

Evaluation of prokinetic and laxative effects of *Hippophae rhamnoides* in rodents

Muhammad Hanif^{1,2}, Malik Hassan Mehmood^{1,3}, Ghazala Isirat⁴, Ahmed Abdullah¹, Samar Sohail¹, Mansoor Ahmed^{2*} and Anwarul-Hassan Gilani¹

¹Natural Product Research Division, Department of Biological and Biomedical Sciences, The Aga Khan University Medical College, Karachi, Sindh, Pakistan

²Department of Pharmaceutical Chemistry & Pharmaceutical Sciences, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan

³Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University, Faisalabad, Pakistan

⁴Department of Pharmaceutics, Faculty of Pharmacy, Barrett Hodgson University, Karachi, Pakistan

Abstract: *Hippophae rhamnoides* (Family; Elaeagnaceae) fruit extract was investigated for prokinetic and gut excitatory effects to rationalize its therapeutic utility in gastrointestinal complaints like delayed gastric emptying and constipation. The fruit extract of *Hippophae rhamnoides* (Hr.Cr) prepared in hydro-methanol (30:70) was verified for flavonoids, tannins, coumarins and terpenes as plant constituents. In mice, Hr.Cr administration caused an increased in faecal production and charcoal meal transport (50-300mg/kg, per-oral.), similar to activity pattern of carbamylcholine (1 mg/kg). Laxative and prokinetic effects of Hr.Cr were found partially atropine-sensitive. On challenge with isolated intestinal tissues, Hr.Cr charged a dose-dependent spasmogenic effect on jejunum (0.01-1mg/mL) preparations of rabbit and in ileal tissues (guinea-pig) at the dose range of 0.03 to 3mg/mL, following predominant relaxing impact at increased concentrations. Unlike carbamylcholine, stimulant effect of Hr.Cr was partly antagonized in atropine incubated tissues. These data attest the laxative, prokinetic and gut excitatory activities of *Hippophae rhamnoides* probably mediated through partial activation of muscarinic receptors. Further in agreement of the current findings with earlier reports on gastric emptying effects of *Hippophae rhamnoides* seed oil, this is the first study of its kind providing insight into mechanism to the laxative potential of *Hippophae rhamnoides* fruit, thus rationalizing its medicinal use in constipation.

Keywords: *H. rhamnoides*, spasmodic, laxative, prokinetic, muscarinic receptor activation.

INTRODUCTION

Due to modern lifestyle practices, gastrointestinal problems including impaired gut motility are the common health issues of current era. Among gastrointestinal problems, bowel movement dysfunctions predominant with constipation and/or diarrhea are commonly prevailing in the society (Ryu and Choi, 2015). Chronic constipation affects almost 27% of the population, being more common in men than women (Cheng *et al.*, 2009). Constipation is usually mediated because of insufficient luminal quantity of stool, impaired colonic contractility and uncoordinated anorectal movements. Modification in current lifestyle patterns like preferred intake of high-fiber diet, exercise and use of plenty of fluids are considered the prime steps for the treatment of constipation (Costilla and Foxx-Orenstein, 2014). Conventional treatment of constipation includes the use of laxatives and bulking agents, neither of these is recommended for long term treatment due to their side-effects (Foxx-Orenstein *et al.*, 2008). Herbal remedies are usually preferred to relieve gut hypo- or hyper-motility complaints like diarrhea, constipation and indigestion. In natural herbs, the existence of several phytochemical constituents (Gilani and Rahman, 2005) with desired effect improving and/or unwanted effect diluting

potential, hence their long-term use is deemed safe and effective. Psyllium husk seed (*Plantago ovata*) is commonly used effective herbal intervention equally familiar among modern and traditional practitioners for alimentary ailments of opposing mechanisms like diarrhea and constipation (Mehmood *et al.*, 2010).

