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Original Article

Gastrouterine Smooth Muscle Tone Modulatory Action of Neem Fruit Extract is Mediated via Muscarinic Receptors and Ca^{2+} Channels

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ABSTRACT

Introduction: Neem (scientific name *Azadirachta indica* A. Juss) is a popular medicinal plant in South Asia. We show here the smooth muscle stimulant and relaxant properties of the neem fruit extract. **Materials and Methods:** Neem fruit were soaked in 70% aqueous-methanol, then dried to obtain a thick brown extract (Ai.Cr). Different isolated gastrointestinal and uterine tissue preparations were obtained and were maintained in tissue baths with a physiological salt solution. **Results:** The extract exhibited a stimulant effect in increasing concentrations (0.03 to 3.0 mg/ml) in isolated rabbit jejunum. When this activity of the extract was challenged with a ganglion blocker hexamethonium, it was not blocked. It was completely antagonized when challenged with atropine, a cholinergic blocker. Once the spasmogenic effect was blocked, Ai.Cr exhibited a spasmolytic effect (1-10 mg/ml). Ai.Cr was also able to suppress K^+ (80 mM) sustained contractions in jejunum in increasing concentrations (0.03-3 mg/ml), indicating calcium channel blockade (CCB). Likewise, Ai.Cr exhibited an atropine-sensitive stimulant response (0.03-5 mg/ml) followed by a CCB-type relaxant effect (0.3-3 mg/ml) in stomach fundus and ileum preparations of rat. In rat uterus, the crude extract in increasing concentrations (0.1-10 mg/ml), exhibited uterotonic activity that was partially sensitive to atropine. This indicated that there is an additional stimulant component(s) active in uterine tissues. **Conclusion:** These results show a combination of spasmogenic and spasmolytic effects of Ai.Cr on smooth muscles from rabbits and rats mediated possibly via muscarinic and CCB mechanisms.

Key words: *Azadirachta indica*, Rabbit jejunum, Rat stomach fundus, Rat ileum, Rat uterus.

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INTRODUCTION

Neem (scientific name *Azadirachta indica* A. Juss; family: Meliaceae), as its commonly known in Urdu and Hindi languages, is an evergreen tree belonging to South Asia. Neem is a popular plant with its description and uses known for thousands of years. Its medicinal benefits have been exploited by many of the world's traditional medical systems including Ayurvedic, traditional Greek medicine, and homeopathy. Many parts of the plant including seeds, roots, bark, leaves, flowers and fruit are known for their medicinal benefits.[1,2] Neem has been traditionally used by healers in a variety of diseases, which is why it is also referred to as the 'sacred tree' or 'heal all' and has been described as a panacea for all diseases. It is used in diseases of the dental, rheumatologic, dermatologic, endocrine, gastrointestinal (GI) and urogenital systems and is also used in pyrexia, inflammation, infections, and cancers.[1,2] In particular, neem is traditionally used for its benefits in constipation, diarrhea, dysentery, and nausea.[2] Many bioactive constituents are chemically and pharmacologically known from this plant with azadirachtin being one of the most prominent ones.[3] Several studies are available in the literature providing pharmacological data and claims on this plant. There are scientific studies on the antioxidant,[4] anti-inflammatory,[5] antibacterial,[6] antianxiety,[7] hepatoprotective,[8] and anticancer[9] properties of neem.

In this manuscript, we present our findings regarding the pharmacology of neem fruit extract. We prepared a 70% aqueous-methanolic crude extract of fresh fruits of neem and then tested it on several isolated GI whole tissue preparations including small intestinal jejunum tissue from rabbit while stomach fundus, ileum and uterus from rats. We found that the neem crude extract exhibited GI spasmogenic and spasmolytic activities, while also having a stimulant effect in the rat uterine preparation. Mechanistically, muscarinic and Ca^{2+} channel blocking (CCB) components were identified as possibly responsible for the smooth muscle tone modulatory activities of this plant extract.

MATERIALS AND METHODS

Animal population

Local rabbits (male and female; around 1 kg) and Sprague-Dawley rats (male and female; 170-200 g) were kept in Aga Khan University's animal house. The temperature inside the animal house was maintained around 23°C and the environment was pathogen-free. The animals were provided water as needed and food was withheld a day before running the experiments. The diet formulated and given to animals had in it: (g/kg): white flour 380, fiber 380, molasses 12, table salt 5.8, nutrivet L 2.5, $K_2S_2O_5$ 1.2, fat 38, seafood 170 and milk powder 150. Steps were taken to prevent the suffering of animals at every step of this study. Experiments were performed ethically as per the laboratory animal use and care guidelines, detailed in European Community guidelines, EEC Directive 86/609/EEC.

