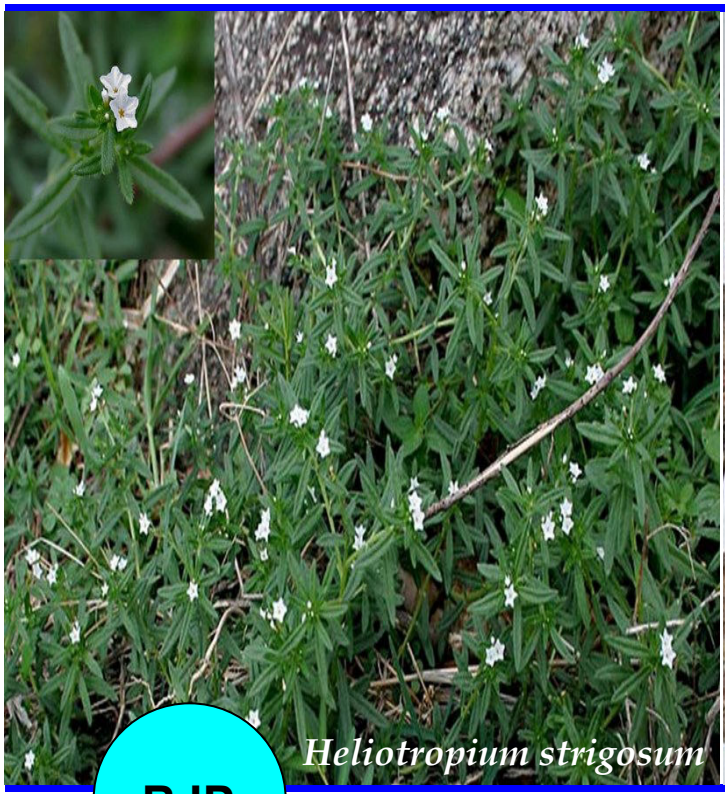
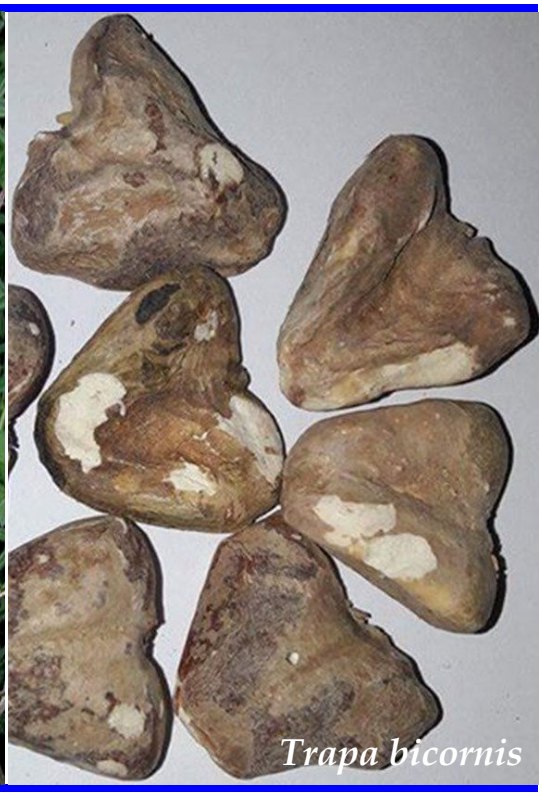


Heliotropium strigosum



Trapa bicornis

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Effects of *Heliotropium strigosum* and *Trapa bicornis* in hyperactive gut disorders

