrations of 0.01, 0.03, 0.1, 0.3 and 1.0 mg/mL respectively (Figure 2) which indicate that either the crude extract has a partial agonist activity or the spasmogenic effect is accompanied by spasmolytic effect.

It has been previously observed that spasmogenic (contractile) effect of the plants is usually mediated through cholinergic mechanism (acetylcholine like) (Gilani et al., 2000; Gilani et al., 2005), so for the conformation of this mechanism, spontaneous contracting isolated jejunum preparations were pretreated with 0.1 μM of atropine (anti-muscarinic agent) and waited for 25 min (tissue equilibration) before the administration of the plant extract (Arunlakhshana and Schild, 1959). Spasmogenic effect of the crude extract was completely abolished in atropinized tissue (Figure 1), while stimulatory effect of histamine remained unchanged, suggesting that the stimulatory effect is mediated through an acetylcholine like mechanism. This acetylcholine-like effect was further authenticated when pyrilamine (histamine (H₁) receptor blocker) pretreated tissue, did not alter the response of the extract or acetylcholine (Sharif et al., 1994), and while completely block the effect of the histamine as expected. Acetylcholine (neurotransmitter) plays an important physiological role to regulate the peristaltic movement of the gut by acting on muscarinic (M₃) receptor and atropine (antagonist) blocks

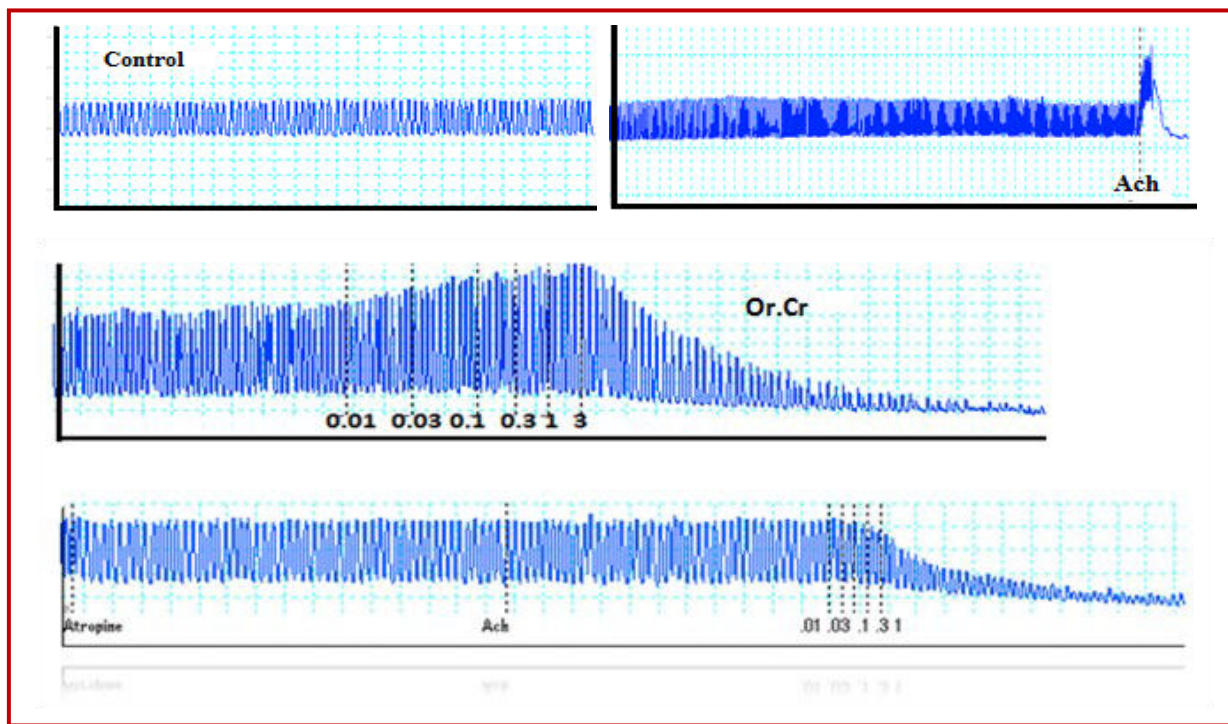


Figure 1: Typical tracing showing the spasmolytic effect of the crude extract of *O. ramosa* in comparison to acetylcholine in the absence and presence of atropine (0.1 μ M) on spontaneously contracting isolated rabbit jejunum

