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Original Article

Anxiolytic and Antistress Potentials of Ethanol Stem-Bark Extract of *Milicia Excelsa* (Moraceae) in Mice

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Abstract

Milicia excelsa stem bark is applied in traditional medicine in some African societies, primarily as a tonic to rejuvenate the body after demanding episodes. However, there is a paucity of scientific evidence to support this usage. Therefore, this research aimed to evaluate the anxiolytic and anti-stress potentials of ethanol extract of the stem bark of *Milicia excelsa* (ESBME) in mice. The central nervous system inhibitory effect of the extract was determined using novelty-induced rearing, grooming, and locomotion behaviors while the anxiolytic effect was investigated by using a hole board and elevated plus maze (EPM) test models. The extract's ability to alleviate the anxiety-like and depressive-like behaviors triggered by acute restraint stress was evaluated with the use of EPM and tail suspension test respectively. The ESBME significantly ($p<0.05$) decreased the novelty-induced rearing, grooming, and locomotion behaviors indicating a central nervous system inhibitory effect. At 37.5 mg/kg, ESBME significantly ($p<0.05$) increased the number of head poking in the hole board test designating anxiolytic potential. Subsequently, the ESBME significantly ($p<0.05$) increased the percentage of open-arm entries and percentage open-arm duration as well as reducing the anxiety index on elevated plus Maze consistent with the antianxiety effect. The extract also significantly ($p<0.05$) alleviated the anxiety-like and depressive-like behaviors triggered by acute restraint stress suggesting an anti-stress effect. In conclusion, ESBME possesses central nervous system inhibitory, anxiolytic, and anti-stress effects thereby providing scientific evidence to the ethnomedicinal claim of the plant as an anti-stress agent.

Keywords: Acute restraint stress, Antidepressant, Central nervous system, Elevated plus Maze, Hole board test, Novelty-induced behaviors.

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1. Introduction

In the 21st Century globally, stress has been on the increase and has affected our daily accomplishments [1]. As a result, long-term

accumulation of psychological stress may lead to different stress-related disorders such as anxiety [2–4] and depression [5, 6], which are the two most prevalent types of mental disorders [7], in addition to cognitive dysfunction, post-stress traumatic disorder, endocrine disorders among others [8]. The existing benzodiazepine anxiolytics, used as an antistress remedy have not proved advantageous against stress-induced negative effects on immunity, peptic ulcer, hypertension, and cognition [9]. It has also been reported that these agents have teratogenic effects on the unborn child and adverse effects on infants during breastfeeding [10]. This has challenged scientists to make new anti-stress drug discoveries that are used in the body while circumventing the undesirable effect of the existing remedy [11].

Milicia consists of 2 species namely *Milicia regia* and *Milicia excelsa*, both of these species are found in the tropical regions of Africa. 'Iroko tree' is the widely accepted name for *Milicia excelsa* in South Western Nigeria particularly amid Yorubas, is also known as African teak in English, and is a member of the Moraceae family [12]. All the morphological parts are used locally [13]. In traditional medicine, the leaves are used in treating insanity [12], as antimalarial [14], while the stem bark is used as an antipsychotic [15], as a sedative for treating mental illness [16] and among others.

Milicia excelsa (stem bark extract) has been utilized in traditional African medicine for treating mental derangement [15, 16]. Continuous use of synthetic drugs has a variety of neurological side effects [17]. Therefore, there is a need to search for new and cost-effective therapeutic remedies that will circumvent the untoward effects of the existing drugs. *Milicia excelsa* stem bark has been used traditionally to revive the body from stress and stress-induced mental illnesses. Therefore, this research aimed to evaluate the anxiolytic and anti-stress potentials of ethanol extract of the stem bark of *Milicia excelsa* (ESBME) in mice.

2. Materials and Methods

2.1. Plant materials

Milicia excelsa stem bark was collected from the botanical farmland of the Pharmacognosy Department, Faculty of Pharmacy, Obafemi Awolowo University (OAU), Ile-Ife. The plant was authenticated at the Herbarium Unit, Department of Botany, Faculty of Science, (OAU) Ile-Ife and the latter was assigned herbarium number, "Ife 17482".

2.2. Extraction of the Milicia excelsa stem bark

The extraction protocols used in this study followed the method earlier described [18]. The stem barks were cut into pieces to hasten to dry. Thereafter the chopped stem barks were air-dried for 2 weeks in the laboratory. The dried stem barks were mechanically ground into powder and 8.29 kg of the powdered material was macerated in the cold fifteen liters (15 L) (w/v) of seventy percent (70%) ethanol for 48 hrs. The resulting filtrate was concentrated *in vacuo* and after freeze-drying the concentrated extract yielded 220.7 g (2.65%) crude extract coded ESBME.

2.3. Drugs

Tween 80, imipramine hydrochloride (Sigma Aldrich, USA), diazepam (Roche, Basel, Switzerland), and physiological saline (Fidson Healthcare Plc, Ogun State, Nigeria). The ESBME was initially solubilized with 3% Tween 80 before the addition of normal saline to the required concentrations for ingestion into the experimental mice. Fresh drugs and plant extracts were prepared every day.