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Abstract

The current study was aimed to investigate the extracts from *Heliotropium strigosum* and *Trapa bicornis* phytochemically for various constituents and pharmacologically for gastro-intestinal disorders. Phytochemical analysis indicated the presence of alkaloids, flavonoids, glycosides and tannins in both the extracts. In castor oil-induced diarrhea, *H. strigosum* and *T. bicornis*, at 100-1000 mg/kg dose provided protection of 10-83% and 14-76% respectively. In isolated rabbit jejunum preparations, *T. bicornis* (0.01-5 mg/mL) relaxed the spontaneous and K^+ (80 mM)-induced contractions with EC_{50} values of 1.2 mg/mL and 2.6 mg/mL respectively, suggesting that spasmolytic effect was possibly mediated through calcium channel blockade. This was further authenticated when pretreatment of tissues with *T. bicornis* (1-5 mg/mL) caused rightward shift of Ca^{++} concentration–response curves, similar to verapamil. In acute toxicity test, both extracts were safe up to 10 g/kg dose. These results indicated the usefulness of *H. strigosum* and *T. bicornis* in the treatment of hyperactive gut disorders.

Introduction

Heliotropium strigosum (family; Boraginaceae), locally known as Gorakh pan or Panjabooti, has folkloric medicine uses in the diarrhea, gum boils, sore eyes, and insects and snake bite (Hussain et al., 2010). It has been reported as anti-oxidant (Modak et al., 2007), antimicrobial (Hussain et al., 2010), antinociceptive, anticonvulsant and anti-inflammatory (Khan et al., 2013). Phytochemical analysis of *Heliotropium* showed the presence of strigosine (pyrrolizidine) and necine (trachelanthamidine, supinidine and retronecine) alkaloids in most species (Mattocks, 1964). Trachelanthamidine and 3 phthalic acid esters have been isolated from *H. strigosum*. Similarly, chromotropic acid, quercetin, trans -4-hydroxy-3-methoxy cinnamic acid, vanillic acid, gallic acid, caffeic acid, *m*-coumaric acid, *p*-coumaric acid, syringic acid, sinapic acid and ferulic acid have also been detected in this plant (Qayyum et al., 2016).

Trapa bicornis (family; Lythraceae), locally known as Singhara, is used in the gastrointestinal, thyroid and cardiovascular disorders, bronchitis, diabetes mellitus, and gout (Rahman et al., 2001). It possesses analgesic (Agrahari et al., 2010), anti-inflammatory (Patel et al., 2010), antidiabetic (Das et al., 2011) and antimicrobial (Parekh and Chandana, 2007) activities. Carbohydrates, phytosterols, saponins, fixed oils, fat, tannins, flavonoids and glycosides have been reported in this plant (Bhatiwal et al., 2012).

Although these plants are used in traditional medicine for the treatment of different gastrointestinal disorders, no scientific information are available regarding the use of *H. strigosum* (in diarrhea) and *T. bicornis* (in diarrhea

and gut spasms). Therefore, the current study was undertaken to explore the pharmacological basis of these plants in hyperactive gut disorders using *in vivo* and *in vitro* approaches.

Materials and Methods

Plant material

H. strigosum was collected from Bhakkar District, Punjab, Pakistan while *T. bicornis* was purchased from the local market. The plants were authenticated by Dr. Abdul Nazir, Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad, Pakistan. The voucher specimen of *H. strigosum* (DB-GC (HSB)-0030) and *T. bicornis* (DB-GC (TBL)-0031) was deposited in the Department of Botany, Government Postgraduate College, Abbottabad, Pakistan.

Processing and extraction

The fresh plant was shade dried at ambient temperature. The dried plant material (300 g each) was pulverized and soaked in 1.5 L aqueous-methanol (70%) for 15 days with occasional shaking. The extracts were, then, filtered through a muslin cloth and then through a Whatman filter paper. Similar protocol was also repeated thrice. The resultant filtrate was concentrated to a thick, semi-solid mass using rotary evaporator (HAHN VAPOR HS-2005S-N, Korea) coupled with an electric aspirator (HS-3000, Korea) and recirculation chiller (RW -0525G, Jiec Tech, Korea) at 45°C under reduced pressure.

