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Anxiolytic-*like* Effects of *Xylocarpus moluccensis* Methanolic Bark Extract in *Swiss* mice

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Abstract

Scientific reports suggest that *Xylocarpus moluccensis* has numerous important biological activities, including antioxidant, anti-inflammatory, anti-microbial, anti-cancer, anti-diarrheal, insecticidal, antifeedent, neuropharmacological (e.g., CNS depressant), anti-atherosclerotic, and lipid-lowering activity. Anxiety disorders are common and disabling psychiatric conditions that are often associated with depressive symptoms. The aim of the present study was to investigate the anxiolytic-*like* effects of the methanolic bark extract of *X. moluccensis* (MEXM) in different behavioral paradigms in *Swiss* albino mice. For this, adult mice of either sex were treated with MEXM (250 and 500 mg/kg, p.o.) and/or diazepam (2 mg/kg, i.p.), and subjected to a number of behavioral studies. In the open-field test, the number of square field cross, grooming, and rearing, was counted, while in the light/dark and swing tests, the time spent in the dark portion and the number of swings were calculated, respectively. Findings from this investigation revealed the presence of flavonoids, phenols, saponins, terpenes (including triterpenes), and gums in MEXM. In addition, MEXM caused a significant ($p < 0.05$) anxiolytic-*like* effect in experimental animals, where it dose-dependently increased the total time in the center and decreased the number of rearing and grooming's responses in the open field test. The MEXM also increased the dark residence time and decreased the number of swings in a dose-dependent manner. A dose of 500 mg/kg of MEXM caused the highest calming effect was when combined with the diazepam group. Taken together, these results extend our understanding of the effects of *X. moluccensis* on the central nervous system and suggest that this plant may be useful for the management of neurological diseases and disorders, especially anxiety.

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Xylocarpus moluccensis, phytochemicals, anxiety, behavioral study, Mus musculus, non-clinical study.

Introduction

Herbal drugs (also called green medicine) are relatively safe and dependable health care paradigms. In recent years, traditional herbal medicine has drawn the attention of researchers worldwide due to its several pharmacological activities, economic viability, and fewer side effects (Chew *et al.*, 2012).

Drugs acting on the central nervous system (CNS), such as CNS depressants including barbiturates and benzodiazepines (BDZs), among others, exert their effect

via interaction with the postsynaptic gamma aminobutyric acid (GABA) receptor (Rang *et al.*, 1996). The most serious drawback of these kinds of drugs are the narrow margin of safety and higher doses which present a higher risk. For example, benzodiazepines and Z-drugs (zopiclone, zolpidem) can cause cognitive problems, even at low doses (Seldenrijk *et al.*, 2017). Moreover, these drugs can produce both psychological and physiological dependence (Isbell, H. and Fraser, 1950; Essig, 1964). Along this line, BDZs are the most commonly used CNS depressants, leading to tolerance and physical dependence.

For example, diazepam (DZP) produces sedation (O'Brien, 1996), whereas ethanol develops tolerance and physical dependence (Schuckit, 1994). Therefore, natural CNS depressants with reduced or no toxicity are essential. However, several undesirable side effects as well as tolerance and physical dependence associated with use of BDZs for the management of anxiety are important challenges which motivated scientists to search for safer and better tolerated anxiolytic compounds devoid of dependence. In this context, Luvah and coworkers isolated active constituents from methanol underground parts of Ajuga remota (Lamiaceae) with anxiolytic-like effects (Luvah *et al.*, 2014).

Similarly, research by Schier and colleagues suggested that cannabidiol (CBD) exhibits anti-anxiety and antidepressant effects in animal models. In addition, experiments with CBD indicated non-activation of neuroreceptors CB1 and CB2, and most studies demonstrated a good interaction between CBD and the 5-hydroxytryptamine-1A (5-HT1A) neuro-receptor (Schier *et al.*, 2014). Moreover, recent studies clearly suggest an anxiolytic-like effect of CBD in both animal models and healthy volunteers, and that CBD seems to be a promising drug for the treatment of panic disorder (PD). However, more work is needed to establish the mechanism of action of CBD and the safe and ideal therapeutic doses of this compound (Soares and Campos, 2017).

