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Research Article

Antispasmodic and Antidiarrheal Effect of *Hippeastrum puniceum*; Involvement of Antimuscarinic and Calcium Antagonism Pathways

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Abstract

Hippeastrum puniceum has been distributed worldwide, possesses spasmolytic activity, and is used for treating asthma and other ailments. This study was conducted to rationalize its spasmolytic activity and antiasthmatic uses. After the phytochemical screening, the plant extract was analyzed by using different ex-vivo and in-vivo techniques. For the evaluation of the spasmolytic effect, the extract was applied to isolated rabbit jejunum, rat ileum, and rabbit trachea suspended in an organ bath. Castor oil-induced diarrhea and charcoal meal transit test models were used to study the extract's antidiarrheal and gut inhibitory effect. Crude extract of *H. puniceum* (Hp.Cr) and its dichloromethane fraction (Hp.DCM) showed spasmolytic effects on isolated rabbit jejunum and trachea. To explore the mechanism of the spasmolytic effect, concentration-response curves of Carbachol and calcium were framed, and it was found that the spasmolytic effect may be due to the antagonism of muscarinic receptors and calcium channels. In addition, significant dose-dependent diarrheal protection and inhibition of gastrointestinal motility were observed. These results indicate that *H. puniceum* possesses spasmolytic, tracheorelaxant, antidiarrheal, gut motility inhibitory activities mediated by muscarinic receptor and calcium channel antagonism.

Keywords: *Hippeastrum puniceum*, spasmolytic, antidiarrheal, asthma, antimuscarinic

1. Introduction

Hippeastrum puniceum (Lam.) Voss (Family: Amaryllidaceae) is an ornamental plant commonly called Easter lily (Kumar Patel 2015). It is widely grown worldwide but is indigenous to Central and South America (Jotham Ziffer-Berger 2015). *H. puniceum* is an orange-red attractive trumpet-shaped flowered herb with green, saber-like leaves and a bitter taste (C.P Deepa 2014). Offsets or seeds propagate it, and annually, flowers open during October (Bradacs, Heilmann, and Weckerle 2011). It has been reported that this plant contains proteins, tannins, alkaloids, amino acids, saponins, flavonoids, terpenoids, and carbohydrates (C.P Deepa 2014). Bioactive alkaloids of *H. puniceum* known as 3- O-acetylnarcissidine, Vittatine and 11-hydroxyvittatine, and narciclasine 11,12-didehydroanhdrolycorine and Lycorine (Cortes et al. 2015).

Brazilian communities have used different parts of this plant to cure various ailments. Tea is prepared from the roots and bulbs of *H. puniceum* and used to treat asthma (Coelho-Ferreira 2009; C.P Deepa 2014). The bulb is used to treat immune disorders like allergies and rheumatoid arthritis (Sangtam et al. 2012). The leaves are used for earaches in some communities (Bradacs,

Heilmann, and Weckerle 2011). Flowers of this plant possess spasmolytic properties and are locally utilized to cure whooping cough. Bulbs are irritant poisons and emetics and are consumed to treat tumors, swelling, piles, sores, wounds, and various inflammatory diseases (Deepa 2014). Roots are used to promote fertility and to cure female health-related issues (Yazbek et al. 2016). It has been used for the treatment of skin and soft tissue problems(Sen et al. 2011). The genus *Hippeastrum* is known to possess acetylcholinesterase inhibitory, apoptosis-inducing, and proliferation-inhibitory activities. It has been reported that *H. puniceum* possesses growth inhibitory, antimitotic activity (Santana et al. 2008), and insecticidal activity (C.P Deepa 2014). The presence of acetylcholinesterase activity in *H. puniceum* has been reported as a basic reason for its use in Alzheimer's disorder (Cortes et al. 2015). A glycosylated alkaloid, narciclasine-4-*O*-*β*-D-xylopyranoside of *H. puniceum* possesses anticancer activity against breast and colon cancer(Feu et al. 2021). Lycorine and galantamine alkaloids present abundantly in *H. puniceum* possess neuroprotective and anticholinesterase inhibitory activity(Costa et al. 2019). Due to the presence of lycorine and galantamine and the presence of acetylcholinesterase inhibitory activity, *H. puniceum* can be used for the treatment of Alzheimer's disease (Tundis et al., n.d.). Bioactive alkaloid (3-O- acetylnarcissidine) has the ability to depress root progression and root proliferation and fertilization of various weeds and showed antifeedant against polyphagous pest *Spodoptera littoralis* (Santana et al. 2008). However, this plant contains a number of important bioactive molecules and possesses beneficial biological properties but still not been explored extensively for its pharmacological importance. This study aimed to elucidate the scientific basis of spasmolytic activity and antiasthmatic use of *H. puniceum* using *ex-vivo* and *in-vivo* techniques in the gastrointestinal and respiratory systems.

2. Materials & Methods

2.1. Plant Material Collection and Extraction

H. puniceum was collected and identified by a taxonomist Prof. Dr. Zafar Ullah Zafar vide voucher specimen No. kew-278253. Adulterants were removed by manual picking, and the collected flowers were dried under a shed and then ground to a coarse powder. One (01) kg of dried flowers were soaked with 80% aqueous methanol for 7 days at 25 ± 2ºC with repeated shaking. The macerated material was cleaned of herbal remain through a double-layered muslin cloth and filtered thrice via Whatman filter paper. These maceration procedures were consequently repeated two times. The combined macerated filtrate was evaporated by using a rotary evaporator (Rotavapor®, Buchi. R-200, Switzerland), which was connected to a recirculation chiller and vacuum pump. A dark brown semisolid extract was obtained with an 18% yield and kept in a refrigerator at -20ºC. About 50g of this crude extract was subjected to fractionation. For this process, 50g crude extract was dissolved in a sufficient volume of water and an equal volume of dichloromethane (DCM) in a separating funnel with gentle shaking. This mixture was left for 20 minutes, and two layers (aqueous and DCM) were separated, dried, and named aqueous fraction (Hp.Aq) and dichloromethane fraction (Hp.DCM) (Aleem and Janbaz 2018).

2.2. Chemicals and Drugs

This study's solvents, drugs, and chemicals were high purity and analytical grade. Stock solutions and physiological solutions were prepared freshly on the day of the experiments. Sodium hydroxide (NaOH,) ammonium hydroxide (NH_4OH), and sodium chloride were provided by BDH Laboratory supplies, England. Magnesium sulfate ($MgSO_4$), glucose, methanol, sodium bicarbonate ($NaHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), calcium chloride, sodium dihydrogen phosphate (NaH_2PO_4) was managed by Merck, Germany.

Verapamil hydrochloride, acetylcholine chloride (AchCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), and Carbachol (CCh) have been brought from the Sigma-Aldrich Co. USA.

