



**BJP**

**Bangladesh Journal of Pharmacology**  
**Research Article**

## Calcium entry blocking activity of the *Elaeagnus umbellata* fruit extract explains its use in diarrhea and gut spasm

Nayyab Rafique, Taous Khan and Abdul Jabbar Shah

Department of Pharmacy, COMSATS Institute of Information Technology, University Road, Abbottabad 22060, KPK, Pakistan.

### Article Info

Received: 26 December 2015

Accepted: 20 February 2016

Available Online: 7 June 2016

DOI: 10.3329/bjp.v11i3.26101

### Cite this article:

Rafique N, Khan T, Shah AJ. Calcium entry blocking activity of the *Elaeagnus umbellata* fruit extract explains its use in diarrhea and gut spasm. Bangladesh J Pharmacol. 2016; 11: 585-92.

### Abstract

This study was aimed to explore the pharmacological basis of potential medicinal use of *Elaeagnus umbellata* in gut disorders. Crude extract of *E. umbellata*, which was found positive for flavonoids, terpenoids and tannins, provided 9.9-71.9% protection in castor oil-induced diarrhea in mice, like verapamil. In isolated rabbit jejunum preparations, crude extract caused inhibition of spontaneous and high K<sup>+</sup>-induced contractions, with respective EC<sub>50</sub> values of 0.3 (0.1-0.5) and 0.5 mg/mL, suggesting Ca<sup>2+</sup>-channel blockade (CCB). Pretreatment of tissue with crude extract (0.1-1 mg/mL) caused a rightward shift in Ca<sup>2+</sup>-concentration-response curves. With the exception of aqueous fraction, *n*-hexane, chloroform and ethyl acetate inhibited spontaneous and high K<sup>+</sup>-induced contractions and displaced rightward Ca<sup>2+</sup>-concentration-response curves. Extract was found safe up to 10 mg/kg in mice. Our data shows that anti-diarrheal effect of crude extract of *E. umbellata* is due to CCB-mediated spasmolytic effect, concentrated in the ethyl acetate fraction and suggests its medicinal importance in diarrhea and spasm.

### Introduction

*Elaeagnus umbellata* Thunb (Autum olive, family; *Elaeagnaceae*) is a wild shrub abundant in northern areas of Pakistan (Hussain, 2011). Locally it's known as "Kankoli". Fruits, flowers and seeds of *E. umbellata* are used for medicinal purposes (Ahmad et al., 2005). Medicinal uses have been reported for various species of *Elaeagnaceae*; fruit of *E. phillypinus* have been used in the treatment of amoebic dysentery, flatulence, nausea and vomiting (Mohammed et al., 2006). Fruits of *E. aungustifolia* found useful in diarrhea and dysentery (Mohammed et al., 2006). In Chinese traditional medicine, fruits of *E. multiflora* are useful in itch, diarrhea, foul sours, cough and cancer (Lee et al., 2007). However, medicinal reputation of *E. umbellata* in hyperactive gut disorders, such as diarrhea and spasm is lacking.

The plant contains different phytochemicals, such as vitamins (A, C and E), flavonoids, essential fatty acids, lycopene, β-carotene, lutein, phytofluene, and phytoene (Fordham et al., 2001). β-Carotenes are effective in cancers. Lutein is an anti-oxidant and is as smooth muscle relaxant (Johnson et al., 2000). Phytoenes are anti-oxidant and anti-inflammatory (Khachik et al., 2002).

*E. umbellata* has not been explored pharmacologically in the past. However, few studies are available on its fruit extract such as antibacterial, against *Staphylococci* and *Escherichia coli* and anti-oxidant. *E. umbellata* was ignored in the past as a potential for pharmacological investigation.

The present study aimed at exploring the underlying pharmacological mechanism of the fruit extract of *E. umbellata*.

## Materials and Methods

### Plant materials

Fresh fruits of *E. umbellata* (6-7 kg) were collected from Sherwan, Abbottabad, Pakistan in the month of July, 2013 and was authenticated by Dr. Ghulam Mujtaba Shah, Department of Botany, Government Postgraduate College No. 1, Abbottabad. A voucher specimen (Eu-F-07-13) was deposited at herbarium located in the Research Lab of Pharmacology and Pharmacognosy, Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, Pakistan.