Xylocarpus moluccensis is a glabrous, medium sized tree. It grows in the tropical mangroves spanning from East-Africa to the Philippines, Australia, and the Pacific Islands. It also grows in the Sundarbans located in Bangladesh and India (Uddin *et al.*, 2011). Traditionally, its bark is used as an astringent and a febrifuge, and in the treatment of fever, dysentery, diarrhea, and other abdominal disorders (Uddin *et al.*, 2005; Raja and Ravindranadh, 2014). Fruits of this plant are used in the treatment of swellings of the breast and elephantiasis (Uddin *et al.*, 2011), and diarrhea, whereas the seed ash is used to manage itching (Sarker *et al.*, 2007). Sarker *et al.*, (2007) indicated that the methanol bark extract of this plant exhibits a dose-dependent CNS depressant effect in mice.

Phytochemical studies have demonstrated that *X. moluccensis* contains protein, minerals, and numerous important secondary metabolites, including flavonoids, proanthocyanidins, terpenoids, and fatty acids (Lakshmi and Gupta, 2008; Wangensteen *et al.*, 2013). Crude extracts and the isolated compounds from different parts of this plant have been reported for important biological activities, such as antioxidant, anti-inflammatory, anti-diarrheal, anti-microbial, anticancer, insecticidal, pesticidal, anti-feedant, and anti-osteoclastogenesis effects (Wangensteen *et al.*, 2013; Zhang *et al.*, 2018). Moreover, the methanol and aqueous extracts of *X. moluccensis* exerted cytotoxic effects on two human cancer cell lines, but were inactive against normal mouse fibroblast cells (Uddin *et al.*, 2011). Recently, Zhang and coworkers have isolated and identified 29 new limonoids from the seeds of *X. moluccensis* (Zhang *et al.*, 2018). In light of the previous discussion, the aim of the present study was to examine the anxiolytic-like effect of the methanol bark extract of *X. moluccensis* in *Swiss* mice.

Materials and Methods

Plant collection and authentification

Fresh bark of *X. moluccensis* was collected from Sundarbans, Bangladesh in July, 2019. The plant was identified and authenticated by a plant taxonomist at the Bangladesh National Herbarium, and a voucher specimen (DACB30320) was deposited.

Extraction and fractionation

Approximately 250 g of powdered air-dried plant material was soaked in 1.2 L of absolute methanol and kept for a period of one week with occasional shaking and stirring. Then the solution was filtered through cotton wool and Whatman No. 1 filter paper, and the methanol extract was concentrated and dried by means of rotary evaporation at the Pharmacognosy Laboratory of the Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj, Bangladesh. Dried dark tan colored extracts thus obtained (30.03%) were screened for their pharmacological properties.

Preliminary phytochemical study

The screening was performed for triterpenes/steroids, alkaloids, flavonoids, saponins, tannins, and gums (Harborne, 1984; Trease and Evans, 2002). The color intensity or the precipitate formation was used as analytical responses to these tests.

Experimental animals

Adult albino *Swiss* mice (22-28 g) obtained from the Animal Centre, Bangladesh Council of Scientific and Industrial Research (BCSIR), Chittagong, Bangladesh, were used throughout this investigation. These animals were housed in plastic cages at constant room temperature (23–25 $^{\circ}$C) with a 12:12 h day/light cycle. They were given free access to a standard rodent pellet diet and water *ad libitum*, and were acclimatized for one week before starting the experiments. Behavioral studies were conducted between 9:00 am and 1:00 pm. Experimental procedures involving animals were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Ethical approval No. T/PHR-BSMRSTU-08.2019).

Drugs and chemicals

Diazepam (DZP, Square Pharmaceuticals Ltd.) was used as a standard. Tween 80 and all the other reagents and chemicals were purchased from Sigma-Aldrich, Denmark.

Experimental design

Dose selection for this study was carried out according to the procedure outlined by Sarker *et al.,* (2007) According to this method, thirty five mice were randomly divided into seven groups of five animals each (n = 5). Group I: Negative control (vehicle: 0.05% tween 80 dissolved in 0.9% NaCl solution); group II: Positive control (DZP); groups III and IV were treated with MEXM: 250 and 500 mg/kg), respectively; groups V and VI were treated with a combination of DZP2 and MEXM (DZP2 + MEXM 250 and DZP2 + MEXM 500).

