Pharmacological validation of the folkloric uses of *Cyperus rotundus* L. in different ailments: A*n in vivo* and *in vitro* research

Musaddique Hussain[1,2]*, Hafiz Muhammad Waqas[1], Ikhlaq Hussain[3], Abdul Majeed[2], Shahid Masood Raza[1] and Khalid Hussain Janbaz[2]

[1]School of Pharmacy, the University of Faisalabad, Faisalabad, Pakistan
[2]Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan
[3]Ghazi Khan Medical College, Dera Ghazi Khan, Pakistan

Abstract: *In vivo* and *in vitro* research study was conducted on *Cyperus rotundus* to evaluate the sound mechanistic background in the treatment of gastrointestinal, bronchial and vascular disorders as well as in pain, emesis, pyrexia and bacterial infections. Results showed that crude extract of *Cyperus rotundus* (Cr.Cr) exhibited the dose-dependent spasmolytic effect in rabbit jejunum by inhibiting the spontaneous and K^+ (80 mM)-induced contractions. Pretreatment of tissue with Cr. Cr caused the rightward shift of calcium concentration response curves, similar to verapamil. Cr. Cr also caused the relaxation of K^+(80 mM)- and carbachol (1 µM)-induced contractions of trachea preparations, similar to that of verapamil. Moreover, Cr. Cr also relaxed the contraction induced by the K^+ (80 mM) and phenylephrine (1 µM) of aorta preparations. Data show that *C. rotundus* possess the spasmolytic, bronchodilator and vasodilator activities possibly through calcium channels blockade; validating its folkloric use in diarrhea, dyspepsia, bronchitis, asthma and hypertension in addition to antibacterial, antiemetic, antipyretic and analgesic activities.

Keywords: Spasmolytic, bronchodilation, vasodilatation, Ca^{+2} antagonist, antiemetic.

INTRODUCTION

Cyperus rotundus Linn. (*Cyperaceae*) is known by vernacular names of purplenut-sedge and nut-grass (English), muthaghas (Bengali), motha (Hindi) and mustaka (Urdu). *Cyperus rotundus* also known as "Worlds worst weed" is widely distributed throughout India and Pakistan (Anonymous, 1990). Rhizomes are dark brown and woody, while stems are trigonous, green and smooth.

The roots and rhizomes of *C. rotundus*are useful in diarrhea, dyspepsia, cholera, inflammation, dysentery, skin rashes and excess bleeding while fresh tubers in the form of paste or plasters are applied on the breast, scorpion sting and spreading ulcers (Kirtikar and Basu, 2002; Sharma and Gupta, 2007). The tubers of *C. rotundus*are traditionally used as; antiemetic, anthelmintic, antipyretic, hypertensive and smooth muscle relaxant (Nadkarni and Nadkarni, 1996; Kirtikar and Basu, 2002; Khare, 2007). The plant has also been reported to possess antimalarial, hepato-protective, tranquillizing activity and reduce the obesity by suppressing appetite centre (Gupta, 2003).

Photochemical investigations revealed the presence of sesquiterpene 4a-, 5a-, cyperene-1 (tricyclic sesquiterpene), oxidoeudesm-11-en-3a-ol, cyperenone, a-cyperone, cyperene-2 (bicyclic sesquesterpene hydrocarbon) (Anonymous 1990, 1992), β-selinene, sugetriol triacetate (sesquesterpenoid), mustakone (sesquesterpene ketone), sugenol (sesquesterpenicketol) (Rastogiand Mehrotra, 1969); the essential oil including eugenol, copadiene,a-and β-rotunol, epoxyguaienerotund one, calamenone, cyperenol, cyperolone, isocyperol, cyperol, d-cadinene, kobusone and isokobusone (Anonymous, 1992); a flavonol glycoside, β-sitosterolrhamnetin 3-O-rhamnosyl-(1-4) rhamnopyrano-side (Gupta,2003); myrtenol, α-cyperone, caryophyllenen oxide, β-pinine, flavonoids, alkaloids, ascorbic acid and poly-phenols (Lawal and Oyedeji, 2009).

