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Pharmacological Studies on Antidiarrheal, Gut Modulatory, Bronchodilatory and Vasodilatory Activities of *Myrica nagi*

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ABSTRACT

This study was aimed to rationalize medicinal use of *M. nagi* in gut, airways and cardiovascular disorders. The crude extract of *M. nagi* bark (Mn.Cr), its aqueous (Mn.Aq) and ethyl acetate (Mn.EtAc) fractions were prepared. Antidiarrheal activity of *M. nagi* was carried out in mice, while its gut modulatory, bronchodilatory and vasodilatory activities were evaluated in isolated tissues of different animals using tissue bath assembly coupled with PowerLab. Orally administered doses (100-500 mg kg^{-1}) of Mn.Cr produced protection against castor oil and $MgSO_4$-stimulated diarrhea in mice. In guinea-pig ileum, Mn.Cr and Mn.Aq exhibited atropine-sensitive contractile effect. In rabbit jejunum, Mn.Cr and Mn.Aq exerted atropine-sensitive stimulant effects at 0.01-1 mg mL^{-1} followed by relaxation, while Mn.EtAc (0.03-0.1 mg mL^{-1}) exhibited only inhibitory effect. Mn.Cr and Mn.EtAc inhibited high K^+ (80 mM) induced contractions and shifted Ca^{++} Concentration Response Curves (CRCs) rightwards in rabbit jejunum. The Mn.Cr inhibited high K^+, carbachol (CCh) and phenylephrine (P.E) induced contractions and shifted CRCs of Ca^{++} rightwards in rabbit trachea and endothelial-intact or denuded aortic tissues of rats. These results indicate the presence of combination of cholinergic and Ca^{++} antagonist constituents in *M. nagi*, which may provide scientific basis to its medicinal use in gut, airways and cardiovascular disorders.

Key words: *Myrica nagi*, antidiarrheal, gut modulatory, bronchodilator, vasorelaxant, cholinergic, Ca^{++} antagonist

INTRODUCTION

Plants used in herbal medicines are accepted as one of the important source for drug discovery and development. Pakistan is rich in medicinal and aromatic plants which are used as natural health care products in traditional system of medicine prevalent in Pakistan (Ahmad *et al*., 2003).

Myrica nagi, Thunb. (family, Myricaceae), known commonly as "Box myrtle" and locally as kaiphal, is a sub-temperate evergreen tree, which can grow up from 3-15 m. The tree yields a drupaceous fruit which is one of the tastiest wild fruits of the sub-Himalayan region (Panthari *et al*., 2012; Parmar and Kaushal, 1982). *Myrica nagi* is geographically found in Khyber Pakhtunkhwa, sub-tropical Himalayas, Simla districts, Singapore, China and Japan (Nadkarni, 1976).

Biological survey indicates that the stem bark has been widely use in asthma, coughs, bronchitis, throat complaints, nasal catarrh, cardiac debility, cardiac edema, anemia, fever,

piles, diarrhea, chronic dysentery, ulcers, tumors and as carminative, gut stimulant and diuretic (Kumar and Rana, 2012; Panthari *et al*., 2012). The oil from the flowers is considered as a tonic and is used for the treatment of earache, headache, diarrhea and paralysis. The bark has high tannin content because of this it acts as a detoxifier, also used for wounds and ulcers (Kumar and Rana, 2012: Usmanghani *et al*, 1997). The juice of unripe fruit with its anthelmintic property is used in various herbal preparations (Parmar and Kaushal, 1982).

Various studies have been conducted to reveal the ethnopharmacological aspects of *M. nagi* including antioxidant (Goyal *et al*., 2013), anti-inflammatory (Patel *et al*., 2011a) anti-allergic, mast cell stabilizing, antihelmintic, antimicrobial against *Escherichia coli* and *Streptococcus pyogenes*, anxiolytic, antihypertensive, hepatoprotective and chemopreventive (Alam *et al*., 2000; Kumar and Rana, 2012; Patel *et al*., 2011b). Bark of this popular medicinal plant has been known to contain myricanol, myricanone, epigallocatechin 3-O-gallate, prodelphinidin dimers, gallic acid and tannin (Dawang *et al*., 1988; Patel *et al*., 2011a), while its fresh fruit juice is reported to possess sufficient phenol, flavonoid and flavonol contents (Goyal *et al*., 2013).

The widespread medicinal use of *M. nagi* demands thorough scientific investigations of therapeutic claims with elucidation of possible mechanisms of action to rationalize its variety of folkloric uses. In this study, we have investigated the antidiarrheal, gut modulatory, bronchodilator and vasodilator activities of *M. nagi* to provide pharmacological basis to its medicinal use in gut (diarrhea, abdominal colic), airway (bronchitis, asthma) and cardiovascular (hypertension, arteriosclerosis) disorders. Activity-directed fractionation revealed separation of gut stimulatory and inhibitory constituents of *M. nagi* in the aqueous and organic fractions, respectively.

MATERIALS AND METHODS

Chemicals: Acetylcholine Chloride (ACh), atropine sulphate, carbachol (CCh), phenylephrine (P.E), loperamide and verapamil hydrochloride were purchased from Sigma Chemicals Company, St. Louis, MO, USA. Chemicals used for making physiological salt solutions were: Potassium chloride (Sigma Chemicals Co, St Louis, MO, USA), calcium chloride, glucose, magnesium chloride, magnesium sulfate, potassium dihydorgen phosphate, sodium bicarbonate, sodium dihydrogen phosphate (Merck, Darmstadt, Germany) and sodium chloride (BDH Laboratory supplies, Poole, England). All chemicals used were of the analytical grade available and solubilized in distilled water.

Animals: Animals used in this study such as BALB/c mice (20-25 g), local bred rabbits (1.0-1.5 kg), guinea-pigs (400-500 g), Sprague Dawley rats (180-200 g) of either sex were housed at the Animal House of The Aga Khan University, maintained at 23-25°C and were given a standard diet and tap water. Animals had free access to water, but food was withdrawn 19-18 h prior to the experiments. For isolated tissue experiments, the animals were sacrificed by cervical dislocation. Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 2011). This study was the part of the M.Phil thesis of Ms. Ambreen Aleem, approved by the Board of Studies, Bahauddin Zakariya University, Multan.