Drugs and standards

Acetylcholine, potassium chloride, calcium chloride and verapamil were acquired from the Sigma Chemical Company (USA). Water solubility of the extracts was improved with 10% Tween 80 or dimethyl sulfoxide (DMSO). Castor oil was obtained from the Karachi Chemical Industries, Pakistan. The chemicals of maximum purity grade were used. Stock solutions were prepared in distilled water/suitable solvent and dilutions were made fresh on the day of experiment.

Animals

Balb^c mice (20-25 g) were used for *in vivo* antidiarrheal experiments while male rabbits (1-1.5 kg) of local breed were purchased from the local market and used for *in vitro* studies. The animals used in current study were kept at the animal house of the Department of Pharmacy, CIIT, Abbottabad under controlled environment and were given standard diet and tap water *ad libitum*.

Phytochemical analysis

Preliminary screening for various groups of natural products

The crude extracts obtained from *H. strigosum* and *T. bicornis* were screened for the presence of saponins, flavonoids, tannins, phenols and alkaloids using the methods described elsewhere (Edeoga et al., 2005).

Thin layer chromatography (TLC)

TLC was used for the separation and analysis of different components of extracts. TLC plates coated with silica gel (60 F254) (Merck, Germany) were used for this purpose. *n*-Hexane : ethyl acetate (1:1) were used as mobile phase and the retardation factor (Rf values) of various constituents were calculated using the following formula:

$$Rf = \frac{\text{Distance moved by substance}}{\text{Distance moved by solvent front}}$$

Ultraviolet light (wavelength 254 and 365 nm) was employed for UV active constituents of the extracts. The various constituents were also visualized on TLC using phosphomolybdic acid (10%) and ceric sulfate as the locating reagents.

High Performance Liquid Chromatographic (HPLC) analysis

HPLC (Perkin Elmer Series 2000 auto samples) was used to detect the major components of extracts. Stock solution (10 mg/mL) of *H. strigosum* and *T. bicornis* were prepared in ethanol : water (60:40) and filtered with 0.45 µm syringe filter. HPLC was performed using C18 column (150 × 4.6 mm) and injection volume was 20 µL. The mobile phase (acetonitrile : water : acetic acid) in ratio 60 : 39.5 : 0.5 and ultraviolet visible detector (200-700 nm) was used. The sample was injected at the rate of 1 mL/min.

Acute toxicity test

The acute toxicity assay was performed as described earlier using the mice model (Gilani et al., 2005). Briefly, the animals were divided into 7 groups (five mice/group). Group I (negative control) was given normal saline at 10 mL/kg dose. Group II-IV were given different doses of *H. strigosum* while Group V-VII were treated with different doses of *T. bicornis*. All the extract treated groups were given oral dose of 3, 5 and 10 g/kg. The animals were given food and water *ad libitum* during the experiment and different effects like flatness, shivering and diarrhea were observed for 24 hours.

Castor oil-induced diarrhea

In vivo antidiarrheal activity of both extracts was performed on Balb^c albino mice as described previously (Awouters et al., 1978; Shah et al., 2010). Animals were divided into 11 groups (5 mice/group) and kept in separate cages. Group I (vehicle control) was given normal saline. Group II was treated with castor oil and groups III-V were given doses of 100, 300 and 1000 mg/kg of *H. strigosum*, respectively. Group VI-VIII were

treated with *T. bicornis* at respective doses of 100, 300 and 1000 mg/kg. Verapamil (standard drug) was orally administered at 1, 3 and 10 mg/kg to Groups IX-XI, respectively. After 1 hours of the treatment, Groups III-XI were treated with castor oil. The castor oil treated groups were observed for defecation after 4 hours of the oil administration. The protection percentage was determined by comparing wet and dry fecal count in each cage.