Despite the *C. rotundus* ancient history of use for GIT, respiratory and vascular system disorders, as well as its use as antipyretic, analgesic and antiemetic remedy, it's *in vivo* anti-diarrheal study has been performed (Uddin *et al*., 2006), just validating its pharmacological effectiveness but no further studies have been reported with respect to its underlying pharmacological mechanism of actions. The present study was carried out to rationalize and to explore mechanistic background validating the folkloric uses of *C. rotundus*.

MATERIALS AND METHODS

Collection and extraction of plant

Rhizomes and roots of *C. rotundus* were collected during the month of May-June, 2012 and were identified by Curator of Department of Botany, G.C University, Lahore, with voucherspecimen labeled as GC. HERB.BOT. 206. Triple maceration process was adopted for the extraction of coarsely powdered material (#40) using 70% methanol-aqueous mixture (Hussain *et al*.,

**Corresponding author:* e-mail: musaddique.ph@gmail.com

2014) and resulting in dark brown thick syrup consistency; named Cr. Cr, with an approximate yield of 18.25%.

Animals and housing conditions

Animals including, rabbits (1.0-1.5kg, 6-9 months old), Swiss albino mice (38-48g) and chicks (7-10 days old,70-90g) of either sex were locally purchased; provided with tap water *ad libitum* and standard diet under controlled environmental condition (25-28°C), while all chicks were housed at room temperature with 12h/12h dark-light cycle. Whereas, *Staphylococcus aureus, Bacillus pumilus, Escherichia coli* and *Citro bacterfreundii* were collected from National Institute of Biotechnology & Genetic Engineering, Faisalabad. All experiments were performed by following the ruling of Institute of Laboratory Animal Resources, Commission on Life Sciences National Research Council 1996).

Standard drugs, chemicals, and solvents

All Standard drugs, chemicals and solvents such as carbachol, potassium chloride, EDTA, verapamil, phenylephrine, chlorpromazine, copper sulphate, dimethyl sulfoxide, glucose, magnesium sulphate, calcium chloride, sodium bicarbonate, dichloromethane, potassium dihydrogen phosphate, polyoxyethylen-esorbitan mono-oleate (tween-80), sodium dihydrogen phosphate, and methanol were of research grade.

Determination of phytochemical constituents

The crude extract was initially subjected to screening with different reagents to detect the presence of possible important constituents, according to a standard method (Tona *et al*., 1998; Evans, 2006).

In vitro experiments

Isolated rabbit jejunum

The crude extract was tested on jejunum preparations for assessment of spasmolytic activity (Hussain *et al*., 2015a; Hussain *et al*., 2016). A weight of 1g was applied to jejunum segments (2-3 cm) hanged in tissue bath filled with 15 mL of Tyrode's solution, aerated with carbogen (pH 7.4;37°C) and tissue sensitivity was recorded via displacement transducers attached with PowerLab data acquisition system (Janbaz *et al*., 2012). For spasmolytic activity, the crude extract was applied to the spontaneous contractions in a cumulative fashion, without prior addition of any agonistic agent (Janbaz *et al*., 2013).

Assessment and conformation of calcium channel blocking activity

To assess whether the spasmolytic response was mediated through calcium channel blockade (CCB), the tissue preparations were depolarized by exposing to K^+(80mM) as KCl, resulting in appearance of sustained contraction (Farre *et al*., 1991), where as control calcium concentration-response curves (CRCs) were constructed for the confirmation of calcium channels blockade (Gilani *et al*., 2005; Janbaz *et al*., 2013; Hussain *et al*., 2015b).

Isolated rabbit trachea

For the assessment of the bronchodilator effect of crude extract, isolated rabbit trachea preparations (2-3mm width) were hanged in tissue baths filled with 15mL of normal Kreb's solution, aerated with carbogen (pH 7.4;37°C) (Gilani *et al*., 2005; Janbaz *et al*., 2013). Afterwards, tissue preparations were exposed to K^+(80 mM)- and carbachol (1µM)-for stabilization (45 min). The crude extract was applied to stabilized tissues to obtained sustained contractions for possible relaxant effect. For the confirm of the possible mechanism of action, verapamil (standard Ca^{2+} channel blocker) was tested on K^+(80mM)- and carbachol (1µM)-induced spastic contractions (Hussain *et al*., 2015c).

