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CITRULLUS COLOCYNTHIS;

FRACTIONATION OF METHANOLIC EXTRACT OF CITRULLUS COLOCYNTHIS FOR SPASMOGENS.

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ABSTRACT... Objectives: To evaluate the methanolic extract of Citrullus colocynthis and its different fractions for possible spasmogenic effects on rat's ileum model. **Study Design:** An experimental study. **Setting:** Department of Pharmacology, Institute of Basic Medical Sciences, Khyber Medical University, Hayatabad, Peshawar. **Period:** August 2016 to March 2017. **Methodology:** Different fractions i.e n-hexane, chloroform, Ethyl acetate, n-butanol and aqueous fractions of methanolic extract were tried in concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 5.0, 10 and 15 mg/ml to identify the portion with maximum spasmogens. **Results:** Aqueous and n-Butanol fractions showed spasmogenic effects on the spontaneous contractions of rat's ileal preparations at the concentrations of 0.1, 0.3, 1.0, 3.0 and 5.0 mg/ml while at 10 and 15 mg/ml, they showed spasmolytic effects. Ethyl acetate and chloroform fractions did not show significant spasmogenic effects, rather showed spasmolytic effects in rabbit's jejunal preparations with respective EC50 values of 0.1283 ± 0.007 mg/ml and 2.703 ± 0.235 mg/ml. Respective EC50 values for spasmolytic effects of Ethyl acetate and chloroform fractions upon KCl induced contractions were 5.37 ± 0.515 mg/ml and 12.36 ± 1.484 mg/ml. Respective EC50 (log [Ca++] M) of ethyl acetate fraction at 1mg/ml vs control were -2.563 ± 0.04 and -2.846 ± 0.035. Similarly, respective EC50 (Log[Ca++]M) in the absence and presence of 0.1 μM verapamil were -2.45 ± 0.06 and -1.7 ± 0.07. EC50 (log [Ca++] M) for control vs chloroform fraction at 3 mg/ml and 5 mg/ml were -2.95 ± 0.035 vs -3.02 ± 0.03 and -3.06 ± 0.0264, respectively. Similarly, respective EC50 (Log[Ca++]M) in the absence and presence of 0.1 μM verapamil were -2.45 ± 0.06 and -1.7 ± 0.07. **Conclusion:** Thus, it is concluded that spasmogens concentrated in the residual aqueous fraction followed by n-butanol fraction while spasmolytic constituents are concentrated in the ethyl acetate fraction followed by chloroform fraction.

Key words: Fractionation, Citrullus Colocynthis, Spasmogens.

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INTRODUCTION

Citrullus Colocynthis is an ancient herbal plant, commonly called as the Colocynth, Bitter cucumber or Bitter apple. Basically, it is a perennial herb which is mostly found in Khyber pakhtunkhwa, Punjab and Sindh regions of Pakistan.[1] It is also found in Central and Southern parts of India, various parts of Arabia, western Asia and tropical Africa. It usually exists in Mediterranean regions and Coromandal coast. As far as phytochemical constituents of Citrullus colocynthis are concerned, several experimental studies have shown that pulp of fruit contains colocynthin, colocynthein, colocynthetin, iso-vitexin, iso-orientin and pectin gum. Seed contains 17% fixed oils (stearic, myristic, palmitic, oleic, linoleic acid), 6% albuminiods, 8.25% proteins (lysine, leucin, methionine), vitamins (B1, B2, niacin) and minerals (Mg, Ca, K, Mn, P, Fe, Zn).[2,3] Pharmacologically, C. colocynthis has got anti-inflammatory, anti-candidal, anti-bacterial, anti-oxidant,anti-proliferative,anti-fertility, anti-alopecia and growth inhibitory activity on breast cancer cells.[4,5,6] It is also useful as hypoglycemic, mosquito larvicidal, hypolipidemic and analgesic agent.[7,8,9] C. colocynthis is of great medicinal value. Fruits of Citrullus colocynthis are effective as anti-pyretic, anti-ulcer, anti-helmintic, anti-asthmatic, purgative and carminative. It is useful in the treatment of jaundice, ascites, spleenomegaly,

leucoderma, tuberculous glands, elephantiasis, urinary incontinence, bronchitis and sore throat. Roots are effective in cough and rheumatoid arthritis. Its root poultice is used for treating boils, mastitis and other skin lesions.[10]

There are some other traditional uses of this plant like in diarrhea, which proposed the presence of spasmogenic principles in its constituents. Using rabbit's Jejunal model, spasmogenic activity of the crude methanolic extract of C. colocynthis has been reported. Our work was an attempt to isolate those spasmogens by activity guided fractionation of methanolic extract of C. colocynthis.