### Preparation of crude extract and fractionation

Extraction and fractionation was carried out as described previously (Williamsons et al., 1996). About 5 kg of fresh fruit was soaked in methanol at room temperature (23–25°C) for 15 days with occasional shaking. It was filtered through a muslin cloth and then through a Whatman qualitative Grade 1 filter paper. This procedure was repeated twice and the combined filtrates were evaporated on rotary evaporator under reduced pressure to a thick, semi-solid pasty mass of dark brown color; i.e. the crude extract, yielding approximately 16.8%. The crude extract was solubilized in 10% Tween 80. Fractionation was carried out, using solvents of increasing polarity. The extract was dissolved in distilled water, *n*-hexane was added to it and shaken vigorously in a separating funnel. The *n*-hexane layer (upper) was collected and evaporated on rotary evaporator to give the *n*-hexane fraction (yielding about 13%). The lower layer was taken in a separating funnel, chloroform was added. The chloroform layer (lower) was collected and evaporated on rotary evaporator to obtain the chloroform fraction (yielding about 21%). The other layer (upper) was again taken into a separating funnel, ethyl acetate was added into it, separated and was also evaporated in rotary evaporator to give the ethyl acetate fraction (yielding about 24%). The remaining lower layer was collected and evaporated to yield the aqueous fraction (yielding about 42%).

### Drugs and standards

The following reference chemicals were obtained from the sources specified: Castor oil (Karachi Chemical Laboratories, Pakistan), acetylcholine chloride, potassium chloride, Ca<sup>2+</sup> chloride and verapamil hydrochloride (Sigma Chemical Company, USA). All chemicals used were of the highest purity grade. Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh on the day of experiment.

### Animals

Balb<sup>c</sup> mice (20-25 g) and local rabbits (1.5-2 kg) used in the study were bred and housed in the animal house of CIIT Abbottabad under controlled environment (23-25°C). Animals were given tap water *ad libitum* and a standard diet.

### Preliminary phytochemical analysis

The crude extract of *E. umbellata* was screened for the presence of saponins, flavonoids, tannins, phenol, terpenes, terpenoids and alkaloids using the methods described by Edeoga et al. (2005).

### Acute toxicity test

The test was performed as described formerly (Gilani et al., 2005b). Groups containing five mice each were made. Group I acted as negative control was administered normal saline (10 mL/kg). Groups II-IV were treated with crude extract applied in incrementing doses (3, 5, and 10 g/kg) and were observed regularly for time period of 24 hours for lethality or toxic effects as flatness, gastrointestinal cramps and diarrhea.

### Castor oil-induced diarrhea

Previously described methods were modified for the determination of *in vivo* anti-diarrheal activity of the extract (Awouters et al., 1978; Shah et al., 2010a). Balb<sup>c</sup> albino mice after being starved for 18 hours were confined in nine groups with five mice in each individual cage, with their bases covered with blotting sheets. Group I, received normal saline (10 mL/kg, p.o) acted as vehicle control. Group II was administered castor oil. Groups III-VI were treated with four doses (3, 10, 30 and 100 mg/kg), selected on trial basis, of the crude extract of *E. umbellata*. Three doses of verapamil (1, 3 and 10 mg/kg) were given to mice placed in groups 7-9. All of the doses were administered orally through an intragastric feeding tube. Castor oil (10 mL/kg) was administered, except the saline treated group after one hour following the above treatments. All the groups were kept under observation for 4 hours after castor oil administration for defecation pattern. Percentage protection against the diarrhea induced by castor oil was calculated by comparing wet and dry fecal count in each cage.