Rabbit jejunum

The rabbit jejunum preparations were used to determine possible spasmolytic activity following the previously reported protocols (Gilani et al., 2005; Shah et al., 2010). The rabbits were fasted for 24 hours before the experiments and sacrificed by cervical dislocation. The abdomen was cut open and jejunal portion was isolated. The video component of the methodology was published earlier (Rafique et al., 2016). About 2 cm long jejunum preparations were mounted in tissue baths containing Tyrode's solution (10 mL) maintained at 37° C and continuously aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The composition of Tyrode's solution was (mM): KCl 2.7, NaCl 136.9, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.6 and $CaCl_2$ 1.8 (pH 7.4). A preload of 1 g was applied to each tissue and was allowed to stabilize for 30 min. Tissues were treated with a submaximal dose (0.3 µM) of acetylcholine to attain control response and presumed stable after the reproducibility of said response. After stabilization, different concentrations of *T. bicornis* were added cumulatively and the response was calculated as a percent of control (untreated).

The calcium channel blocking activity was determined by depolarizing the tissues with a high dose of K^+ (80 mM) (Farre et al., 1991). After plateau formation, *T. bicornis* was added in a concentration dependent manner to obtain concentration-dependent inhibitory curves (van Rossum, 1963). The inhibitory effect of the extract was calculated as percent of control response exhibited by high K^+. The calcium channel blocking activity of *T. bicornis* was further confirmed by tissue stabilization in Tyrode's solution followed by replacement with Ca^{++}-free Tyrode's solution containing ethylenediaminetetra-acetic acid (EDTA) (0.1 mM). The solution was further replaced with Ca^{++}-free and K^+-rich Tyrode's solution. Calcium response curves were obtained when tissue was pretreated with different concentrations of *T. bicornis*. The calcium response curves were reproduced and compared with control. Similar protocol was also performed for verapamil, a standard calcium channel blocking drug.

Statistical analysis

All the data expressed are mean ± SEM, and the median effective concentrations (EC_{50} values) are given with 95% confidence intervals. The statistical parameter applied was the Student's t-test with $p<0.05$ noted as significantly different (GraphPad Prism, version 5).

Results

Phytochemical analysis

The crude extracts were subjected to primary phytochemical analysis to find out their chemical composition. Initial analysis of *H. strigosum* revealed the presence of alkaloids, glycosides, flavonoids, steroids, tannins and terpenoids while *T. bicornis* indicated the presence of alkaloids, glycosides, flavonoids, tannins and saponins. Saponins were absent in *H. strigosum* while *T. bicornis* was devoid of steroids and terpenoids. Quinones were absent in both the extracts (Table I).

Table I

Phytochemicals analysis of *H. strigosum* and *T. bicornis* extracts

Phytochemical constituents	Results	
	H. strigosum	*T. bicornis*
Alkaloids	+	+
Glycosides	+	+
Flavonoids	+	+
Saponins	–	+
Steroids	+	–
Tannins	+	+
Terpenoids	+	–
Quinones	–	–

Chromatographic analysis

TLC plates were developed for *H. strigosum* and *T. bicornis*. None of the components were located under UV-light for *H. strigosum* on TLC developed in the mentioned mobile phase. The components, however, became visible (Figure 1A) by spraying the TLC plate with ceric sulphate. Various constituents in *T. bicornis* were located on TLC using UV-light (254 and 365 nm). No spot of constituents was visualized in 254 and 365 nm light but spraying the TLC plate with 10% molybdic acid clearly indicated the separation of the various components (Figure 1D). The Rf values of different compounds for *H. strigosum* and *T. bicornis* are shown in Figure 1B and E, respectively.

HPLC analysis of *H. strigosum* at a wavelength of 271 nm displayed two major peaks at the retention time of 2.1 and 3.1 min (Figure 1C). The chromatogram for *T. bicornis* (at 267 nm) also showed two major peaks at 1.9 and 3.1 min (Figure 1F), respectively.