MATERIAL AND METHODS

Collection and authentication of plant materials

The study was carried out at the department of Pharmacology, Institute of Basic Medical Sciences, Khyber Medical University, Peshawar. After collection of fresh whole plant from Malakand division of Khyber Pakhtunkhwa, it was properly washed and air dried under shade.

Extract preparation and fractionation

The air dried whole plant of Citrullus colocynthis was evenly grinded and macerated in methanol (80%) with occasional shaking. After 15 days, the whole material was filtered through ordinary filter paper. It was subjected to evaporation using rotary evaporator. Finally, a dark brown chocolate like extract was obtained. Methanolic extract of Cc(72.2 g) was fractionated successively, yielded 0 gram of n-hexane (Cc. n-hex), 6.8 grams of chloroform (Cc. CHCl3), 6.3 grams of ethyl acetate (Cc. EtOAc), 3 grams of n-butanol (Cc. n-BuOH) and 11.7 grams of residual aqueous fraction (Cc. Aq).[11]

Drugs and animals

All chemicals/drugs used in the experiments were of analytical grade and prepared on the same day of experiment. New Zealand rabbits of either sex weighing 2-2.5 kg and Dawley wistar rats of either sex weighing 180-230 grams were utilized. The rabbits were fasted for 24 hours before the experiment. However, they had free access to water.[11]

Preparation of extract portions of different strengths

For each fraction of extract, stock solutions of strength 300 mg/ml, 30 mg/ml and 3 mg/ml were prepared for pharmacological screenings.

Data recording

Force Transducer (Model No: MLT 0210/A Pan Lab S.I.) attached with Power lab AD Instruments, were used for recording the data of intestinal responses. Lab chart was set at the rate of 40/S, with range 20 mv, low pass and 5 Hz $\times$ 10 gain.

Statistical analysis

Microsoft excel sheet was used for calculation of means, standard deviations and construction of bar charts. Graphpad prism was used for the construction of graphs and determination of EC_{50}. Student 't' test was used to determine the level of significance i.e. at 95% of confidence level, $p \leq 0.05$.

Preparation of Rat's ileum

The spasmogenic activity of Citrullus Colocynthis was performed on rat's ileum model as per standard protocol.[12] After obtaining small segments (1-1.5 cm each) of ileum, they were mounted in Tyrode's solution containing organ bath, supplied with carbogen gas at temperature of 37 $\pm$ 1 °C. After stabilization, it was treated with 0.3 μM of Ach 2 to 3 times to get the control maximum of Ach. Different fractions of plant extract (residual water, n-butanol, ethyl acetate and chloroform) were tested in concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 5, 10 and 15 mg/ml at small intervals and responses were recorded.

Effects on calcium channels, cholinergic and histamine receptors

In order to determine the possible mechanisms of spasmogenic activity, tissues were incubated sequentially with 0.3 μM atropine (a cholinergic receptor antagonist), 0.08 μM loratadine (a standard H1 blocker) and 0.1 μM verapamil (a standard calcium channel blocker) each for 30 minutes. Then dosing of the test samples were applied in similar concentrations and responses in the absence and presence of standard blockers were recorded.

Preparation of Rabbit's jejunum

As fractions of methanolic extract of Citrullus colocynthis also showed relaxant effects at higher doses and we know that ileal preparations merely tells us about the relaxant effects, therefore, anti-spasmodic activity of C. Colocynths was performed on rabbit's jejunal model.[12] Different fractions of plant extract were tested in similar concentrations and responses were recorded.