2.3. Animals

Sprague Dawley rats, Balb C mice, and locally bred healthy rabbits of both sexes were used in this study. These animals were placed in controlled environmental conditions. Standard diet and water were provided to them. This study was approved by the departmental ethical committee.

2.4. Phytochemical Screening

The extract (Hp.Cr) was subjected to phytochemical screening to identify the presence of different phytochemicals such as saponins, alkaloids, sterols, flavonoids, anthraquinones, phenols, coumarins, and terpenes; by using the procedures described earlier [18-19].

2.4.1. Total Phenolic Content

Folin–Ciocalteu spectrophotometric method was adopted for the determination of phenolic contents in Hp.Cr by using gallic acid as a positive control. Sodium carbonate (2.5mL), Folin-ciocalteu (0.1mL), and plant extract (0.5mL) were mixed and incubated for 30 minutes. Using a UV-spectrophotometer, the optical density of the incubated mixture was measured at 760nm. Total phenolic contents present in the extract were expressed as mg equivalents of gallic acid (GAE/g).

2.4.2. Total Flavonoid Contents

An aluminum chloride spectroscopic procedure was adopted by using quercetin as a positive control to determine the total flavonoid contents in the plant extract. A solution of plant extract (1mg/mL) was taken, and 1.0mL of this solution was mixed with 1.0mL of 10% AlCl3. This mixture is left for half an hour for incubation. After incubation, absorbance was measured at 415nm. Measured total flavonoid contents were expressed as mg QU/g of Hp.Cr (El Far and Taie 2009; Rahman et al. 2020).

2.5. Ex vivo Experiments

Experiments on isolated tissue preparations of the jejunum and trachea were performed in an organ tissue bath according to the protocol previously explained (Abdur Rahman et al. 2017; Aleem and Janbaz 2018).

2.5.1. Spasmolytic Study on Isolated Rabbit Jejunum

Crude extract of *H. puniceum* (Hp.Cr) and its fractions were applied to rhythmical contracting isolated jejunum of rabbit. For this purpose, about a 2.0cm piece of jejunum was hung in an organ bath containing Tyrode's solution. A 1.0g of preload tension, a temperature of 37 ºC, and a continuous supply of carbogen were maintained during the experiment. Untreated jejunum was equilibrated for 30 minutes then plant extract was administered cumulatively to check their spasmolytic activity. The response was recorded by using isotonic transducers connected to PowerLab.

2.5.2. Antimuscarinic Activity

To study the antimuscarinic activity of the plant extract, isolated ileum was incubated with different concentrations of plant extract. Carbachol (CCh) 1µM was applied to the incubated tissue, and the response was recorded with the help of an isotonic transducer (MLT0015) and compared with the control response of Carbachol. Furthermore, concentration-response curves (CRCs) of CCh were built in the absence and presence of plant extract to confirm antimuscarinic activity (Bashir et al. 2018; Gilani et al. 2013).

2.5.3. Calcium Channel Blocker Activity

Concentration-response curves (CRCs) of calcium were built for the confirmation of the calcium channel-arresting activity of plant extract by following the procedure described by Abdur Rahman et al. 2013 (Abdur Rahman, Bashir, and Gilani 2013a).

2.5.4. Tracheorelaxant Study

A 2 cm piece of the isolated tracheal strip containing 2-3 cartilages was incised longitudinally to form a tracheal strip with a central smooth muscle layer and cartilage on dual

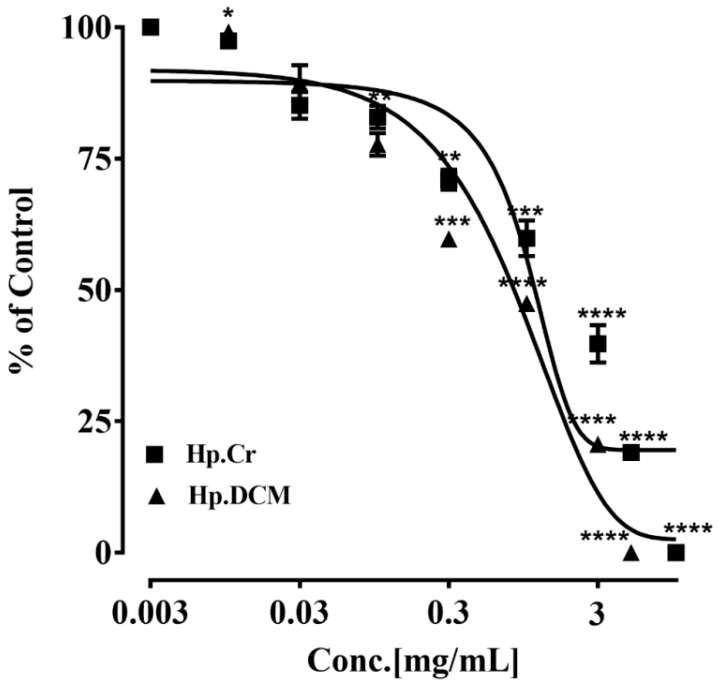

Figure 1. Relaxant effect of Hp.Cr and Hp.DCM on spontaneous contracting jejunum. Data values are presented as mean ± SEM, n=6 and *P<0.05, **P<0.01, ***P<0.001 (Two-way ANOVA followed by Tukey's test).

edges. The tracheal preparation was suspended in ventilated Kreb's solution with a continuous supply of carbogen in a tissue organ bath. The Kreb's solution composition is (mM): glucose 11.7, NaCl 118.2, KH_2PO_4 1.3, $MgSO_4$ 1.2, KCl 4.7, $CaCl_2$ 2.5, and $NaHCO_3$ 25. A preload tension of 1g and temperature of 37 ºC was maintained during the experiment. Untreated tissue was equilibrated for 1 hour and stabilized by repeated exposures to Carbachol (1µM) or K^+ (80mM). After stabilizing the tissue, the extract was cumulatively applied to this tissue to study the tracheal relaxant effect. Cumulative response curves of carbachol concentrations were constructed. Tissue was washed after achieving maximum response of Carbachol then this curve was rebuilt in the presence of different concentrations of Hp.Cr. Isometric transducers, which were attached to Power Lab (AD Instruments, Australia), were used to record tracheal responses after the cumulative addition of plant extract.

2.6. In vivo Experiments

2.6.1. Antidiarrheal Activity

To study the antidiarrheal effect of Hp.Cr, SD rats (100-200g) were divided into six animals (n= 6). These rats were individually housed in cages lined with blotting paper. Group 1 was treated with normal saline (10mL/kg), whereas other groups 2 to 6 were treated with castor oil (10 mL/kg) via oral route to induce diarrhea. One hour after the first treatment, groups 1 and 2, which served as the normal and negative control, respectively, were treated with normal saline (10mL/kg), and group 3, the positive control, was treated with Loperamide (3mg/kg), while groups 4, 5 and 6, the test groups were treated with respective doses of Hp.Cr at 100, 300, and 500 mg/kg. Afterward, every rat was inspected for the absence and presence of diarrheal signs for 6.0 hours, and the total number of diarrheal spots for each group was calculated(Jahan et al. 2019; Rahman et al. 2020).