Acute toxicity test

The results for *H. strigosum* and *T. bicornis* (3, 5 and 10 g/kg), administered to mice and observed for beha-

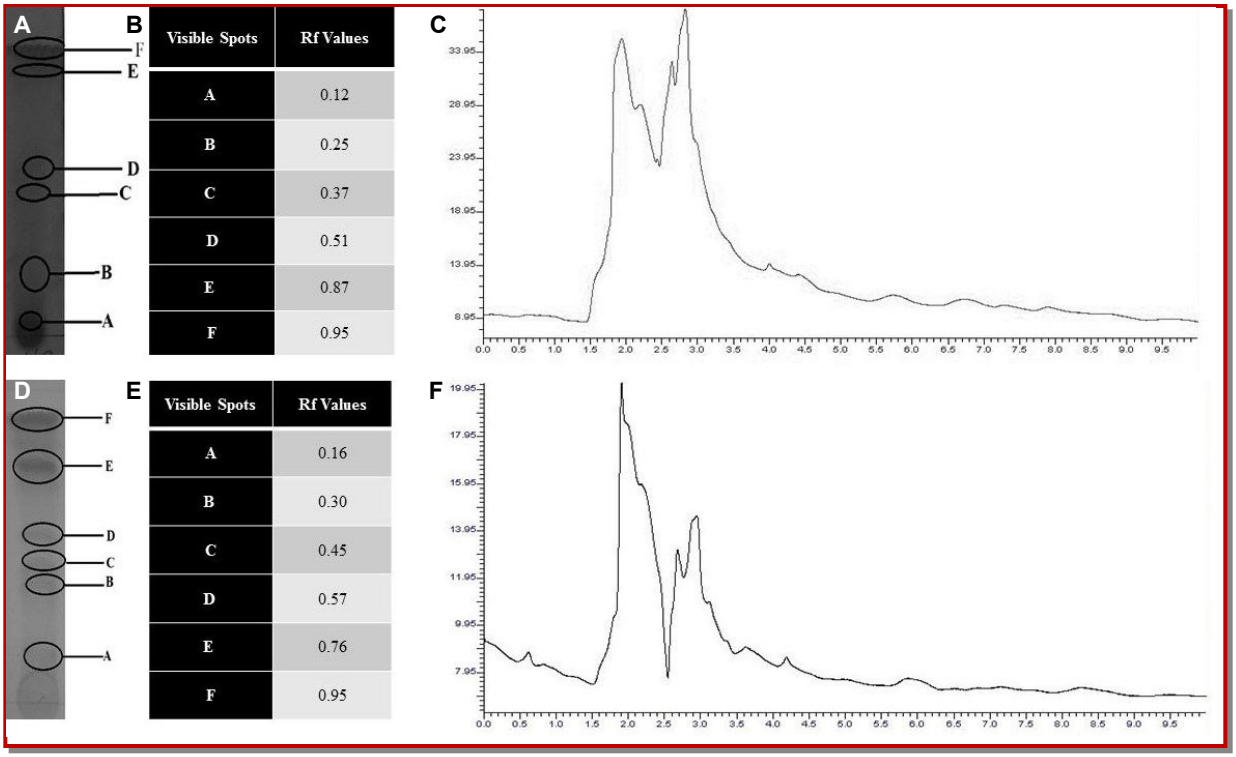

Figure 1: TLC and HPLC analysis of *H. strigosum* and *T. bicornis* crude extracts; (A) TLC chromatogram, (B) Rf values of the detected compounds, and (C) HPLC chromatogram of *H. strigosum*. (D-F) respectively, shows TLC chromatogram, Rf values of the detected compounds and HPLC chromatogram of *T. bicornis*

vioral changes and mortality for 24 hours, showed that both the extracts were safe up to 10 g/kg dose, as no lethality was observed.

Antidiarrheal activity

The antidiarrheal activity of *H. strigosum* showed that it provided 10.3, 37.1 and 83.3% protection at 100, 300 and 1000 mg/kg doses, respectively (Table II). Similarly, *T. bicornis* displayed 14.2, 39.6 and 76.4% protection against diarrhea at respective similar doses (Table II). The results were compared with verapamil (1, 3 and 10 mg/kg), a standard calcium channel blocker, which controlled diarrhea up to 31.6, 60.1 and 85.3%, respectively (Table II).