Preparation of crude extract and the fractions: The dried bark of *M. nagi was* purchased from a local herbal store at Multan. The plant material was identified by expert taxonomist (Prof. Altaf Ahmad Dasti) at the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan. The plant material was made free from soil and other adulterants and vegetative debris. The dried plant material (1 kg) was ground into coarse powder by electrically driven device and soaked into aqueous-ethanol (80% v/v) for three days with occasional shaking. The soaked material was filtered through a muslin cloth and then through a Whatman qualitative grade no. 1 filter paper. This procedure was repeated twice and the combined filtrate was evaporated on rotary evaporator under reduced pressure to a semisolid mass i.e., the crude extract of *M. nagi* (Mn.Cr), yielding approximately 8%.

For the purpose of fractionation, around 10 g of the crude extract was dissolved in water and shaken with ethyl acetate in a separating funnel. The layers of the immiscible solvents were allowed to separate. The ethyl acetate layer was collected whereas the aqueous layer was re-extracted with fresh ethyl acetate for a total of three times. The ethyl acetate layers, so collected, were combined and then dried on a rotary evaporator to yield the ethyl acetate fraction (Mn.EtAc) weighing 1 g. The aqueous layer left after extraction with ethyl acetate was evaporated separately to obtain the aqueous fraction (Mn.Aq) weighing 9 g. The Mn.Cr, Mn.EtAc and Mn.Aq were solubilized in normal saline for all experiments.

Castor oil-induced diarrhea in mice: The antidiarrheal activity of Mn.Cr was studied on castor oil-induced diarrhea in mice as described previously (Mehmood *et al*., 2011). Thirty mice of equal body weight were randomly divided into six groups (n = 5/group), and kept individually in cages lined with blotting paper. The first group served as negative control and was treated with normal saline (10 mL kg^{-1}). The second to fourth groups received 100, 300 and 500 mg kg^{-1} of Mn.Cr, respectively. The fifth and sixth groups were administered loperamide (10 mg kg^{-1}) and verapamil (100 mg kg^{-1}), respectively. One h after respective treatment, all the animals received castor oil (10 mL kg^{-1}, p.o.) through a feeding needle. After 5 h of castor oil administration, the cages were inspected for the presence of typical diarrheal droppings; the absence was regarded as a positive result, indicating protection from diarrhea.

Magnesium sulfate ($MgSO_4$)-stimulated diarrhea in mice: The antidiarrheal effect of Mn.Cr was also evaluated against $MgSO_4$-induced diarrhea in mice as described previously (Izzo *et al*., 1994). A similar process as of castor oil-induced diarrheal study was followed. Diarrhea was induced by oral administration of $MgSO_4$ at the dose of 2 g kg^{-1} to all 5 groups (n = 5 animals/group) of animals one h after pretreatment of normal saline (10 mL kg^{-1}, p.o.) to the negative control group, Mn.Cr at 100, 300 and 500 mg kg^{-1} to the treatment groups, and loperamide (10 mg kg^{-1}) and verapamil (100 mg kg^{-1}) to the positive control groups. After 5 h of $MgSO_4$ administration, total number of feces (fecal output) in each group were counted and expressed as Mean±SEM.

Isolated tissue preparations: Experiments on isolated tissue preparations were performed by methods employed previously in our laboratory (Gilani *et al*., 2007).

Guinea-pig ileum: The ileum was identified and isolated out. The tissues (2-3 cm) were prepared and suspended in a 10 mL tissue bath containing Tyrode's solution, maintained at 37°C and aerated with a mixture of 95% oxygen and 5% carbon dioxide (carbogen). The composition of Tyrode's solution in mM was: KCl 2.68, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.55. Intestinal responses were recorded isotonically using bioscience transducers coupled with PowerLab data acquisition system. Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug and then stabilized with a sub-maximal concentration of acetylcholine (ACh, 0.3 μM) with a 3 min interval until constant responses were recorded. To characterize the spasmodic effect of the test material, tissues were pretreated with antagonist (atropine), 30 min before re-determining the stimulatory effect of test substance (Rehman *et al*., 2013).

Rabbit jejunum: The jejunum was dissected out, kept in Tyrode's solution and cleaned off mesenteries. Each segment of about 2-3 cm length was suspended in a 10 mL tissue bath containing Tyrode's. The tissues equilibrated and stabilized by following the procedure described above (Guinea-pig ileum). The contractile responses of the tissues were recorded using similar isotonic transducers as used for guinea-pig ileum. Under these experimental conditions, rabbit jejunum exhibits spontaneous rhythmic contractions, allowing the testing of relaxant (spasmolytic) activity directly without the use of an agonist (Mehmood and Gilani, 2010).

For the determination of Ca^{++} antagonist activity, high K^+ (80 mM) was used to depolarize the preparations as described by Farre *et al*. (1991). The K^+ was added to the tissue bath, which produced a sustained contraction. Test materials were then added in a cumulative fashion to obtain concentration-dependent inhibitory responses.

To confirm the Ca^{++} antagonist action of the test substance, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with Ca^{++}-free Tyrode's solution containing EDTA (0.1 mM) for 30 min in order to remove Ca^{++} from the tissues. This solution was further replaced with K^+-rich and Ca^{++}-free Tyrode's solution, having the following composition (mM): KCl 50, NaCl 91.04, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, glucose 5.55 and EDTA 0.1. Following an incubation period of 30 min, control Concentration Response Curves (CRCs) of Ca^{++} were obtained. When the control Ca^{++} CRCs were found super-imposable (usually after two cycles), the tissue were pretreated with the plant extract for 60 min to test the possible Ca^{++} antagonist like effect. The CRCs of Ca^{++} were reconstructed in the presence of different concentrations of the test material (Mehmood and Gilani, 2010; Janbaz *et al*., 2015).