Isolated rabbit aorta

The vasodilator/vasoconstrictor effect were studied by application of crude extract to tissue bath containing isolated rabbit thoracic aorta preparations (2-3 mm wide) in a cumulative manner, already exposed to K^+(80 mM)- and phenylephrine (1 µM)- for stabilization with a dose interval of 45 min (Janbaz *et al*., 2014).

Antibacterial potential assessment

For the assessment of antibacterial potential, disc diffusion method was used by measuring the zone of inhibition around sample disc (crude extract), positive (Gentamicin) and negative (DMSO) control (Taylor *et al*., 1995). The relativepercentage of inhibition (RPI) with respect to positive control was derived from following equation (Ajay *et al*. 2002).

$$RPI=[(A-B)/(C-B)]x\ 100$$

In this equation A, B and C represent the total area of inhibition of sample discs, negative control and positive control respectively.

In vivo experiments

Antiemetic potential assessment

Chick emesis model was slightly modified for the assessment of the antiemeticpotential of crude extract (Akita *et al*., 1998; Ahmed and Patrricia, 2013). Chicks were placed in 4different groups and each group has 6 chicks. Each chick was set aside in a large beaker for the 15 min to acclimatize. Chicks of control group (group 1), experimental group (group 2 and 3) and standard group (group 4) were subjected to administration of normal saline (0.9% NaCl; 10mL/kg), crude extract (10 mL/kg of 100 and 150 mg/kg) and Chlorpromazine (150 mg/kg), respectively. After 10 min, Copper sulphate (50 mg/kg of body weight) was given orally to chicks of all groups in order to stimulate the peripheral nervous system for emesis, then numbers of retches were counted for next 15 min and %age inhibition was calculated by this equation:

Retching inhibition (%) = [(A-B)/A] x 100
A and B represents the frequency of retching in control and experimental groups respectively.

Antipyretic potential assessment
Antipyretic activity was performed on albino rabbits by the slight modification of previously reported method (Grover, 1990; Naveed *et al*., 2012). Albino rabbits were divided into 3 groups of 6 each. Pyrexia was induced by i.p. injecting brewer yeast (0.5mL/kg) to each group. After 2 h of pyrexia, rabbits of group 1, 2 and 3 were subjected to i.p. administration of 2mL/kg normal saline (0.9% NaCl), 2 mL of 150mg/kg of crude extract properly dissolved in saline and 2 mL of 10mg/kg of Aspirin properly mixed in normal saline respectively. After administration of the doses, rectal temperature was measured for 1 to 4 h. Decrease in rectum temperature (%) was noted by using this equation:

Decrease in rectum temperature (%)= $[(B\text{-}C_n)/ (B\text{-}A)]x 100$

In this equation, A, B and C represent the normal rectum temperature, rectum temperature after 2 h of injecting brewer yeast and rectum temperature after 1^{st}, $2^{nd,}$ 3^{rd}, and 4^{th} h of treatment respectively.

Analgesic potential assessment
Analgesic activity was performed on albino mice by modifying the previously reported tail flick method (Asongalem *et al*., 2004; Basar *et al*., 2010; Fan *et al*., 2014). Albino mice were divided into3 groups of 6 each. Tails of albino mice were dipped in water having atemperature of 60°C in vertical position. After 10 min of noting the reaction time (tail's withdraw time), group 1,2 and 3 mice were subjected to i.p. administration of normal saline (0.9% NaCl; 10mL/kg), crude extract (200 mg/kg of extract properly dissolved in saline) and Aspirin (75 mg/kg) respectively. Reaction time was noted after 20 min.

Acute toxicity assessment
For acute toxicity assessment mice were used; fasted 24 h prior to test but had free access to water. All albino mice were divided into 4 groups of 6each.Albino mice of group 1 (control group) were subjected to oral administration of normal saline (0.9% NaCl; 10 mL/kg), whereas mice of group 2, 3 and 4 (experimental groups) were orally administered with 1, 3 and 5g/kg of crude extract, respectively. All 4 groups were keenly observed for 24 h.