### Determination of mechanism of anti-diarrheal action

#### Isolated tissue preparations (Video Clip)

The isolated tissue experiments were carried out as described previously (Shah et al., 2010b). The animals had free access to water but were fasted for 24 hours before the experiment. The animals were killed by cervical dislocation, the abdomen was cut open and the jejunal portion isolated out. Preparations 2 cm long were mounted in 10 mL tissue baths containing Tyrode's solution maintained at 37°C and aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The composition of Tyrode's solution, in mM, was: KCl 2.7, NaCl 136.9, MgCl<sub>2</sub> 1.1, NaHCO<sub>3</sub> 11.9 and NaH<sub>2</sub>PO<sub>4</sub> 0.4, glucose 5.6 and CaCl<sub>2</sub> 1.8 (pH 7.4). A preload of 1 g was applied and the tissues kept undisturbed for an equilibrium period of 30 min after which control responses to a submaximal dose of acetylcholine (0.3 mM) were obtained and the tissue presumed stable



only after the reproducibility of the said responses. Under these experimental conditions, rabbit jejunum exhibits spontaneous rhythmic contractions, allowing the testing of the relaxant (spasmolytic) activity directly without the use of an agonist (Gilani *et al.*, 1992).

#### Determination of calcium antagonist activity

To assess whether the spasmolytic activity of the test substances was mediated through  $\text{Ca}^{2+}$  channel blockade, a high concentration of  $\text{K}^+$  (80 mM), was used to depolarize the preparations and produced a sustained contraction. Plant extract and standard were then added in a cumulative fashion to obtain concentration-dependent inhibitory responses (van Rossum, 1963). The relaxation of intestinal preparations to plant extract and standard, precontracted with high  $\text{K}^+$  (80 mM), was expressed as the percent of the control precontraction. To confirm the  $\text{Ca}^{2+}$  antagonist activity of test substances, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with  $\text{Ca}^{2+}$ -free Tyrode's solution containing EDTA (0.1 mM) for 30 min in order to remove  $\text{Ca}^{2+}$  from the tissues. This solution was further replaced with  $\text{K}^+$ -rich and  $\text{Ca}^{2+}$ -free Tyrode's solution, having the following composition: KCl 50, NaCl 91.04,  $\text{MgCl}_2$  1.05,  $\text{NaHCO}_3$  11.90,  $\text{NaH}_2\text{PO}_4$  0.42, glucose 5.55 and EDTA 0.1 mM. Following an incubation period of 30 min, control concentration-response curves of  $\text{CaCl}_2$  were obtained. When the control concentration-response curves of  $\text{CaCl}_2$  were found super imposable (usually after two cycles), the tissue was pretreated with the plant extract for 60 min to test the possible  $\text{Ca}^{2+}$  channel blocking (CCB) effect. The concentration-response curves of  $\text{CaCl}_2$  were reproduced in the presence of different concentrations of the extract and standard.

#### Statistics

All the data expressed are mean  $\pm$  standard error of the mean (SEM), and the median effective concentrations ( $\text{EC}_{50}$  values) are given with 95% confidence intervals (CI). The statistical parameter applied is the student's *t*-test with  $p < 0.05$  noted as significantly different (Graph pad prism).

## Results

#### Effect on castor oil-induced diarrhea

Crude extract from the fruit of *E. umbellata* inhibited significantly ( $p < 0.05$ ) the frequency of defecation as well as wetting of feces compared with the untreated group, similar to verapamil. Both crude extract and verapamil reduced greatly the wetness of the fecal droppings and provided around 9.6-71.9% and 34.1-80.9% protection, respectively (Table I).

#### Effect on isolated rabbit jejunum preparation

Crude extract of *E. umbellata* inhibited isolated rabbit jejunum spontaneous contractions, similar to that observed with verapamil (Figure 1). This inhibition was concentration-dependent and maximum inhibition occurred at 5 mg/mL, with  $\text{EC}_{50}$  value of 0.3 mg/mL (0.1-0.5). Verapamil also inhibited spontaneous contractions (Figure 1). Crude extract caused a concentration-dependent inhibition of high  $\text{K}^+$ -induced sustained contraction with  $\text{EC}_{50}$  value of 3.4 mg/mL (2.6-4.2;  $n=5$ ) (Figure 2). Pretreatment of the jejunal tissues with crude extract (3-10 mg/mL) caused a rightward shift in the  $\text{CaCl}_2$  concentration-response curves. Verapamil also inhibited high  $\text{K}^+$ -induced contractions and shifted