Rabbit trachea: The trachea was dissected out and kept in Kreb's solution. The tracheal tube was cut into rings, 2-3 mm wide, each containing about two cartilages. Each ring was opened by a longitudinal cut on the ventral side opposite to the smooth muscle layer, forming a tracheal strip with a central part of smooth muscle in between the cartilaginous portions on the edges. The preparation was then mounted in a 20 mL tissue bath containing Kreb's solution, maintained at 37°C and aerated with carbogen. The composition of Kreb's solution was (mM): NaCl 118.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.3, $MgSO_4$ 1.2 and glucose 11.7 (pH 7.4). A tension of 1 g was applied to each of the tracheal strip and was kept constant throughout the experiment. The tissue was equilibrated for 1 h before the addition of any drug. For stabilization, the tissues were repeatedly exposed to carbachol (CCh, 1 μM) until constant responses of each agonist were achieved (usually 3-4 treatments). The sustained contractions of CCh or high K^+ were then obtained and relaxant effect of the test substance was assessed on the induced contractions by adding in a cumulative fashion. Isometric responses were recorded using isometric transducers coupled with PowerLab data acquisition system.

Endothelium intact and denuded rat aortic tissues: To study the effect on vascular resistance, the thoracic aorta ring preparations from rat were used. Due care was employed while isolating the thoracic aortae to avoid any damage to endothelium. Aortic rings 2-3 mm wide were individually mounted in 5 mL tissue baths containing Kreb's solution, at 37°C and aerated with carbogen gas. A resting tension of 2 g was applied to each tissue and an equilibrium period of 1 h was allowed before studying the effect of test materials. To study effect of the plant material on endothelial denuded preparations, the endothelium lining of the tissues was removed by gentle rubbing. The changes in isometric tensions of the rings were measured via a force-displacement transducer (FT-03) using PowerLab data acquisition system. To study the effect, phenylephrine (PE, 1 μM) and high K^+ were used to induce sustained contractions and the vasodilator

effect of the extract was assessed by adding in a cumulative fashion in endothelium-intact and denuded tissues (Aziz *et al*., 2009).

Statistical analysis: Data are expressed as Mean±SEM (n = number of experiments) and median effective concentrations (EC_{50} values) with 95% Confidence Interval (CI). Statistical analysis was performed using Graph Pad Instant software. For comparison, One-way ANOVA followed by Dennett's test or Chi-test were used. $p<0.05$ was considered statistically different.

RESULTS

Antidiarrheal effect of *M. nagi* against castor oil-induced diarrhea in mice: Castor oil treatment induced diarrhea in all 5 mice of the control group. Treatment with 100 and 300 mg kg^{-1} doses of Mn.Cr caused 20 and 40% protection, respectively. A further increase in the dose (500 mg kg^{-1}) of Mn.Cr did not cause an increase in the number of mice protected from diarrhea; instead the effect was declined to 20%. The positive control drugs, verapamil and loperamide caused 80 and 100% protection at respective doses of 100 and 10 mg kg^{-1} (Table 1).

Antidiarrheal effect of *M. nagi* against $MgSO_4$-stimulated diarrhea in mice: Administration of $MgSO_4$ treatment enhanced fecal output in all animals of saline treated group. Treatment of Mn.Cr at the doses of 100 and 300 mg kg^{-1} markedly inhibited $MgSO_4$-stimulated fecal output by offering 26.9 and 46.2% protection vs. saline+$MgSO_4$ treated group, respectively. Next higher dose (500 mg kg^{-1}) of Mn.Cr did not produce further increase (30.8%) in inhibition of the number of feces stimulated by $MgSO_4$ administration. The positive control drugs, verapamil and loperamide produced 58.5 and 41.5% protection, at respective doses of 100 and 10 mg kg^{-1} (Table 2).

Table 1: Antidiarrheal activity of the crude extract of *M. nagi* (Mn.Cr) against castor oil (10 mL kg^{-1}) induced diarrhea in mice

Treatment (p.o.), dose (mg kg^{-1})	No. of mice with diarrhea	Protection (%)
Saline (10 mL kg^{-1})+Castor oil	5/5	0
Mn.Cr+Castor oil		
100+10		4/520
300+10		3*/540
500+10		4/520
Verapamil+Castor oil		
100+10	1*/5	80
Loperamide+Castor oil		
10+10	0**/5	100

* $p<0.05$ and ** $p<0.01$ vs. Saline+Castor oil treated group (Chi-square test)

Effect of *M. nagi* and its fractions on guinea-pig ileum: The crude extract of *M. nagi* at the dose range of 0.3-1 mg mL^{-1} caused spasmodic effect reaching its maximum of 51.67±6.38% (Mean±SEM; n = 6) of ACh (0.3 µM)-induced contraction at 0.3 mg mL^{-1}. However, at next higher dose (1 mg mL^{-1}), its contractile effect was reduced to 18.33±3.53% followed by no contraction at the dose of 3 mg mL^{-1}. To characterize the stimulatory effect of Mn.Cr, when tissues were pretreated with atropine (0.1 µM) before studying the stimulant effect of Mn.Cr, it was completely blocked (Fig. 1a). Its aqueous fraction (Mn.Aq) at 0.1-5 mg mL^{-1} also caused dose-dependent atropine-sensitive stimulatory effect, reaching its maximum of 51.11±1.10% (Mean±SEM, n = 4) at 5 mg mL^{-1} (Fig. 1b). However, its ethyl acetate fraction (Mn.EtAc) was devoid of any stimulant activity at tested doses of 0.03-3 mg mL^{-1} (Fig. 1c).