Drugs and reagents

Several standard pharmacological agonists and antagonists were used such as atropine sulphate, acetylcholine chloride (ACh), carbachol chloride (CCh), hexamethonium chloride, and estradiol. All these were obtained from the Sigma Co., USA. Ethylenediaminetetra-acetic acid (EDTA) was acquired from BDH Lab, UK. Other chemicals for making physiologic salt solutions (Dejalon's, Kreb's, and Tyrode's) were bought from either Sigma Co., USA, or Merck, Germany. The composition of salt solutions was as follows:

Tyrode's (mM): 2.68 KCl, 136.9 NaCl, 1.8 $CaCl_2$, 5.55 glucose, 11.9 $NaHCO_3$, 1.05 $MgCl_2$, 0.42 NaH_2PO_4

Kreb's (mM): 4.7 KCl, 118.2 NaCl, 2.5 $CaCl_2$, 11.7 glucose, 25.0 $NaHCO_3$, 1.3 KH_2PO_4, 1.2 $MgSO_4$

Dejalon's (mM): 5.6 KCl, 154.0 NaCl, 0.5 $CaCl_2$, 2.8 glucose, 6.0 $NaHCO_3$

Preparation of the crude extract

Unripe (fresh) fruit of neem (1 kg) were obtained from a market in the city of Karachi, Pakistan. For authentication purposes, neem fruits were confirmed and catalogued at the Natural Products Research Unit Herbarium located within the Department at Aga Khan University, Pakistan.

Before starting the extraction process, the neem fruit were washed with water and then lightly mashed in a mortar and pestle. To make the extract, a couple of litres of 70% aqueous methanol (analytical grade from Merck Co., Darmstadt, Germany) were used. The plant material was immersed in the solvent for 72 hr. At the end of this period, the solvent was passed through a piece of cloth and filtered. The neem fruit were further extracted in fresh solvent for a further 72 hr and this step was repeated twice. Finally, all the filtrates were combined and filtered through a filter paper (Whatman qualitative Grade 1). The filtrate was concentrated in a rotary evaporator. The resultant extracts were labelled Ai.Cr.

Rabbit jejunum tissues

The effect of the extract on smooth muscle contraction was evaluated using standard methods.[10] Briefly, pieces of rabbit jejunum (~1-2 cm in length) were hung in tissue baths containing Tyrode's solution bubbled with a mixture of 95% O_2 and 5% CO_2 (carbogen) at 37°C. Isotonic changes were noted using Harvard equipment (oscillographs and force transducers). Tissues were left to normalize in the solution for 30 min before any drug was added. In such a situation, preparations of rabbit jejunum showed spontaneous contractions. The benefit of this is that we can check for the spasmolytic activity of an agent on the spontaneous contractions of the isolated preparation. Stimulant or spasmogenic effects of test material were tested on the baseline of tissue and the effect compared to one produced by a standard spasmogenic agent like ACh (10 µM). Spasmolysis was calculated as a percent change in spontaneous behaviour of the tissue preparation.

To further categorize the spasmolytic effect, tissues were given K^+ (80 mM). This acts as a strong contractile agent and induces contractions upon which a potential CCB action can be tested.[11] On these K^+ contractions, the concentration-dependent relaxant effect can be quantified.

Isolated rat stomach fundus preparation

The effect of the extract on stomach tissue was evaluated using previously described methods.[12] Briefly, longitudinal stomach fundus strips (1.5-2 cm long) were obtained from rats and were then hung with a thread in organ baths (10 ml volume) filled with Kreb's solution. The temperature was set at 37°C and O_2 and CO_2 mixture were bubbled continuously throughout the experiment. Tissue strips were kept under 1 g tension. Isotonic changes were noted using Harvard equipment (transducers and student oscillographs). The tissues were left to stabilize for 1 hr and then a standard agonist was used (CCh 0.3 µM) to get consistent stimulant responses. Once the tissues were stable, Ai.Cr was tested on the flat baseline for any stimulant effect. A relaxant effect was noted by using contractions induced with high K^+.

