

Anxiolytic-like effects of *Moringa oleifera* in Swiss mice

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Abstract: *Moringa oleifera* is evident to act against many neurological diseases, including muscle spasm, epilepsy, nervousness, fatigue, memory impairment, convulsion, and epilepsy. Anxiety represents the most common and disabling psychiatric condition, being often associated with depressive symptoms. This study investigated the anxiolytic-like effects of crude organic fractions of *M. oleifera* leaves in different behavioral paradigms that evaluate anxiety in mice. To this end, mice were administered with crude extracts (500 mg/kg, p.o.) and/or diazepam (2 mg/kg, p.o.), and submitted to behavioral tests. In the open-field test, the number of square field cross, grooming and rearing were calculated, while in light-dark and swing test were, respectively, the time spent in dark portion and number of swings. Each test was performed for 3 min. *M. oleifera* leaf methanol and n-hexane extracts elicited an anxiolytic-like effect observed by increased total time in the center and decreased number of rearings and groomings responses in the open field and swing tests, and residence in the dark portion in the light-dark box, similar to the diazepam group. A moderate anxiolytic effect was observed in the aqueous fraction group, while insignificant effects were recorded in the ethyl acetate fraction group in all test paradigms. In addition, both extracts potentiate the calming effects of diazepam in experimental animals. Preliminary phytochemical reports suggest that *M. oleifera* contains alkaloids, flavonoids, phenols, steroids, glycosides, saponins, tannin, terpenes, and gums. Of note, the results expand the understanding of *M. oleifera* effects in central nervous system and suggest that plant metabolites may be helpful for anxiety-related disorders management.

Key words: Anxiety; Organic fractions; Behavioral study; *Mus musculus*.

Introduction

Herbal drugs (also called green medicine) are relatively safe and dependable health care paradigms. Recently, traditional herbal medicines have drawn a high attention due to their incredible pharmacological activities, economic viability and less side effects (1).

Drugs acting on central nervous system (CNS), such as depressants (e.g., barbiturates, benzodiazepine, among others) give their effects by interaction with post-synaptic gamma aminobutyric acid receptor subunit A (GABA_A) (2). The most serious drawback of these kinds of drugs are narrow margin of safety, and only 10 times of their therapeutic dose may be lethal (3). Moreover, they can produce both psychological and physiological dependence (4,5). Specifically, benzodiazepines are the most often used CNS depressant, leading to tolerance and physical dependence, with for example, diazepam producing sedation, develops tolerance and physical dependence (6). Therefore, natural CNS depressants with

reduced or no toxicity are essential.

Moringa oleifera (Family: Moringaceae), widely distributed in Bangladesh, is used in folk medicine since several years ago (7). It has nutritional value and its leaves and fruits are widely used as vegetables in Bangladesh. The plant is used to treat various diseases, including muscle spasm, epilepsy (7), nervousness, and fatigue (8). *M. oleifera* is also able to improve brain development (8), and ameliorate memory (9). In a recent study, it has been found that *M. oleifera* ethanol leaf extract exerted anticonvulsant and anti-epileptic effects (10). In this sense, the present experiment aims to assess the anxiolytic effects of organic fractions from *M. oleifera* leaves in Swiss mice.

Materials and Methods

Plant collection and authentication

M. oleifera leaves were collected from the Jessore district in August, 2013. The leaves were identified and

authenticated by a plant taxonomist at Bangladesh National Herbarium, where a voucher specimen with the number (DACB32494) was deposited.

Extraction and fractionation

The leaves were air-dried, pulverized and 300 g was macerated for two weeks in 1.2 L of absolute methanol. Filtration was performed using cotton wool and Whatman's No.1 filter paper and concentrated at room temperature in the Pharmacognosy Laboratory of the Southern University of Bangladesh. The percentage yield of the dark tan colored extract was 11.33%. Fractionation was done using modified Kupchan Partitioning process (Figure 1).

Preliminary phytochemical study of *M. oleifera* leaves

The screening was performed for triterpenes/steroids, alkaloids, flavonoids, saponins, tannins, and gums (11,12). The color intensity or the precipitate formation was used as analytical responses to these tests.