Effects on KCl induced contractions

In order to determine the possible mechanisms of relaxation, effects of test samples on KCl (80mM) induced contractions were determined in rabbit's jejunal preparations.[13,14]

Effects on calcium channels

Because of relaxant effects on KCl (80mM) induced contractions, different fractions were further tested for possible blockage of voltage gated calcium channels. Calcium chloride curves were constructed in the absence and presence of test samples.[15,16] After stabilizing the tissues in normal tyrode's solution, they were decalcified with potassium normal tyrode's solution and then potassium rich tyrode's solution respectively, 2-3 times. Using calcium chloride, in the concentration range of 1×10^{-4} - 256×10^{-4} M, standard calcium curves were constructed for the tissues that served as control. Following same procedure, medium was again decalcified and tissues were treated with different doses of different fractions of C. Colocynthis. After incubation period of an hour, again calcium curves were constructed in similar fashion. The curves were compared to its respective control curve. Similar curves were constructed for verapamil as well and both were compared for possible right shift.[11]

RESULTS

Effects on rat's ileal preparations

According to Figure-1(A), Cc. Aq causes concentration dependent increase in the amplitude of spontaneous contractions with maximum effect of 138.7% of ach max (0.3 μM) at the concentration of 5 mg/ml. However, it started relaxing the spontaneous activity at 10 mg/ml with maximum relaxation of 74.8 % of control maximum at 15 mg/ml. In the presence of atropine (0.3 μM), the spasmogenic activity of extract got markedly reduced to 29.1 % of ach max at 5 mg/ml dose. When extract dosing was applied after 0.08 μM loratadine and 0.1 μM verapamil, the spasmogenic activity of extract got completely lost. The EC50 value for spasmogenic effect of Cc. Aq, in the absence of atropine was 3.07 ± 0.340 mg/ml. According to Figure-1(B), Cc. n-BuOH increased the amplitude of spontaneous contractions with maximum effect of 117.9 % of ach max (0.3μM) at the concentration of 5 mg/ml. It started relaxing the spontaneous activity at 10 mg/ml i.e. 58.75 % of ach max (0.3μM). In the presence of atropine (0.3 μM), the spasmogenic activity of extract got markedly reduced to 48.35 % of ach max at 5 mg/ml dose. When extract dosing was applied after 0.08 μM loratadine and 0.1 μM verapamil, the spasmogenic activity of extract got completely lost. The EC50 value for spasmogenic effect of Cc. n-BuOH, in the absence of atropine was 3.143 ± 0.310 mg/ml. According to Figure-1(C), in the absence of atropine, Cc. CHCl3 showed maximum spasmogenic effect of only 4.4 % of Cmax but showed relaxant activity at 1.0 mg/ml. When extract dosing was applied after 0.3 μM Atropine, 0.08 μM loratadine and 0.1 μM verapamil, the spasmogenic activity of the extract got completely lost. It is clear from Figure-1(D) that Cc. EtOAc did not show any possible spasmogenic activity on spontaneous rat's ileum preparations.

Effects on rabbit's jejunal preparations and KCl induced contractions

As shown in Figure-2(A), both ethyl acetate and chloroform fractions completely relaxed the spontaneous jejunal contractions. Respective EC50 values for spasmolytic effects of Cc. CHCl3 and Cc. EtOAc on spontaneous jejunum contractions were 0.1283 ± 0.007 mg/ml and 2.703 ± 0.235 mg/ml. According to Figure-2(B), Cc. EtOAc completely while Cc. CHCl3 partially relaxed the KCl (80 mM) induced contractions. Respective EC50 values for spasmolytic effects of Cc. CHCl3 and Cc. EtOAc on KCl induced contractions were 5.37 ± 0.515 mg/ml and 12.36 ± 1.484 mg/ml.

