Original Research Article

Investigation of the laxative, spasmolytic and prokinetic properties of aqueous methanol extract of Buxus sempervirens Linn (Buxaceae)

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Abstract

Purpose: To investigate the spasmodic and laxative properties of Buxus sempervirens Linn (Buxaceae) in rabbits and mice.

Methods: Aqueous methanol extract (AqMeBS) as well as the dichloromethane (DCMF) and aqueous (AqF) fractions of Buxus sempervirens were investigated on isolated rabbit jejunum to explore its antispasmodic effect, relative to the standard drug, verapamil. Laxative and prokinetic potentials of 250 and 500 mg/kg doses of AqMeBS extract were evaluated in mice and compared to that of negative (normal saline) and positive (carbachol) control groups. The effects of AqMeBS and carbachol were also tested in mice pretreated with atropine (10 mg/kg). Single dose, acute oral toxicity study on AqMeBS was also executed in mice at 4000, 8000 and 12000 mg/kg doses.

Results: AqMeBS, DCMF and AqF significantly inhibited the rhythmic contractility of jejunum with 0.961, 0.0327 and 0.242 mg/mL, respectively, as median effective concentrations (EC50). In addition, AqMeBS, DCMF and AqF significantly relaxed the contractions due to K+ with EC50 of 1.85, 0.05 and 1.07 mg/mL, respectively. Ca2+ concentration response curves (CCRCs) were shifted to the right by AqMeBS and DCMF, in the same manner as verapamil. In the in vivo experiments, AqMeBS produced significant (p < 0.0001) laxative and prokinetic effects at 250 and 500 mg/kg doses and was comparable to that of carbachol. The acute toxicity study showed that AqMeBS was associated with one mortality at the highest tested dose (12000 mg/kg).

Conclusion: These results provide the pharmacological basis for the traditional use of B. sempervirens Linn as a laxative and prokinetic remedy in the management of constipation.

Keywords: Buxus sempervirens, Calcium channel blocker, Prokinetic, Laxative, Spasmolytic

INTRODUCTION

Buxus sempervirens known in Pakistan as paapari and is an evergreen tree widespread, especially in the Swat area of Pakistan. This Himalayas plant is characterized by its elliptic-shaped leaves and tetragonal shoots [1]. The evergreen plants of B. sempervirens are popularly used for the decorative purpose. The plant is used in folkloric medicine for the treatment of alopecia, neoplasm, seizures, liver diseases (Hepatitis), malaria, pyrexia, pneumonia, skin irritation, arthritis and stomatitis. It is also used as an astringent, cardiac stimulant and purgative. It is also used for the management of constipation, skin disorders, hyperuricemia, hypertension, immobility, tetanus and infections caused by mycobacterium [2]. Several alkaloids e.g. semperviraminol, buxamine F, 17-oxocycloprotopubxine, buxoxy-
produce an extract yield of 10.075 g.

Critical grade constituent was then substituted with calcium chloride. This was followed by overnight prior to commencement of the study. The rabbits were anesthetized with chloroform and sacrificed by cervical dislocation to excise jejunum for use in in vitro experimentations. All experimental procedures were according to the directives of the Institute of Laboratory Animal Resources, Commission on Life Sciences [8]. Approval (EC/05PhDL/2013) was obtained from the Animal Ethics Committee of Bahauddin Zakariya University, Multan.

**EXPERIMENTAL**

**Chemicals and drugs**

Ethylene-diamine-tetra-acetic acid (EDTA), potassium chloride (KCl), carbacchol, atropine and acetylcholine were products of Sigma®. All other chemicals were also of analytical grade and acquired from BDH® and Merck®.