Animals

Adult albino *Swiss* mice (22-28 g) were obtained from the Animal Centre, Bangladesh Council of Scientific and Industrial Research, Chittagong, Bangladesh, and were housed in plastic cages at room temperature with a 12:12 h day/light cycle. Mice were fed with standard rodent pellet diet and water *ad libitum*. The animals were acclimatized for 1 week before starting the experiments. The experimental procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals (Ethical approval No. T/PHR-SUB-03.2013).

Drugs and chemicals

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

Experimental design

Fifty mice were randomly divided into ten groups ($n = 5$): Group 1: Negative control (NC; vehicle: 0.05% tween 80 dissolved in 0.9% NaCl solution); Group 2: Positive control (DZP); Groups 3-6: four groups of *M. oleifera* extract/fractions [aqueous (AMO), methanol (MMO), n-hexane (HMO), and ethyl acetate (EAMO) extract of *M. oleifera*], Groups 7-10: four combined treatment groups (DZP + AMO, DZP + MMO, DZP + HMO, DZP + EAMO).

Open-field test

The open-field test (OFT) was performed to assess the animals' exploratory activity (13). Animals received controls and test extract/fractions 30 min prior to the test. Then, animals were placed individually in the center of the arena, and allowed to explore freely. The ambulation or crossing (the number of squares crossed with all 4 paws), numbers of rearings, and number of grooming were recorded for 3 min (testing period). In the combined treatment groups, DZP (2 mg/kg, i.p.) was administered 15 min prior to extract/fractions administration and the above-mentioned parameters were tested

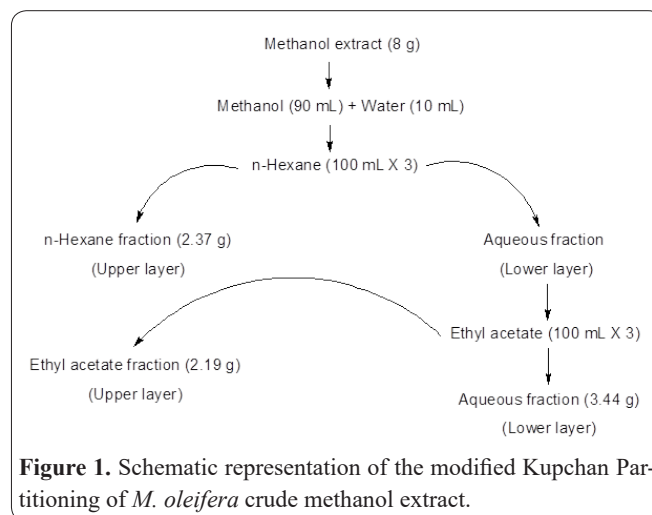


Figure 1. Schematic representation of the modified Kupchan Partitioning of *M. oleifera* crude methanol extract.

similarly.

Light-dark test

In the light-dark test (LDT), after 30 min of administration of controls and test groups, mice were placed in the middle of the open compartment of light-dark board. The animals were then observed for 3 min and the time spent in the dark compartment was recorded (14). In the combined treatment groups, DZP (2 mg/kg, i.p.) was administered 15 min prior to the extract/fractions administration and the above-mentioned parameters were tested similarly.

Swing test

In the swing test (ST), the number of swings of each animal were recorded after 30 min of administration of controls and test groups for 3 min (15). DZP (2 mg/kg, i.p.) was administered 15 min prior to the extract/fractions administration in the co-treatment groups, and number of swings were recorded similarly.

Statistical analysis

Values are expressed as mean \pm standard error of mean (SEM). The significance of differences between groups was determined using one-way analysis of variance (ANOVA) followed by Neuman Keuls test, with $p < 0.05$ considered statistically significant, and at 95% confidence intervals, using GraphPad Prism (version: 6.0) software.

Results

The preliminary phytochemical report suggests that *M. oleifera* (MMO) methanol extract possesses alkaloids, flavonoids, steroids, glycosides (including cardiac glycosides), saponins, tannin, terpenes (including triterpenes), and resins or gums (Table 1).