2.6.2. Gastrointestinal Motility Study

Effect of Hp.Cr on gastric motility was studied by using a gastrointestinal charcoal meal transit time test. Balb/C mice were equally segregated into five groups and kept on fasting

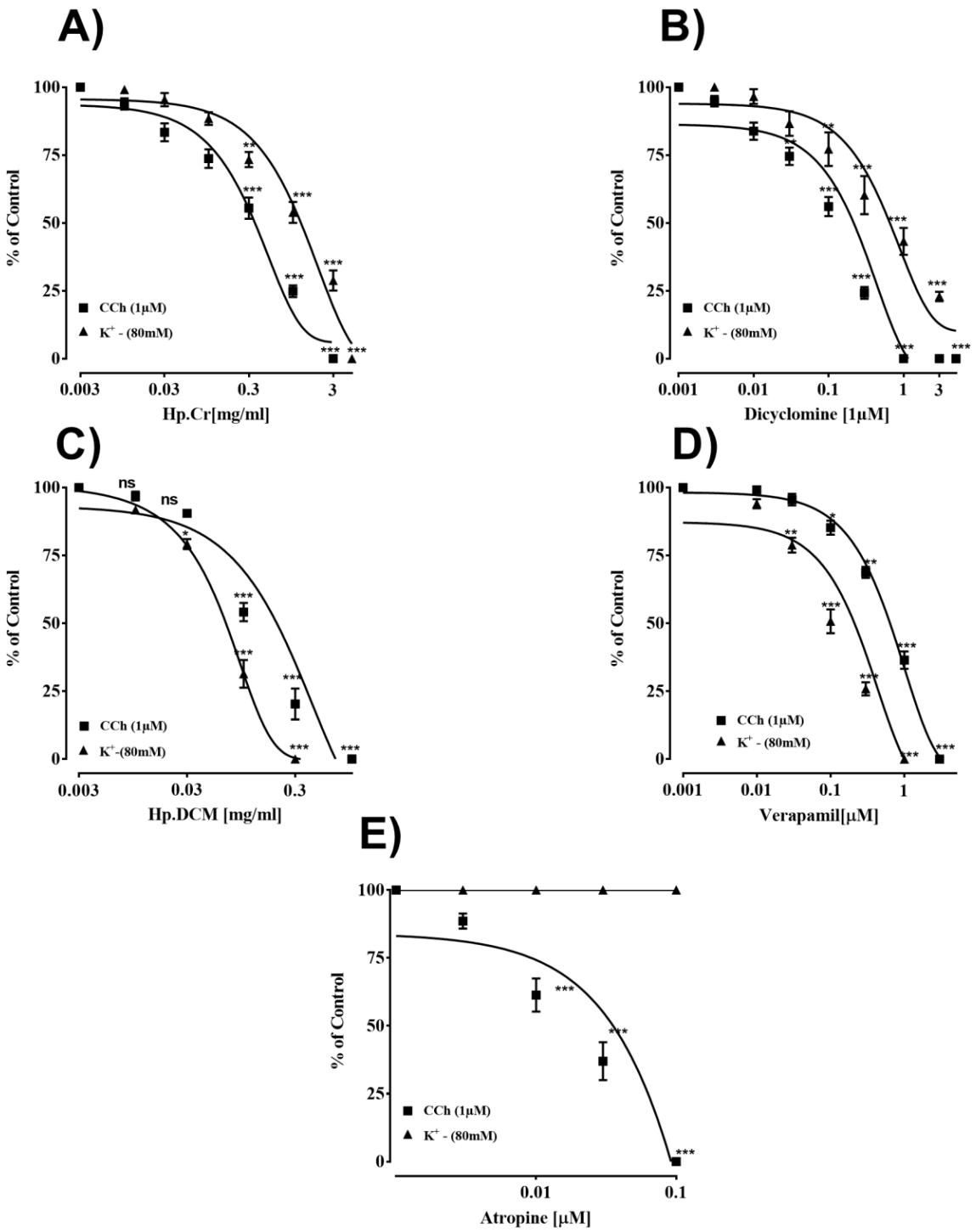

Figure 2: Concentration-based spasmolytic effect of A) Hp.Cr, B) Dicyclomine, C) Hp.DCM, D) Verapamil, and E) Atropine on isolated jejunum pretreated with K^+-80mM and CCh. Data values of the result are represented as mean±SEM, n=6-8 (*P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA followed by Tukey's test).

overnight. The next morning after 12 hours, these animals were administered respective treatments. Control group animals were intraperitoneally treated with normal saline (10 mL/kg), and the standard group (2nd group) was treated with atropine (10 mg/kg/i.p.),

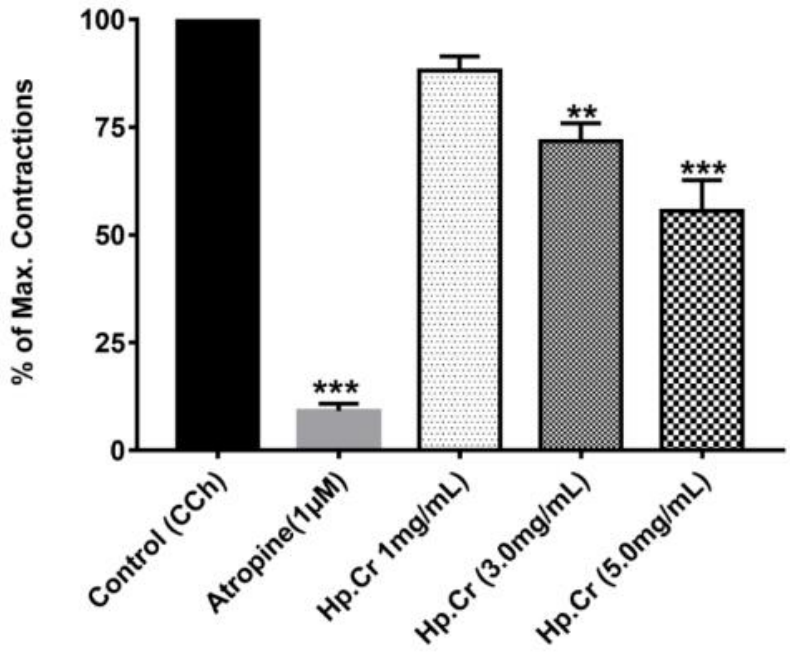

Figure 3: Inhibitory effect of Hp.Cr on CCh induced contractions in isolated rat ileum. Data values are indicated as mean±SEM and n=6 (*P<0.05, **P<0.01, ***P<0.001, One way ANOVA followed by Dunnet's test).

respectively. The test groups were treated with Hp.Cr 100, Hp.Cr 300 and Hp.Cr 500mg/Kg (p.o). Fifteen minutes later, these animals were orally treated with 0.3mL of the charcoal meal. After half an hour, all of these animals were killed, followed by the opening of the abdomen, and the whole small intestine was removed from the abdomen. The total length of the small intestine and the distance traveled by the charcoal meal from the pylorus were measured (Aleem and Janbaz 2018).