Hippophae rhamnoides, familiar locally as sea buckthorn or sea berry, belongs to the family Elaeagnaceae (oleaster family) is a valuable medicinal plant worldwide considered popular for its therapeutic and nutritious potential. The plant is found in Northern areas of Pakistan (Sabir *et al.*, 2005). It is a shrub which rises up to 4 meter in height (Suryakumar and Gupta, 2011; Li, 2003). *H. rhamnoides* flowers between April and May. It produces small orange coloured fruits which are sour to taste (Kallio *et al.*; 2002). Different bioactive phytochemical constituents were purified from the fruits of *H. rhamnoides* such as palmitic acid, oleanolic acid, octacosanoic acid, cirsiumaldehyde, 1-O-hexadecanoleninopolysaccharides, Hippophae cerebroside, ursolic acid and dulcic acid (Wang *et al.*, 2016; Zheng *et al.*, 2009), isorhamnetin (Kim *et al.*, 2019), phenolics and ellagi-tannins (Ma *et al.*, 2019). *H. rhamnoides* is used as phytotherapy by the tribes of Central Himalayas, Western and North Western India, Tibet, China and Northern areas of Pakistan (Lalitkumar and Sundriyal, 2007) to treat a variety of ailments. Its berries are

*Corresponding author: e-mail: hanifbuzdar@gmail.com

commonly employed in herbal medicines across Europe and Asia. *H. rhamnoides* is popular for its medicinal utility for respiratory tract, cardiovascular and gut disorders in Tibetan and Mongolian traditional medicines. In Asia, *H. rhamnoides* is used to treat jaundice, skin issues, asthma, cardiovascular and gastrointestinal disorders like constipation (Suryakumar and Gupta, 2011; Singh, 2005; Li and Wang, 1998). Furthermore, literature review confirms the intestinal absorptive (Xin *et al.*, 2019), anti-biofilm effect in oral cavity (Smida *et al.*, 2019), anti-neuroinflammatory (Kim *et al.*, 2019), anti-melanogenic (Zhang *et al.*, 2018), cardioprotective (Skalski *et al.*, 2018), antimutagenic and antioxidant (Ma *et al.*, 2019; Tsybikova *et al.*, 2018; Kim *et al.*, 2011), antiviral (Singh *et al.*, 2018), antiplatelet (Olas *et al.*, 2017), hepatoprotective (Geetha *et al.*, 2008; Hsu *et al.*, 2009; Zhang *et al.*, 2018), antihypertensive (Pang *et al.*, 2008) and anxiolytic properties (Saggu *et al.*, 2007) of *H. rhamnoides*. Despite being popular indigenous medicinal plant used to relieve constipation, to the best of our knowledge, *H. rhamnoides* has not been well studied in this regard except a few reports on its effectiveness in delayed gastric emptying (Xing *et al.*, 2012) and children with functional dyspepsia (Iao *et al.*, 2013) without sharing precise mechanism(s). The current study is the first report elucidating the insight into mechanism in support of the laxative effect of *Hippophae rhamnoides* fruit.

MATERIALS AND METHODS

Preparation of crude extract from plant material

H. rhamnoides dried fruit were collected from District Skardu, (Gilgit) province of Pakistan and were validated by Dr. Muniba Nadir, a plant Taxonomist, Department of Botany, Karachi University, Sindh Pakistan. Specimen of fruit part of plant material was submitted in Department of Botany herbal museum, University of Karachi. A sample receipt (G.H. No 92097) was submitted in the herbarium of the respective Department.

Around 772 gram of *H. rhamnoides* was taken (after the removal of adulterant material) and washed thoroughly. The cleaned fruit material was soaked in methanolic-aqueous (70:30) solution for 72 h. The solution was shaken occasionally during time period of three days. The solution was then passed by a muslin cloth, followed by filtration using Whatman qualitative grade # 1 filter paper (Williamson *et al.*, 1998). After repeating this procedure thrice, the crude extract of *H. rhamnoides* (Hr.Cr) was obtained by evaporating the collective filtrate on rotary evaporator (Rota vapor BUCHI model RE-111). The final residue was the crude extract of *H. rhamnoides* with total amount of 69.72 g i.e. approximately 9 % yield.