**Preparation of crude extract and fractions**

Fresh aerial parts of *B. sempervirens* were from the Swat valley in 2014. Authentication of the plant was done in University of the Punjab, Lahore by a taxonomist Dr. Niazi, (who assigned a voucher no. 252014) and the sample was submitted to the departmental herbarium. The aerial portion was dried and pulverised. The powdered sample was then extracted in aqueous-methanol (30:70 volume ratio of water : methanol) to produce an extract yield of 10.075 %. The extract (AqMeBS, 50 g) was then dissolved in enough distilled water and an equal volume of dichloromethane (DCM) was added to it. The extraction was repeated thrice, and the resultant extracts were combined and concentrated with a rotary evaporator (model 9230, BUCHI Switzerland) at 37 °C. The aqueous part was lyophilized to yield AqF. The yield of AqF was 21.6 %, while that of DCMF was 78.4 % [6].

**Phytochemical screening**

Phytochemical evaluation of AqMeBS was performed according to the standard methods described earlier [7].

**Experimental animal conditions**

Mature, local breed of rabbits (mean weight = 1.5 kg) and albino mice (Swiss breed, weight: 20 - 25 g) of both sexes were procured from a supplier in Multan. The rabbits and mice were housed under standardized conditions at 25 ± 1 °C and provided unrestricted access to balanced diet (Hi-Tech Feeds Pvt. Ltd. Lahore) and clean drinking water. The animals were fasted overnight prior to commencement of the study. The rabbits were anesthetized with chloroform and sacrificed by cervical dislocation to excise jejunum for use in in vitro experimentations. All experimental procedures were according to the directives of the Institute of Laboratory Animal Resources, Commission on Life Sciences [8]. Approval (EC/05PhDL/2013) was obtained from the Animal Ethics Committee of Bahauddin Zakariya University, Multan.

**Evaluation of in vitro anti-spasmodic effect**

The anti-spasmodic activities of AqMeBS, DCMF and AqF were investigated in isolated rabbit jejunum preparation. A 2-3 cm portion of the jejunum was placed in an organ bath containing 15 mL Tyrode’s solution. The composition of Tyrode’s solution (mM) was: sodium chloride (136.9), KCl (2.68), sodium bicarbonate (11.90), magnesium chloride (1.05), sodium dihydrogen phosphate (0.42), calcium chloride (1.8) and glucose (5.55). The tissue was kept at normal body temperature and aerated using carbogen gas (95 % oxygen and 5 % carbon dioxide). Each jejunum tissue was subjected to pre-load of 1g and allowed to stabilize for half an hour. Then doses of test material were administered in a cumulative manner after every 10 min. A rhythmic contractility was detected in each jejunum preparation which allowed the assessment of relaxing potential of the extracts.

**Determination of in vitro calcium channel blocking effect**

The aqueous methanolic extract (AqMeBS) and its dichloromethane fraction (DCMF) were further investigated to ascertain the mechanism underlying their effects. On exposure to 80mM K⁺, the isolated jejunum produced sustained contractility through voltage-dependent calcium channels (VDCs) [10]. Isolated jejunum preparations were subjected to the solutions of test extracts which exerted concentration dependent inhibition in contractility. This inhibition in contractility was compared to that of verapamil, a calcium channel blocker. The inhibition in contractility with calcium channel blocker was validated in isolated jejunum. The isolated jejunum was steadied in Tyrode’s solution and was then substituted with calcium free Tyrode’s solution (comprising of the constituents as that of Tyrode solution, however, lacks in calcium chloride). Thereafter, the...
calcium free Tyrode’s solution was replaced with EDTA-rich, Ca²⁺-free Tyrode’s solution (comprising of the same constituents as that of calcium free Tyrode solution, however, contains EDTA, 0.1 mM). Calcium chloride (0.01 and 0.1 mM) was added to the organ bath after 30 minutes, in accumulative manner to attain control Ca²⁺ concentration response curves (CRCs). The trial was repeated multiple times until alike Ca²⁺ control CRCs were obtained. The tissues were then rinsed and equilibrated with AqMeBS and DCMF for 45 mins. Thereafter, Ca²⁺ CRCs produced in response to varying dilutions of AqMeBS, DCMF and verapamil were constructed and matched with control CRCs [11].

