

Spasmogenic Activity of the Seed of *Terminalia chebula* Retz in Rat Small Intestine: In Vivo and In Vitro Studies

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Abstract

Background: *Terminalia chebula* Retz is traditionally used to relieve constipation. The current study was performed to investigate the pharmacological action of aqueous extract of *Terminalia chebula* seeds (ATC) in vitro and in vivo.

Methods: Terminal pieces of rat ileum were suspended in organ bath containing Tyrode solution. The ileum spontaneous motility frequency and contractility were recorded isotonicly. To induce ileal contraction, carbachol and ATC were added to the organ bath. In addition, the effect of hexamethonium, indomethacin, atropine, and verapamil on the ATC-induced ileal contractions was also investigated. The effectiveness of ATC on relieving morphine-induced constipation was investigated in an in vivo study by measuring the faecal number, faecal water content, and intestinal transit ratio.

Results: ATC increased the frequency of ileum motility and tension of contraction dose-dependently ($P < 0.05$). Responses induced by ATC were inhibited by pre-treatment of the tissue with verapamil. The ATC activities were not affected by atropine, hexamethonium, and indomethacin. The faecal number and faecal water content were increased dose-dependently by ATC ($P < 0.05$).

Conclusion: The excitatory effects of ATC on ileal contractile frequency and tension are possibly mediated through Ca^{2+} channels activation. The results of the present study support the traditional usage of ATC for the treatment of constipation.

Keywords: calcium channels, gut, intestinal motility, morphine, plant extracts, rat, seed, *Terminalia*

Introduction

Terminalia chebula Retz. (Combretaceae) is commonly known as chebulic myrobalan. It grows throughout central Asia and some other parts of the world (1). Seeds of *T. chebula* is used in traditional medicine to treat kidney and urinary disorders (2) and can also be used as homeostatic, laxative, antitussive, diuretic, and cardiogenic remedy (3). The semi-ripe seed is considered as a purgative (4), and its gastroprotective effect against indomethacin-induced gastric ulceration has been reported (5). High performance liquid chromatography analysis revealed that *T. chebula* contains gallic acid, punicalagin, chebulic acid, ellagic acid, chebulagic acid, chebulinic acid, chebulanin, corilagin, neochebulinic acid, and 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (6). Antioxidant and hypocholesteremic properties of *T. chebula* have been scientifically demonstrated

(7–9). Moreover, it is effective against several pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* (10), and *Helicobacter pylori* (11). *T. chebula* also inhibits reverse transcriptase of human immunodeficiency virus-1 (12). It has been reported that *T. chebula* decreases the plasma glucose level both in normal and diabetic rats (11–13) and has cardioprotective effect against isoproterenol-induced myocardial injury in rats (14). In addition, *T. chebula* aqueous extract delays rat gastric emptying (15).

Morphine has been used to alleviate the chronic pain of cancer. However, a major side effect of morphine is constipation that often detracts from the patient's quality of life. Since *T. chebula* is used in traditional medicine in southern part of Iran to treat constipation, current work aimed to examine the excitatory effect of ATC on isolated ileum and its effect on morphine-induced constipation in rat.

Materials and Methods

Preparation of aqueous extract

The whole seed of *T. chebula* was purchased from a local market in Ahwaz, Iran, and was authenticated by Dr N Alemzadeh Ansari, Department of Horticultural, Faculty of Agriculture, Shahid Chamran University, Ahwaz, Iran. The voucher specimen (No. TCH-10-21) was deposited in the herbarium of Ahvaz Jundishapur University of Medical Sciences. The seeds (50 g) were crushed and soaked overnight in distilled water (500 mL) at room temperature. The mixture was filtered through a cheesecloth and centrifuged at 3500 rpm for 15 minutes. The supernatant was concentrated and lyophilised (yield: 13%) for preservation and stored at 4 °C until used.

Drugs

Carbachol (CCh), hexamethonium, atropine sulphate, indomethacin, verapamil, and charcoal were purchased from Sigma (USA), and morphine from Darou Pakhsh Company (Iran). All materials used for the preparation of Tyrode solution were purchased from Merck (Germany). Indomethacin was dissolved in Na₂CO₃ (100 mM) according to a published method (16). Other chemicals used were of the analytical grade available and dissolved in Tyrode solution.