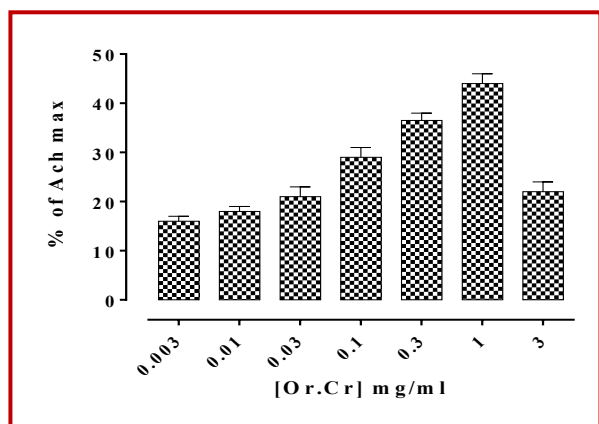


Figure 2: Bar diagram showing the effect of the crude extract of *O. ramosa* in comparison to the acetylcholine maximum response in isolated rabbit jejunum preparation (values are expressed as mean \pm SEM; n = 3)

muscarinic receptors (Brown and Taylor, 1996).

When crude extract of *O. ramosa* applied on hyperactive jejunum preparation, spasmogenic effect was less marked followed by the spasmolytic effect at dose concentration while in the atropinized preparation, spasmolytic effect was noted at lower concentration, shifting the concentration-response curve to the left (Figure 3). Spasmolytic effect of the medicinal plants is mostly mediated through calcium channel blocker (s) like activity (Gilani et al., 2000; Gilani et al., 2005). When crude extract was tested for its possible Ca^{2+}

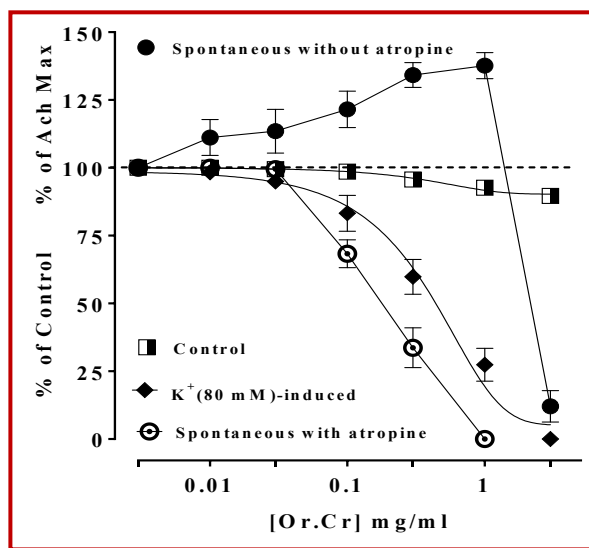


Figure 3: Concentration-response curves, showing the effect of crude extract of *O. ramosa* in the absence and presence of atropine (0.1 μ M) on spontaneous and K^+ (80 mM) contracted isolated rabbit jejunum (values are expressed as mean \pm SEM; n = 3)

antagonistic mechanisms, it caused the dose dependent relaxation of the K^+ (80 mM)-induced contraction of the rabbit jejunum (Figure 3). High K^+ (>30 mM) cause the smooth muscle contraction through the opening of voltage-dependent L-type Ca^{2+} channels (VDLCs), thus allowing influx of extracellular Ca^{2+} causing contractile effect (Bolton, 1979; Godfraind et al., 1986). Sufficient

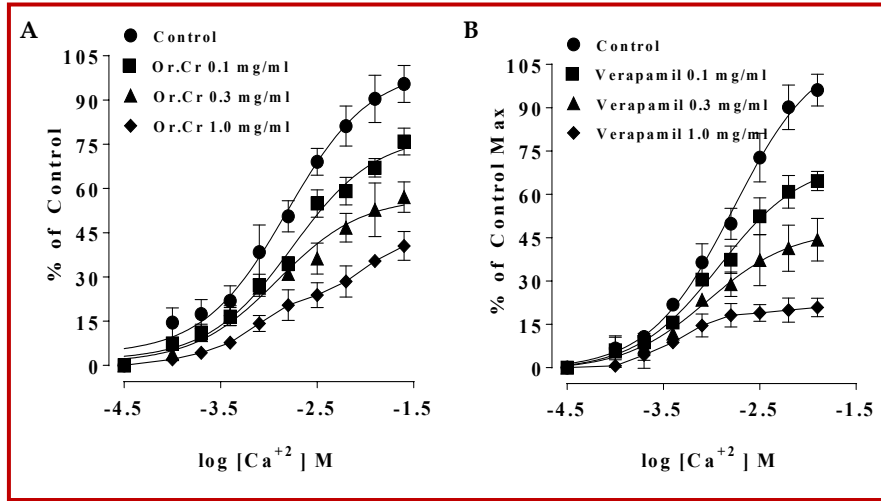


Figure 4: Concentration–response curves of Ca^{2+} showing the inhibitory effect of increasing concentrations of: (A) crude extract of *O. ramosa* and (B) verapamil in isolated rabbit jejunum (values are expressed as mean \pm SEM; n = 3)