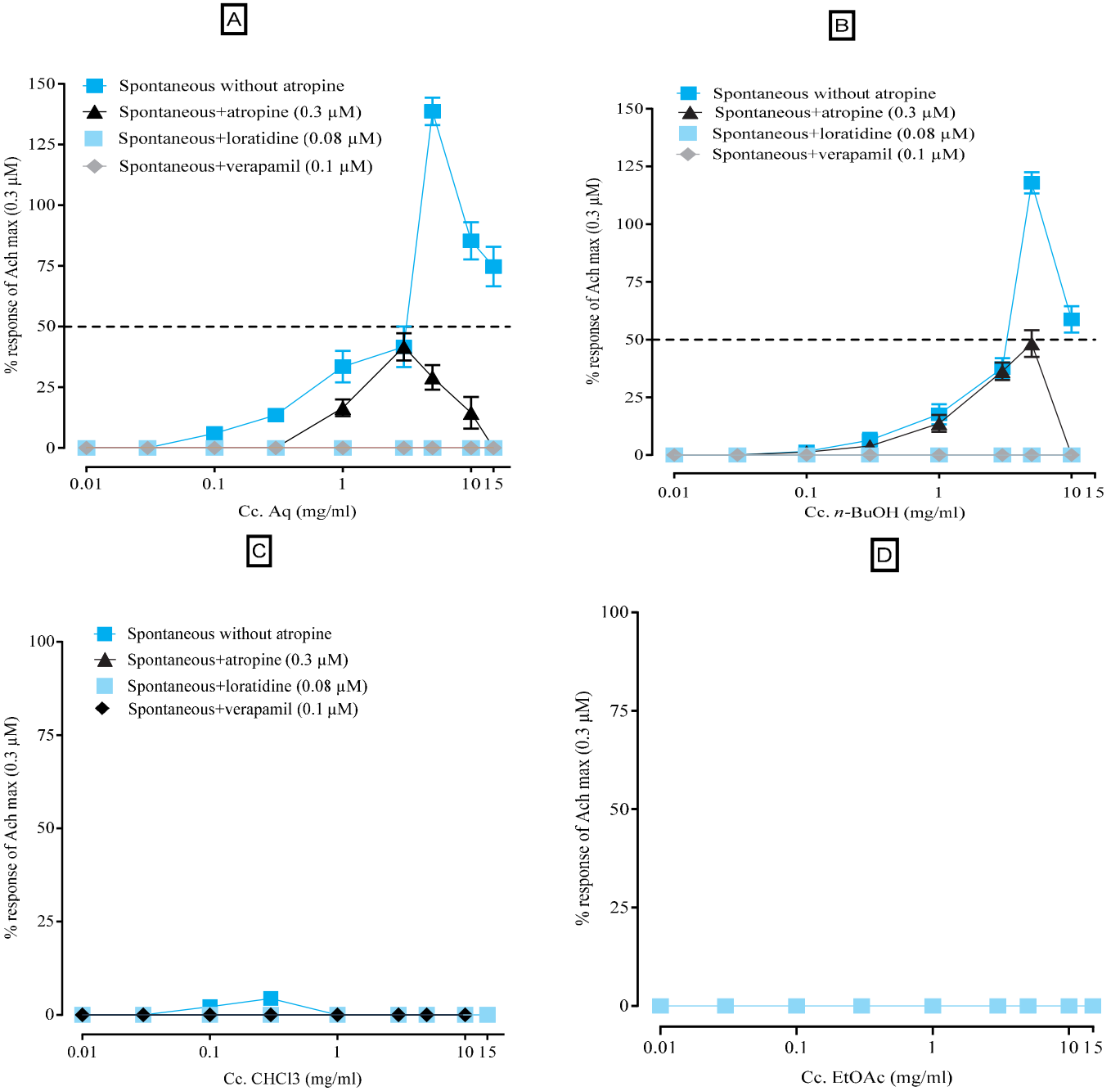

Figure-1. Effects of various concentrations of different fractions of Citrullus colocynths on rat's ileum. Spasmogenic effects are expressed as % response of Ach max (0.3 µM), n=3.

According to Figure-3(A), respective EC50 (log [Ca++] M) of ethyl acetate fraction at 1mg/ml vs control were -2.563 ± 0.04 and -2.846 ± 0. Similarly, respective EC50 (Log[Ca++]M) in the absence and presence of 0.1 µM verapamil were -2.45 ± 0.06 and -1.7 ± 0.07 as shown in Figure-3(B).