Animals

Male adult Wistar rats (weighed 150–200 g) were prepared from the animal house of Ahvaz Jundishapur University of Medical Sciences. Animals were fed conventional diets and tap water ad libitum. Rats were maintained under standard conditions with temperature of 20–24 °C and 12 hours light/dark cycle. All animal experiments were carried out in accordance with Ahvaz Jundishapur University of Medical Sciences Ethical Committee Acts (PRC52).

In vitro study

Animals were deprived from food but not water 24 hours prior to experiments and killed by an overdose of diethyl ether. The ileum segment of intestine was dissected out and kept in oxygenated Tyrode solution; then, its surrounding mesenteries were dissected (17). Each segment (about 2 cm in length) was mounted under 1 g of resting tension in a 10 mL tissue chamber containing Tyrode solution, maintained at 37 °C and aerated with a mixture of oxygen and carbon dioxide (95% and 5%, respectively). The Tyrode solution composition was 136.90 mM NaCl, 2.68 mM KCl, 1.05 mM MgCl₂, 11.90 mM NaHCO₃,

0.42 mM NaH₂PO₄, 1.80 mM CaCl₂, and 5.55 mM glucose. An isotonic transducer (Bioscience, UK) coupled to Harvard Oscillograph (UK) was used for recording the ileum contractility.

After 30-minute equilibration period, CCh (1 µM) was applied to the tissue chamber, and the contractile response was assumed as control. In the first series of experiment, the effect of ATC on basal tone and frequency of spontaneous contractions of ileum were examined. Then, the effect of cumulative concentrations of ATC (0.01, 0.02, and 0.04 mg/mL) were recorded and responses were compared with CCh (1 µM). At the end of each experiment, response to CCh was retested to confirm the viability of tissue preparation. To study the mechanism of ATC spasmogenic activity, tissue was first incubated either with atropine (5 minutes, 1 µM), hexamethonium (5 minutes, 100 µM), or indomethacin (20 minutes, 1 µM) (18); then, ATC (0.04 mg/mL) was applied. The intervention of extracellular Ca²⁺ in the ATC-induced responses was evaluated by incubating the tissue with verapamil (5 minutes, 1 µM) or by recording responses in Ca²⁺-free Tyrode solution (18). Results were expressed as percentage of response induced by CCh. Contractile frequency was calculated as the numbers of contractile waves per minute. To confirm the Ca²⁺ agonist property of ATC, the tissue was allowed to stabilise in Ca²⁺-free Tyrode solution containing EDTA (0.1 mM) for 30 minutes in order to remove Ca²⁺ from the tissue (17), and then contractions induced by cumulative concentrations of CaCl₂ (0.45–2.25 mM) were recorded. The same experiments were repeated in the presence of ATC (0.04 mg/mL). All tested substances were applied in volume not exceeding 1% of the bath volume.

In vivo experiments

To test the effect of ATC on normal rats, animals were divided into control and 3 ATC-treated groups. Animals in control group received saline (1 mL/rat, p.o.) and the 3 treated groups received ATC at 62.5, 125, or 250 mg/kg, respectively (1 mL/rat, p.o.). This experiment was carried out within 3 consecutive days.

To examine the effect of ATC on morphine-induced constipation, animals were randomly assigned to control, negative control, and 3 ATC-treated groups of 7 rats each. Animals in control group, negative control, and the 3 ATC-treated groups received saline (1 mL/rat, p.o.), morphine (2 mg/kg, s.c.), and ATC (62.5, 125, or 250 mg/kg, p.o.), respectively. The animals in ATC-treated groups received ATC (p.o.) 4 hours

after the first trial of morphine administration. Morphine was injected subcutaneously, twice per day (19). All administrations were carried out for 3 consecutive days.

To carry out the aforementioned protocols, rats were housed in individual cages for 3 days. Every day at around 10 AM, faeces were collected from each cage. The number of the faeces pellets was counted, and weighed. Faeces of each rat were then lyophilised and weighed to determine faecal water content. The total number and water content of the faecal pellets were calculated as the average per 3 consecutive days for each rat.