Isolated rat ileum preparation

A detailed methodology for testing the effect of the extract on rat ileum tissue preparations is outlined in a previous report.[12] Ileum tissues (~2 cm long) were cut from rat small intestines and were hung in baths containing Tyrode's buffer, aerated with a O_2 and CO_2 mixture at 37°C under a 1 g tension. All contractile changes were noted isotonically with the use of Harvard equipment. The preparation was left for 30 min to normalize, then challenged with a standard agonist like ACh (0.3 µM) repeatedly. A gap of 3 min was allowed between repeated doses. To test Ai.Cr on this preparation, the extract was applied to tissue at baseline and stimulant effects were noted. To test for a relaxant effect via CCB, high K^+-induced contractions were utilized.

Isolated rat uterus preparation

The effect of the extract on rat uterus tissue was evaluated using previously described methods.[13] To obtain the estrogenized uterus, 0.1 mg/kg β-estradiol was administered to female rats, under the skin a day before the experiment. The abdomen was cut open by a longitudinal cut and the two horns of the uterus were dissected. These were hung with a thread in baths with Dejalon's solution at 32°C. One end of the horn was fixed at the lower side while the opposite end was fixed with cotton thread to an isotonic Harvard transducer on top and aerated with O_2 and CO_2 as above. A tension of 0.5 g was used for these tissue preparations. The tissues were left for 30 min in the salt solution and a 0.3 µM concentration of ACh was subsequently used as a standard agonist. This was repeatedly administered to the tissue to obtain consistent responses. Once this was achieved, the tissue was ready for testing.

Data analysis

We have used mean ± standard error of mean (SEM; 'n' shows the number of observations) to represent the results. Median effective concentrations or EC_{50} are with 95% confidence intervals (CI). To determine a statistical difference, we used a two-way analysis of variance (ANOVA) test. Statistical difference was noted when $p < 0.05$. These statistical calculations were done using the GraphPad program.

RESULTS

Activity on jejunum from rabbit

Ai.Cr, once administered on jejunum, exhibited a stimulatory effect from concentrations of 0.01-5 mg/ml (Figure 1). The EC_{50} value for this was 0.39 mg/ml (0.08–1.97, 95% CI, n=6). Beyond the maximum tested stimulant concentration, the extract showed a relaxant effect. The stimulant response was calculated to be 69.1 ± 9.0% (n=6) of ACh maximum effect (Figure 1). To possibly determine the mechanism of this spasmogenic effect, tissues were left with 0.3 mM hexamethonium for 0.5 hrs. When the extract response was repeated in the presence of hexamethonium, a similar ($p > 0.05$, compared to the absence of blocker) extract response was noted (Figure 1). The EC_{50} of this effect was 0.66 mg/ml (0.47–0.92, n=3), indicating no blockade. Alternatively, when Ai.Cr was administered in presence of atropine (0.03 µM) that was in contact with tissues for 30 min, the extract mediated stimulation was flattened. Following blockade of Ai.Cr's spasmogenic component, a spasmolytic component became visible (Figure 1) and this was also elicited in increasing concentrations (0.3-10.0 mg/ml). The EC_{50} of this antispasmodic action was calculated to be 3.09 mg/ml (0.92–10.40, n=3).

To classify the mechanism of this spasmolytic effect, Ai.Cr concentrations were added cumulatively up on contractions sustained with K^+ (80 mM). The extract exhibited relaxation (Figure 1) in increasing concentrations (0.03-3.0 mg/ml). The EC_{50} was calculated to be 0.19 mg/ml (0.10–0.36, n=3).