Table I

#### Effect of *Elaeagnus umbellata* methanolic crude extract of the fruit on castor oil-induced diarrhea in mice

Group	Dose	Total number of feces in 4 hours	Total number of wet feces in 4 hours	Protection (%)
Control (saline)	10 mL/kg	4.8 $\pm$ 0.4	4.0 $\pm$ 4.0	9.6 $\pm$ 4.0
Castor oil	10 mL/kg	10 $\pm$ 0.6	98.2 $\pm$ 2.3	2.6 $\pm$ 2.3
+ crude extract	3 mg/kg	4.0 $\pm$ 0.5	90.0 $\pm$ 6.6	9.98 $\pm$ 6.6
+ crude extract	10 mg/kg	5.0 $\pm$ 2.5	82.7 $\pm$ 7.3	17.2 $\pm$ 7.3
+ crude extract	30 mg/kg	5.8 $\pm$ 0.5	65.8 $\pm$ 3.7	40.7 $\pm$ 3.7
+ crude extract	100 mg/kg	8.4 $\pm$ 0.2	28.6 $\pm$ 2.9	71.9 $\pm$ 3.0
+ Verapamil	1 mg/kg	5.8 $\pm$ 0.5	65.8 $\pm$ 3.7	34.1 $\pm$ 3.7
+ Verapamil	3 mg/kg	7.2 $\pm$ 0.3	43.4 $\pm$ 6.0	56.5 $\pm$ 6.0
+ Verapamil	10 mg/kg	8.4 $\pm$ 0.2	28.6 $\pm$ 2.9	80.9 $\pm$ 2.9

Data are Mean  $\pm$  SEM;  $n=5$  in each group;  $^a p < 0.05$ ,  $^b p < 0.01$  and  $^c p < 0.001$  vs control, Student's *t*-test

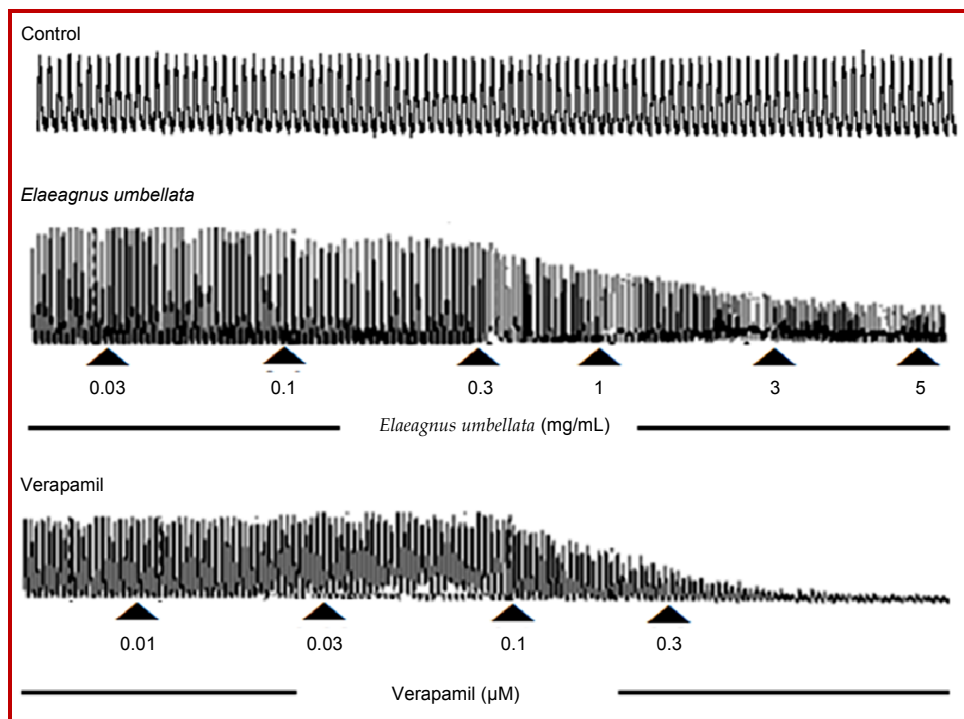


Figure 1: Typical tracing showing spasmolytic effect of *Elaeagnus umbellata* crude extract and verapamil in isolated rabbit jejunum

rightward  $\text{CaCl}_2$  concentration–response curves.