Effect of *M. nagi* and its fractions on rabbit jejunum: Mn.Cr caused a concentration-dependent (0.01-0.3 mg mL^{-1}) contractile effect followed by relaxant effect at the higher concentrations (1 and 3 mg mL^{-1}) in rabbit jejunum. Pretreatment of the tissue with atropine (0.1 µM) blocked the contractile effect of Mn.Cr while the inhibitory effect was evident at lower concentration with EC_{50} value of 0.65 mg mL^{-1} (0.42-1.0, 95% CI, n = 5). Mn.Cr also inhibited high K^+-induced contraction with EC_{50} value of 0.34 mg mL^{-1} (0.18-0.61, n = 5) as shown in Fig. 2a. The aqueous fraction (Mn.Aq) of *M. nagi* produced spasmogenic response at lower doses (0.03-1 mg mL^{-1}), followed by weak inhibitory response at 3-10 mg mL^{-1}. The spasmogenic effect of Mn.Aq was blocked in the presence of atropine (0.1 µM). The Mn.Aq exhibited only a weak inhibitory effect against high K^+ induced contraction with maximum relaxation of 35.92±7.36% (Mean±SEM, n = 4) at highest tested dose of 10 mg mL^{-1}

Table 2: Antidiarrheal activity of the crude extract of *M. nagi* (Mn.Cr) against $MgSO_4$ stimulated diarrhea in mice

Treatment (p.o.), dose (mg kg^{-1})	Mean (Mean±SEM) Fecal output/group in 4 h	Protection (%)
Saline (10 mL kg^{-1})+$MgSO_4$ (2 g kg^{-1})	13.0±1.02	0.0
Mn.Cr+$MgSO_4$		
100+2	9.5±0.76*	26.9
300+2	7.0±0.85**	46.2
500+2	9.0±0.94*	30.8
Verapamil+$MgSO_4$		
100+2	5.4±0.87**	58.5
Loperamide+$MgSO_4$		
10+2	7.6±0.67**	41.5

*$p<0.05$ and ** $p<0.01$ vs. Saline+Mg SO_4 treated group (One way ANOVA followed by Dunnet's test)

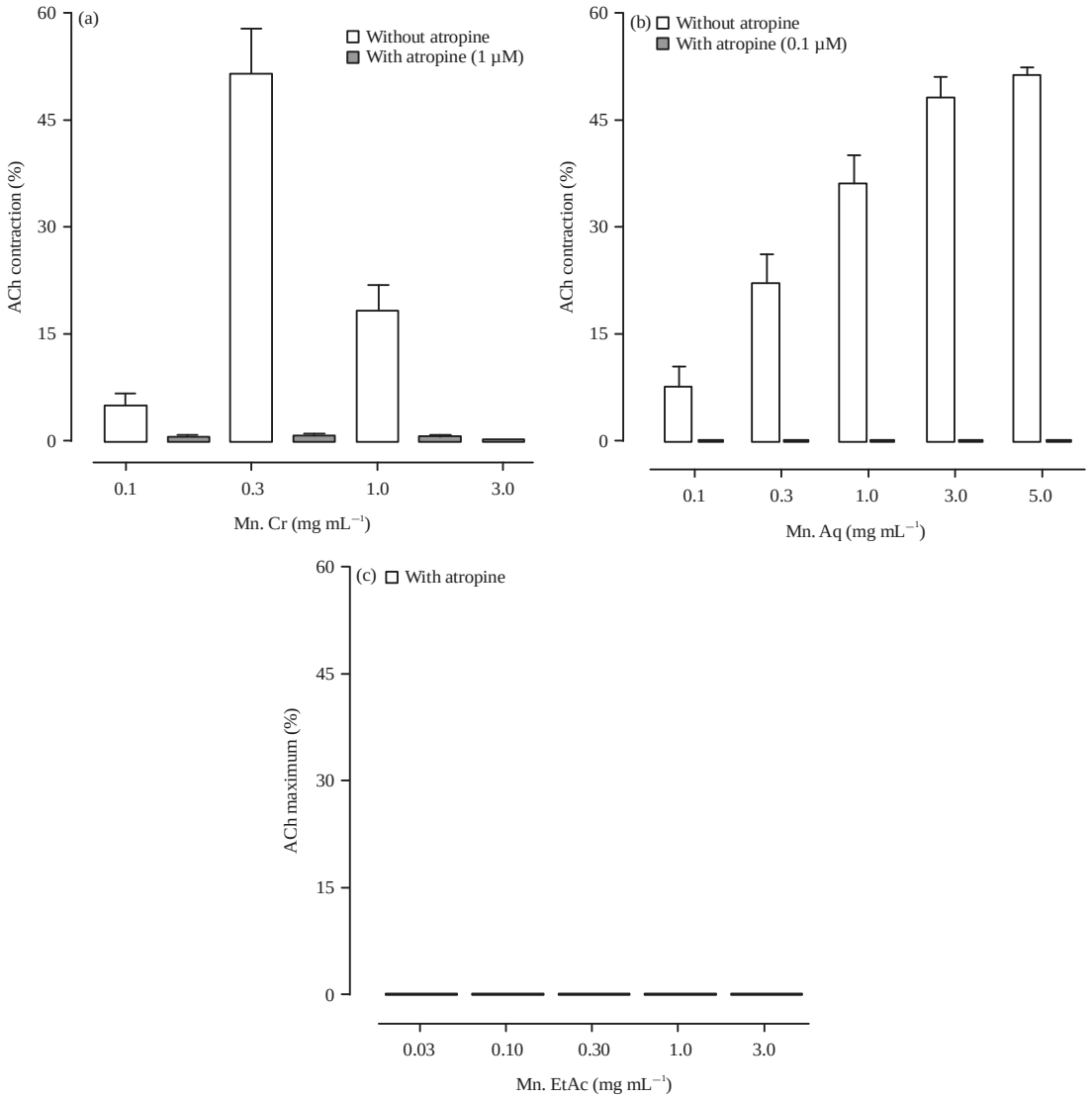

Fig. 1(a-c): Dose-dependent stimulatory effect of (a) Crude extract of *M. nagi* (Mn.Cr), (b) Its aqueous (Mn.Aq) and (c) Ethyl acetate (Mn.EtAc) fraction without and with atropine (0.1 µM) in isolated guinea-pig ileum. The values shown are Mean±SEM, n = 4-6

(Fig. 2b). The ethyl acetate fraction (Mn.EtAc) relaxed both spontaneous and high K^+-induced contractions with respective EC_{50} values of 0.02 (0.01-0.04, n = 3) and 0.30 mg mL^{-1} (0.18-0.52, n = 3) as shown in Fig. 2c. Similarly, verapamil inhibited both spontaneous and high K^+-induced contractions with respective EC_{50} values of 0.24 (0.16-0.36, n = 5) and 0.06 µM (0.03-0.11, n = 5) as shown in Fig. 2d. Pretreatment of jejunal tissues with Mn.Cr (0.1 and 0.3 mg mL^{-1}) and Mn.EtAc (0.1 and 0.3 mg mL^{-1}) shifted the Ca^{++} CRCs to the right as shown in respective Fig. 2e and f, respectively, similar to the effect of verapamil (Fig. 2g).