Preliminary phytochemical evaluation

Phytochemical evaluation of *H. rhamnoides* extract was estimated to detect coumarins, alkaloids, flavonoids, anthraquinones, saponins, terpenes and sterols by

previous methodologies as described in literature (Evans, 2006).

Standard drugs and animals

Carbamylcholine, atropine sulfate, acetylcholine perchlorate, potassium chloride and activated charcoal were obtained from Sigma Chemicals Co., St. Louis, MO, USA. NaHCO₃, NaCl, CaCl₂, MgCl₂, NaH₂PO₄, KH₂PO₄, MgSO₄ and D-glucose were acquired from Merck, Darmstadt, consumed for preparation of physiological solutions. All analytical grade compounds were dissolved in distilled water.

Guinea-pigs of weighing 450-550g and rabbits (1500-2000 g) were used. BALB/c mice (weight ranging 25-30 g) were used in this research work. These were kept in animal house of the Aga Khan University in controlled environment at 23°C-25°C. The animals were placed in plastic boxes and were given normal drinking water and laboratory animal in routine. To dissect out the tissue from the animals, excess doses of isoflurane (2-7%) were challenged till attainment of deep anesthesia. The experiments were performed as per approved method by the Ethical Committee for Animal Care and Use (ECACU) at Aga Khan University, Karachi, Sindh, and also are in line with the rules of the institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC, 1996).

Charcoal meal propulsion in small intestine

Similar to the study of Mehmood *et al.* (2013), mice (20-30 g) were kept fasted one day before the experimentation and were separated into six groups each having five mice. First group was treated with normal saline (10mL/kg, per-oral.) acting as the negative control. Remaining four groups were treated with increasing amounts (50-500 mg/kg) of the plant extract, serving as the test groups. Carbamylcholine (1 milligram/kg), a parasympathomimetic agent, was administered to the last group which acted as (+ve) control. After 15 min, animals were fed with 0.3ml of freshly prepared charcoal meal orally (suspension comprising 10% gum acacia, 20% starch and 10% vegetable charcoal). After an hr of charcoal meal administration, the mice were euthanized as described in "Drugs and animal section" followed by dissection of abdomen. The small intestine was uncoiled and the distance charcoal travelled from the point where charcoal thread was first observed on the small intestine to the point where charcoal thread ended on the small intestine was recorded and % age movement was calculated.

Laxative activity

A previously reported method of Haruna (1997) was adopted for this experiment with some modification. Mice male or female of weighing, 20-25 g were fasted for one day before the assay. Each mice was then placed in separate plastic box which had been lined with bloating

sheet and separated into eleven groups of five animals, respectively, receiving the succeeding treatments. The 1st group was administered 0.9 % NaCl (10 mL/kg, per-oral) acting as the (-ive) control. The 2nd group, received carbamylcholine (1 mg/kg, per-oral) which acted as the (+ive) control. Further four groups received orally, 50, 100, 300 and 500 mg/kg of Hr.Cr, served the test groups. Remaining five groups already treated by atropine (10 mg/kg) thirty minute before administered the above doses of Hr.Cr and carbamylcholine (CCh) to restudy the laxative effects of Hr.Cr and carbamylcholine. The feces count was observed after six hours in all sets for the total defecation. The total amount of feces, dry feces & wet feces in the given treatment was counted for each mice and the increase in wet feces was used as marker for the laxative property of *H. rhamnoides*.

Isolated Tissue Preparations

Assays were derived following established methods of Gilani *et al.* (2000), Mehmood and Gilani (2010), Mehmood *et al.* (2011) and Malik *et al.* (2017).