STATISTICAL ANALYSIS

The data are expressed as mean ± S.E.M and EC_{50}values are given with 95% confidence intervals (CI 95%). Logarithmic dose response curves of different treatments were plotted by using Graph pad Prism. $p<0.05$ and $p<0.005$ representing significant and most significant responses by using student's *t*-test.

RESULTS

Phytochemical screening
Phytochemical study of *C. rotundus* showed the presence of saponins, tannins, alkaloids, flavonoids, terpenes and sterols.

In vitro experiments
Spasmolytic effect of *C. rotundus*
The crude extract of *C. rotundus* caused the relaxation of both spontaneous and K^+(80 mM)-induced contraction EC_{50} values of 1.06 mg/mL (0.66-1.68, CI 95%: n=4) and 0.21 mg/mL (0.11-0.39, CI 95%: n=4)respectively suggesting Ca^{+2} channel blockade activity(fig 1A). Verapamil also relaxed the spontaneous and K^+(80 mM)-induced contractions with EC_{50} values of 0.34 µM (0.17-0.67, CI 95%: n=4) and 0.02µM (0.01-0.04, CI 95%: n=4) respectively, (fig 1B). The Cr.Cr caused the rightward shift of Ca^{+2} concentration-response curves (0.3-1.0 mg/mL) in a manner similar to verapamil, thus confirming the Ca^{+2} channel blockade activity (fig. 2).

Broncho-relaxant effect of C. rotundus
The crude extract of *C. rotundus* showed concentration-dependent relaxant effect when tested on K^+(80mM)- and carbachol (1µM)-induced contractions with EC_{50} values of 0.41 mg/mL (0.23-0.74, CI 95%: n=4) and 0.83 mg/mL (0.4-1.13, CI 95%: n=4) respectively, (fig 3A). Similarly, verapamil also relaxed the K^+(80mM)- and carbachol (1µM)-induced contractions with EC_{50} values of 0.27 µM (0.53-1.4, CI 95%: n=4) and 1.15 µM (1.18-2.1, CI 95%: n=4) respectively, (fig 3B).

Vasodilator effect of C. rotundus
The crude extract of *C. rotundus* exhibited the concentration-dependent relaxant effect when tested on K^+ (80mM)- and phenylephrine (1µM)-induced contractions with EC_{50} values of 0.24 mg/mL (0.12-0.36, CI 95%: n=4) and 1.18 mg/mL (0.9-1.43, CI 95%: n=4) respectively, (fig 4A). These relaxant effects were comparable to verapamil, which relaxed the K^+ (80 mM)- and phenylephrine (1µM)-induced contractions with EC_{50} values of 0.11 µM (0.09-0.25, CI 95%: n=4) and 1.35 µM (1.08-1.73, CI 95%: n=4) respectively (fig. 4B).

Antibacterial activity of C. rotundus
The crude extract of *C. rotundus* (150mg/mL) showed zone of inhibition (mm) of 20.12, 18.25, 17.65, and 18.95, while Gentamicin at the dose range of 20µg/disc showed the zone of inhibition (mm) of 22.45, 20.84, 19.41 and 22.67 with relative percentages of inhibition (RPI) of 80.35, 76.73, 19.41 and 69.90 against *S. aureus*, *B. pumilus, E. coli* and *C. freundii* respectively (table 1).