Effect of *M. nagi* on isolated rabbit trachea: In tracheal preparations, Mn.Cr had no effect on the resting baseline tension. When tested against CCh (1 µM) and high K^+-induced contractions it caused inhibition of both at similar doses (Fig. 3a) with respective EC_{50} values of 1.08 (0.72-1.60, n = 5) and 0.90 mg mL^{-1} (0.59-1.38; n = 5). Verapamil, similarly, inhibited both CCh and high K^+-induced contractions with respective EC_{50} values of 0.11 (0.05-0.23, n = 4) and 0.02 µM (0.01-0.03, n = 3) as shown in Fig. 3b.

Pretreatment of the tissues with Mn.Cr at the doses of 0.3 and 1.0 mg mL^{-1} shifted the Ca^{++} CRCs to the right, similar to the effect seen with verapamil (Fig. 3c and d).

Effect of *M. nagi* on isolated rat aorta: In endothelium intact rat aortic preparations, Mn.Cr caused relaxation of both P.E (1 µM) and high K^+-induced contractions with respective EC_{50} values of 0.24 (0.18-0.34, n = 3) and 0.06 mg mL^{-1} (0.04-0.10, n = 4), as shown in Fig. 4a. In endothelium

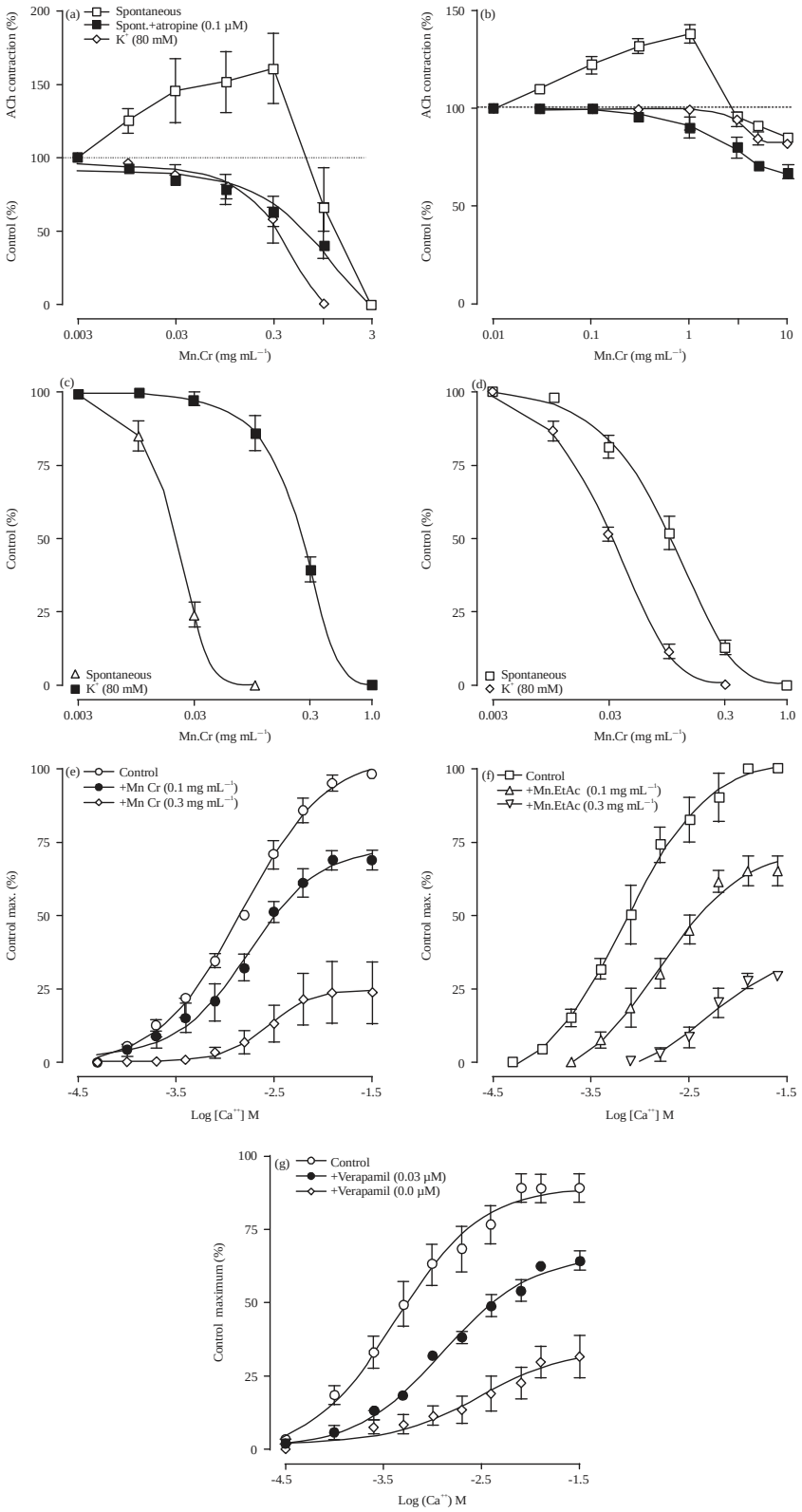

Fig. 2(a-g): Dose-dependent stimulant/inhibitory effect of (a) Crude extract of *M. nagi* (Mn.Cr), (b) Aqueous fraction (Mn.Aq), (c) Ethyl acetate fraction (Mn.EtAc), (d) Verapamil on spontaneous and K^+ (80 mM)-induced contractions and the concentration-response curves of Ca^{++} in the absence and presence of (e) Mn.Cr, (f) Mn.EtAc and (g) Verapamil in isolated rabbit jejunum. The symbols represent Mean±SEM, n = 3-6