Determination of laxative effect in albino mice

Seven groups of mice were used (n=5). Mice in negative control group were orally administered with 10 ml/Kg normal saline. Mice in positive control Group were given the laxative, cabachol (1 mg/kg) through intraperitoneal route. Doses of 250 and 500 mg/ Kg AqMeBS were administered to the mice in group 3 and 4 respectively. Mice, pretreated with intraperitoneal atropine (10 mg/Kg) were subdivided into group 5, 6 and 7 that subsequently received 1 mg/Kg carbachol and 250 and 500 mg/Kg AqMeBS extract respectively. The total number of droppings and total number of moist droppings in all the groups were recorded after 6 hours. The increase in moist droppings comparative to the total droppings was an indicative of laxative effect [12].

Evaluation of gastrointestinal motility

The mice were randomly divided into seven groups. These were kept overnight with an access to water, however, were devoid of any food. In group 1 (negative control), 1 mL normal saline was given to the mice p.o. Group II mice (positive control) received 1 mg/kg CCh i.p., while mice in groups 3 and 4 received 250 and 500 mg /Kg of AqMeBS (p.o), respectively. The acetylcholine-like prokinetic properties of carbachol and AqMeBS were also studied. Mice in groups 5, 6 and 7 received 10 mg/kg atropine i.p 15 min before treatment with carbachol (1 mg/Kg) and AqMeBS (250 and 500mg/Kg) respectively. 30 minutes post administration of initial therapy, each mouse also received 1 mL (5 % w/v) charcoal suspension consisting of (0.5 %w/v) methyl cellulose. These were anesthetized with chloroform and sacrificed 30 minutes after the charcoal meal. A percentage of intestinal propulsion (an index of distance moved by the charcoal) was calculated as outlined earlier [13].

% Intestinal propulsion = Distance traveled by charcoal diet (cm)/total length of small intestine (cm) ×100

Acute toxicity Study

Four groups of mice were used (n=5, one group per cage). The acute toxicity study was carried out at 25 ± 01 °C and 12 hour in a well-aerated room with 12 h/12 h day/night cycle. The mice were fasted overnight however, had free access to water. Mice in group 2, 3 and 4 were given a single oral dose of 4000, 8000 and 12000 mg/Kg respectively, while, mice in group 1 received 10 ml/Kg normal saline orally. Mice in all the groups were monitored non-stop for the first 48 h, for toxicity signs such as mortality, behavioral changes and weight changes. The monitoring continued for 14 days [14]. All observed toxicity signs were recorded.

Statistical analysis

Data from laxative and prokinetic studies were analyzed with one-way ANOVA using Graph Pad Prism® version 6 (San Diego, California USA). P < 0.0001 was taken as indicative of statistically significant differences. Data from in-vitro experiments were shown as mean ± Standard error of mean (SEM). EC₅₀ was determined from the logarithmic dose response curves with GraphPad Prism®.

RESULTS

Phytochemical profile

The results of phytochemical investigations are presented in Table 1.

<table>
<thead>
<tr>
<th>Secondary metabolites</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Present</td>
</tr>
<tr>
<td>Cardiotonic glycosides</td>
<td>Present</td>
</tr>
<tr>
<td>Saponins</td>
<td>Present</td>
</tr>
<tr>
<td>Tannins</td>
<td>Present</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Present</td>
</tr>
<tr>
<td>Coumarins</td>
<td>Present</td>
</tr>
</tbody>
</table>

Assessment of in-vitro antispasmodic effect

AqMeBS induced spasmodic activity at 0.1-1 mg/mL dose, which was followed by antispasmodic action at 3 mg/mL dose. The spasmodic effect was entirely blocked by pre-treatment with 3 µM atropine (Figure 1).
AqMeBS and AqF relaxed the rabbit jejunum contractions at doses of 0.01 - 0.1 mg/mL, showed 0.032 mg/mL EC$_{50}$ (95% CI = 0.022 - 0.04) while 0.01 - 3 mg/mL showed EC$_{50}$ = 0.24 mg/mL (95% CI = 0.15 - 0.38), respectively.