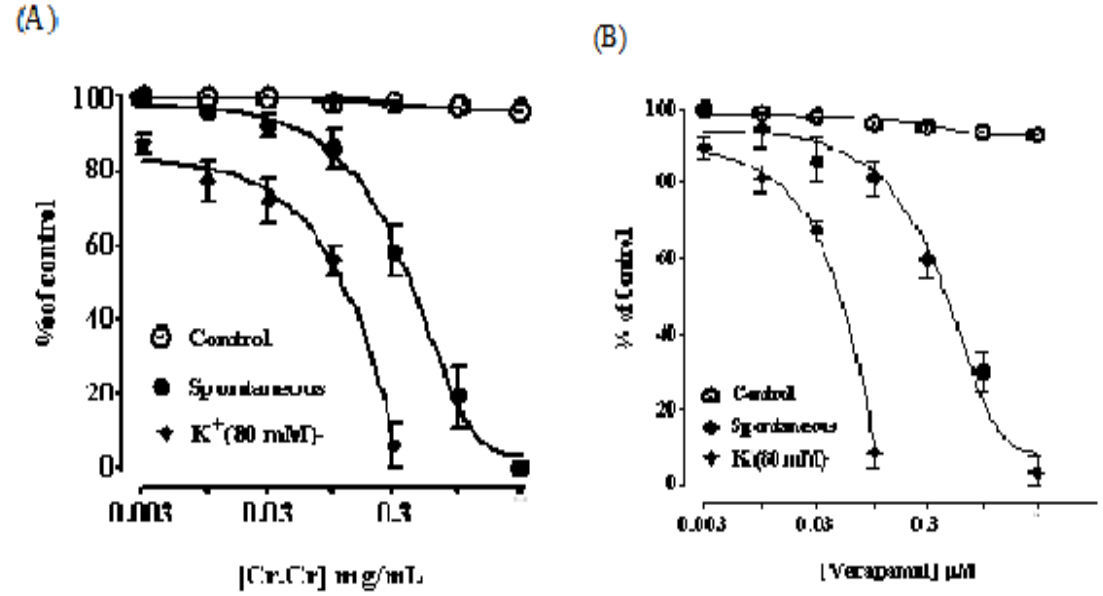

Fig. 1: Concentration-response curves showing inhibitory effect of: (A) crude extract of *C. rotundus* and (B) verapamil against spontaneous and K^+(80 mM)-induced contractions in isolated rabbit jejunum preparations. Values are expressed as mean ± SEM., n=3.

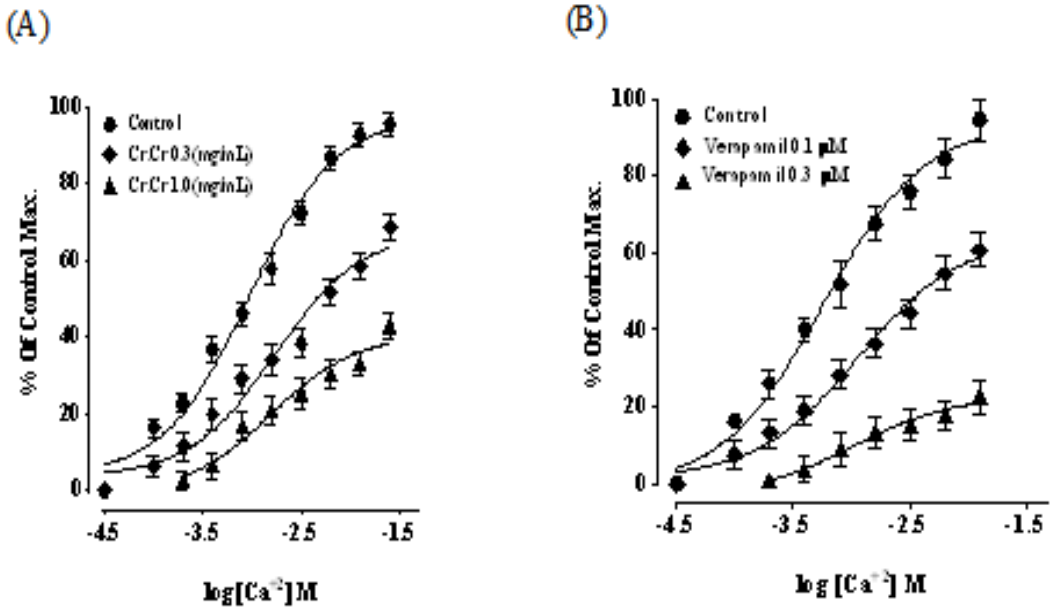

Fig. 2: Concentration–response curves of Ca^{+2} in the absence and presence of different concentrations of (A) crude extract of *C. rotundus*and (B) verapamil in isolated rabbit jejunum preparations. Values are expressed as mean ± SEM., n=3.