2.6.3. Acute Toxicity Test

In order to check the safety of Hp.Cr, Balb/C mice of either sex were used and divided into 4 groups of 5 mice each. Group 1 (negative control) was given 10mg/kg normal saline orally, and the rest groups were administered oral Hp.Cr 3, 5, and 10mg/kg respectively in 10mL/kg volume at increasing doses pattern. The mice stayed with free access to food and water and were observed for 6 hrs. Lethality was noted after 24 hrs (Sajjad Haider et al. 2021; Aslam and Janbaz 2019).

2.7. Statistical Analysis

Data from this work were analyzed by using GraphPad Prism (version 6.01). Data values of *ex- vivo* studies were analyzed using non-linear regression and represented as mean ± SEM. The antidiarrheal and gastrointestinal motility effects of Hp.Cr were analyzed by using One-Way ANOVA followed by Dunnett's multiple comparison test. In all cases, P values < 0.05 were considered statistically significant.

3. Results

3.1. Preliminary Phytochemical Analysis

Crude extract of *H. puniceum* (Hp.Cr) was subjected to phytochemical analysis, and the presence of alkaloids, anthraquinones, saponins, flavonoids, tannins, and sterols was observed.

3.1.1. Total Phenolic Contents

Folin–Ciocalteu spectrophotometric method revealed that the total phenolic contents in the

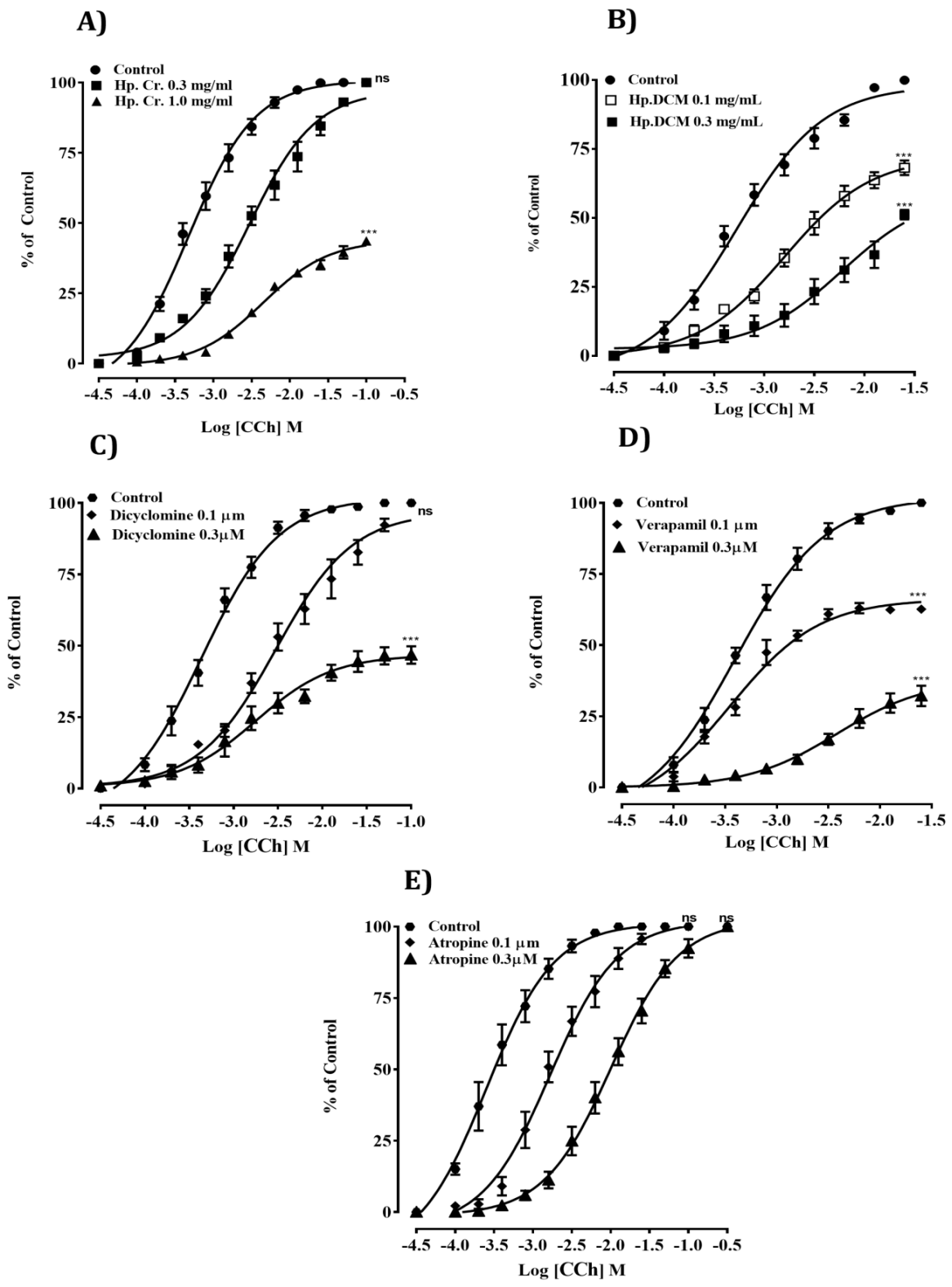

Figure 4: Inhibitory effect of A) Hp.Cr, B) Hp.DCM, C)Dicyclomine, D) Verapamil, and E) atropine on concentration-response curves of Carbachol. Results are represented as mean ± SEM, n=5-8(*P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA followed by Dunnett's test).

crude extract of H.puniceum were 103.21 mg GAE/g of the extract.

3.1.2. Total Flavonoid Contents

The aluminum chloride spectrophotometric method was used for the determination of total flavonoid contents in the plant extract. An amount of 41.23mg QE/g of Hp.Cr total flavonoids were found to be present in the plant extract.