Spasmolytic effect of T. bicornis

The spasmolytic effect of *T. bicornis* was tested on isolated rabbit jejunum strips. *T. bicornis* (0.01-5 mg/mL)

Table II

Effect of the crude extract of *H. strigosum and T. bicornis* on castor oil-induced diarrhea in mice

Group	Dose	Total number of feces in 4 hours	Wet feces in 4 hours (%)	Protection (%)
Control (saline)	10 mL/kg	5.2 ± 0.5	0 ± 0	9.6 ± 4.0
Castor oil	10 mL/kg	16 ± 0.7	96.2 ± 2.3	2.6 ± 2.3
H. strigosum	100 mg/kg	6.3 ± 0.7	76.0 ± 1.0	10.3 ± 1.0
	300 mg/kg	5.0 ± 1.5	56.0 ± 1.2	37.1[a] ± 0.6
	1,000 mg/kg	4.6 ± 1.9	20.4 ± 1.8	83.3[c] ± 5.9
T. bicornis	100 mg/kg	10.2 ± 3.2	71.0 ± 2.2	14.2 ± 4.9
	300 mg/kg	8.6 ± 2.8	53.6 ± 2.0	39.6[a] ± 6.0
	1,000 mg/kg	5.4 ± 3.0	27.2 ± 0.9	76.4[b] ± 0.9
Verapamil	1 mg/kg	8.4 ± 0.6	66.9 ± 3.7	31.6[a] ± 3.3
	3 mg/kg	6.2 ± 0.4	47.2 ± 5.0	60.1[b] ± 4.2
	10 mg/kg	4.2 ± 0.2	24.6 ± 1.7	85.3[c] ± 6.0

n=5 in each case; Mean ± SEM; [a]p<0.05, [b]p<0.01 and [c]p<0.001 vs control; Student's t-test

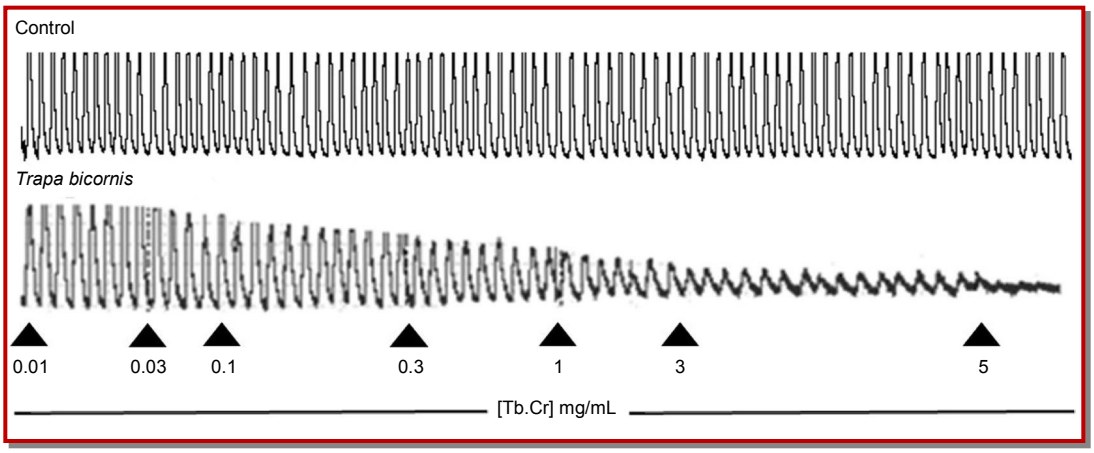

Figure 2: Tracing showing normal contraction and relaxation (control) of rabbit jejunum and relaxant effect of *Trapa bicornis* crude extract (Tb.Cr) on spontaneous contractions in isolated rabbit's jejunum preparation

suppressed the spontaneous (Figure 2) and high K^+ (80 mM) induced contractions (Figure 3A) in a dose-dependent fashion with EC_{50} values of 1.2 mg/mL (1-1.3) and 2.6 mg/mL (1-4.6), respectively. Verapamil also inhibited spontaneous and high K^+ (80 mM) induced contractions (Figure 3B) with respective EC_{50} values of 0.1 mg/mL (0.1-0.13) and 0.02 mg/mL (0.01-0.03). Pretreatment of tissue with *T. bicornis* (1-5 mg/mL) shifted calcium concentration response curves towards right (Figure 3C) in a comparable manner to verapamil (Figure 3D).