In vitro experiments

Antiemetic activity of *C. rotundus*

In the chicks of control group (treated with 10 mL/kg of normal saline followed by Copper sulphate) numbers of retches were 70±0.17, while in the chicks of group 2 (100 mg/kg of Cr. Cr), group 3(150 mg/kg of Cr.Cr) and group 4 (150 mg/kg of Chlorpromazine), the number of retches were reduced with %age inhibition of emesis of 51.45, 78.60 and 45.75 respectively (table 2). It is clear from the results that all tested *C. rotundus* have significant antiemetic potential (p<0.05) which were comparable to Chlorpromazine in Copper sulphate induce chick emesis model.

Antipyreticactivity of C. rotundus

The crude extract of *C. rotundus*(150 mg/kg)decreased the rectum temperature of pyrexia induced rabbit from 40.73±0.98 to 38.35±0.76°C (77.27%) after 4 h,

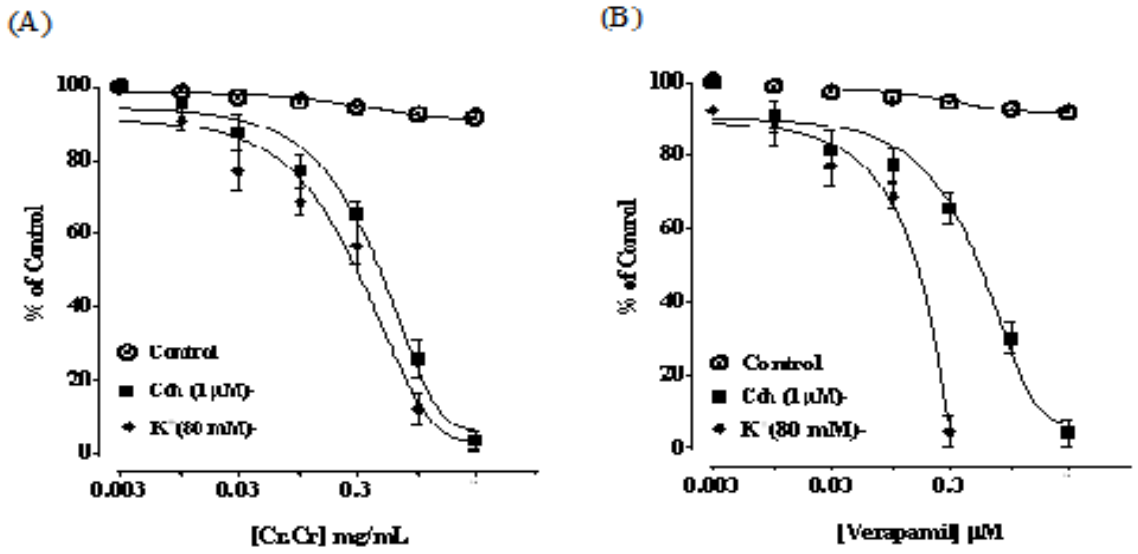

Fig. 3: Concentration-response curves showing inhibitory effect of: (A) crude extract of *C. rotundus* and (B) verapamil against K^+(80 mM)- and Cch(1µM)-induced contractions in isolated rabbit trachea preparations. Values are expressed as mean ± SEM., n=3.