Gastrointestinal transit ratio

Four groups of rats (7 in each) were fasted overnight. Control group received saline (1 mL/rat, p.o.) and 3 other groups received ATC at 62.5, 125, and 250 mg/kg, respectively (1 mL/rat, p.o.). After 90 minutes, all groups received 3% active charcoal suspension in saline (1 mL/rat, p.o.) as charcoal meal. One hour after the marker administration, the animals were sacrificed by an overdose of diethyl ether, and the small intestine from pylorus sphincter to ileocaecal sphincter was removed. The intestinal transit ratio was calculated by the percentage of the small intestine length in which the charcoal had travelled.

Statistical analysis

All values were expressed as the mean \pm SEM. The significant difference was tested by one-way ANOVA followed by Student's *t* test. Differences with *P* values of less than 0.05 were considered statistically significant.

Results

In vitro study

Effects of ATC on ileal spontaneous contraction

Ileum showed regular regular spontaneous phasic contractions, and ATC at 0.01, 0.02, and 0.04 mg/mL increased the contractile frequency and tension in a concentration-dependent manner ($P < 0.01$, Table 1). Figure 1 represents the actual recordings of CCh and cumulative concentrations of ATC on basal tone and ATC-induced ileal contractions, in which the maximal amplitude of the ATC-induced contraction was observed at 0.04 mg/mL, occurring at about 60% of the maximal response evoked by CCh (1 μ M).

Effect of hexamethonium, indomethacin, atropine, verapamil, and extracellular Ca²⁺ on ATC-induced contraction

The ATC-induced contractions were not inhibited by atropine (muscarinic receptor antagonist), hexamethonium (nicotinic receptor blocker), or indomethacin (cyclooxygenase inhibitor). In the Ca²⁺-free Tyrode solution, however, the spasmogenic effect of ATC was abolished. Verapamil (Ca²⁺ channel blocker) inhibited the ATC-induced contraction completely (Figure 2). Table 2 demonstrates the changes of frequency of spontaneous ileal contractile activity in these protocols.

Effect of ATC on CaCl₂-induced ileal contractions in Ca²⁺-free Tyrode solution

In Ca²⁺-free Tyrode solution, ATC was unable to evoke ileal contraction. However, applying cumulative concentrations of CaCl₂ (0.45–2.25 mM) evoked ileal contraction in a concentration-dependent manner ($P < 0.05$). In the presence of ATC (0.04 mg/mL), the CaCl₂-induced ileal contractions were potentiated ($P < 0.05$), as shown in Figure 3.

In vivo study

Effect of ATC on number of faecal pellets in the morphine treated and untreated rats

In morphine-untreated rat, ATC at doses of 125 and 250 mg/kg significantly increased the number of faecal pellets ($P < 0.05$, Figure 4). On the other hand, morphine decreased the number of faecal pellets significantly ($P < 0.05$), as shown in Figure 5. ATC at 625.5 mg/kg was unable to induce any change; however, ATC at 125 and 250 mg/kg significantly antagonised the morphine effect dose-dependently ($P < 0.05$), and the number of faecal pellets in these 2 groups were not different from control group, which received saline (Figure 5).