Open-field test (OFT)

The open-field test was performed according to a published procedure with slight modification (Archer, 1973). According to this procedure, animals received controls and test extract 30 min prior to the test. Then the animals were individually placed in the center of the arena and allowed to explore freely. The number of squares crossed with all four paws, rearings, and number of grooming were recorded for 3 min (testing period). In the combined treatment groups, DZP (2 mg/kg, i.p.) was given 15 min prior to the administration of MEXM, and the aforementioned parameters were similarly recorded.

Light-dark test (LDT)

After 30 min of administration of controls and test sample, mice were placed in the middle of the open compartment of the light-dark board. Animals were then observed for 3 min and the time spent in the dark compartment was recorded (Hascoët and Bourin, 1998). In the combined treatment groups, DZP (2 mg/kg, i.p.) was administered 15 min prior to the administration of MEXM; the above-mentioned parameters were similarly recorded.

Swing test (SWT)

In this test, the number of swings of each animal was recorded for a duration of 3 min, after 30 min of administration of controls and MEXM (Islam *et al.*, 2014). In the combined treatment groups, DZP (2 mg/kg, i.p.) was given 15 min prior to the administration of the MEXM, and the number of swings was recorded in a similar fashion.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Data were subjected to one-way analysis of variance (ANOVA), and Statistical analysis was performed with the aid of Neuman Keul's test for significance, using GraphPad Prism (version: 6.0) software; differences were considered significant at $p \leq 0.05$ at 95% confidence intervals.

Results and Discussion

Results of our phytochemical study indicate that MEXM contains flavonoids, phenols, saponins, terpenes (including triterpenes), and gums as shown in Table 1.

Shown in Table 2 are results of our investigation pertaining to the anxiolytic-*like* effects of the MEXM. Results revealed that DZP (2 mg/kg) and the extract (250 and 500 mg/kg) significantly ($p < 0.05$) reduced the number of field crosses, rearings, and groomings in OFT.

Table.1 Phytochemical relevance of crude extract of *X. moluccensis*

Metabolites	Relevance
Alkaloids	-
Flavonoids	+++
Phenols	++
Steroids	-
Glycosides	-
Cardiac glycosides	-
Saponins	++
Terpenoids	+++
Triterpenoids	++
Tannins	++
Resins/Gums	++

+++: Strong intensity reaction
++: Medium intensity reaction
+: Weak intensity reaction
-: No intensity reaction

Table.2 Effects of test sample and controls on mice

Treatment groups		Open-field test (3 min)			Light-dark test (3 min)	Swing test (3 min)
		Field cross	***Rearing***	***Grooming***	***Residence in dark (Sec)***	***Number of swing***
NC (10 mL/kg, p.o.)		35.22 ± 0.87	7.08 ± 1.13	9.03 ± 1.25	53.13 ± 2.17	37.25 ± 2.19
DZP (2 mg/kg, i.p.)		17.05 ± 1.46*	3.57 ± 0.64*	4.41 ± 0.56*	156.12 ± 2.97*	9.33 ± 1.29*
MEXM (mg/kg, p.o.)	**250**	22.64 ± 1.75*	4.31 ± 1.28*	4.39 ± 1.64*	152.65 ± 2.87*	11.54 ± 2.63*
	500	21.75 ± 2.61*	4.09 ± 2.03*	4.49 ± 1.29*	163.02 ± 2.43*	11.12 ± 2.43*

Values are the mean ± SEM (n = 5). ANOVA followed by Neuman Keuls test, *p < 0.05 when compared to NC group. NC: Negative control (Vehicle: 0.05% tween 80 dissolved in 0.9% NaCl solution). DZP: Diazepam. MEXM: Methanol extract of *X. moluccensis*.

Table.3 Combined effects of test sample and DZP in *Swiss* mice

Treatment groups	Open-field test (3 min)			Light-dark test (3 min)	Swing test (3 min)
	Field cross	***Rearing***	***Grooming***	***Residence in dark (Sec)***	***Number of swing***
DZP	17.05 ± 1.46	3.57 ± 0.64	4.41 ± 0.56	156.12 ± 2.97	9.33 ± 1.29
DZP2 + MEXM250	15.04 ± 2.03*	3.39 ± 0.24	4.01 ± 1.67	161.61 ± 3.07*	8.13 ± 1.03*
DZP2 + MEXM500	13.05 ± 2.68*	2.99 ± 1.07*	2.04 ± 2.13*	171.11 ± 2.39*	8.01 ± 2.03*

Values are the mean ± SEM (n = 5). ANOVA followed by Neuman Keuls test, *p <0.05 when compared to positive control (DZP) group. DZP: Diazepam. Methanol extract of *X. moluccensis*.