AqMeBS, DCMF and AqF relaxed dose-dependently, the potassium (80 mM)-induced contraction with EC$_{50}$ values of 1.85 mg / mL (95% CI = 1.5 - 2.1), 0.05 mg / mL (95% CI = 0.03 - 0.09), and 1.07 mg / mL (95% CI = 0.75 - 1.5), respectively. The corresponding EC$_{50}$ for verapamil was 0.82 mg / mL (95% CI = 0.82 to 0.82). These results are shown in Figure 2.

In vitro calcium channel blocking effect

AqMeBS and DCMF shifted the CRCs to the right at 0.3 - 3 mg/mL and 0.03 - 0.3 mg/mL, respectively, in a manner similar to the shift produced by 0.1 - 1µM verapamil (Figure 3).

Laxative effect

A high number of wet droppings resulted from administering 250 and 500 mg/Kg doses of AqMeBS [51.67 ± 3.73 % (**p < 0.001) and 64.70 ± 2.59 % (**p < 0.0001)], respectively. However, carbachol administration resulted in the highest wet droppings [73.9 ± 3.85 % (**p < 0.0001)]. In comparison, prior administration of atropine resulted in significant decreases in the amount of wet droppings in the AqMeBS group. The atropine-induced reduction in wet droppings was most pronounced in the CCh group [9 ± 5.18 % (**p < 0.0001)]. These results are shown on Table 2.

Effect of extracts on intestinal transit

Prokinetic effect was produced by AqMeBS at 250 and 500 mg/kg as evidenced by the intestinal movement of the charcoal meal. The charcoal meal traveled the least distance in the group given saline (56.6 ± 0.4 %). Intestinal transit was significantly increased by CCh (92.2 ± 1.11 %, p < 0.0001). 250mg/kg dose of AqMeBS produced 64 ± 0.32 % (p < 0.0001) whereas, 500mg/Kg dose of AqMeBS produced 77.4 ± 0.6 % intestinal propulsions (p < 0.0001). However, the prokinetic activities of AqMeBS and carbachol were significantly attenuated by prior treatment with 10 mg/kg atropine. These results are presented in Figure 4.
Figure 3: Effect of (a) AqMeBS: control ( ), 0.3 mg/mL I mg/mL (△) (▼), 3mg/mL (O); (b) DCMF: control (□), 0.03mg/mL (▲) 0.1 mg/mL (Δ), 0.3 mg / mL (◇); (C) Verapamil: control ( □), 0.03 µM/mL (▲), 0.1 µM / mL (◇) and 0.03mg/mL (◇) on CRCs of rabbit jejunum.

Table 2: Laxative property of AqMeBS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean defecation/ group</th>
<th>Mean no of wet feces/ group</th>
<th>Mean wet feces (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline p.o. (10 mL/kg)</td>
<td>3.4 ± 0.24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carbachol intraperitoneal (1 mg/kg).</td>
<td>13.8 ± 0.37****</td>
<td>10.2 ± 0.37****</td>
<td>73.9 ± 3.85</td>
</tr>
<tr>
<td>AqMeBS p.o. (250 mg/kg)</td>
<td>6.2 ± 0.37****</td>
<td>3.2 ± 0.2****</td>
<td>51.67 ± 3.73</td>
</tr>
<tr>
<td>AqMeBS p.o. (500 mg/kg)</td>
<td>11.6 ± 0.24****</td>
<td>7.5 ± 0.15****</td>
<td>64.70 ± 2.59</td>
</tr>
<tr>
<td>CCh intraperitoneal (1mg/kg) + atropine intraperitoneal (10mg/kg)</td>
<td>4.4 ± 0.24****</td>
<td>0.4 ± 0.1****</td>
<td>9 ± 5.18</td>
</tr>
<tr>
<td>AqMeBS p.o. (250mg/kg) + atropine intraperitoneal (10 mg/kg)</td>
<td>4.7 ± 0.2**</td>
<td>1.6 ± 0.1***</td>
<td>34.17 ± 4.49</td>
</tr>
<tr>
<td>AqMeBS p.o. (500mg/kg) + atropine intraperitoneal (10mg/kg, i.p.)</td>
<td>4.2 ± 0.37****</td>
<td>0.7 ± 0.12****</td>
<td>16.33 ± 3.75</td>
</tr>
</tbody>
</table>