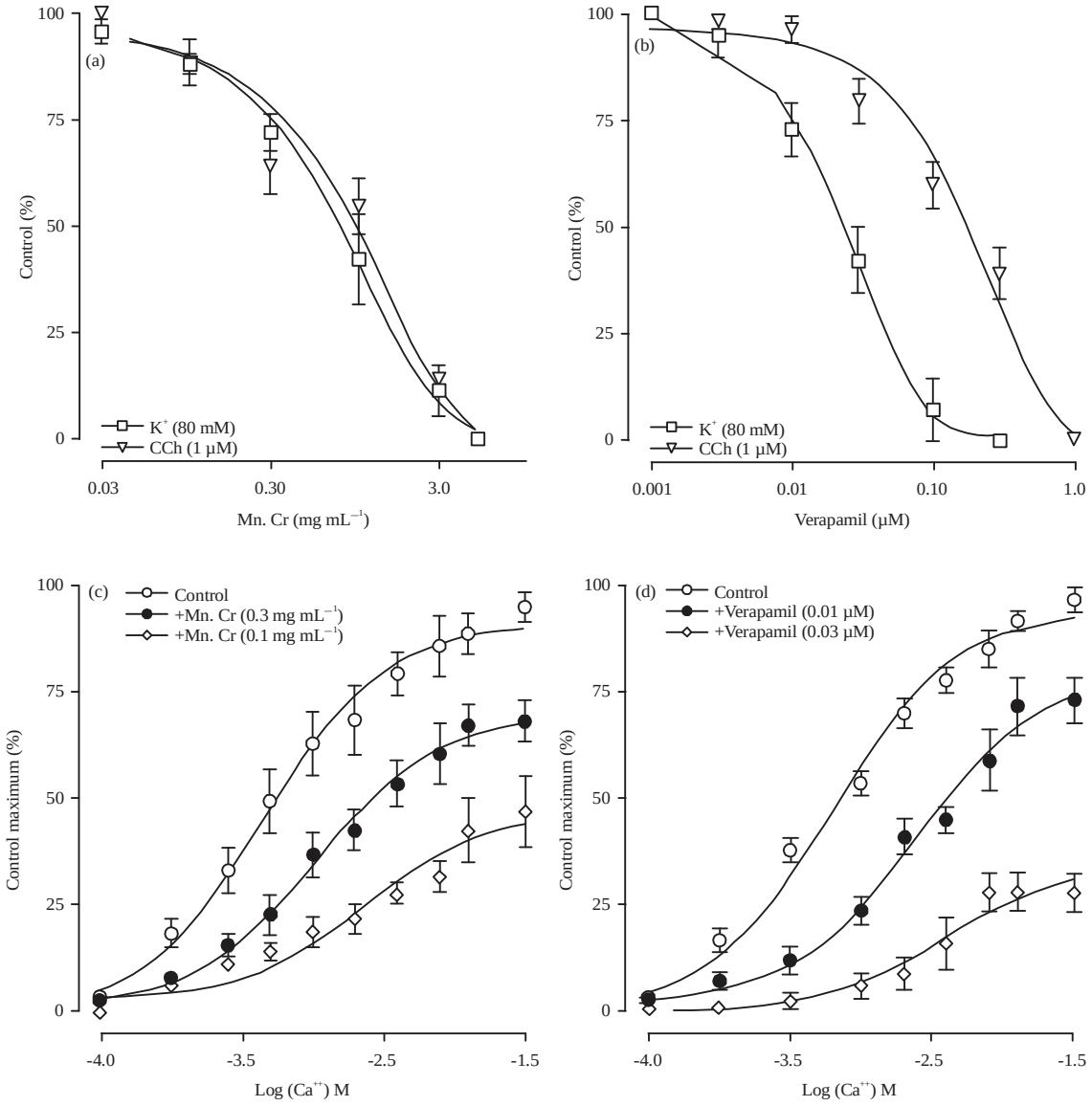

Fig. 3(a-d): Dose-dependent inhibitory effect of (a) Crude extract *M. nagi* (Mn.Cr), (b) Verapamil on carbachol (CCh) and K^+ (80 mM)-induced contractions, the concentration-response curves of Ca^{++} in the absence and presence of, (c) Mn.Cr and (d) Verapamil in isolated rabbit trachea. Values shown are Mean±SEM, n = 4-5

denuded aorta, higher concentration of Mn.Cr was required to relax P.E-induced contraction with resultant EC_{50} value of 1.48 mg mL^{-1} (0.96-2.29, n = 3).

Verapamil also relaxed both P.E and high K^+-induced contractions in endothelium intact aorta with respective EC_{50} values of 0.24 (0.18-0.34, n = 4) and 0.06 µM (0.04-0.10, n = 4), as shown in Fig. 4b. In endothelium denuded aorta, P.E-induced contraction was inhibited by verapamil with EC_{50} value of 0.32 µM (0.21-0.47, n = 4), which was not significantly different from the concentration required to relax the contraction induced in the endothelium intact aorta preparations. Pretreatment of the rat aortic tissue with Mn.Cr at the doses of 0.03 and 0.1 mg mL^{-1} shifted the Ca^{++} CRCs to the right, like that caused by verapamil (Fig. 4c and d).