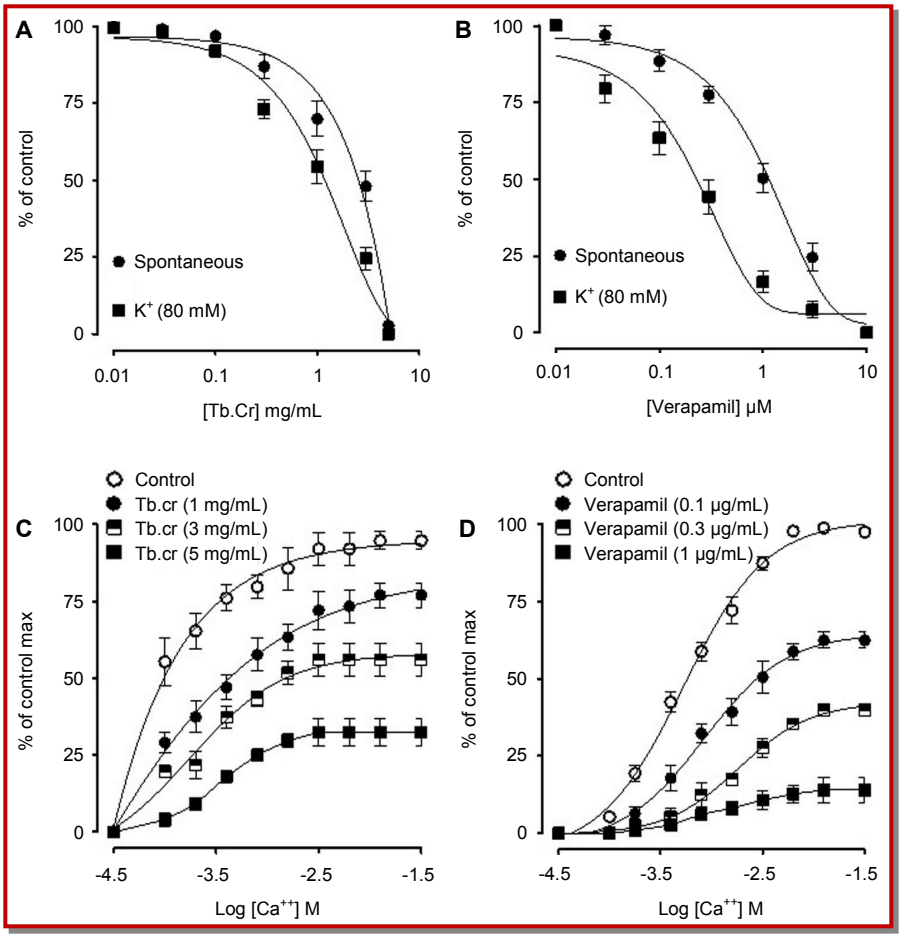

Figure 3: Graphical representation of (A) relaxant effect of *Trapa bicornis* crude extract (Tb.Cr), (B) verapamil on spontaneous and K^+-induced contractions in isolated rabbit's jejunum preparation, (C) concentration-response curves of calcium by different concentrations of *T. bicornis*, and (D) verapamil on isolated rabbit's jejunum preparation. Values shown are mean ± SEM (n=3)

Discussion

H. strigosum and *T. bicornis* significantly inhibited the frequency of defecation as well as wetting of feces compared to the untreated group, the effect was similar to verapamil. The induction of diarrhea by castor oil results from the action of ricinoleic acid, which is formed by the action of lipase on triricinolein (a major constituent of the castor oil) in the duodenum. Ricinoleic acid in turn causes 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., 2010). A potential antidiarrheal agent may exhibit its effect by inhibiting the gut motility (spasmolytic) and/or electrolyte out flux in the form of wet droppings (Croci et al., 1997). The protective effect of *H. strigosum* and *T. bicornis* against the castor oil-induced diarrhea, similar to verapamil, suggests that these extracts may exert an inhibitory effect on contractions.