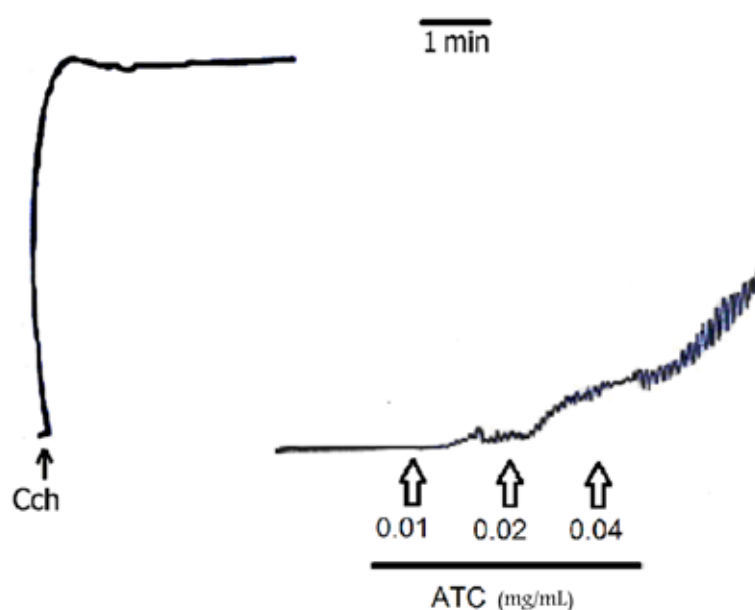
Effects of ATC on faecal water content

Table 3 shows that, in normal animals, the administration of ATC increased the faecal water content as compared with in the saline administration ($P < 0.05$). The mean percentage (SEM) of faecal water content in saline and ATC-treated (62.5, 125, and 250 mg/kg) groups were 30% (1.2), 31.2% (1.6), 36.2% (2.1), and 39.8% (2.5), respectively. As shown in Table 3, faecal water content in the ATC-treated groups (125 and 250 mg/kg) was significantly more than in the control group ($P < 0.01$ and $P < 0.001$, respectively).

Table 1: Effect of different concentrations of aqueous extract of *T. chebula* (ATC) on the contractile frequency and tension of rat ileum

	ATC (mg/mL)			
	0	0.01	0.02	0.04
Frequency (cycle/min)	7.25 (0.25)	7.36 (0.31)	10.00 (0.85)**	15.54 (1.50)***
Tension (% of CCh response)	2.24 (0.35)	6.00 (1.42)*	25.20 (2.43)**	63.00 (4.12)**

Data are presented as mean (SEM). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control (0 mg/mL). Abbreviation: CCh = carbachol.

**Figure 1:** Representative tracing showing the contractions evoked by carbachol (CCh, 1 μ m) and aqueous extract of *T. chebula* (ATC) to rat isolated ileum.

In the morphine-treated animals, although ATC increased the animals' faecal water content, the differences were not significant when compared with the animals that received morphine alone.

Effect of ATC on intestinal transit ratio

Figure 6 demonstrates that oral administration of ATC at doses of 125 mg/kg and 250 mg/kg has increased the intestinal transit ratio as compared with the saline group ($P < 0.05$ and $P < 0.01$, respectively).

Discussion

Our results showed that ATC increases the frequency of ileal contraction in a concentration-dependent manner. The excitatory effect of ATC on the contractile frequency of ileum was unaffected by pre-treatment with atropine, hexamethonium, and indomethacin. Furthermore, the ATC excitatory effect was dependent on extracellular Ca^{2+} . In addition, ATC increased the intestinal transit ratio, number of faecal pellets, and faecal water content.

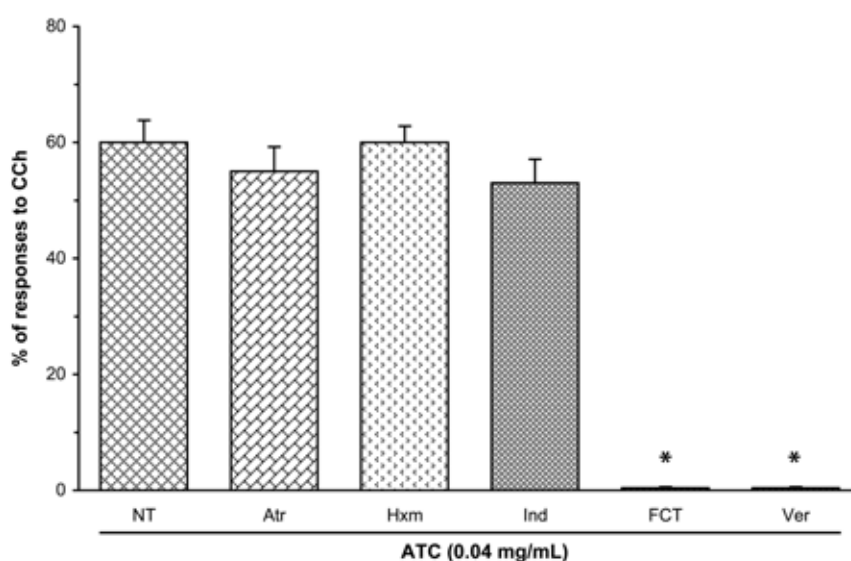


Figure 2: Spasmogenic effect of aqueous extract of *T. chebula* (ATC, 0.04 mg/mL) on rat ileum in normal Tyrode solution (NT) and after tissue incubation with atropine (Atr, 1 μM), hexamethonium (Hxm, 100 μM), indomethacin (Ind, 1 μM) and verapamil (Ver, 1 μM). ATC spasmogenic effect was also recorded in the Ca²⁺-free Tyrode solution (FCT). Data are presented as mean (SEM) percentage of responses induced by carbachol (CCh, 1 μM). **P* < 0.05 versus NT.