According to Figure-4(A), EC50 (log[Ca++] M) for control vs chloroform fraction at 3 mg/ml and 5 mg/ml were -2.95 ± 0.035 vs -3.02 ± 0.03 and -3.06 ± 0.0264, respectively. Similarly, respective EC50 (Log[Ca++]M) in the absence and presence of 0.1 µM verapamil were -2.45 ± 0.06 and -1.7 ± 0.07 as shown in Figure-4(B).

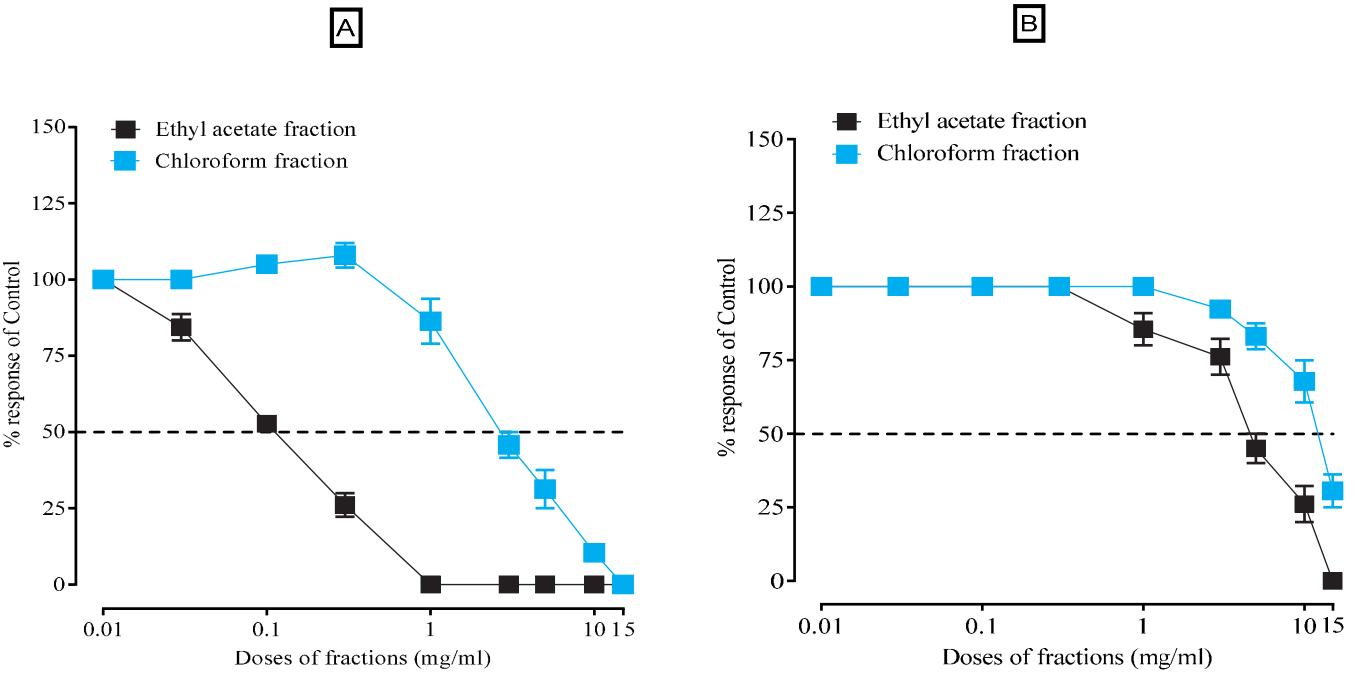

Figure-2(A). Effects of Cc. EtOAc and Cc. CHCl3 on spontaneous jejunal contractions.
(B) Effects of Cc. EtOAc and Cc. CHCl3 on KCl (80mM) induced contractions in rabbit's jejunal preparations, n=3.