DZP (2 mg/kg) and the crude fractions (at 500 mg/kg) AMO, MMO and HMO significantly ($p < 0.05$) reduced the number of field cross, rearing, and grooming in OFT. Additionally, in these groups the dark residence and number of swings in LDT and ST, respectively, also decreased. EAMO (500 mg/kg, p.o.) did not show significant effects in such kind activities in the experimental animals when compared to the NC group. However, HMO and MMO were found to reduce all the test parameters better than the AMO and EAMO (Table 2).

Table 1. Phytochemical relevance of methanolic leaf extract of *M. oleifera*.

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

+++ : Strong intensity reaction. ++ : Medium intensity reaction. + : Weak intensity reaction.. MMO, Methanol extract of *M. oleifera*.

The extract/fractions, co-treated by the DZP (2 mg/kg, i.p.) group, also elicited an anxiolytic-like effect through modifying the test parameters in OFT, LDT and ST. In this case, HMO and MMO also showed better activity than AMO and EAMO fractions. The number of field cross, rearing, grooming and swings were significantly ($p < 0.05$) reduced, while increasing in the dark residence time by DZP+MMO group. On the other hand, HMO (500 mg/kg, p.o.) co-treated with DZP (2 mg/kg, i.p.) led to a significant reduction in field cross and swings. The best raise in dark residence time was also recorded in this combined treatment group. However, it did not reduce the number of rearing and grooming in experimental animals. All extracts were found to modify the test parameters when combined with DZP than their individual treated groups (Table 3).

Table 2. Effects of crude extract and organic fractions of *M. oleifera* and controls in 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 swings
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*
AMO	25.01 ± 2.47*	5.78 ± 2.24	7.56 ± 2.18	123.02 ± 3.17*	17.67 ± 1.63*
500 mg/					
kg (p.o.)					
MMO	22.64 ± 1.75*	4.31 ± 1.28*	4.39 ± 1.64*	152.65 ± 2.87*	11.54 ± 2.63*
HMO	21.75 ± 2.61*	4.09 ± 2.03*	4.49 ± 1.29*	163.02 ± 2.43*	11.12 ± 2.43*
EAMO	29.41 ± 2.78*	9.14 ± 1.56	12.34 ± 2.01	87.03 ± 2.02	35.03 ± 2.04

Values are 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. AMO: Aqueous extract of *M. oleifera*. MMO: Methanolextract of *M. oleifera*. HMO: n-Hexane extract of *M. oleifera*. EAMO: Ethyl acetate extract of *M. oleifera*.

Table 3. Combined effects of crude extract and organic fractions of *M. oleifera* and DZP in 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 swings
DZP	17.05 ± 1.46	3.57 ± 0.64	4.41 ± 0.56	156.12 ± 2.97	9.33 ± 1.29
DZP + AMO	20.13 ± 1.07	4.71 ± 0.82	5.51 ± 1.10	153.01 ± 1.19	13.09 ± 2.01
DZP + MMO	15.04 ± 2.03*	2.39 ± 0.24*	3.01 ± 1.67*	161.61 ± 3.07*	8.13 ± 1.03*
DZP + HMO	13.05 ± 2.68*	3.99 ± 1.07	5.04 ± 2.13	171.11 ± 2.39*	8.01 ± 2.03*
DZP + EAMO	22.15 ± 1.70	6.11 ± 2.53	9.31 ± 2.13	103.02 ± 2.13	21.45 ± 2.49

Values are mean ± SEM (n = 5). ANOVA followed by Neuman Keuls test, * $p < 0.05$ when compared to positive control (DZP) group. DZP: Diazepam. AMO: Aqueous extract of *M. oleifera*. MMO: Methanol extract of *M. oleifera*. HMO: n-Hexane extract of *M. oleifera*. EAMO: Ethyl acetate extract of *M. oleifera*.

Discussion

It is evident that a number of novel psychiatric drugs have been introduced for the treatment of anxiety over the past two decades, but all drugs have so far failed to minimize side effects. In this respect, herbal drugs, considered alternative remedies, could be attractive candidates as therapeutic strategies for these conditions (16,17). Plant-derived compounds have immunomodulatory effects; therefore, these kinds of lead compounds have been subject to rigorous scientific analysis to determine their efficacy and safety (18).