Among the fractions tested; the *n*-hexane, chloroform and ethylacetate inhibited spontaneous con-tractions, the later fraction was most potent with  $\text{EC}_{50}$  value 0.2 mg/mL (0.1-0.3) (Figure 3). *n*-hexane, chloroform and ethyl acetate inhibited high  $\text{K}^+$ -induced contractions. The later fraction was more potent than the parent crude extract and fractions, with  $\text{EC}_{50}$  value of 0.8 mg/mL (0.5-1.3). Pretreatment of the jejunal preparations with extract of *n*-hexane (3-10 mg/mL), chloroform (1-5 mg/mL) and ethyl acetate (0.1-1 mg/mL) caused a right ward shift in the  $\text{Ca}^{++}$  concentration-response curves. Interestingly, the aqueous fraction did not affect spontaneous and high  $\text{K}^+$ -induced contractions.

## Discussion

In castor oil-induced diarrheal model in mice, crude extract from the fruit of *E. umbellata* inhibited significantly ( $p < 0.05$ ) the frequency of defecation as well as wetting of feces compared with the untreated group, similar to verapamil (Karaki and Weiss, 1983; God-fraind et al., 1986). Both crude extract and verapamil reduced greatly the wetness of the fecal droppings and provided around 9.6-71.9% and 34.1-80.9% protection. The induction of diarrhea by castor oil results from the action of ricinoleic acid, which is formed as a result of the hydrolysis of the oil resulting changes in the transport of water and electrolytes in the intestine and leads to a hypersecretory response and generation of a

giant contraction of the intestine (Shah et al., 2011). This provides a base to understand that a potential anti-diarrheal agent may exhibit its anti-diarrheal effect by inhibiting gut motility (spasmolytic) and/or electrolyte outflux, the latter is evident in the form of diarrheal droppings. The protective effect of the crude extract of *E. umbellata* against the castor oil-induced diarrhea in mice, similar to verapamil, suggests that it might exert an inhibitory effect on intestinal smooth muscle contraction (intestinal motility) and or secretion. To see its possible inhibitory effect on gut motility, the crude extract was further studied in isolated rabbit jejunum.

Spontaneously beating isolated rabbit jejunum preparations are usually used to test possible antispasmodic effect of test substance because rabbit jejunum contracts spontaneously and does not require agonists to induced rhythmic contractions (Gilani et al., 1992). Crude extract suppressed rabbit jejunum spontaneous contractions in a concentration-dependent manner, similar to that caused by verapamil.

The contraction of smooth muscle preparations, including rabbit jejunum, is dependent upon increase in the cytosolic free  $\text{Ca}^{++}$  concentration, which activates the contractile elements and ensure contraction (Karaki and Weiss, 1983). The increase in intracellular  $\text{Ca}^{++}$  occurs either via influx through voltage-dependent  $\text{Ca}^{++}$  channels (VDCs) or its release from intracellular stores in the sarcoplasmic reticulum. Periodic depolarization and repolarization regulates the spontaneous movements of the intestine and at the height of depolarization, the action potential appears as a rapid influx of