DISCUSSION

Due to its widespred folkloric use in diarrhea, *M. nagi* bark extract was studied for its possible antidiarrheal effect against castor oil and $MgSO_4$ stimulated diarrhea in mice. The Mn.Cr caused protection against induced diarrhea in mice of both models at 100 and 300 mg kg^{-1}. However, its antidiarrheal effect was observed dose-specific in both animal models as at the higher dose of 500 mg kg^{-1}, a decline in the antidiarrheal effect of *M. nagi* was observed, which might be due to result of the presence of combination of gut inhibitory and gut stimulant constituents in this plant.

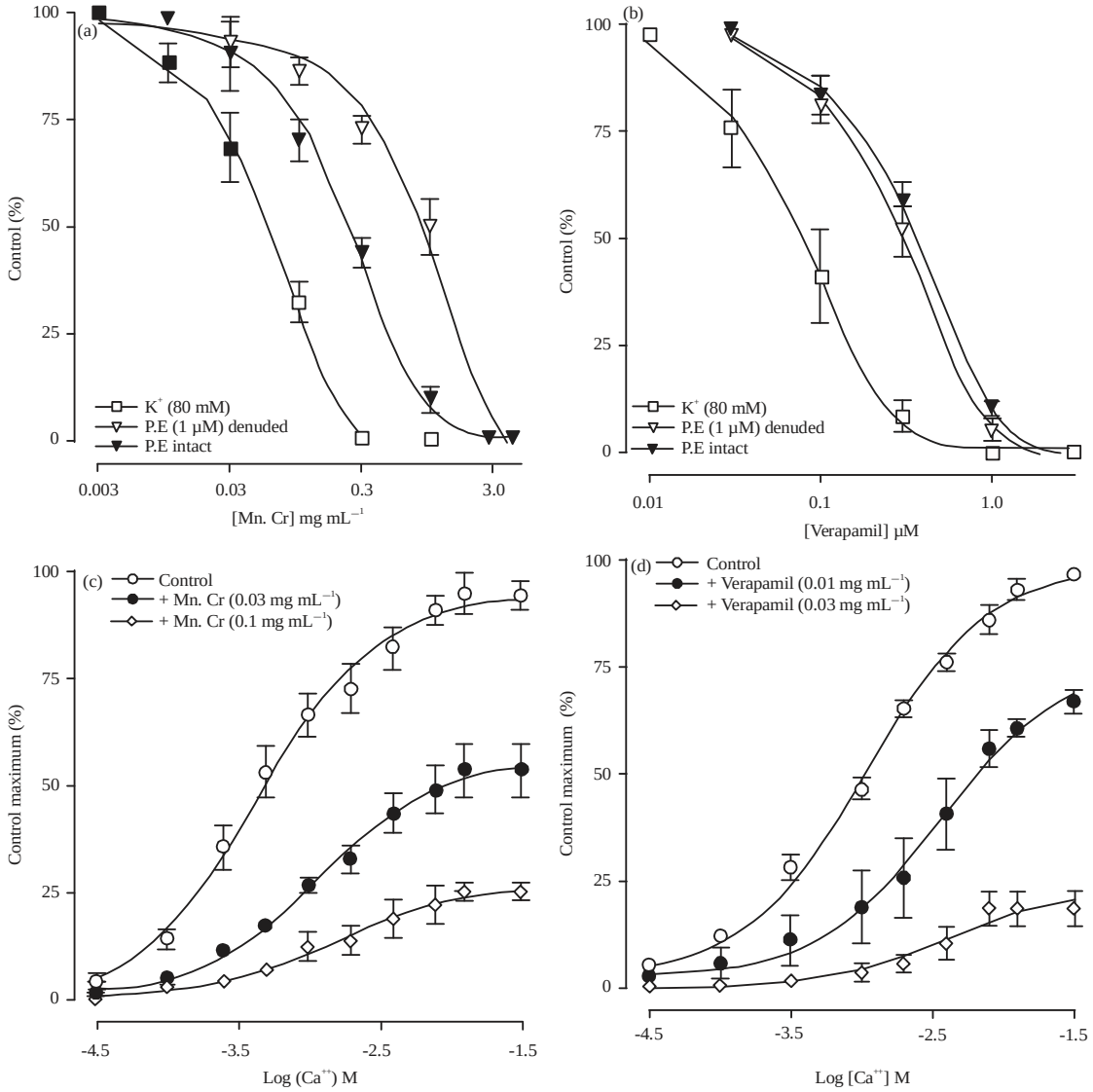

Fig. 4(a-d): Dose-dependent effect of (a) Crude extract of *M. nagi* (Mn.Cr), (b) Yerapamil on phenylephrine (P.E, 1 µM) and K^+ (80 mM)-induced contractions in isolated rat aortic preparations with intact or denuded endothelium. The symbols represent Mean±SEM, n = 3-4

In small intestine of mice, castor oil is degraded by lipases to glycerol and ricinoleic acid. The ricinoleic acid enhances intestinal secretions, fluid accumulation and electrolytes loss and accelerates the intestinal transit (Iwao and Terada, 1962). On the other hand, $MgSO_4$ is known to produce its laxative effect casing osmotic imbalance (Stewart *et al*., 1975) and Nitric Oxide (NO) release (Izzo *et al*., 1994). Thus, a substance producing protection against such diarrheagenic models not only indicates its promising antidiarrheal activity but also provides clue about its direct inhibitory effect on intestinal secretions and NO releasing potential.

To address the possibility of the presence of gut stimulant components in *M. nagi*, isolated guinea-pig ileum, a quiescent preparation known to be suitable for gut stimulatory effect (Rehman *et al*., 2013) was used. The plant extract and its aqueous fraction exhibited atropine-sensitive gut stimulant effect, which was evident by its blockade in the presence of atropine, a muscarinic receptor antagonist (Gilani *et al*., 1997). Interestingly, the plant extract also showed co-presence of some gut inhibitory constituents as reflected by reduction in resultant contractile response of Mn.Cr at 1 mg mL^{-1} compared to its effect on lower dose (0.3 mg mL^{-1}) followed by no contraction at further higher tested dose of 3 mg mL^{-1}. Its aqueous fraction was concentrated with gut stimulant constituents, while ethyl acetate fraction did not produce any stimulant effect. Thus, suggesting that Mn.Cr possesses acetylcholine (ACh) like-gut stimulant constituents in addition to its gut inhibitory component(s).