Table 2: Effect of aqueous extract of *T. chebula* (ATC, 0.04 mg/mL) on the contractile frequency of rat ileum after pre-treatment with antagonists

	ATC	Ind + ATC	Hxm + ATC	Atr + ATC	Ver + ATC
Frequency (cycle/min)	15.54 (1.5)	15.1 (0.85)	15.8 (1.43)	14.24 (1.5)	0.00***

Data are presented as mean (SEM). ATC was applied without or after tissue incubation with indomethacin (Ind, 1 μM), hexamethonium (Hxm, 100 μM), atropine (Atr, 1 μM), or verapamil (Ver, 1 μM). ****P* < 0.001 versus ATC alone.

In smooth muscle, there are several factors that increase the frequency of contraction, such as increased pacemaker activity and shortened action potential (20). The extracellular Ca²⁺ and Ca²⁺ influx are essential for excitability and contractility in smooth muscle cells. Intracellular Ca²⁺ elevation is the main regulating factor of smooth muscle tension (21); therefore, in Ca²⁺-free Tyrode solution, the contractile activity was appeared and augmented after adding cumulative concentrations of CaCl₂. However, in the presence of ATC (0.04 mg/mL), the stimulatory effect of Ca²⁺ was increased, indicating that ATC, in some way, has potentiated Ca²⁺ spasmogenic effect. To elucidate the mechanism of ATC activity, ileal preparations were incubated with atropine, hexamethonium, and indomethacin. It has been shown that prostaglandins (22)

and acetylcholine at pre-ganglionic (23) and post-ganglionic levels (24) stimulate small intestine contractility. However, our results showed that ATC spasmogenic effect was unaffected by indomethacin, atropine, and hexamethonium, indicating that neither of these stimulatory pathways were involved in ATC activity. On the other hand, ATC was unable to induce ileal contraction in Ca²⁺-free Tyrode solution. The same results were obtained when ileal tissue was incubated with verapamil. Therefore, our results indicated that Ca²⁺ channels and Ca²⁺ influx were essential for inducing ileal contractions by ATC. The same conclusion could be obtained from the results of ileal frequency being affected by ATC alone or in the presence of applied antagonists and inhibitors.

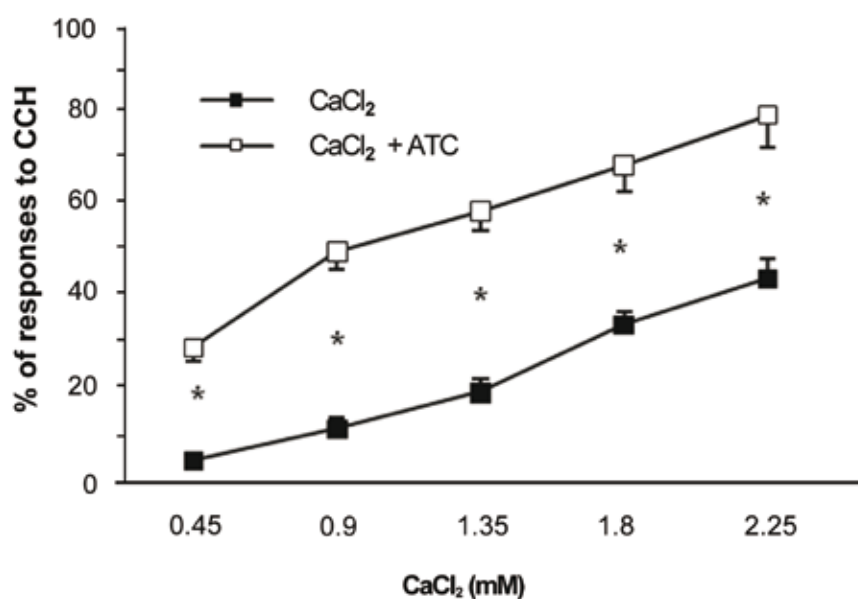


Figure 3: Spasmogenic effect of cumulative concentrations of CaCl_2 in rat ileum in the absence and presence of aqueous extract of *T. chebula* (ATC, 0.04 mg/mL). Data are presented as mean (SEM) percentage of responses induced by carbachol (CCh, 1 μM). * $P < 0.05$ CaCl_2 versus CaCl_2 + ATC of the same concentration.