Around 10% of the world population are afflicted by anxiety in one or many of its forms, including panic attacks, social phobias or generalized anxiety disorders (19). Briefly, anxiety is characterized by excessive or irrational fear associated with a real or anticipated stimulus, and it is often accompanied by phobic avoidance and a constellation of somatic symptoms. The former one may be seen as an adaptive mechanism enabling the individual to minimize exposure to situations that may be anxiety provoking. But such avoidance can become maladaptive when it leads to significant behavioral changes, including social isolation and agoraphobia (20). In general, the most common somatic manifestations of anxiety are cardiovascular (palpitations, non-cardiac chest pain), respiratory (dyspnea), neurological (dizziness, headache, tremulousness), laryngeal (lump in the throat), and gastrointestinal (diarrhea, abdominal cramps) symptoms. The somatic complaints are often the impetus for the individual to seek treatment, usually with a primary care provider, and can result in the extensive medical work-up that fail to find an underlying medical etiology for the symptom (19,20).

Anxiety behavior is triggered by animal separation from its social group and agoraphobia (21). In the OFT,

a decrease in locomotion (diminished exploration) was observed, which might also be an implication for anxiogenic effect. In this study, *M. oleifera* extracts were found to reduce the OFT and ST parameters, while increased residence time in the dark portion of the light-dark box in the experimental animals.

Benzodiazepines (e.g., DZP) are positive allosteric GABA_A modulators. GABA, the major inhibitory neurotransmitter in the brain, after binding to benzodiazepines, increases the total chloride ions conduction across the neuronal cell membrane and causes chloride ion influx and hyperpolarizes neuron's membrane potential. Therefore, the difference between resting and threshold potential is increased and firing is less likely. As a result, the arousal of cortical and limbic systems in CNS is reduced (22). To exert an anxiolytic effect, DZP appears to intervene on areas of the limbic system, thalamus, and hypothalamus. In this study, *M. oleifera* extracts, especially the MMO and HMO fractions, may elicit a calming effect on the Swiss mice, which can be confirmed by the reduction of animals' movements in the OFT and ST, as well as on the time spent in the dark chamber of the LDT.

Plant-derived compounds, such as alkaloids (23), glycosides (24), and other phytochemicals, like flavonoids, terpenes, triterpenes, and saponins isolated from various plant extracts and the mixture of them have shown interesting anxiolytic effects in a wide range of animal models of anxiety (25).

The leaf extract of *M. oleifera* contains vitamins C and E, that play a significant role in improving memory in patients with Alzheimer's disease (26,27). Scientific reports suggest that the ethanolic extract of leaves exhibited both CNS depressant and muscle relaxant activities (28-30) and also showed significant anxiolytic activity in experimental animals in a dose-dependent manner (31,32). The leaves of the plant contains many important phytochemicals, including quercetin-3-O-glucoside, quercetin-3-O-(6"-malonyl-glucoside), 4-alpha and gamma-tocopherol-2 (33). The flowers of the plant contain quercetin, and isoquercetin, while stem contains beta-sitosterol. Alpha-tocopherol exerted a protective effect against high-caffeine induced anxiety effects in Zebrafish (34). Quercetin is evident to reduce cortical GABAergic transmission (35) and exert an anxiolytic effect on experimental animals (36). Beta-sitosterol is evident to provide anxiolytic effect through interacting with GABA_A receptor (37). Here, the MMO and other *M. oleifera* leaf fractions composed of a mixture of different secondary metabolites seems to be responsible for the anxiolytic-like effects in the studied mouse model.

M. oleifera contains some important plant secondary metabolites, including alkaloids, flavonoids, glycosides, and tannins. MMO and HMO fractions showed better anxiolytic-like effects in Swiss mice. Interestingly, all crude extracts produced a more calming effect when combined with the standard drug, DZP. It was stated that when DZP is used in combination with *M. oleifera* extracts the calming effects of each one was potentiated. Thus, *M. oleifera* may be one of the best sources of plant-based anxiolytic phytoconstituents. Further studies are needed to isolate main responsible active constituent of *M. oleifera* for anxiolytic effects.

Conflict of interests

The author declares no conflict of interest.

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