Guinea-pig ileum. Ileum was promptly separated out and was then placed in physiological tyrode's solution. A small piece of around 2-3 centimeter long, were mounted in a 10 mL tissue organ bath, filled with tyrode's solution, maintained at 37°C and bubbled with carbogen (95% oxygen and 5% carbon dioxide). The initial tension 0.7-1 g was supplied to each tissue piece followed by equilibration period for at least half an hour before administration of any test material or standard. In given experimental circumstances, guinea-pig ileum performs as a smooth preparation and is considering much suitable to assess contractile activity (Gilani *et al.*, 2004). Sub-maximum concentrations of acetylcholine (0.3 µM) was used to stabilize the tissues at intervals of three minute till similar responses were recorded. The tissues were first subjected to 0.01, 0.03, 0.1, 0.3, 1, 3, 5 and 10 mg/ml doses of Hr.Cr. The ileum was then antagonized with atropine (0.1 µM) prior to restudy of the gut stimulant effects of same doses of *H. rhamnoides* using Power Lab set up.

Rabbit jejunum. Tissue of rabbit jejunum were extracted, maintained and equilibrated using the protocol mentioned for guinea-pig ileum. Under the same experimental conditions as used for ileum (guinea pig), rabbit jejunum displays pendular spontaneous movements. Hr.Cr was administered in increasing doses. Jejunum tissues were then antagonized with atropine (0.1 µM) and the doses of the extract were repeated. Tissue responses were recorded as mentioned in guinea-pig ileum section.

Data analysis and Statistics

GraphPad Prism (GraphPAD, San Diego, California) was applied to construct the graphs and to analyze the results. Results were represented as mean ± standard error of

mean (SEM., n= no. of observations) and the median effective concentrations (EC₅₀) were taken as the geometric mean with 95% confidence intervals (CI). ANOVA (one-way) tailed by Tukey's test and/or Dunnett's test was applied. P value <0.05 was observed as significant statistically.

RESULTS

Preliminary phyto-chemical evaluation

Hr.Cr extract were investigated for the flavonoids, carotenoids, sterols, saponins, coumarins and terpenes detection.

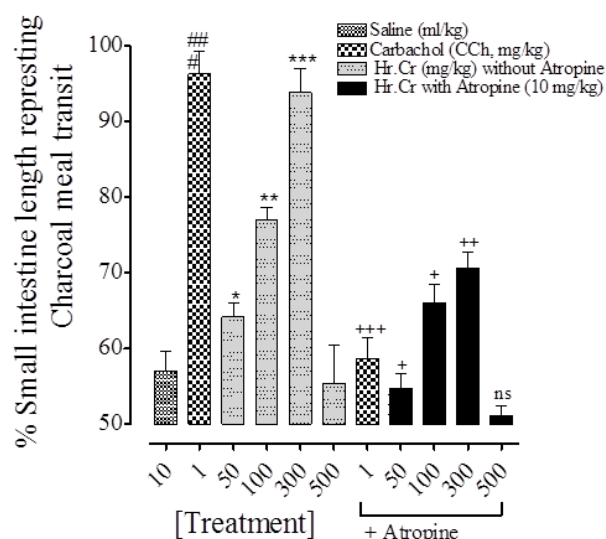


Fig. 1: Fig. presenting dose-dependent effect of the crude extracts of *H. rhamnoides* (Hr.Cr) in mice on propulsion of charcoal meal in small intestine of experimental animals (mice), both with and without atropine. */*[†]p<0.05, **/*^{††}p<0.01 and ***/^{†††}p< 0.001, One-way ANOVA and Tukey's tests were applied, ns shows non-significant. Bars represent mean ± SEM the experimental animal's results per group.

Effect on charcoal meal transit

H. rhamnoides fruit extract dose-dependently (50–500 mg/kg) enhanced movement of charcoal in mice all the way through the small intestine. 57.1±2.60% distance was covered (of entire length of small intestine) by normal saline treated group (mean ± SEM., n=6), whereas positive control group i.e. CCh (1mg/kg) significantly (p <0.001 vs. saline) enhanced the movement of the charcoal meal with respective value of 96.3±2.9%. The crude extract of plant at the different doses of 50, 100 and 300mg/kg accelerated the transit of charcoal meal with respective values of 64.24±1.82, 77.04±1.64 and 93.86±7.07% compared to saline treated (57.1±2.60%) group, however at maximum dose of 500 mg/kg, the effects were found relatively attenuated (55.40±5.16%). All tested doses of the experimental plant samples were re-studied for their impact against transportation of

charcoal meal in mice, pretreated with atropine (10 mg/kg), their respective effects were markedly attenuated ($p < 0.001$ vs. effects of test material administered in animals without atropine pretreatment) as shown in fig. 1.