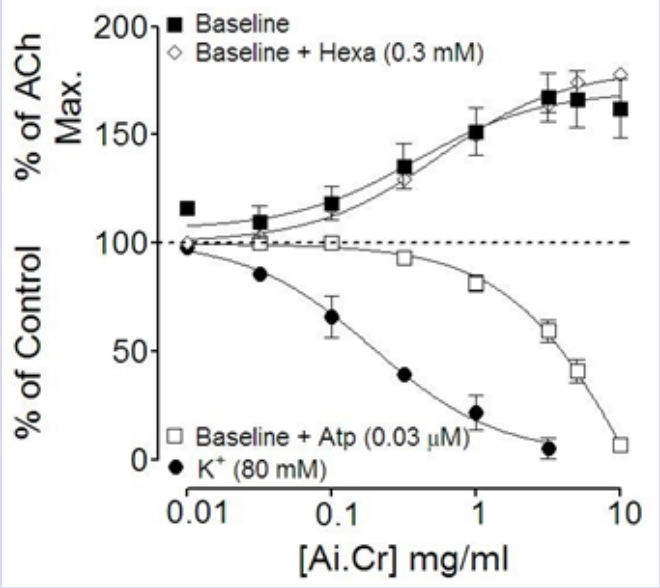

Figure 1: Figure showing effects of *Azadirachta indica* extract (Ai.Cr) when tested on rabbit jejunum preparations: (above the dotted line) stimulant effect (% ACh/acetylcholine maximum) with or without hexamethonium 0.3 mM (Hexa); and (below the dotted line) spasmolytic response of Ai.Cr with atropine 0.03 µM (Atp) pretreatment and on K^+ (80 mM) contractions. Data is represented as mean ± SEM, *n*=3-6. The curves above the dotted line are the same ($p > 0.05$). The curves below the dotted line are distinct from one another ($p < 0.0001$).

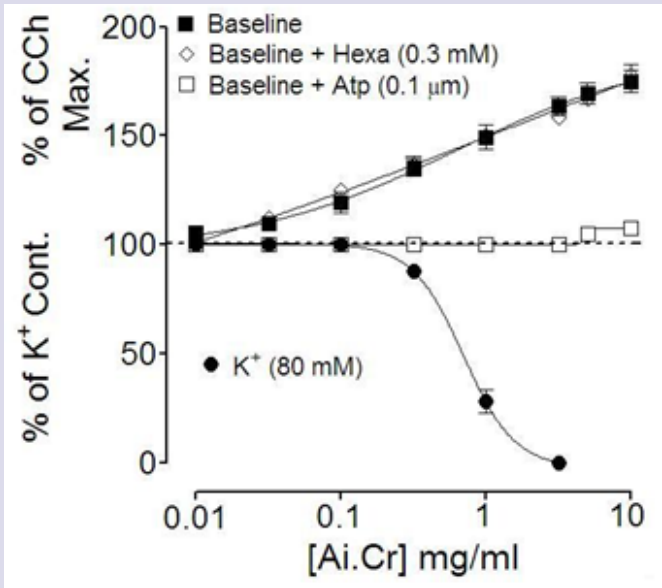

Figure 2: Graph representing the effect for *Azadirachta indica* fruit extract (Ai.Cr) on stomach fundus preparations from rat: (above the dotted line) stimulant effect (% of carbachol or CCh maximum) on the tissue baseline (in the absence of antagonists) and in the presence of antagonists hexamethonium 0.3 mM (Hexa, $p > 0.05$ when compared to without hexamethonium) and atropine 0.1 µM (Atp); and (below the dotted line) relaxant effect of the extract on high K^+ (80 mM) contractions. Data is represented as mean ± SEM, *n*=3-5.

Activity on stomach fundus from rat

Ai.Cr showed a stimulant effect in increasing concentrations (0.03 to 10.0 mg/ml; Figure 2). The EC_{50} for this effect was 0.63 mg/mL (0.17–2.35, *n*=5). This stimulant effect was determined to be 74.8 ± 5.1% (*n*=5) of the effect produced by the standard CCh (Figure 2). This spasmogenic response of extract was repeated with hexamethonium (0.3 mM), no change was observed ($p > 0.05$; Figure 2). Atropine (0.1 µM), on the other hand, completely abolished the stimulant action of Ai.Cr (Figure 2).

We wanted to see if the extract was exhibiting any spasmolytic activity in stomach tissues, like jejunum. For this, Ai.Cr was administered upon K^+ (80 mM) contractions. Like in jejunum, Ai.Cr in increasing concentrations (0.3 to 3 mg/ml), suppressed K^+ contractions (Figure 2). The EC_{50} for this effect was determined to be 0.7 mg/ml (0.62–0.80, *n*=3).