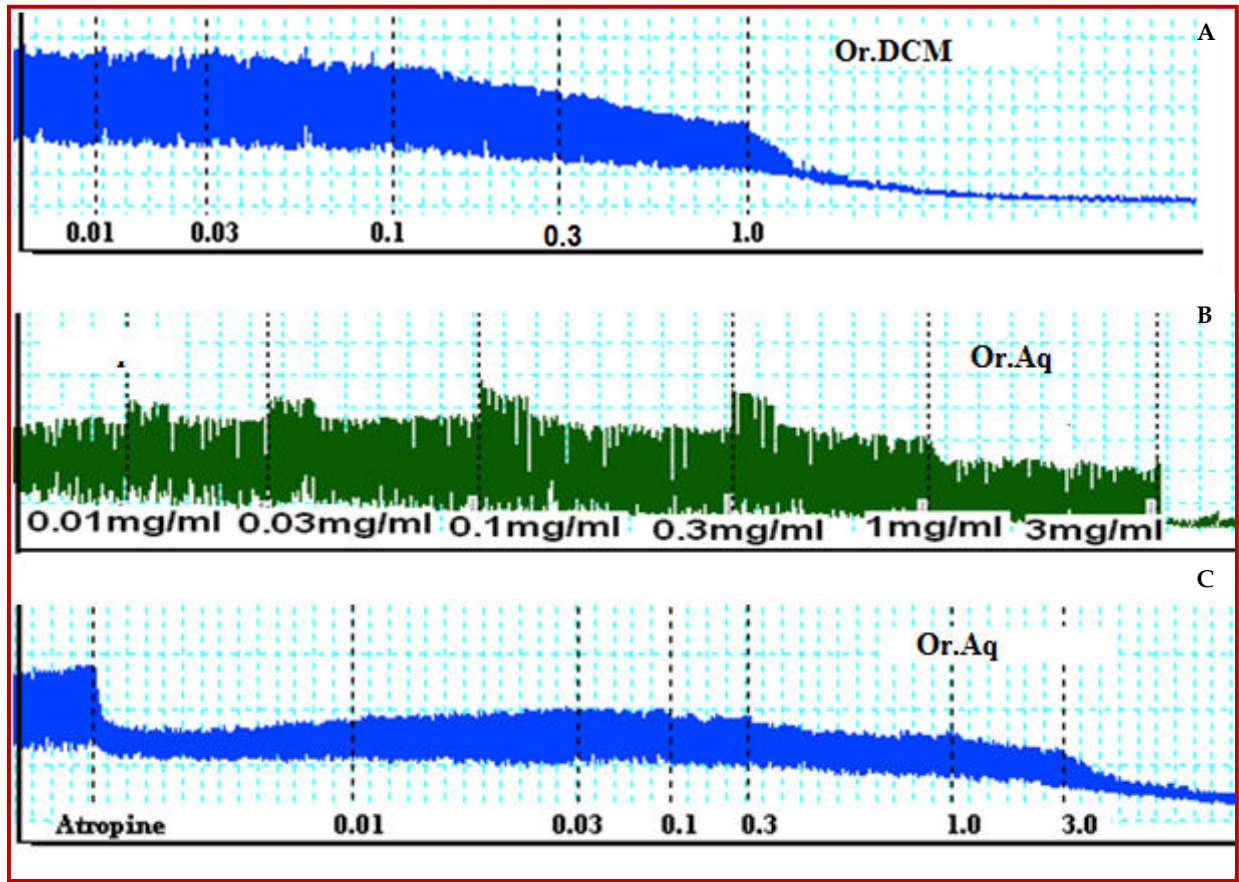


Figure 5: Typical tracing showing the (A) spasmolytic effect of the organic fraction of *O. ramosa*, (B) spasmogenic effect of the aqueous fraction of *O. ramosa* and (C) spasmolytic effect (on atropinized tissue) of aqueous fraction of *O. ramosa*, on spontaneously contracting isolated rabbit jejunum