2.4. Laboratory animals

Adult mice (18-25 g) of both sexes were purchased from the Animal House Department of Pharmacology, OAU, Ile-Ife, Nigeria. They were maintained under 12 hr natural daylight/12 hr natural night cycle. Mice were housed in cages with bed shavings and had free access to food and clean, drinkable water. The experimental procedures used in this study were approved by the Ethical Committee of the Faculty of Pharmacy Postgraduate Research Committee vide the approval number PHA13/14/H/2467 in compliance with the United States National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication No 85-23). The experiments were conducted between 10.00 am to 3.00 pm on each day of the experiments to circumvent the changes in biological rhythms.

2.5. Dose Selection for the experimental study

Earlier scientific endeavor has indicated that the LD_{50} of ESBME is greater than or equal to 5000 g/kg body weight [18]. Therefore, to access the gross effect of the extract on the central nervous system, doses of 125, 250, 500, and 1000 mg/kg were used for the evaluation of novelty-induced rearing, grooming, and locomotion behaviors in mice. Based on the outcome of the above, doses of 37.5, 75, 150, and 300 mg/kg were selected for the anxiolytic and anti-stress experiments.

2.6. Evaluation of ESBME on novelty-induced rearing, grooming, and locomotion in mice

Thus, thirty-six mice were randomized into 6 groups (n=6). Group I (control): mice received physiological saline (10 mL/kg, p.o.). Groups II to V (ESBME administered groups): mice received single oral treatment of ESBME at 125, 250, 500, and 1000 mg/kg, while group VI (positive control group): mice received intraperitoneal injection of diazepam at 1 mg/kg [19]. Sixty minutes following oral administration in groups I to V, and 30 minutes post diazepam injection in group VI, each mouse was singly and gently put in an observation cage for its frequency of rearing (raising of the fore paws on air or against the wall of the observation cage while standing on the hind limb), grooming (face and mouth washing, body scratching, body cleansing or cleansing of the pubic parts with mouth or paws) and locomotion (number of squares crossed with the four paws in each square) were counted and recorded for 20 minutes duration in a novelty-induced cage measuring 45cm x25cm x25cm evenly divided into 16 squares [20, 21]. The novelty-induced cage was cleaned after behavioral assessment of each mouse with seventy percent (70%) ethanol and cotton wool.

2.7. Effect of ESBME on the number of head dipping in hole board (HBT) tests in mice

Mice were divided and treated as above for groups I to VI. Sixty minutes following oral

administration in groups I to V, and 30 minutes post diazepam injection in group VI. Each mouse was gently placed on the hole board apparatus (40X40) with 16 holes that are symmetrically distributed in four rows. The frequency of head dipping into the holes was counted in 5 minutes duration [22]. The hole board apparatus was cleaned with seventy percent (70%) ethanol after the head dipping duration by each mouse.

2.8. Effect of ESBME on the anxiolytic parameters on elevated plus maze (EPM)

The anxiolytic potential of ESBME was assayed on EPM as earlier described [21]. Mice were divided and treated as earlier described above for groups I to VI. Sixty minutes following administration in groups I to V, and 30 minutes post diazepam injection in group VI. All the mice in every group were positioned individually in the middle part of the maze, with each mouse pointing towards the open-arm and allowed to survey the maze for a period of 5 minutes. The experiment was performed in an isolated and quiet area. The number of open-arm entrances and the duration for each mouse in the open-arm of the EPM were estimated and recorded for each mouse during the 5 minutes duration of continuous exploration of the EPM. The index of open-arm avoidance scores [23], which has been earlier interpreted as the level of anxiety of each mouse was calculated as

$$(100 - (\frac{\%\ \text{time in open-arm}}{\%\ \text{entries into open-arm}} + \%\ \text{entries into open-arm})/2$$

2.9. General experimental design following acute restraint stress (ARS) model

Forty-two mice were randomized into seven experimental groups (n=6). Group-I (unstressed control) mice received physiological saline (10 mL/kg, p.o.) but were not subjected to ARS; Group-II (stressed control) mice received physiological saline (10 mL/kg, p.o.) and were subjected to ARS. Group-III (standard drug-treated group) mice received intraperitoneal administration of imipramine (15 mg/kg) for tail suspension test (TST) or Diazepam (2 mg/kg, i.p.) for behavioral assay on the EPM 1 h before subjecting to ARS. Group-IV-VII mice were pretreated with oral administration of ESBME at 37.5, 75, 150, and 300 mg/kg 1 h before subjecting to ARS [24].