Note: * showed the significance in comparison of group 2, 3 and 4 against control group 1 at p < 0.05; ** showed the significance in comparison of group 5 against carbachol group at p < 0.01; *** showed the significance in comparison of groups 2, 3 and 4 against control group 1 at p < 0.001; **** showed the significance in comparison of group 6 against 250 mg/kg AqMeBS group and group 7 against 500 mg/kg AqMeBS group at p < 0.0001. Data were expressed as mean ± SEM (n = 5); p.o. = per oral.

Acute toxicity

No signs or symptoms of toxic effects were seen in the mice treated with AqMeBS at a dose of 4000 mg /kg. However, minor toxicity signs were noticed in the groups that received doses of 8000 and 12000 mg/kg, although these signs receded by the 14th day of the acute toxicity test (Table 3).

DISCUSSION

The traditional use of *B. sempervirens* as a laxative and spasmolytic [2,15] formed the bases of this study. Results from photochemical analysis showed that AqMeBS contains flavonoids, tannins, alkaloids and saponins. These phytochemicals may be responsible for the traditional and medicinal application of this plant [16]. This study has demonstrated that *B. sempervirens* possesses antispasmodic and prokinetic activities. Similar results were obtained in studies on *Buxus wallichiana* (a plant in the same genus) which is used for treating gastrointestinal, cardiovascular and respiratory illnesses [15].

Studies have established unequivocally that potassium ions open voltage-gated calcium channels, a process that triggers influx of...
extracellular Ca\(^{2+}\) resulting in enhancement of smooth muscle contractility [19]. The effect of AqMeBS was evident from the shifting of the CRCs to right, an effect resembling that of the calcium channel blocker, verapamil. More potent effects were produced by DCMF and verapamil than AqMeBS fraction. This is consistent with the superior jejenum contractile responses seen in this fraction, relative to AqMeBS. Again, this effect suggests that DCMF contains more potent calcium channel-blocking agents than AqMeBS.

The results acquired in this study showed that AqMeBS possesses laxative properties comparable to that of carbachol, an agent which enhances motility of the small intestine [20]. The laxative effect of AqMeBS was inhibited partially by atropine, an established antagonist of muscarinic receptor. Atropine blocks cholinergic nicotinic receptor in the intestine where the release of acetylcholine from myenteric plexus activates cholinergic receptors [21]. The inhibition of the laxative effect of AqMeBS by atropine suggests that the extract may contain compounds that exert the same effects as acetylcholine. Acetylcholine increases gastrointestinal contraction by stimulating muscarinic receptors in a process mediated by G-protein, phospholipase C and inositol triphosphate [22]. Previous studies have also reported that B. sempervirens extract inhibited the activities of butrylcholinesterase and acetylcholinesterase [23]. These findings strongly suggest that some components of AqMeBS may have cholinergic properties, which are responsible for blockage of the activity of acetylcholinesterase [24]. Indeed, some parasympathomimetic alkaloids and tannins have been reported in B. sempervirens [16]. Thus the presence of acetylcholine-like components may be responsible for the cholinergic activity of B. sempervirens [25].
Results from oral acute toxicity study in mice revealed that the lethal dose for 50 percent animals was more than 12000 mg/kg. The weight loss recorded may be due to the saponins in the extract. It has been reported that saponins may be implicated in loss of weight in some experimental animals [26].

**CONCLUSION**

The findings of this study show that B. sempervirens Linn exhibit laxative, prokinetic and antispasmodic potential. These results may lend some pharmacological justification for the traditional use of the plant in constipation and other gastrointestinal problems.

**DECLARATIONS**

**Acknowledgement**

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**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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