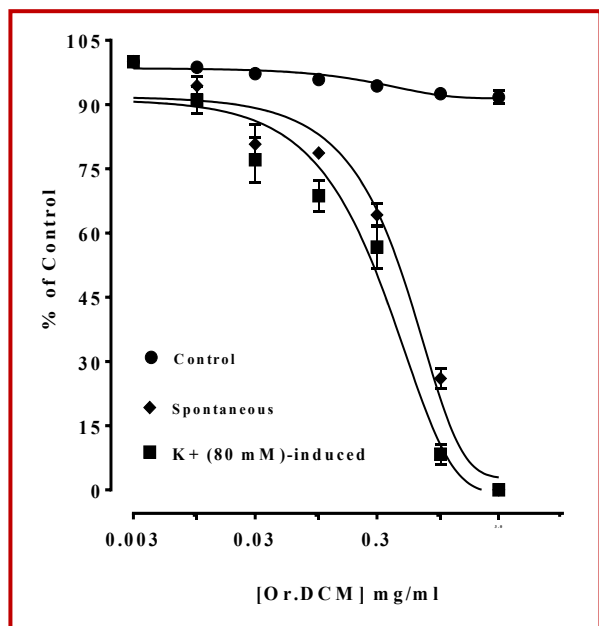


Figure 6: Concentration–response curves showing the effect of organic fraction of *O. ramosa* on spontaneous and K⁺ (80 mM) contracted isolated rabbit jejunum (values are expressed as mean ± SEM; n = 3)

amount of Ca²⁺ enters through Ca²⁺ channels to produce smooth muscle contraction by activating the intercellular contractile protein (Bolton, 1979) and K⁺ (80 mM)-induced contraction utilize Ca²⁺ influx (Chiu et al., 1986). Standard calcium channel blockers drugs (nifedipine, verapamil and diltiazim) prevent depolarization-induced Ca²⁺ influx by binding to voltage sensitive calcium channels (Fleckenstein, 1977). Thus inhibitory effect of the crude extract against K⁺ (80 mM)-induced contraction can be visualized as an outcome of restricted Ca²⁺ entry through voltage dependent calcium channels. Calcium channel blockers-like activity was further confirmed when pretreatment of tissue with crude extract caused a rightward shift of the Ca²⁺ response curve in a manner similar to that of verapamil (Figure 4), a standard calcium channel blocker (Hamilton et al., 1986). The observed spasmolytic effects at higher tissue bath concentrations are in good agreement with the phytochemical analysis of the extract, indicating presence of flavonoids among plant constituent (Jalpure et al., 2002). Oral dose of crude extract of *O. ramosa* as high as 7 g/kg, did not produced the lethality among the treated groups of mice.

In order to separate the constituents responsible for the spasmogenic and spasmolytic activities, activity directed fractionation was carried out which revealed that spasmolytic effect was separated in organic fraction (DCM) (Figure 5A) which cause the inhibition of spontaneous as well as K⁺ (80 mM)-induced contraction at the dose range of 0.01– 1.0 mg/mL, with EC₅₀ of 0.707 mg/mL (95% CI: 0.487 – 1.029; n = 5) and 0.54 mg/mL (95% CI: 0.384 – 0.75, n = 5) respectively (Figure 6). The

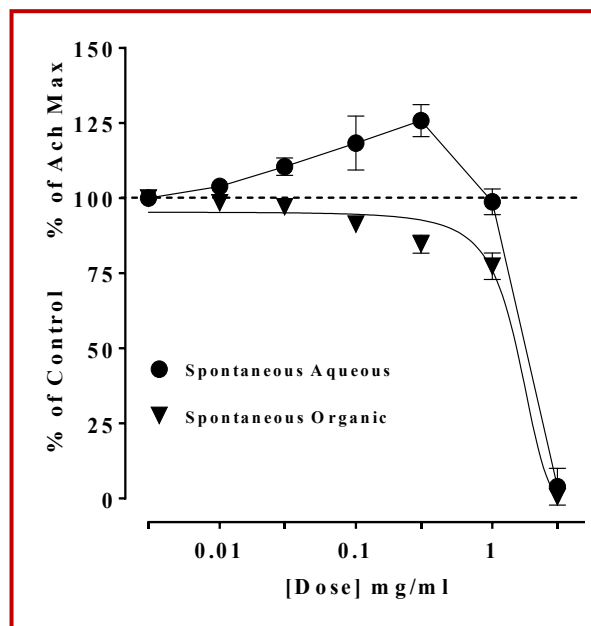


Figure 7: Concentration–response curves showing the effect of aqueous and organic fraction of *O. ramosa* on spontaneous contraction of isolated rabbit jejunum (values are expressed as mean ± SEM; n = 3)

spasmogenic effect was found to be exhibit in the aqueous fraction (Figure 5B), followed by the relaxation at higher concentration, while pretreatment of the tissue with atropine (0.1 μM) abolish the spasmogenic effect, conforming the cholinergic effect (Figure 5C). The aqueous fraction could not be tested against K⁺ (80 mM)-induced contraction due to limited supplies. The aqueous fraction was found to possess a combination of weak spasmolytic and spasmogenic activities (Figure 7).

This studied data clearly indicate the presence of two components (calcium antagonistic and cholinomimetic) in crude extract of *O. ramosa*. The calcium antagonistic activity provides pharmacological validation for its use in diarrhea, while cholinomimetic activity is likely to play role as laxative (aperient) and provides mechanistic basis for its possible use in constipation.

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