3.2. Ex vivo Experiments

3.2.1. Spasmolytic Effect of Plant Extract on Jejunum

Crude extract of *H. puniceum* (Hp.Cr) and its fractions were evaluated for their possible spasmolytic effect. Complete inhibition of spontaneous contractions of jejunum was observed by the application of Hp.Cr and Hp.DCM with respective EC_{50} values of 2.83 mg/mL (1.90 to 4.23, *CI*=95% and n=5). While Hp.DCM showed inhibition of jejunal contractions with EC_{50} value of 0.82 mg/mL (0.61 to 1.09, *CI*=95% and n=5), but the aqueous extract did not show any relaxant effect (Figure1).

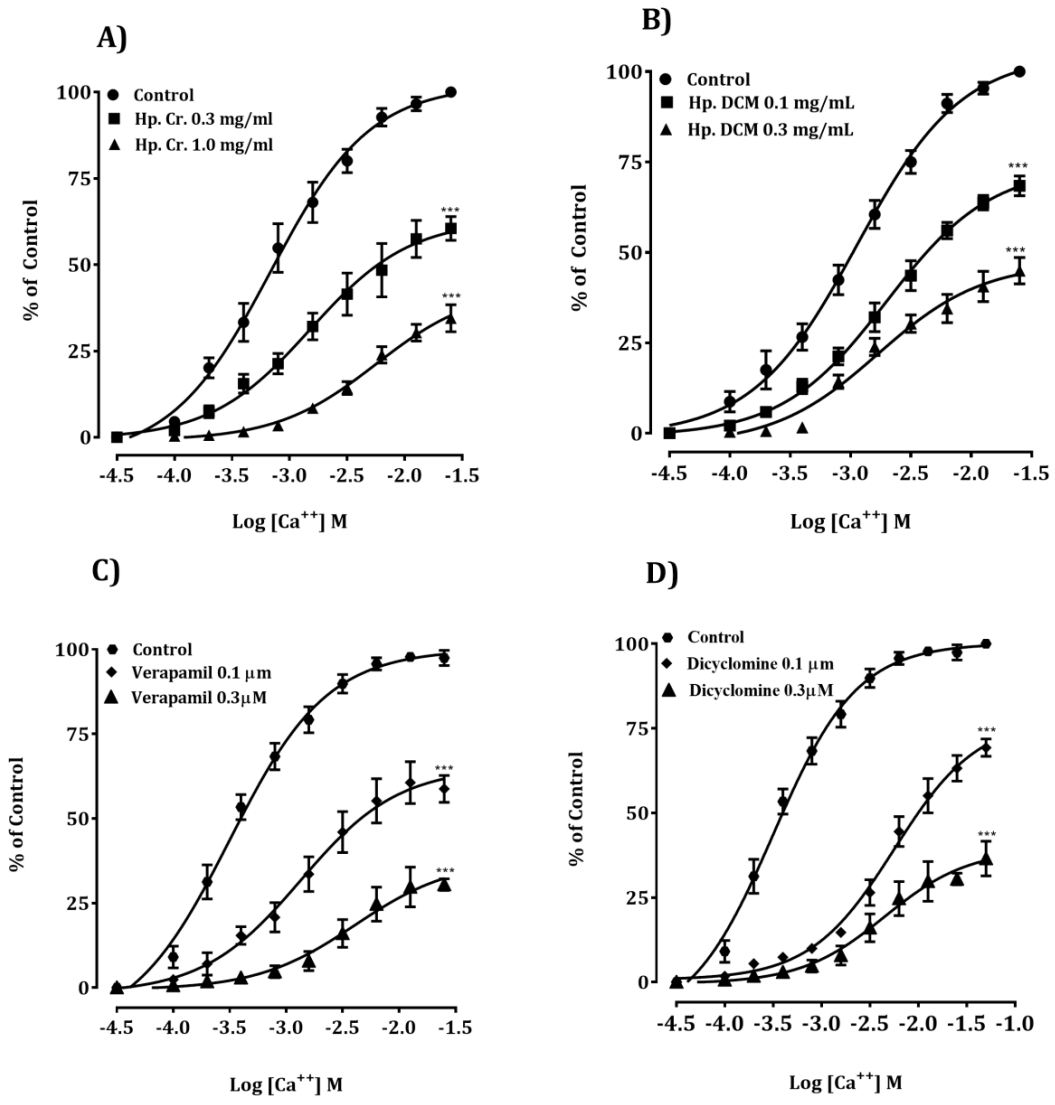

Figure 5: Inhibitory effect of A) Hp.Cr, B) Hp.DCM, C) Verapamil, and D) Dicyclomine against concentration-response curves of calcium on isolated rabbit jejunum. Result values are represented as mean±SEM, n=5-8 (*P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA followed by Dunnett's test).

To explore the mechanism of action of spasmolytic effect, Hp.Cr and Hp.DCM both were tested on Carbachol (CCh), and K^+-80mM mediated contractions in isolated jejunum of rabbit. Relaxation of CCh and K^+-80mM induced contractions was observed by Hp.Cr with EC_{50} of 0.49 ± 0.06mg/mL (0.36-0.68; *CI*= 95% and n= 4-5) and 1.93 ± 0.08 mg/mL (1.27-2.93; *CI*=95% and n= 4-5) respectively. Spasmolytic effect of Hp.Cr was similar to dicyclomine, which has a potent spasmolytic effect against CCh-mediated contractions as compared to K^+-80mM induced contractions with EC_{50} values of 0.09 ± 0.03 mg/mL (0.08-0.11; *CI*= 95% and n= 5) and 0.50 ±0.05mg/mL (0.39-0.65; *CI*= 95% and n= 5) respectively. Similarly, Hp.DCM showed complete relaxation of CCh and K^+-80mM induced contractions, but its effect was more potent against K^+-80mM induced contractions. Respective EC_{50} values of Hp.DCM against CCh and K^+-80mM were 0.11±0.02 mg/mL (0.10-0.12; *CI*= 95% and n= 4) and 0.06±0.02 mg/mL (0.05-0.06; *CI*= 95% and n= 5). Spasmolytic effect of Hp.DCM was comparable to verapamil which has a more potent effect against K^+-80mM induced contractions as compared to CCh-induced contractions having EC_{50} values of 0.10±0.02 mg/mL (0.09-0.11; *CI*= 95% and n= 4) and 0.53±0.03mg/mL (0.47-0.61; *CI*= 95% and n= 5) respectively. These results indicate that antimuscarinic activity is dominant in crude extract (Hp.Cr). While Hp.DCM possesses calcium channel blocking activity but Hp.Aq is devoid of any spasmolytic or spasmogenic activity. Atropine, a pure muscarinic antagonist, when applied to CCh and K^+-80mM induced contractions in the jejunum, caused complete inhibition of CCh induced contractions while no effect against K^+-80mM induced contractions was observed (Figure 2).