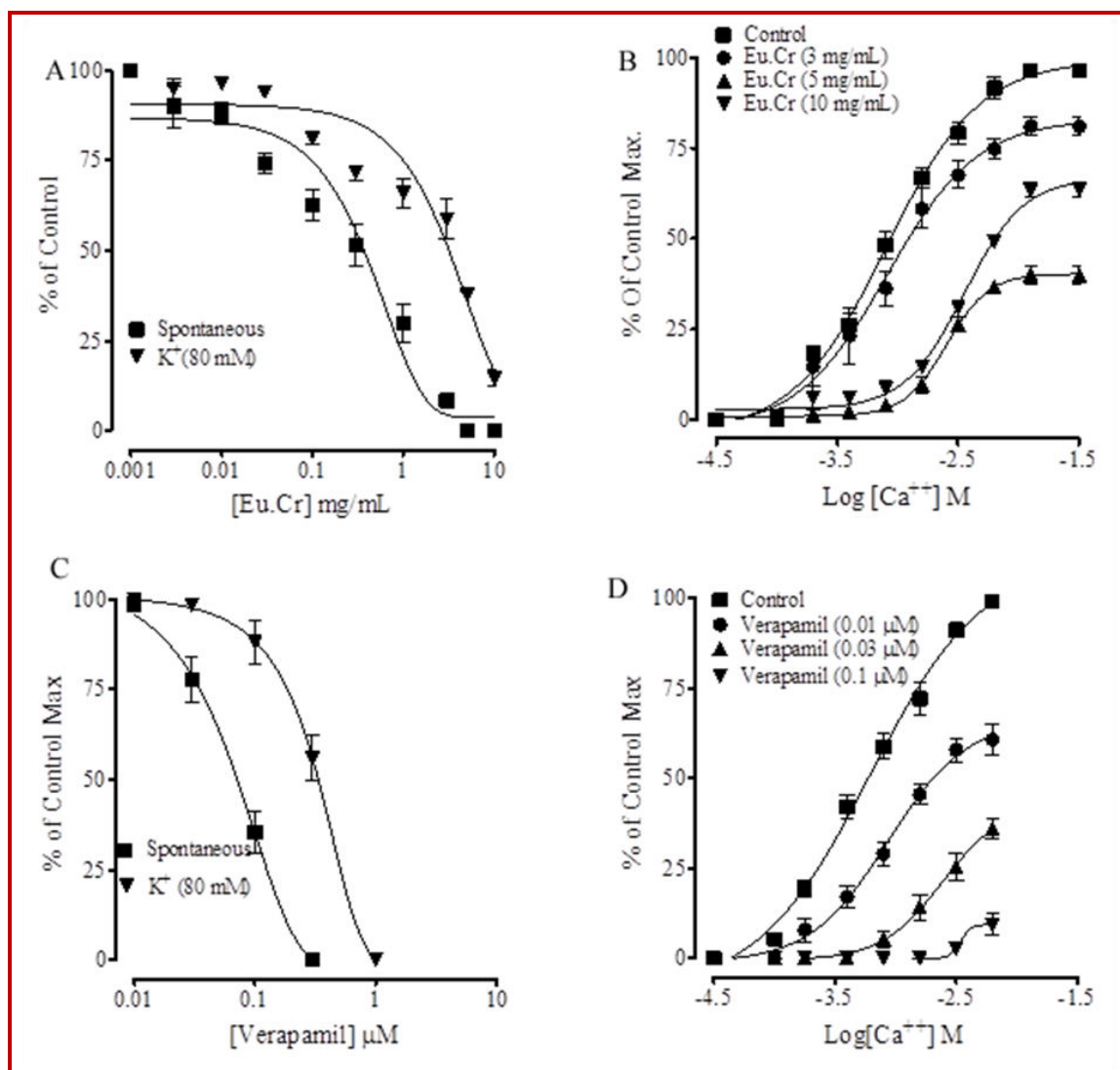


Figure 2: Concentration–response curves of the crude extract of (A) *Elaeagnus umbellata* (Eu.Cr) and (C) verapamil on spontaneous and high K<sup>+</sup> (80 mM)-induced contractions. Figure B and D show effect on CaCl<sub>2</sub> concentration–response curves in the absence and presence of different concentrations of the Eu.Cr and verapamil, respectively, in isolated rabbit jejunum preparations. Values shown are mean ± SEM (n= 6-7)

Ca<sup>2+</sup> via VDCs (Brading, 1981). Thus, the inhibitory effect of the crude extract on isolated rabbit jejunum spontaneous contraction seems due to a possibly interference with Ca<sup>2+</sup> influx through VDCs. We have previously observed that the spasmolytic constituents present in different medicinal plants mediate their effect through an inhibitor effect on Ca<sup>2+</sup> movement (Gilani et al., 2005a, 2005b). To see whether the spasmolytic effect of the crude extract is mediated through an inhibitor effect on Ca<sup>2+</sup> influx through VDCs, a high concentration of K<sup>+</sup> (80 mM) was introduced to depolarize the tissue. This depolarization produced sustained contraction of the jejunal preparation and allowed to see the effect of the crude extract and verapamil. Crude extract caused a concentration-dependent inhibition of high K<sup>+</sup>-induced sustained contraction in the isolated rabbit jejunal preparation,