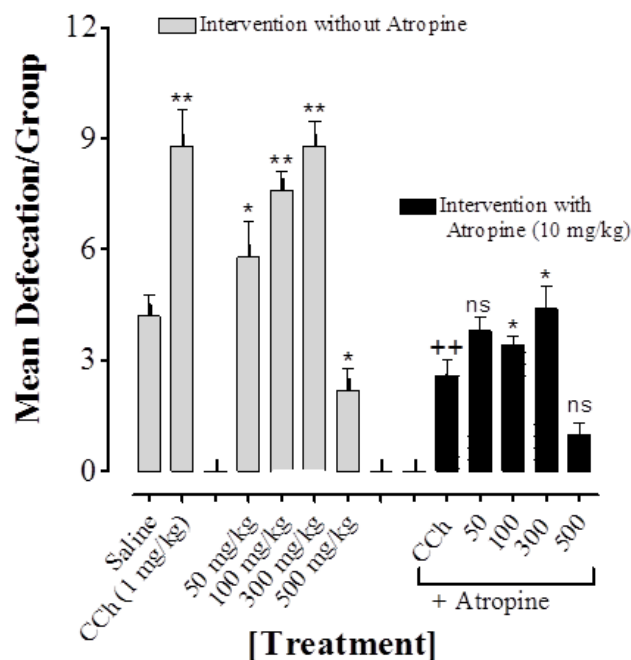


Fig. 2: Fig. presenting the laxative effect of *Hippophae rhamnoides* (Hr.Cr) and carbamylcholin (CCh) measured as fecal output both with & without atropine. $^*p < 0.05$ and $^{**/+}p < 0.01$, One-way ANOVA tailed with Tukey's test was applied, ns shows non-significant. Each bar shown represents mean \pm SEM of the results obtained from 6 different animals per group.

Laxative effect

When Hr.Cr was administered to mice, it exhibited laxative effect in terms of an increase in mean total fecal defecation (fig. 2). Hr.Cr enhanced total fecal out at 50, 100 and 300 and 500 mg/kg with resultant mean (mean \pm SEM, n = 6) number of 5.8 ± 0.97 , 7.6 ± 0.97 and 8.8 ± 0.66 , respectively, compared to saline treated group (3.6 ± 0.58), similar to the effect of carbachol ($9.8 \pm 0.93\%$). At maximal tested dose (500 mg/kg), the effect of Hr.Cr was relatively decreased ($2.2 \pm 0.58\%$) as observed at lower doses. All tested doses of the plant extracts were re administered to animals groups prior fed with atropine (10 mg/kg), their effects on fecal output were noticeable declined ($p < 0.01$ vs. effects of test material administered in animals without atropine pretreatment) as seen in fig. 2).

Effect of Hr.Cr on guinea pig ileum preparation

Hr.Cr produced dose-related (0.01-3.0mg/ml) gut excitation similar to Ach ($1.0 \mu\text{M}$)-evoked contraction, in guinea-pig ileum (fig. 3). The maximal excitatory outcome reaching to $91.33 \pm 4.10\%$ (mean \pm SEM., n=4) at 3mg/ml and then further inhibitory effects at tested doses

of 5.0 and 10mg/ml. Observed gut-stimulant effects of Hr.Cr at all doses was significantly decreased ($p < 0.001$ vs. without atropine) when assessed in atropine pretreated tissues, it can be seen in fig. 3.