3.2.2. Antimuscarinic Activity

To study the antimuscarinic activity of Hp.Cr, isolated rat ileum was pretreated with Hp.Cr and atropine (1μM), and it was observed that the contractile effect of the Carbachol was reduced by Hp.Cr 1, 3, and 5mg/mL in a concentration-dependent way like atropine ($F_{(4,20)}$ =100.6, P<0.0001). Pretreatment of ileum with atropine (1μM) reduced the contractile effect of CCh to 9.2±1.64% (P=0.0001) compared with the control. Pretreatment of tissue with Hp.Cr 1, 3 1nd 5mg/mL also attenuate the contractile effect of CCh to 83.38±2.54% (P=0.01), 51.43±5.73% (P=0.0001), 38.30±4.76% (P=0.0001) respectively compared to control (Figure 3).

Furthermore, the presence of antimuscarinic activity of Hp.Cr was confirmed by inhibition of CRCs of CCh. Pretreatment of tissue with Hp.Cr 0.3mg/mL resulted in the rightward parallel shift of concentration-response curves without decreasing the maximum response, a property of a specific antagonist. But at a higher concentration of 1.0 mg/mL, Hp.Cr caused a non-parallel rightward shift of the curve with the suppression of maximum response like dicyclomine. However, Hp.DCM shifted the CRCs of CCh to the right at both concentrations, like verapamil, a calcium channel blocker (Figure 4).

3.2.3. Calcium Channel Blocker Activity

Presence of calcium channel antagonistic potential of Hp.Cr and Hp.DCM was analyzed by the CRCs of calcium. Both Hp.Cr and Hp.DCM showed a non-parallel rightward shift of CRCs of calcium in a concentration-dependent manner at respective concentrations of Hp.Cr 0.3 and 1.0mg/mL and Hp.DCM 0.1 and 0.3mg/mL like verapamil (Figure 5).

Hp.Cr showed an inhibitory effect against contractions induced by Carbachol (1μM) and K^+-80mM with EC_{50} values of 0.52 ± 0.07 mg/mL (0.37-0.73; *CI*= 95% and n= 5) and 2.20 ± 0.125 mg/mL (1.21-4.01; *CI*= 95% and n= 4) respectively like dicyclomine. Hp.DCM caused inhibition of Carbachol (1μM), and K^+-80mM mediated tracheal contractions with respective EC_{50} values of 1.71 ± 0.17 mg/mL (0.76-3.87; *CI*= 95% and n= 6) and 0.38 ± 0.09 mg/mL (0.23-0.61; *CI*= 95% and n= 5). On the other hand, Dicyclomine showed

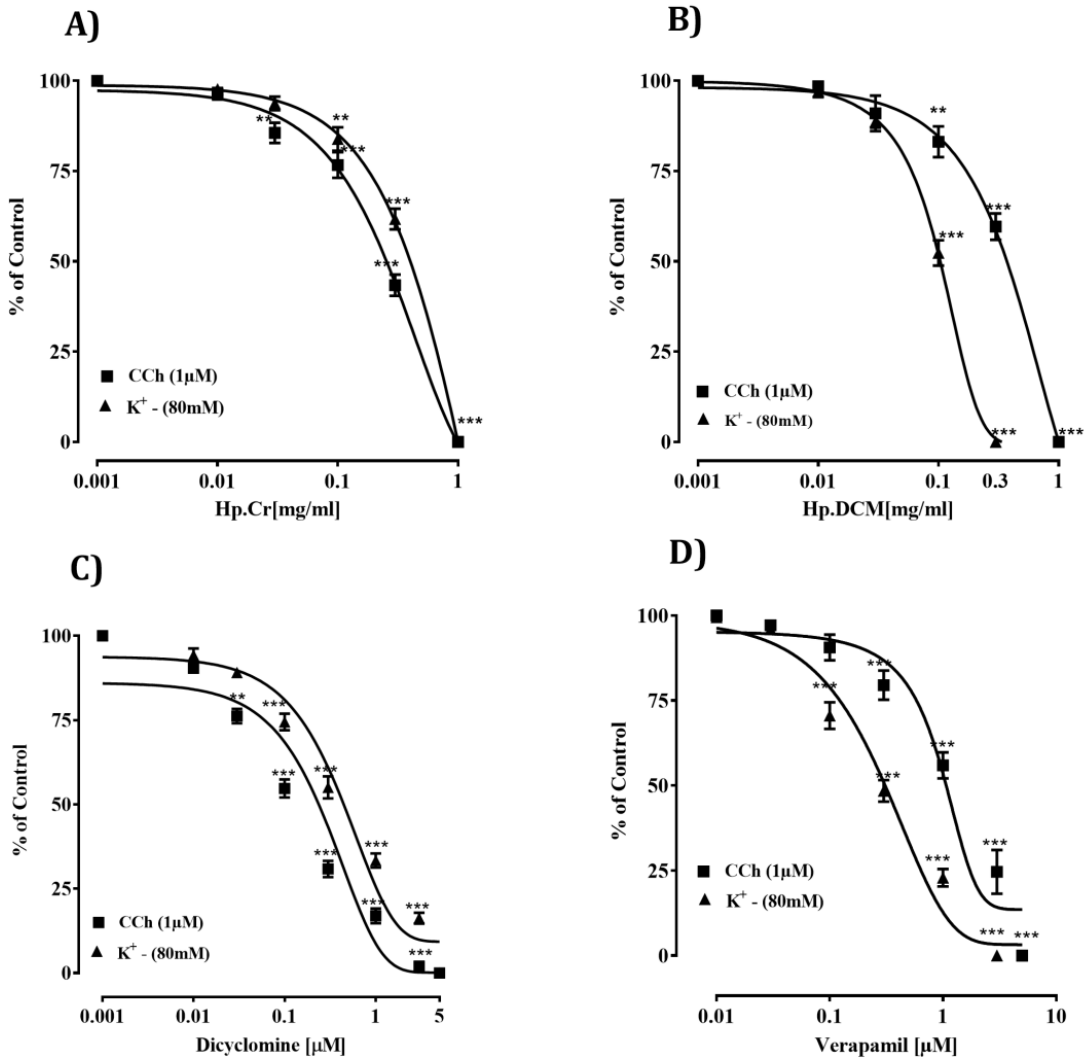

Figure 6: Inhibition of contraction by the application of A) Hp.Cr, B) Hp.DCM, C) Dicyclomine, and D) Verapamil in isolated tracheal strips pretreated with K^+-80mM and CCh. Results are represented as mean±SEM, n=6-8 (*P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA followed by Tukey's test).

tracheal dilation of Carbachol (1μM) and K^+-80mM induced contractions with an EC_{50} value of 0.11 ± 0.04 μM (0.09-0.15; *CI*= 95% and n=6) and 0.43 ± 0.06 μM (0.32-0.59; *CI*= 95% and n= 5) respectively (Figure 6).