To have insight into the nature of effect on intestinal contractions (gut motility), the effects of *T. bicornis* was further studied in isolated rabbit jejunum preparation. Literature lacks spasmolytic studies on *T. bicornis*, however, *H. strigosum* is recently showed as antispasmodic with calcium channel blocking effect (Janbaz et al., 2015). Spontaneously beating isolated rabbit jejunum preparation is considered suitable for testing spasmolytic substances without use of a spasmogen (Gilani et al., 2005). Cumulative addition of *T. bicornis* suppressed the spontaneous contractions of rabbit jejunum, similar to that caused by verapamil, a standard calcium channel blocker (Godfraind et al., 1986). This suggests that *T. bicornis* induces inhibitory effect on intestinal smooth muscle contractions. However, further studies were carried out probing the underlying mechanism.

Contractions of smooth muscles including rabbit jejunum, is dependent on an increased intracellular concentration of free Ca^{++}, which subsequently activate the contractile proteins (Karaki and Weiss, 1988). The intracellular Ca^{++} concentration is elevated either by influx through voltage-dependent Ca^{++} channels (VDCs) or discharge from the Ca^{++} stores present in the sarcoplasmic reticulum. The spontaneous movements of the intestine are regulated by periodic depolarization and repolarization and the action potential appears as a rapid influx of Ca^{++} through VDCs at the height of depolarization (Brading, 1981). Thus, the inhibitory effect of *T. bicornis* on spontaneous movements of rabbit jejunum may be due to an interference with Ca^{++} influx through VDCs. Previously, it was observed that the spasmolytic constituents present in different medicinal plants mediate their effects usually through an inhibitory effect on Ca^{++} movement (Gilani et al., 2005; Shah et al., 2010). In order to verify that the spasmolytic effect of *T. bicornis* is mediated through an inhibition on Ca^{++} influx via VDCs, the tissues were depolarized with a high concentration of K^+ (80 mM) followed by the addition of the crude extract in a cumulative fashion. *T. bicornis* showed concentration dependent relaxation, similar to verapamil, suggesting that the spasmolytic effect is mediated through its inhibitory effect on Ca^{++} influx via VDCs. This hypothesis was further strengthened when pretreatment of the tissues with *T. bicornis* caused a rightward shift in the Ca^{++} concentration response curves, similar to verapamil. These results indicate that extract of *T. bicornis* possesses calcium channel blocking type constituents. Our findings provide pharmacological basis to the medicinal use of *T. bicornis* in hyperactive gut disorders, as calcium channel blockers are clinically useful in diarrhea and gut spasms (Brunton, 1996). Previously reported calcium channel blocking activity of the extract of *H. strigosum* supports our finding on its antidiarrheal potential.

Preliminary phytochemical analysis indicated presence of flavonoids, saponins, tannins, alkaloids and glycosides. Plant derived flavonoids (Zhu et al., 1997), saponins (Kai et al., 1998) and tannins (Zhu et al., 2005) have been found to possess calcium channel blocking effect, which might be the active candidate(s) responsible for the antidiarrheal and spasmolytic effect of *T. bicornis* and also *H. strigosum* . In the acute toxicity study both extracts were found safe up to 10 g/kg dose.

Conclusion

The crude extracts of *H. strigosum* and *T. bicornis* possess various phytochemicals with antidiarrheal effects. Moreover, the crude extract from *T. bicornis* also showed antispasmodic effect possibly mediated through calcium channel blockade.

Ethical Issue

All animals were housed, cared and used according to the rulings of the Ethical Committee of CIIT, Abbottabad, which completely agreed with the recommendations of Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996).

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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