Table 3: Effect of aqueous extract of *T. chebula* (ATC) on the percentage of faecal water content (FWC) in normal and morphine-induced constipated rats

Groups	Dose (mg/kg)	FWC
Normal	Control (saline, 1 mL)	30.0 (1.2)
	ATC (62.5 mg/kg)	31.2 (1.6)
	ATC (125 mg/kg)	36.2 (2.1)**
	ATC (250 mg/kg)	39.8 (2.5)***
Constipated	Control (M)	28.0 (1.4)
	M + ATC (62.5 mg/kg)	29.0 (0.8)
	M + ATC (125 mg/kg)	30.0 (1.8)
	M + ATC (250 mg/kg)	30.8 (2.2)

Data are presented as mean (SEM) percentage. Constipation was induced by administration of morphine (M) at 2 mg/kg, twice per day. ** $P < 0.01$, *** $P < 0.001$ versus control.

In the in vivo experiments, ATC increased the number of faecal pellets, indicating that ATC has augmented intestinal motility, which is also evidenced by the increase in water content of faecal pellets (Table 3). The same results were obtained from intestinal transit ratio, which was increased significantly by ATC. Our results are consistent with the report that aqueous extract of *T. chebula* increased the gastric emptying in rats (15).

It has been reported that morphine (as an opioid receptors agonist) decreases the rat faecal weight significantly (25) and abolishes the propulsive peristaltic waves in the intestine (26,27). The opiate also promotes water absorption in the intestine (28). In the present study, the total number of faecal pellets was significantly decreased by the morphine. Although ATC at 62.5 mg/kg was unable to prevent the effect of

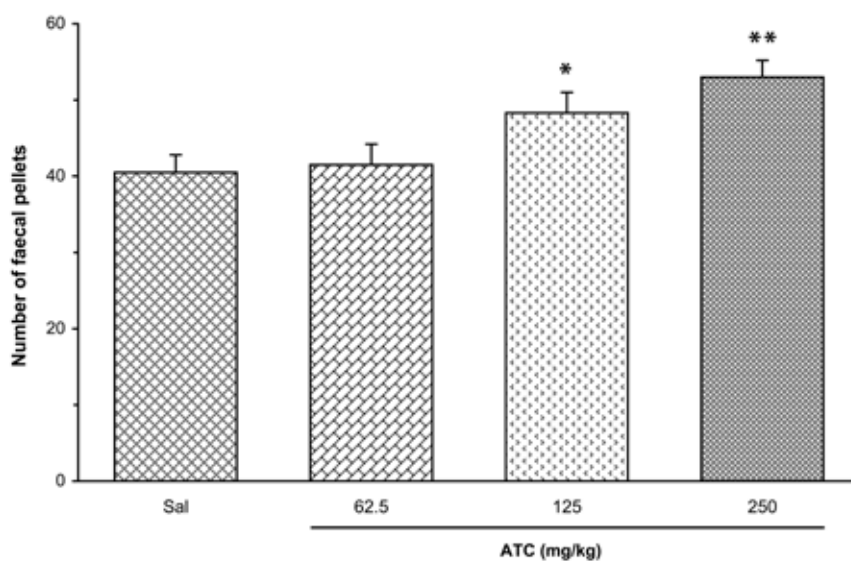


Figure 4: Effect of different doses of aqueous extract of *T. chebula* (ATC) on the number of faecal pellets in rat. Data are presented as mean (SEM). * $P < 0.05$ and ** $P < 0.01$ versus control (Sal).