3.3. In vivo Experiments

3.3.1. Antidiarrheal Activity

Significant protection from diarrhea was observed by the administration of Hp.Cr to rats in castor oil-induced diarrhea model ($F(4,20)=35.4$, $P<0.0001$). Administration of Hp.Cr showed dose-dependent protection from diarrhea by decreasing the diarrheal spots. The number of diarrheal spots seen at Hp.Cr 100, 300, and 500 mg/kg were 13.6 ± 0.92 (P=0.002), 8.40 ± 0.81(P=0.0001), and 6.8 ± 0.58 (P=0.0001), respectively. Loperamide result was (3mg/kg) 5.2 ± 0.86(P=0.0001) compared to the control group animals. Diarrheal spots in control group animals treated with normal saline were 19.2 ±0.86 (Figure 7).

3.3.2. Effect of Plant Extract on Gastrointestinal Motility

Treatment of mice with Hp.Cr resulted in a decrease in gastrointestinal motility as observed by a decrease in the distance traveled

by charcoal as compared to the control (saline-treated) group, and the same as atropine ($F(4,20)=20.89$, $P<0.0001$). The distance traveled by charcoal in control group animals was 46.6±3.14cm. The respective distances traveled by charcoal in atropine, Hp.Cr 100, 300, and 500 treated animals were 11.4±1.03 (P=0.0001), 38±3.49 (P=0.17), 32±2.21 (P=0.009), and 21.4±4.2 (P=0.0001) cm (Figure 8).

3.3.3. Acute Toxicity Test

The Hp.Cr was observed as safe in mice at 3, 5, and 10mg/kg. No abnormal physical or behavioral changes and neither morbidity nor mortality were noted at given doses for 24 hrs.

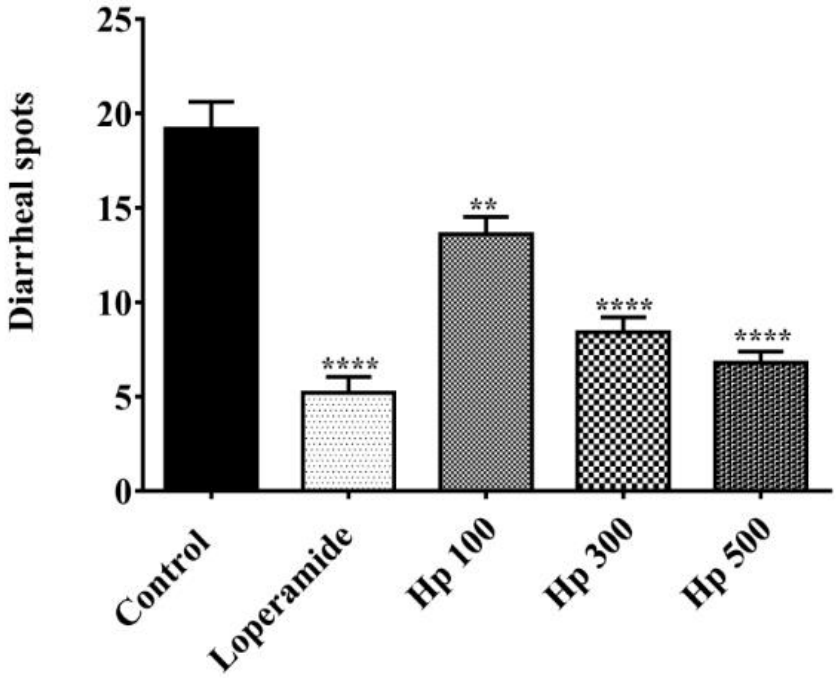

Figure 7: Antidiarrheal effect of Hp.Cr against castor oil-induced diarrhea in rats. Results are shown as mean±SEM and n=5 (*P<0.05, **P<0.01, ***P<0.001, One way ANOVA followed by Dunnett's test).

4. Discussion

In view of traditional antispasmodic uses of *H. puniceum*, this study was planned to explore the pharmaceutical of its spasmolytic basis activity and use in asthma by using *ex-vivo* and *in-vivo* techniques in the gastrointestinal and respiratory systems.

The crude extract of the plant was analyzed for the presence of macromolecules and alkaloids, anthraquinones, saponins, flavonoids, tannins, and sterols were found to be present in it. Alternative depolarization and repolarization occur in smooth muscles, which control their contraction. During the depolarization phase, voltage-gated calcium channels open, and calcium influx occurs rapidly (Aleem and Janbaz 2018). An increase in calcium level in cytosol causes the contraction of smooth muscles, and a decrease in calcium level may lead to the relaxation of smooth muscles. Increased free cytosolic calcium may activate calmodulin-dependent myosin light chain kinase contractile machinery of smooth muscles and cause contractions. Abnormal contraction of smooth muscles may lead to various problems like diarrhea and asthma. It has been reported that the contraction of smooth muscles of the jejunum and trachea may be inhibited by herbal medicines having calcium channel blockers or antimuscarinic activity (Chaudhary et al. 2012;

Rahman et al. 2017). Increased extracellular potassium (>30mM) cause the opening of voltage-dependent calcium level and result in a spasmogenic effect (Abdur Rahman, Bashir, and Gilani 2013b; Bashir et al. 2018). Any substance inhibiting High-K+-induced contractions is considered a calcium channel blocker. Muscarinic receptor agonists like Carbachol (CCh) also induce contractions in smooth muscles and muscarinic antagonists like atropine cause the relaxation of these contractions (Alam and Shah 2019). When applied to spontaneously contracting isolated rabbit jejunum, crude extract (Hp.Cr) and its dichloromethane (Hp.DCM) fraction showed a spasmolytic effect. However, its aqueous fraction did not show any effect on isolated tissues. To study the underlying mechanism of action of observed spasmolytic effect, Hp.Cr and Hp.DCM were applied to isolated jejunum pre-exposed to K^+-80mM and CCh. The application of K^+-80mM causes the induction of contractions in smooth muscles by opening the voltage-operated ion channels, while CCh induces the contractions in smooth muscles by stimulating muscarinic receptors. Inhibition of induced contractions was observed both by the application of Hp.Cr and Hp.DCM.