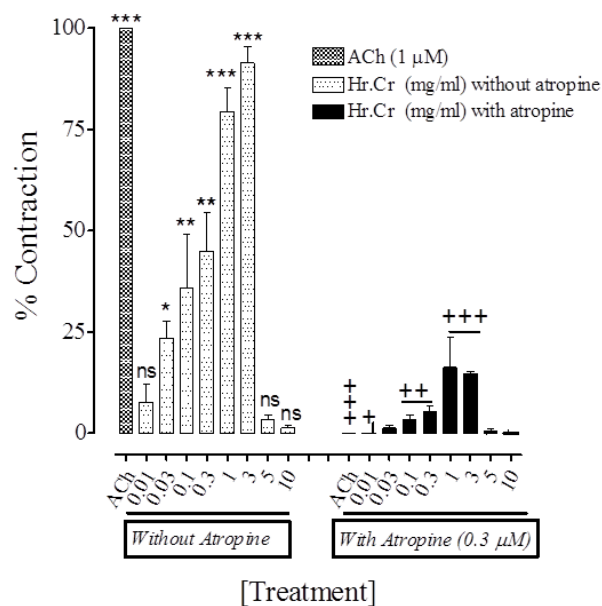


Fig. 3: Histogram presents the conc. dependent spasmogenic effects of the crude extracts of *H. rhamnoides* (Hr.Cr). The results are measured as % of acetylcholine max. Contraction (Ach Max.) denoted as “% Contraction”. The readings are publicized as mean \pm SEM., n= 4-5.

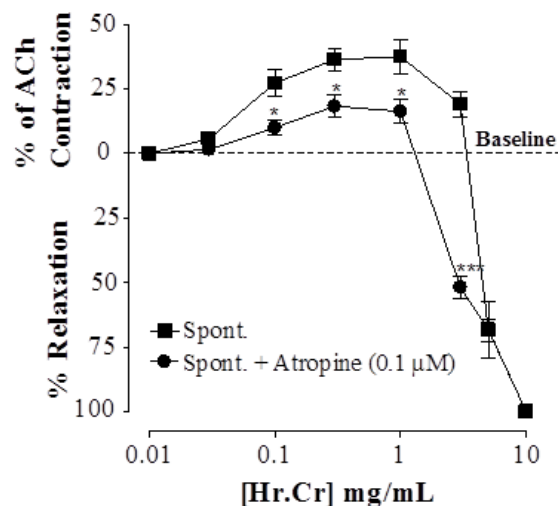


Fig. 4: Concentration based gut excitation effects followed by inhibition of *H. rhamnoides* extract (Hr.Cr) without or with atropine (0.1 μM) in jejunal tissue of rabbit. The responses are assumed as percentage of acetylcholine ($1 \mu\text{M}$)-induced contraction (Ach Max.). The readings are publicized as mean \pm SEM, n=4-6. Baseline represents the normal contraction of rabbit jejunum.

Effect of Hr.Cr on rabbit jejunum preparation

Hr.Cr affected dosage-dependent (0.01-01mg/ml) spasmogenic effects with maximum contraction of $38.7 \pm 9.70\%$ (mean \pm SEM, n= 4 to 6) relative ACh (1 μ M) evoked contraction on rabbit tissues followed by relaxation at greater concentration (fig. 4). Pre-treatment of tissues using atropine (0.1 μ M) significantly (p<0.05 vs. without atropine) attenuated gut stimulant effect (fig. 4).