similar to verapamil. This suggests that the spasmolytic effect of crude extract is, mediated through its inhibitory effect on Ca<sup>2+</sup> influx. Thus, the inhibition of high K<sup>+</sup>-induced contractions of rabbit jejunum by crude extract may reflect the restricted Ca<sup>2+</sup> entry via VDCs. This hypothesis was further strengthened when pre-treatment of the jejunal tissues with crude extract caused a rightward shift in the CaCl<sub>2</sub> concentration–response curves, similar to verapamil.

Further studies were carried out on the fractions to see, if any shift of the activity to any of the fractions. We obtained four fractions; *n*-hexane, chloroform, ethyl acetate and aqueous. When tested in the isolated rabbit jejunum preparations, *n*-hexane, chloroform and ethyl acetate fractions inhibited spontaneous and high K<sup>+</sup>-induced contractions. Ethyl acetate fraction was the

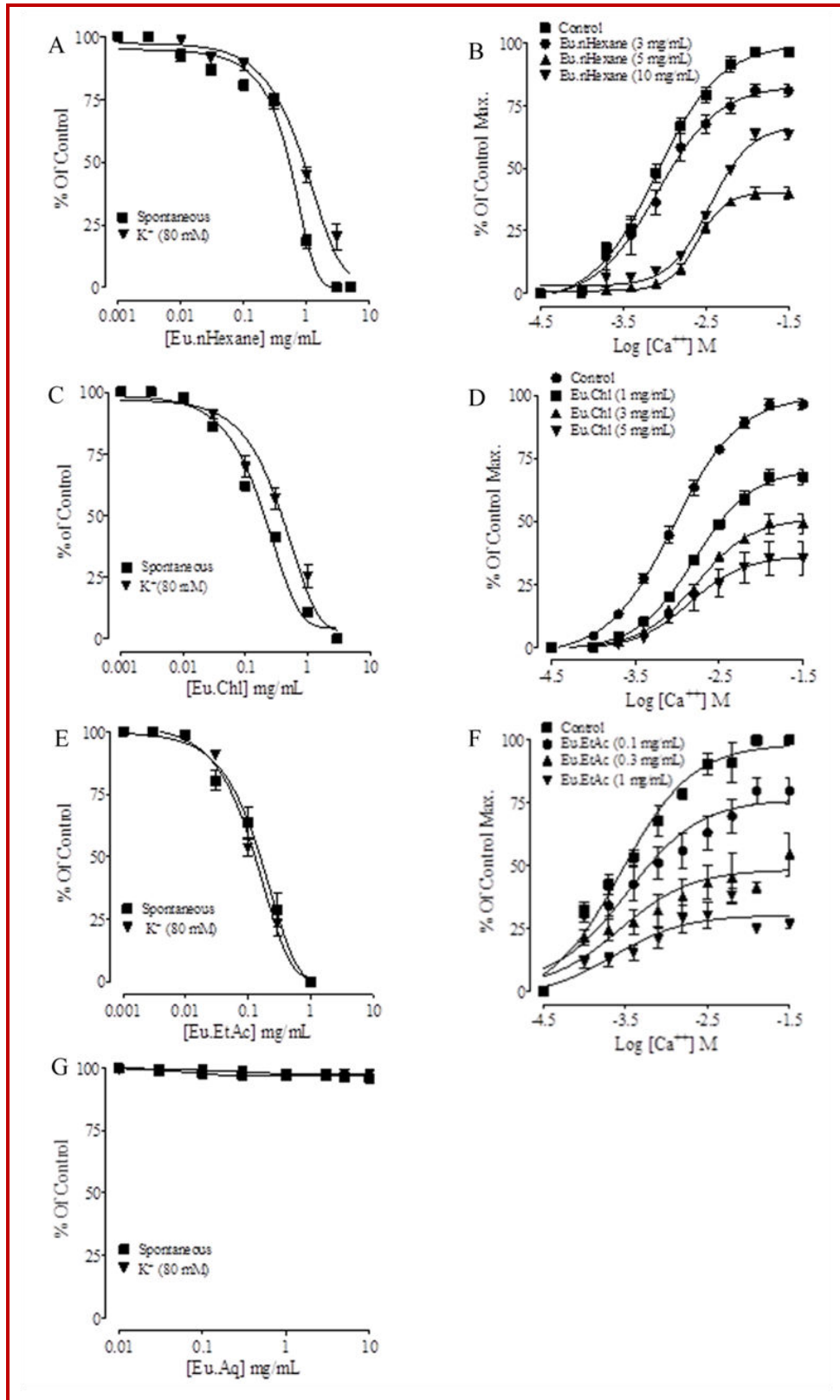


Figure 3: Concentration–response curves of *n*-hexane (Eu. *n*-hexane) (A) chloroform (Eu. Chl) (C) ethyl acetate (Eu. Et Ac) (E) and aqueous (Eu. Aq) (G) fractions on spontaneous and high K<sup>+</sup> (80 mM)-induced contractions. Figure B, D and F show effect on CaCl<sub>2</sub> concentration–response curves in the absence and presence of different concentrations of the Eu. *n*-hexane, Eu. Chl, Eu. EtAc, Eu. Aq, respectively, in isolated rabbit jejunum preparations. Values shown are mean ± SEM (n=6-7)

most potent among the fractions tested, this data suggest that the spasmolytic constituents are more concentrated in this particular fraction. Pretreatment of the jejunal preparations with these fractions caused a right ward shift in the  $Ca^{++}$  concentration response curves, indicating that these fractions possess CCB activity; Ethyl acetate being the most potent. Interestingly, the aqueous fraction did not affect spontaneous and high  $K^{+}$ -induced contraction. This is not surprising as most of the sugar kind of constituents is concentrating in such fraction, which lacks effect.