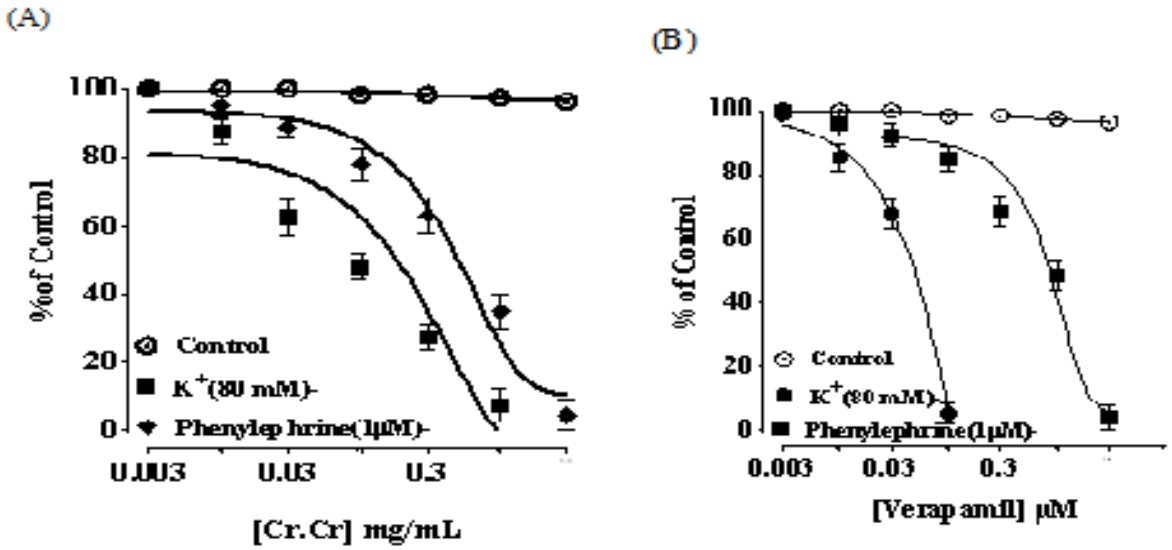

Fig. 4: Concentration-response curves showing inhibitory effect of: (A) crude extract of *C. rotundus*and (B) verapamil against K^+(80 mM)- and phenylephrine (1 µM)-induced contractions in isolated rabbit aorta preparations. Values are expressed as mean ± SEM., n=3.

comparable with Aspirin, which decreased from 40.69±0.94 to 37.90±0.76°C (90.95%). However, normal saline solution induced no prominent response on the elevated rectal temperature of the rabbit. There was a significant rise in temperature in all groups after brewer yeast administration but extract and Aspirin treatment result in a significant decrease (p<0.05) in rectum temperature equal to normal rectum temperature thus expressing that *C. rotundus*has a significant antipyretic effect (table 3).

Analgesic activity of C. rotundus

Prior to administration of *C. rotundus*, deflection time was 3.15±1.11 sec but after i.p. administration of *C. rotundus* (200 mg/kg) and Aspirin (75mg/kg) to mice of group 2 and 3, deflection time was increased up to 9.15± 0.7 and 9.95±1.11 sec respectively. Tail deflection time of *C. rotundus* was comparable to aspirin (p<0.05), thus indicating the significant analgesic effect (table 4).

Acute toxicity of C. rotundus

Acute toxicity of crude extract of *C. rotundus*was tested at 1, 3 and 5 g/kg; there was no behavioral changes and mortality up to 5g/kg which was higher than the normal therapeutic dose, thus indicating that *C. rotundus*is safe.

DISCUSSION

The crude extract of *C. rotundus* was tested for different phytoconstituents and it was found to contain saponins, tannins, alkaloids, flavonoids, terpenes and sterols.

C. rotundus (Linn.) has traditionally been used to treat diarrhea, dyspepsia, bronchitis, and hypertension, as well as have antibacterial, antipyretic, antiemetic and analgesic activities but presently, the crude extract of *C. rotundus*are subjected to evaluate and validate the folkloric uses and underlying possible mechanisms of aforementioned activities. Spontaneously contracting jejunumwas used to study the spasmolytic (inhibitory) mechanism of *C. rotundus,* without the addition of any spasmogen (Farre *et al*., 1991). Contraction of jejunum smooth muscles is mediated through activation of contractile elements caused by increased level of cytoplasmic free Ca^{+2}(Karaki and Weiss, 1983).The increase in intracellular Ca^{+2} occurs either an influx of Ca^{+2} through voltage-dependent Ca^{+2} channels (VDCs) or release of Ca^{+2} from sarcoplasmic reticulum which results in spontaneous rhythmic movement of smooth muscles by periodic depolarization (influx of Ca^{+2} via VDCs) and repolarization (Brading, 1981). Thus, the spasmolytic (inhibitory) effect of *C. rotundus*may appear due to calcium channel blockade (CCB)mediated possibly due to the interference of Ca^{+2} influx through VDCs. Calcium antagonistic activity was further confirmed when pretreatment of tissue with *C. rotundus*shifted the calcium concentration-response curves (CRCs) to the right, in a manner similar to verapamil.