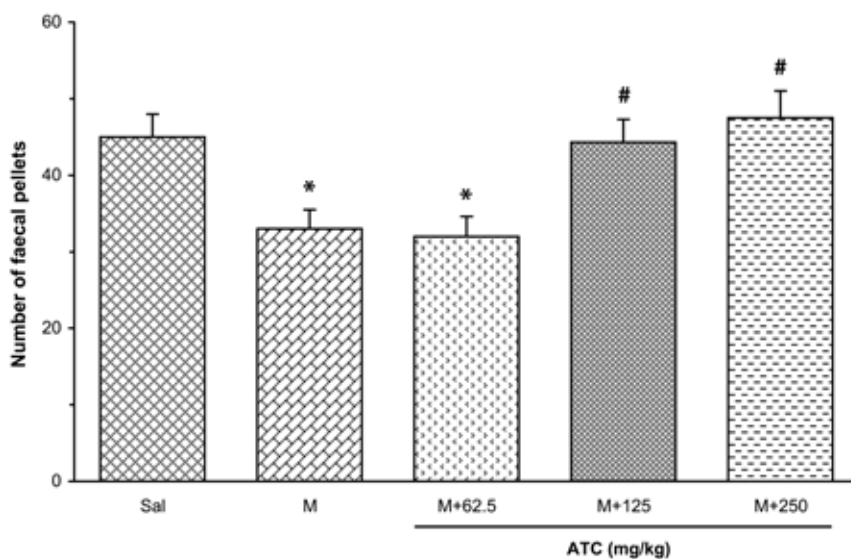


Figure 5: Number of faecal pellets in rats received saline (Sal), morphine (M) only, and morphine plus aqueous extract of *T. chebula* (ATC) at different doses. Morphine reduced the number of faecal pellets. ATC at higher doses (125 and 250 mg/kg) increased the number of pellets similar to that of the saline group. Data are presented as mean (SEM). * $P < 0.05$ versus saline, and # $P < 0.05$ versus morphine only.

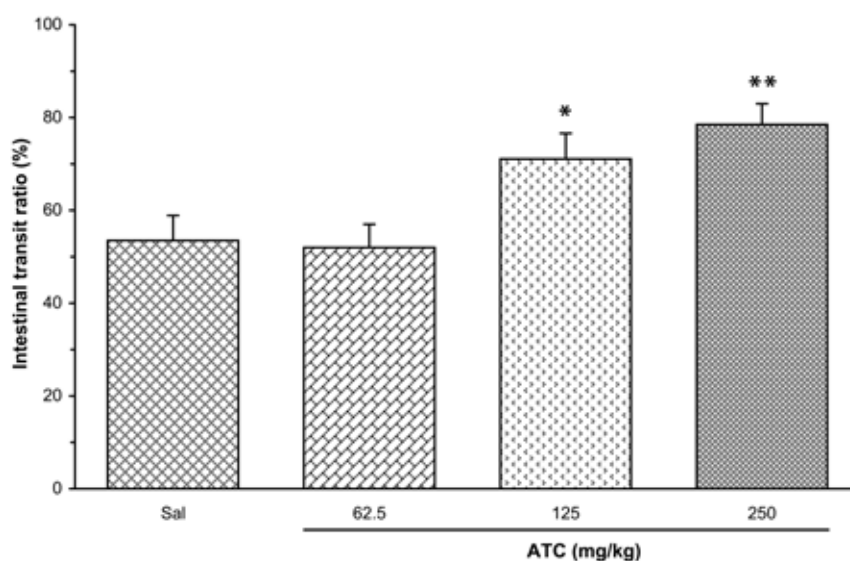


Figure 6: Effect of different doses of aqueous extract of *T. chebula* (ATC) on rat intestinal transit ratio. Data are presented as mean (SEM). * $P < 0.05$ and ** $P < 0.01$ versus saline.

morphine, at higher doses (125 and 250 mg/kg), ATC antagonised the effect and these 2 groups had the similar number of faecal pellets as in the saline group. These results indicate the ability of ATC (at higher doses) to inhibit the morphine-induced constipation. However, the water content of the faecal pellets in groups co-treated with morphine and ATC did not show any significant differences.

Conclusion

Our study showed that aqueous extract of *T. chebula* seed improves constipation induced by morphine and increases the gastrointestinal transit ratio, which supports its traditional use in constipation. The in vitro study revealed that ATC activity was related to Ca^{2+} influx. More studies are necessary to elucidate the mechanism(s) of its action.

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Authors' Contributions

Conception and design, final approval of the article: SAM, MKGN
 Obtaining of funding, analysis and interpretation of the data, drafting and critical revision of the article: SAM
 Collection and assembly of the data: PM
 Administrative, technical, or logistic support: AV, PM

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