These data indicate that crude extract and its organic fractions with the exception of aqueous fraction possess spasmolytic effect, which is possibly mediated through  $Ca^{++}$  channel blockade and the activity is more concentrated in the ethyl acetate fraction than the crude extract and other fractions. The CCB-mediated spasmolytic effect may be responsible, as a mechanism for the anti-diarrheal effect of the extract. Preliminary phytochemical analysis of the crude extract indicated presence of flavonoids, terpenoids and tannins. Plant-derived flavonoids, terpenes and tannins have smooth muscle relaxant effects. The smooth muscle relaxant effect of these natural products is mediated through various mechanisms including CCB (Zhu et al., 1997; de Alencar Cunha et al., 2003; Zhu et al., 2005) as CCBs are considered useful in diarrhea and gut spasms (Brunton, 1996). Kaurenoic acid, a terpene isolated from *Copaifera langsdorffii* possesses  $Ca^{++}$  entry blockade-mediated smooth muscle relaxant effect (de Alencar Cunha et al., 2003). We believe that terpenes/terpenoids present in the extract of *E. umbellata* might be the active constituents that mediate intestinal smooth muscle relaxation through inhibitory effect on VDCs.

## Conclusion

*E. uctmbellata* crude extract possesses CCB-mediated smooth muscle relaxant effect that is concentrated more in the ethylacetate fraction. The active constituents might be terpenoids that could possibly explain the antidiarrheal and spasmolytic effects of the extract and this finding provides a possible pharmacological base to its potential medicinal use in diarrhea and gut spasms. Further studies will be worthwhile to explore its flavonoids, terpenoids and tannins and molecular nature of spasmolytic and antidiarrheal effect.

## Ethical Issue

Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and were approved by the Ethical Committee of CIIT, Abbottabad.

## Conflict of Interest

The authors report no conflict of interest as document in HTML file.

## Acknowledgement

This study was carried out from a grant to the principle investigator from the Higher Education Commission (HEC) of Pakistan. Authors are thankful for assistance of PhD students; Rahila Qayyum, Hafiz Misbah-ud-Din Qamar, Shamim Khan and Umme Salma in finalizing the manuscript.

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**Author Info**

Abdul Jabbar Shah (Principal contact)

e-mail: jabbarshah@ciit.net.pk

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