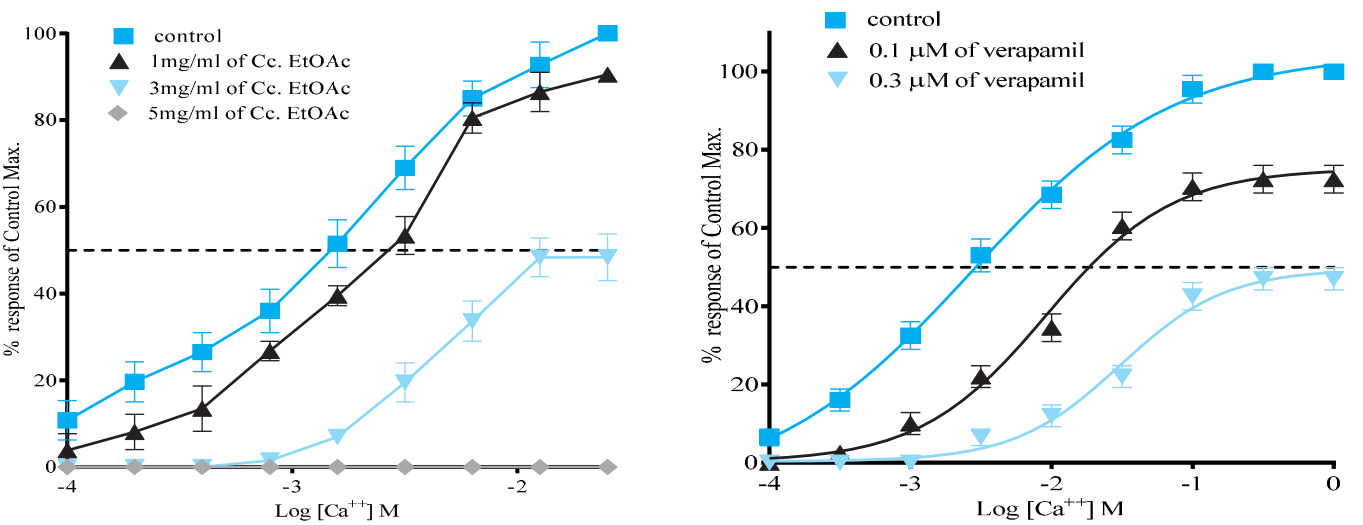

Figure-3(A). Calcium chloride curves in the absence and presence of Cc. EtOAc.
(B) Calcium chloride curves in the absence and presence of verapamil, n=3.

DISCUSSION

Citrullus colocynthis is an ancient herbal plant. Number of scientific experiments revealed its medicinal importance. Spasmogenic activity of the crude methanolic extract of C. colocynthis has been reported so our work was an attempt to isolate those spasmogens by activity guided fractionation of its methanolic extract.

Our study proved that spasmogens are concentrated in different fractions with order of spasmogenic potency as: Aqueous >n-butanol > chloroform > ethyl acetate. Thus, Cc. Aq and Cc. n-BuOH could be the source of