When tested on spontaneously rhythmic contractions of rabbit jejunum, which is considered a suitable preparation to test the dual effect (spasmogenic and spasmolytic) of the test material (Mehmood *et al*., 2011; 2014), Mn.Cr showed a dose-dependent stimulatory effect at lower doses followed by relaxation at higher doses, indicating the co-existence of stimulatory and inhibitory components. Similar to the nature of its gut stimulant effect observed on guinea-pig ileum, Mn.Cr showed ACh-like gut stimulatory effect on rabbit jejunum. Its aqueous fraction also exhibited a similar picture as seen with crude extract. However, the relaxant activity of Mn.Aq was very weak, while its ethyl acetate fraction was potent in its gut inhibitory effect when compared with Mn.Cr. The spasmogenic effect observed in Mn.Cr and its distribution in its aqueous fraction observed in isolated gut preparations of two different animals seems to be responsible for the reduced antidiarrheal effect of Mn.Cr at higher dose (500 mg kg^{-1}) as seen in *in vivo* assays. The presence of ACh-like gut excitatory constituents of *M. nagi* also explains its medicinal use as carminative and gut stimulant (Parmar and Kaushal, 1982) because cholinergic agents are known to stimulate digestive secretions in the gut (Brown and Taylor, 2006).

We previously observed that the spasmolytic activities present in different plants are usually mediated through a Ca^{++} antagonist-like pathway (Gilani *et al*., 2006, 2007; Mehmood and Gilani, 2010; Mehmood *et al*., 2011; 2014). To see, whether the spasmolytic effect of *M. nagi* bark observed in this study is mediated through the same mechanism, the plant extract was tested against sustained contractions induced by high K^+. Interestingly, the crude extract caused a dose-dependent inhibition of K^+-induced contractions. K^+ at high concentrations (>30 mM) is known to cause smooth muscle contractions through opening of voltage dependent slow Ca^{++} channels, thus allowing influx of extracellular Ca^{++} resulting in contractile effect (Bolton, 1979). Thus, the inhibitory effect of Mn.Cr against K^+-induced contractions can be visualized as mediated through blockade of Ca^{++} channels. The presence of Ca^{++} antagonist like constituents in the plant extract was confirmed when it caused a rightward shift in the Ca^{++} CRCs, constructed in Ca^{++}-free and K^+-rich medium. The Ca^{++} Channels Blockers (CCBs) are known to be useful as antispasmodic and antidiarrheal agents (Pasricha, 2006) and the observed CCB-like activity on the part of this plant may explain the medicinal use of *M. nagi* in diarrhea, though the involvement of additional mechanisms cannot be ruled out.

The presence of combination of gut stimulant and relaxant components in *M. nagi* is interesting but not an unusual phenomenon, as we have previously observed such combinations in a number of plants (Gilani *et al*., 2006; Jabeen *et al*., 2007; Mehmood *et al*., 2011; Rehman *et al*., 2013). It is probably meant by the nature to avoid undesirable effects due to excessive inhibition of gut motility from happening, as seen with chemical drugs in clinical use resulting in constipation and mega colon (Pasricha, 2006).

Based on the medicinal uses of *M. nagi* in asthma, bronchitis and cough, the plant extract was further studied on isolated tracheal preparations for the possible bronchodilator effect. The Mn.Cr produced dose-dependent inhibition of CCh and K^+-induced spasms, suggestive of non-specific tracheal relaxant effect as expected from a Ca^{++} antagonist. The observed bronchodilator effect explains the use of *M. nagi* in the airways disorders. Interestingly, the CCBs have also been shown to be useful in such conditions (Mathewson, 1985).

The CCBs constitute an important group of anti-hypertensive drugs which mediate their effect through relaxation of arteriolar smooth muscle resulting in decreasing peripheral vascular resistance (Hoffman, 2006). In view of the medicinal use of *M. nagi* (Kumar and Rana, 2012; Panthari *et al*., 2012) in cardiovascular disorders and its CCB like constituents as observed in gut and tracheal tissues, Mn.Cr was further studied on isolated rat aorta for potential vasodilator effect. In endothelium intact aorta, Mn.Cr inhibited both P.E and high K^+-induced contractions as expected from a Ca^{++} antagonist, like verapamil. When tested in endothelium denuded aorta, interestingly, a significantly higher concentration was required to relax P.E-induced contraction compared to that required for endothelium intact aorta, suggesting the presence of additional endothelium-dependent vasodilator activity most likely mediated though cholinergic mechanism in Mn.Cr and its NO-releasing potential, which was indirectly evident from its *in vivo* antidiarrheal findings. The Ca^{++} antagonist like effect was confirmed when Mn.Cr caused a concentration-dependent shift in the Ca^{++} CRCs. However, the nature of endothelium-dependent relaxant components of *M. nagi* remains to be elucidated.

On activity guided fraction, spasmogenic component of the plant was found to be concentrated in the aqueous and spasmolytic activity in organic fraction, suggesting the polar nature of the former, while organic or non-polar nature of the latter activity. The Mn.EtAc, unlike verapamil, was found more potent against the spontaneous than high K^+-induced contraction, suggesting the presence of some additional gut inhibitory constituents not involving Ca^{++} antagonist like inhibitory pathway. On the basis of the study on fractions, we postulate that cholinergic constituents, on account of its polar nature, will not get absorbed systemically and therefore, will not interfere with bronchodilator effect of the plant.

CONCLUSION

This study clearly show that *M. nagi* crude extract possesses antidiarrheal, gut modulatory, bronchodilator and vasodilator activities mediated possibly through Ca^{++} antagonist, cholinergic and endothelial-dependent relaxant pathways. These findings provide the scientific basis to the medicinal use of *M. nagi* in airways, gut and cardiovascular disorders.

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