They also increased the dark residence and decreased the number of swings in LDT and SWT, respectively. Additionally, MEXM at 500 mg/kg reduced all test parameters better than at 250 mg/kg.

In the meantime, MEXM (250 and 500 mg/kg), co-treated by the DZP (2 mg/kg, i.p.) group, caused an anxiolytic-*like* effect through modifying the test parameters in OFT, LDT, and SWT. In this case, the MEXM at 500 mg/kg showed better activity than its 250 mg/kg counterpart. The number of field crosses, rearings, groomings and swings were significantly ($p < 0.05$) reduced, along with an increase in the dark residence time by the DZP + MEXM groups in comparison to the DZP group. The best augmented in dark residence time was recorded in the DZP2 + MEXM500 treatment group (Table 3).

A number of novel psychiatric drugs have been introduced for the treatment of anxiety over the past two decades. However, all of these drugs have so far failed to minimize side effects. In this context, herbal medicines might be considered as alternative remedies, and could be attractive candidates for the management of these conditions (Calixto, 2000; Fajemiroye *et al.,* 2016). Published research has demonstrated that some plant-derived compounds exert immunomodulatory effects. Therefore, these lead compounds have led to rigorous scientific examination to determine their efficacy and safety (Licciardi and Underwood, 2011). Our findings reveal that treatment of animals with MEXM caused anxiolytic-*like* effects in mice as shown in different tests.

Anxiety behavior is triggered by the separation of an animal from its social group and agoraphobia (Ambavade *et al.,* 2006). In the OFT, there was a decrease in locomotion (diminished exploration), which might also be an implication of the anxiogenic effect. In this study, MEXM was found to reduce the parameters of OFT and SWT, along with an increase in dark residence time of experimental animals.

BDZs (e.g., DZP) are positive allosteric modulators of the $GABA_A$. On the other hand, GABA is the major inhibitory neurotransmitter in the brain, which after binding to BDZs, increases the total conduction of chloride ions across the neuronal cell membrane. This process causes a chloride ion influx, which hyperpolarizes the neuron's membrane potential. Therefore, the difference between the resting potential and the threshold potential is increased, and firing is less likely. Consequently, arousal of the cortical and limbic systems in the CNS is reduced (Ries *et al.,* 2009). To exert a calming effect, DZP appears to act on areas of the limbic system, thalamus, and hypothalamus. In this context, our results show that MEXM at a dose of 500 mg/kg may elicit a better calming effect on the *Swiss* mice. This effect may be confirmed by reduction of the movement of animals in the open-field and swing boxes, as well as in the time spent in the dark chamber of the light-dark box. Moreover, the MEXM was also found to modulate the calming effect of DZP in experimental animals, where the extract at both doses plus 2 mg/kg of DZP groups showed better anxiolytic-*like* effects than the individually treated groups with MEXM or DZP. However, the effect is anxiolytic with a slight depressant undertone.

Research findings show that dietary phytochemicals such as alkaloids, terpenes, flavonoids, phenolic acids, lignans, cinnamates, and saponins, in addition to various plant extracts with a mixture of different phytochemicals, possess anxiolytic-*like* effects in a wide range of animal models of anxiety (Fedotova *et al.*, 2017). Phytochemical screening suggests that MEXM contains flavonoids, phenols, saponins, and terpenes.

In summary, findings from this investigation show that MEXM contains important plant secondary metabolites, including flavonoids and terpenes. In addition, the extract exhibited a calming effect in *Swiss* mice in a dose-dependent manner, and produced a dose-dependent calming effect with the standard drug, DZP. Findings also suggest that the extract may potentiate the anxiolytic-*like* effect of DZP. Further studies are required to isolate the responsible active constituents of the plant and to understand the possible mechanism of action behind the anxiolytic-*like* effect in experimental animals. Furthermore, more detailed studies are required to establish the safety, efficacy, and active constituents of this plant.

Competing interests

The authors declare no conflicts of interest.

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