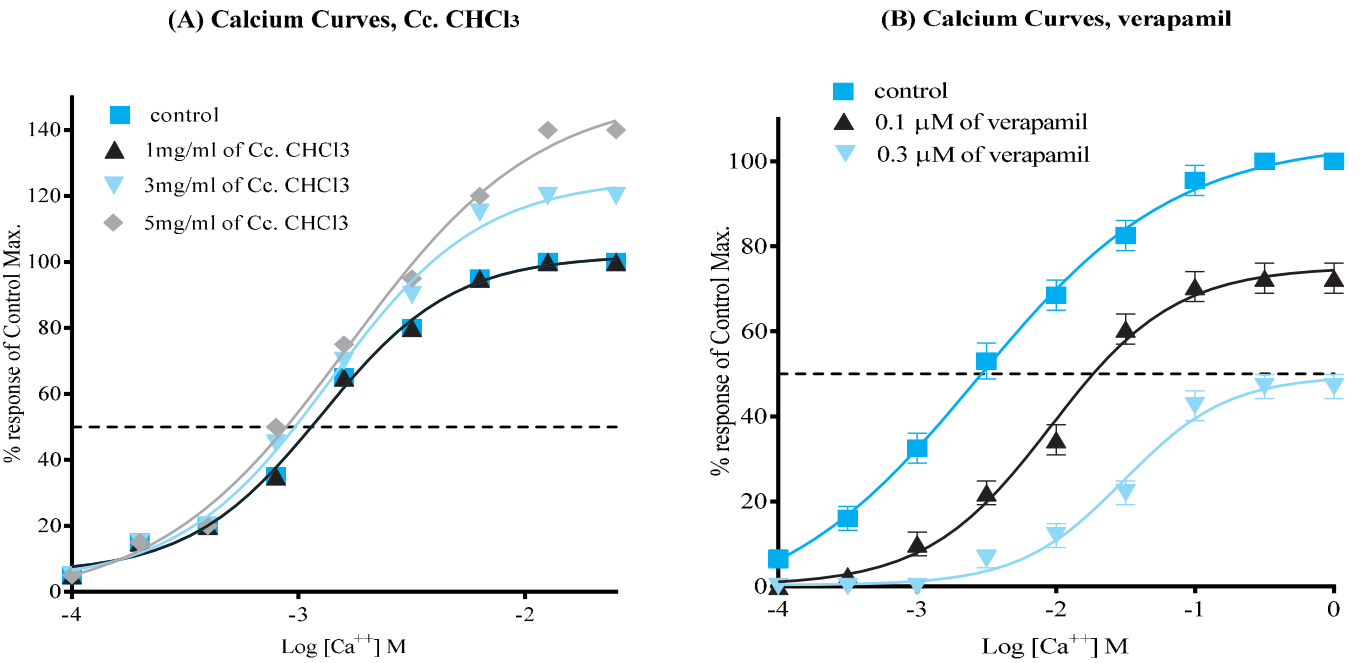

Figure-4 (A). Calcium chloride curves in the absence and presence of Cc. CHCl3. (B) Calcium chloride curves in the absence and presence of verapamil, n=3.

spasmogens of C. colocynthis. Spasmogenic activity in smooth muscles can be carried out by multiple mechanisms/receptors/channels. It is an established fact that cytosolic calcium is the major factor for production of spasmogenic response by contractile proteins. This cytosolic calcium is regulated by different mechanisms like release of calcium from internal stores and entrance of calcium from external sources.[17,18,19] Acetylcholine, a standard spasmogen exerts its spasmogenic effect by increasing intracellular calcium through various mechanisms like release of calcium from calcisomes, cytoplasmic reticulum and also by ionositol tri-phosphate dependant pathways.[20] Histamine receptors in gastro-intestinal tract are also involved in spasmogenic activity when get stimulated.As clearly depicted by our results, spasmogenic activity of all fractions of C. colocynthis extract were partially blocked by atropine and totally blocked by loratadine and verapamil, suggesting that they follows mix pathway i.e. cholinergic receptors, histaminergic receptors and voltage gated calcium channels to produce spasmogenic response.

It is also clear from our results that chloroform and ethyl acetate fractions were devoid of spasmogens, so we expected these portions to have predominant spasmolytic activities for which rabbit's jejunal model was used as per standard procedure.[14] It was found that spasmolytic constituents are concentrated in the Cc. EtOAc followed by Cc. CHCl3.KCl induced contractions mostly follows voltage gated channels.[21] As shown in our results, both Cc. CHCl3 and Cc. EtOAc relaxed the high molar (80 mM) KCl induced contractions, so it is inferenced that they may follow the voltage gated calcium channels. In order to confirm it, calcium chloride curves were constructed.[12,22] As the right shift for verapamil resembled to that of right shift for test sample, so it is deduced that Cc. EtOAc follows voltage gated calcium channels for calcium influx. On the other hand, Cc. CHCl3 doesn't follow voltage gated calcium channels as there was left shift. Further work up need to be done to determine the exact mechanism.

CONCLUSION

The results confirmed that spasmogens are concentrated in residual Aqueous fraction followed by n- butanol fraction. They follows cholinergic receptors, histaminergic receptors and voltage gated calcium channels to produce

spasmogenic response. It was also found that spasmolytic constituents are concentrated in the ethyl acetate fraction followed by chloroform fraction. Ethyl acetate fraction follows voltage gated calcium channels to exhibit its relaxant effect while mechanism for chloroform fraction still need to be confirmed.

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The only thing we have to fear is fear itself.

– Unknown –

AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	M. Saleh Faisal	Planning, Evaluation of study, Data collection, Manuscript writing, Statistical analysis.	
2	Niaz Ali	Planning of study, Data collection, Manuscript writing.	
3	Kashif Ali	Manuscript writing, statistical analysis.	