DISCUSSION

H. rhamnoides is used as a laxative, appetizer and gastrointestinal relief remedy (Suryakumar and Gupta, 2011; Singh, 2005; Li and Wang, 1998). Present study was therefore carried out to determine its GIT excitatory properties in mice and animal tissues to rationalize its medicinal benefits in gut disorders. *H. rhamnoides* extract was prepared to assess its influence on the charcoal meal transfer through the small intestine and total fecal output in mice. Hr.Cr in a dosage-dependent manner boosted the intestinal transport of charcoal and triggered a momentous rise in the total fecal output at initial tested doses followed by gut inhibitory effects at relatively higher doses. Gastrointestinal stimulant activity pattern of Hr.Cr was found comparable to carbamylcholine, a cholinergic agonist and GIT stimulating agent (Brown and Taylor, 1996). It has also been observed that herbal products elicit laxative actions (Gilani et al., 2000, Gilani et al., 2004; Mehmood et al., 2011; Mehmood et al., 2014) co-existed with gut inhibitory constituents. To assess if the GIT excitatory influence of the plant was also facilitated by the connection of an analogous pathway, prokinetic and laxative properties of Hr.Cr were tested in the absence and presence of atropine. Pre-treated mice by atropine significantly inhibited prokinetic and laxative effects of Hr.Cr, unlike to carbamylcholine, thus signifying that the GIT stimulating influence of Hr.Cr is partly facilitated by stimulation of muscarinic receptors plus some additional uncharacterized gut excitatory constituents, yet to be explored. Acetylcholine a known gut neurotransmitter, plays a significant physiological role in regulation bowel movements (Brown and Taylor, 1996).

Further studies were carried out on isolated tissue preparations to check the mechanism of action of Hr.Cr. An inert preparation is considered beneficial like guinea-pig ileum to perform this procedure. (Bashir et al., 2006), Hr.Cr resulted concentration-dependent contractile effect, that was significantly antagonized by atropine, thus depicting the presence of partial ACh like co-existed with some uncharacterized spasmogenic components. A weak partially-atropine sensitive excitatory effect was observed by crude extracts of the *H. rhamnoides* at less concentration and inhibitory effect at higher concentrations, when studied on spontaneously contracting rabbit jejunum, indicating *H. rhamnoides* consists of combination of stimulatory and inhibitory constituents.

Spontaneous gut movements are coordinated through modulation of diverse physiological mediators similar to acetylcholine (ACh), serotonin (5-hydroxytryptamine), histamine, substance P and cholecystokinins. The discharge of these substances increase cytosolic Ca^{++} which ultimately produce stimulatory effect (Burks, 1987). Amongst these gut modulating neurotransmitters, ACh is released on parasympathetic nerve endings and mediates longitudinal and circular smooth muscles excitation in human gut via M_3 subtype of G-protein-coupled muscarinic receptors (Kerr et al., 1995). Clinically, Para sympathomimetic drugs are not used to relief constipation, mainly because of their non-specificity in mechanism of action and known adverse-effects like abdominal cramps observed with all chemical agents when used at a slightly higher dose to alleviate constipation (Pasricha, 2006). Dietary and lifestyle measures are thought to be the primary therapy to regulate bowel movements (Costilla and Foxx-Orenstein, 2014). Whereas, alternate options including the use of medicinal herbs for the treatment of constipation is believed to be relatively safe and cost effective (Pasricha, 2006; Mehmood and Gilani 2010; Mehmood et al., 2011; Mehmood et al., 2013).

The presence of both gastrointestinal excitatory and inhibitory phytochemical ingredients in *H. rhamnoides* are considered valuable to offset excessive gut stimulant effect resulting in abdominal colic and cramps. This combination of constituents is perhaps intended naturally to antagonize the undesired excessive GIT stimulating and/or GIT relaxant effects. Gastrointestinal stimulant effects of *H. rhamnoides* crude extracts observed in this study proved its traditional uses in constipation and indigestion (Baquar, 1989; Prajapati et al., 2003), the conditions outcome from slow GIT transport (Tatsuta and Iishi, 1993). Phytochemical evaluation analysis exposed the occurrence of tannins, flavonoids, terpenes, and coumarins compounds in *H. rhamnoides*.

CONCLUSION

Current research work signifies that *H. rhamnoides* possess prokinetic, laxative and GIT stimulating property mediated in part through activation of cholinergic pathway along with some additional uncharacterized gut stimulant component(s). Thus, this study rationalizes medicinal use of *H. rhamnoides* in delayed gastric emptying and constipation.

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