Activity on ileum from rat

In isolated rat ileal preparations, Ai.Cr showed similar stimulant effect in increasing concentrations (0.03 to 10.0 mg/ml; Figure 3). The EC_{50} for this was calculated to be 0.25 mg/m1 (0.08–0.58, *n*=3). This spasmogenic effect was 92.80 ± 1.85% (*n*=3) of the standard ACh maximum response (Figure 3). This spasmogenicity from Ai.Cr was abolished by the antagonist atropine (0.1 µM; Figure 3). Hexamethonium (0.3 mM; Figure 3) was unable to suppress the stimulant effect of Ai.Cr ($p > 0.05$).

The tissues were administered at high K^+ (80 mM) so that sustained contractions could be obtained. Ai.Cr, in increasing concentrations (0.03 to 5.0 mg/ml), suppressed the K^+ contractility (Figure 3). The EC_{50} of this relaxant effect was 0.22 mg/ml (0.08–0.58, *n*=3).

Activity on uterus from rat

Ai.Cr was able to stimulate uterus tissues from 0.1 to 10 mg/ml (Figure 4). The EC_{50} for this stimulant effect was 0.35 mg/ml (0.23–0.55, *n*=3). This stimulant effect was 96.5 ± 0.86% (*n*=4) of the standard ACh response (Figure 4). When the tissues were challenged with 0.3 mM hexamethonium, no change was detected ($p > 0.05$; Figure 4), although atropine (1 µM) was able to partially block the Ai.Cr stimulant effect (1-10 mg/ml; *n*=4) (Figure 4). The EC_{50} for this response was 1.65 mg/mL (0.21–12.75, *n*=4), while it was 63.8 ± 8.6% (*n*=4) of standard ACh maximum effect.

DISCUSSION

We undertook this project to elucidate the pharmacological activities of the neem plant (particularly its fruit) on some isolated smooth muscle preparations. Neem is an age-old medicinal plant used by traditional healers in several medical conditions. We were specifically interested in the activity of neem fruit extract in GI and uterine smooth muscles as neem is known to be active on smooth muscles. Neem is regarded useful as a laxative, antidiarrheal and an anti-nausea agent.

To investigate the activity of neem extract, it was tested on rabbit jejunum tissues. The extract, in increasing concentrations, exhibited stimulant activity through possible interaction with muscarinic receptors. Atropine is a competitive non-specific muscarinic acetylcholine receptor antagonist[14,15] and it completely blocked the stimulant effect of Ai.Cr. Atropine can also mask the response of nicotine (but not nicotinic receptors) because in the end nicotine leads to the release of ACh that acts on its cholinergic receptors.[16] Thus, to rule out a nicotine-like effect, the stimulant effect of the extract was challenged with hexamethonium, a ganglion blocker.[17] Hexamethonium pretreatment was not able to block the Ai.Cr stimulant effect, thus confirming that the Ai.Cr stimulant effect is a consequence of direct action of the extract on intestinal muscarinic receptors. This atropine-sensitive stimulant effect was not only visible in the rabbit jejunum tissues but was also observed in other GI tissues like

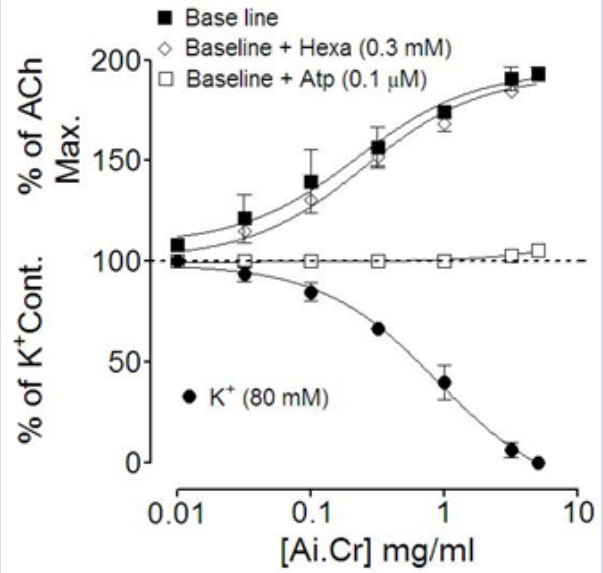

Figure 3: Graph representing effect for *Azadirachta indica* fruit extract (Ai.Cr) on ileal preparations from rat: (above the dotted line) stimulant effect (% ACh/acetylcholine maximum) on the tissue baseline (in the absence of antagonists) and in the presence of antagonists hexamethonium 0.3 mM (Hexa, $p > 0.05$ when compared to without hexamethonium) and atropine 0.1 µM (Atp); and (below the dotted line) relaxant effect of the extract on high K^+ (80 mM) contractions. Data is represented as mean ± SEM, n=3.