Furtherresearch study was performed on tracheal preparations for the assessment of bronchodilator effect. Like in gut preparation, *C. rotundus*produced a concentration-dependent inhibition of K^{+}(80 mM)- and carbachol (1 μM)-induced contractions, similar to that of verapamil, thus indicating that bronchodilator effect was mediated possibly through Ca^{+2}-antagonism (Chad and Eckert, 1984).

Moreover, *C. rotundus*also relaxed phenylephrine (1 μM)- and K^{+}(80 mM)-induced contractions; elaborating calcium channel blockade (Gillani *et al*., 1994), as K^{+}(80 mM)-induced contractions are mediated through activation of calcium channel as well as release of Ca^{+2} from endoplasmic reticulum (Janbaz *et al*., 2012). Whereas phenylephrine (1μM)-induced contractions is mediated through the activation of α-receptors and subsequent Ca^{+2}influx. As *C. rotundus*relaxed the both phenylephrine (1μM)- and K^{+}(80mM)-induced contractions, indicating the calcium channel blockade.

The presence of flavonoids, saponins, and tannins (Kai *et al*., 1998; Zhu *et al*., 2005) support the calcium channel blockade effect of *C. rotundus,* which might be responsible for its traditional use in diarrhea, asthma and hypertension, while additional mechanism (s) cannot be ruled out.

Bacterial infections may result in fever, chill, headache, nausea, vomiting, and organ failures that may lead to physical disabilities, health problems, and mortalities. Almost, all known bacteria have developed resistance to antibiotics, whereas, antibiotics are associated with serious unwanted effects such as antibiotic-associated diarrhea, hypersensitivity, depletion of the gut normal flora, allergic response and immune-suppression(Al-Jabri, 2005).*C. rotundus*exhibited significant response by inhibiting the growth of bacteria on most regulatory levels such as DNA, RNA, peptidoglycan, and protein synthesis that may be due to the presence of secondary metabolites such as tannin, alkaloids, and flavonoids that are responsible for antibacterial activity (Trease and Evans, 1983).

Emesis is caused by the activation of vomiting centre located in the medulla oblongata, either activation of the motor pathway or the following input from four principal areas such as chemoreceptor trigger zone (CRTZ), GIT, cortex (and thalamus),cerebral and vestibular region. The CRTZ is in the proximity to the medulla and it is not surrounded by blood brain barrier (Becker, 2010). So, it can be hypothesized that the antiemetic effect of the *C. rotundus*can be likely mediated through inhibition of chemoreceptor trigger zone.

Pyrexia is caused by the approach of prostaglandins to the thermo-regulator hypothalamic neurons, which stimulates the set point and result in the elevation of body temperature through the retention and/or active generation of heat (Loux *et al*., 1972). *C. rotundus* exhibited a significant antipyretic effect (febrifuge) as it reduces the elevated body temperature of the experimental animals, that may be due to presence of flavonoids and alkaloids which are the known inhibitors of prostaglandins through inhibition of cyclo-oxygenase (Hajare *et al*., 2000; Rajnarayana *et al*., 2001; Ray *et al*., 2006).

Pain is generated by the interaction of prostaglandins with nociceptor (Geusens *et al*., 2013; Kanda *et al*., 2013), whereas, prostaglandins are biosynthesized by the action of the cyclooxygenase 1 & 2 on ω-3 and ω-6 polyunsaturated C-20 fatty acids (Lone and Taskén, 2013). *C. rotundus* exhibited a significant analgesic response in mice by tail flick method that may be mediated through the inhibition cyclo-oxygenase resulting

in unavailability of prostaglandins for action on nociceptors.

An oral dose of *C. rotundus* did not produce the lethality among the treated groups of animals up to 5 g/kg, which is higher than the therapeutic dose. However, chronic and sub-acute tests are to requireto justify the safety of *C. rotundus*.

CONCLUSION

It can be inferred that relaxant effect showed by the *C. rotundus* on isolated rabbit jejunum, trachea and aorta preparations may be attributed due to blockade of calcium channels, which provide sound mechanistic background to validate the folkloric uses of *C. rotundus* in the management of gastrointestinal, respiratory and vascular disorders, though additional mechanism(s) cannot be ruled out. The observed results also validate its uses in bacterial infection, emesis, pain and pyrexia.

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