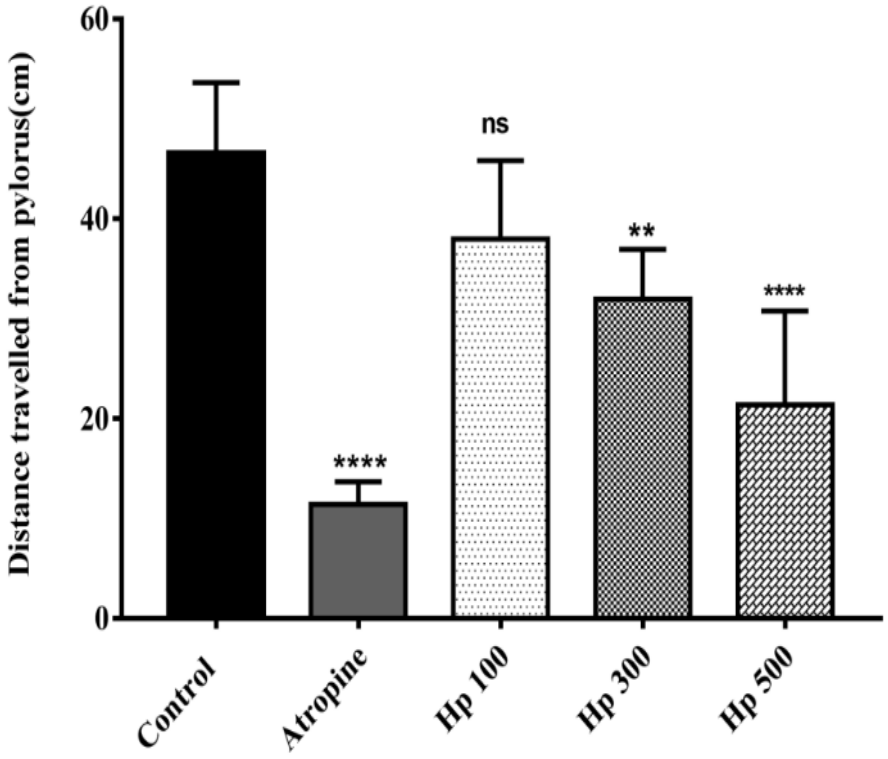

Figure 8: Gastrointestinal motility inhibitory effect of Hp.Cr. Results are shown as mean±SEM and n=6 (*P<0.05, **P<0.01, ***P<0.001, One way ANOVA followed by Dunnett's test).

These results indicated the presence of two different mechanisms of action, like dicyclomine. Dicyclomine is a spasmolytic drug that shows its effect by antagonizing the muscarinic receptors and blocking voltage-gated ion channels (Aleem and Janbaz 2018; Bashir et al. 2018). Pretreatment of the isolated rat ileum tissue with Hp.Cr showed the parallel shift concentration-response curves (CRCs) of CCh without inhibiting the maximum response at a lower concentration, a characteristic of specific antagonism. However, CRCs showed a non-parallel rightward shift at

higher concentrations by inhibiting the maximum response, like dicyclomine. Verapamil, a typical calcium channel blocker, showed a non-parallel rightward shift of CRCs of CCh both at low and high concentrations. But the rightward shift of the CRCs of CCh without inhibition of maximal effect was observed by the application of atropine at both concentrations. Pretreatment of isolated tissue of rabbit jejunum with Hp.Cr, showed the rightward shift of CRCs of Ca^{2+} by inhibiting the maximal effect like calcium channel blocker. Antimuscarinic and calcium channel blockers have an important role in the treatment of hyperactive gut disorders. Due to presence of dual mechanism of action, this plant may provide a beneficial role for the treatment of such disorders along with existing remedies.

To endorse its tracheorelaxant activity, plant extract and its fractions were tested on isolated rabbit tracheal preparations. It relaxed CCh (1μM) and K^+(80mM) induced constrictions similar to dicyclomine, suggesting the presence of antimuscarinic and weak calcium blockage activities(Janbaz et al. 2015; Rahman et al. 2020). The extract's dichloromethane fraction (Hp.DCM) also showed a tracheorelaxant effect against CCh and K^+-80mM induced spasm, but the response was similar to verapamil, while Hp.Aq observed no effect. Anticholinergic drugs are commonly used for respiratory ailments (Gosens and Gross 2018; D'Amato et al. 2017). Therefore, due to its antimuscarinic activity, *H. puniceum* may be proven to be beneficial in treating respiratory ailments like asthma and COPD.

The observed spasmolytic effects of *H. puniceum* were also confirmed by *in-vivo* antidiarrheal and gastrointestinal motility studies. The castor oil-induced diarrheal model was used to study the antidiarrheal effect of Hp.Cr. The administration of Hp observed dose-dependent diarrheal protection.Cr to rats similar to Loperamide, a standard antidiarrheal drug. When castor oil is ingested, it is converted to ricinoleic acid in GIT. Ricinoleic acid induces gastrointestinal motility and hypersecretions from the mucosa, which results in diarrhea (Aslam and Janbaz 2019). Hp also observed dose-dependent gastrointestinal motility.Cr in charcoal meal transit time model. Antidiarrheal and gastrointestinal motility effects of Hp.Cr may increase reabsorption and control diarrhea. A decrease in intestinal motility may result in excessive water and electrolyte reabsorption, leading to diarrheal control(Yu et al. 2019). Antidiarrheal effect of Hp.Cr may be due to the inhibition of secretion.

It has been reported that flavonoids, alkaloids, sterols, terpenes, and saponins possess the spasmolytic activity and are used for the treatment of diarrhea, asthma, and hypertension (Park et al. 2010; Aslam and Janbaz 2019). The current study and the literature indicate the presence of alkaloids, anthraquinones, saponins, flavonoids, tannins, and sterols in *H. puniceum*. Therefore, these macromolecules are likely responsible for the observed spasmolytic activity. However, an extensive study is recommended to isolate the compounds responsible for the spasmolytic and antiasthmatic effects of *H. puniceum*. No acute toxicity was observed when Hp.Cr was administered orally, up to 10mg/kg, in the healthy Balb C mice, indicating the safety of high oral doses.

5. Conclusion

This study indicates that *H. puniceum* spasmolytic, Tracheorelaxant, antidiarrheal, and gut inhibitory activities. The antagonism of muscarinic receptors and calcium channels is responsible for these activities. The crude extract (Hp.Cr) possesses antimuscarinic and calcium channel blocker activity, but in dichloromethane fraction (Hp.DCM), calcium channel blocker activity is dominant. This study provides scientific proof for the spasmolytic activity of *H. puniceum* and its use in asthma treatment.

Conflict of interest

The authors declare that they have no competing interests.

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Study Approval

The protocols and procedures of this study were approved by the ethical committee of the Faculty of Pharmacy Institutional Review Board pertaining to animal experiments vide **No.09/PEC/2015** before the start of work.

Consent Forms

NA.

Authors Contribution

KHS and HMAR conceptualized the study and wrote the final manuscript, HMAR, MSH, and II helped in the analysis and writing the first draft, did the experimental analysis, and II supervised the whole project and wrote the final manuscript.

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