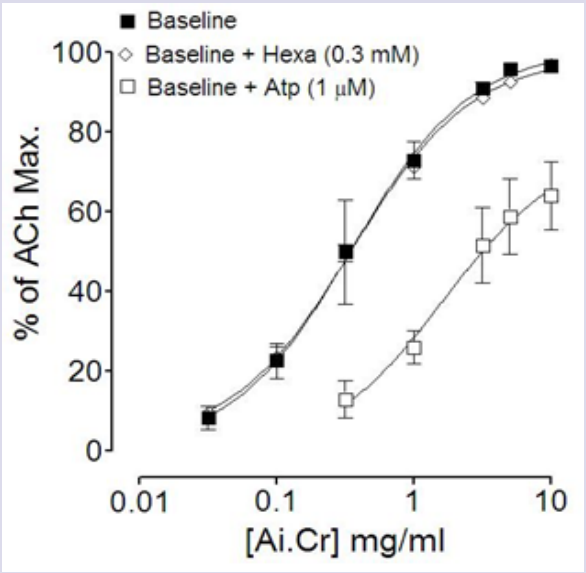

Figure 4: Graph representing the effect for *Azadirachta indica* fruit extract (Ai.Cr) on uterine preparations from rat: Stimulant effect (% ACh/acetylcholine maximum) on the baseline (in the absence of antagonists) and in the presence of antagonists hexamethonium 0.3 mM (Hexa, $p > 0.05$ when compared to without hexamethonium) and atropine 1 µM (Atp). Data is represented as mean ± SEM, n=4. There was significant blockade of stimulant effect of extract in the presence of atropine ($p < 0.0001$).

rat stomach fundus and rat ileum. Among these three tissues, the extract was most efficacious, compared to the ACh/CCh control maximum response, in rat ileum with a 92.80 ± 1.85% effect of ACh.

Muscarinic acetylcholine receptors are known to mediate human GI smooth muscle contractions and tone.[18] They are present in the esophagus, stomach, as well as the small and large intestines,[19] thus helping to control the gut peristaltic movements.[20] As already discussed, neem is traditionally known for its laxative properties and our results confirm this. The traditional use of neem in nausea is thus explained here as the stimulant effect on stomach fundus tissues helps to propagate stomach emptying and thereby helps to reduce nausea on the GI local level.[12] Notably, Senthil Nathan *et al.*[21] have reported an acetylcholinesterase (AChE) inhibitory property of one of the main components of neem, azadirachtin. AChE inhibition is an indirect cholinergic effect compared to a direct cholinergic effect as shown in our results. Azadirachtin has also been reported to modulate cholinergic excitatory currents, on the neuron level in *Drosophila melanogaster* via a patch clamp study.[22] These two studies further strengthen our findings.

Interestingly, when the stimulant action of Ai.Cr was abolished by atropine in GI tissues, a spasmolytic effect was visible. The relaxant effect was recorded on baseline contractility of rabbit jejunum while in the fundus and ileal tissues was exhibited on agonist-induced contractions, as these tissues are devoid of any spontaneous contractions. We have reported many different plant extracts in the past studies that exhibit a relaxant effect via CCB.[23,24] Thus, to further investigate the antispasmodic action of Ai.Cr, high K^+ (80 mM) contractions were induced in isolated smooth muscles (jejunum, stomach fundus, ileum). This administration of K^+ opens Ca^{2+} channels that results in entry of Ca^{2+} from the outside of the cell to the inside. Potential CCBs suppress these induced contractions.[25] Ai.Cr in increasing concentrations suppressed these high K^+-induced contractions, confirming a CCB-like effect.

The neem plant is not only used traditionally for its laxative properties but is also used in diarrhea and dysentery. The spasmolytic effect seen here in this investigation proves the utility of neem for its antidiarrheal actions. A separate study has shown *in vivo* antidiarrheal and antisecretory activity of neem leaves extract in mice[26] and we herein confirm that this antidiarrheal effect of neem may be due to Ca^{2+} antagonism. CCBs are clinically used as GI antispasmodics. One example is of pinaverium bromide which is marketed in North America and exerts its antispasmodic effect via CCB.[27]Another study[28] has also shown an antispasmodic effect of neem extract although that is with an aqueous extract of the flowers (we have used methanol extract of fruits). The authors of that study challenged the spasmolytic effect of neem flower aqueous extract with propranolol (a beta-adrenergic antagonist) and L-NAME (inhibitor of nitric oxide synthase inhibitor). This further confirms the CCB effect seen here in our study. Furthermore, we report herein an atropine-sensitive spasmogenic effect of the extract in addition to the spasmolytic effect. This shows the widespread presence of such smooth muscle tone modulatory properties in different parts of this plant. Azadirachtin is known to possess most of the medicinal properties of neem plant including antimalarial, anthelmintic and insecticidal properties. It is also reported to have AChE inhibitory property,[21] and modulates cholinergic and calcium currents.[22] This indicates that the activities seen in our study may also be due to presence of azadirachtin. Although, this needs to be confirmed on a phytochemical level.

This combination of spasmogenic and spasmolytic effects from plant extracts is novel but we have shown these kinds of profiles in the past too.[10,12,13,23,29] It indicates the effectiveness of the plant not only in constipation but also in diarrheal states. A good clinical situation is what is seen in irritable bowel syndrome where patients need more of a GI tone modulatory agent than a pure laxative or spasmolytic. We have shown the efficacy of the psyllium husk for such a scenario.[30]

The neem extract also caused a concentration-dependent spasmogenic effect in isolated rat uterus preparations. Like the other tissues, this effect was resistant to blockade by hexamethonium. When the uterine preparations were pretreated with muscarinic blocker atropine, the spasmogenic action of Ai.Cr was only partially diminished indicating the presence of an additional stimulant component in the extract (this remains to be characterized). We have shown a similar partial muscarinic utero-stimulant effect from plant extracts in the past.[13] Related to this, neem oil has been reported to have antifertility and post-coital contraceptive activities too.[31,32]

CONCLUSION

This study presents the ability of a 70% methanolic extract of neem fruit to stimulate and relax smooth muscle preparations. The results show a mixture of GI smooth muscle stimulant (muscarinic) and relaxant (CCB) constituents. The extract also showed a utero-stimulant effect. This study has shown pharmacological evidence to the use of this plant by traditional healers in several conditions. More detailed studies are required in the future to further elucidate the unidentified stimulant component in the extract.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ACh: Acetylcholine; **Ai.Cr:** *Azadirachta indica* crude extract; **Atp:** Atropine; **CCh:** Carbachol; **CCB:** Calcium Channel Blocker; **GI:** Gastrointestinal; **Hexa:** Hexamethonium.

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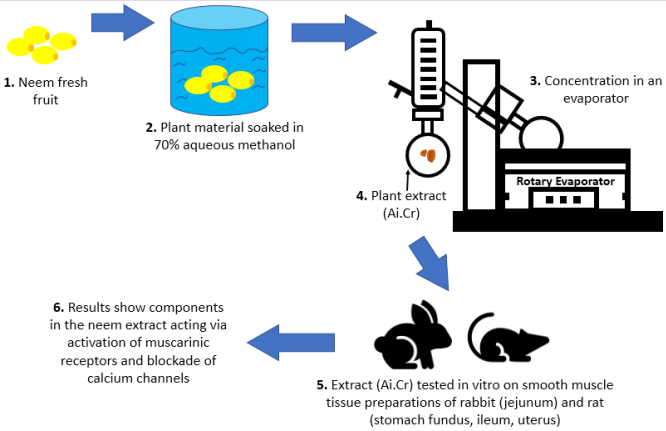
PICTORIAL ABSTRACT



SUMMARY

- Neem is a popular plant of South Asia regarded for its many traditional uses.
- We report the activity of a neem (*Azadirachta indica*) fruit 70 % aqueous methanolic crude extract on different isolated smooth muscle tissue preparations.
- The neem extract exhibited both spasmogenic and spasmolytic properties when tested on isolated rabbit jejunum, rat stomach fundus, and rat ileum preparations.
- These actions were mediated via activation of muscarinic receptors and blockade of calcium channels.
- The extract also showed a uterotonic effect that was partially mediated via activation of muscarinic receptors.
- The study helps to identify some scientific rationale for the traditional use of neem in different medical conditions.

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