2.10. Acute restraint stress (ARS) model procedure

One hour after oral ingestion or 30 minutes after intraperitoneal administration of diazepam, each mouse was subjected to a restraint test in a white transparent and well-ventilated 50 ml tube 4 cm long, with a lid cover that restricted turning or movement for 2 h. After the restraint duration, each mouse was immediately transferred into a clean animal cage and allowed to stay for 20 min [25] before behavioral testing of locomotion on open field test (OFT), arm entries, and duration of stay on the EPM or immobility time on TST respectively.

2.11. Evaluation of anxiety-like behavior post ARS on EPM

The anxiety-like behavior of each mouse post-ARS was assayed on EPM as earlier described [26]. The frequencies of open-arm entries and time spent on the open-arm EPM were noted and recorded for 5 minutes of uninterrupted exploration of the EPM by each mouse. The index of open-arm avoidance scores [23], which has been earlier interpreted as the level of anxiety of each mouse was calculated as

$$(100 - (\%\ \text{time in open-arm} + \%\ \text{entries into open-arm})/2$$

2.12. Evaluation of depression-like behavior following ARS

The depressive-like behavior of each mouse post-ARS was assayed on TST as earlier done [24]. Each mouse was singly suspended on the edge of a table 50 cm above the floor with the use of adhesive tape placed approximately 1 cm from the tip of the tail. The total time spent immobile for the last 4 minutes of a 6-minute test was manually recorded using a stopwatch. Each mouse was considered immobile when it hung passively and completely motionless.

2.13. Statistical Analysis

Data were expressed as mean±S.E.M. The significance of the difference between the different experimental groups was analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. The significance level for all the behavioral assays was set at $p<0.05$.

3. Results and Discussion

3.1. Effects of oral administration of ESBME on novelty-induced rearing, grooming, and locomotion in mice

The ESBME at all the administered doses (125-1000 mg/kg) and diazepam (1 mg/kg) significantly ($p<0.05$) lowered the novelty-induced rearing, grooming, and locomotion compared to the control-treated mice. ESBME reduced rearing and locomotion dose-dependently. The activity of the extract at reducing novelty-induced rearing, grooming, and locomotion is comparable to the standard drug diazepam at 500 and 1000 mg/kg. The result is shown in **Table 1.**

3.2. Effects of oral administration of ESBME on the number of head dipping in hole board tests in mice

At 37.5 mg/kg, ESBME and diazepam (1 mg/kg) significantly ($p<0.05$) increased the number of head dipping compared to the control-treated mice in HBT. The result is shown in **Figure 1**.

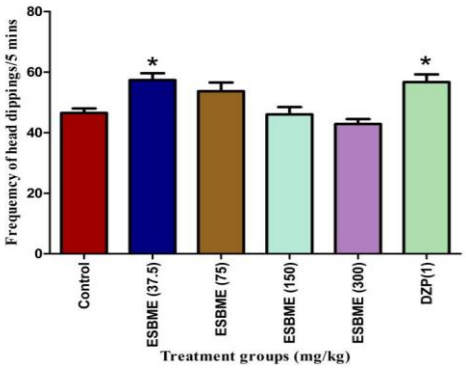

Figure 1. Effect of ESBME on the number of head dippings in HBT in mice. Control: 3% Tween 80 in Normal saline (10 mL/kg, p.o.), ESBME: Ethanol stem bark extract of M. excelsa, DZP: Diazepam (1 mg/kg, i.p.). Each bar denotes Mean ± SEM (n=6). *$p<0.05$ compared to the control treated group.

Table 1: Effect of ESBME on novelty-induced rearing, grooming and locomotion in mice. Each group represents Mean ± SEM (n=6). *p<0.05 compared to the control treated mice.

Group	Doses (mg/kg)	Novelty induced behavior per 20 mins. (Mean±SEM)		
		Rearing	Grooming	Locomotion
Control	10	104.2±7.2	36.2±1.6	191.7±15.1
ESBME	125	67.3±3.0*	14.52±1.1*	146.2±3.5*
ESBME	250	63.5±5.1	12.5±1.1*	134.0±2.5*
ESBME	500	57.7±7.0*	10.7±0.4*	128.2±2.9*
ESBME	1000	39.5±3.7*	18.5±1.6*	87.2±8.5*
Diazepam	1	55.5±1.6*	21.8±1.8*	144.7±3.7*

3.3. Effects of ESBME on the percentage number of open-arm entries and open-arm duration and anxiety status of mice on EPM

At all the administered doses (37.5-300 mg/kg), ESBME and diazepam (1 mg/kg) significantly (p<0.05) increased the percentage number of open-arm entries and open-arm duration on EPM compared to the control-treated mice. The ESBME also significantly (p<0.05) reduced the anxiety status of mice compared to the control-treated mice. The result is shown in **Table 2**.

3.4. Effects of ESBME on percentage number of open-arm s entries on EPM following ARS in mice

There was a significant (p<0.05) decrease in the percentage of open-arm entry of the stressed mice compared to the unstressed treated control group. However, ESBME at all test doses (37.5-300. The result is presented in **Figure 2** (Panel A).

3.5. Effect of ESBME on percentage open-arm s duration of the EPM following ARS in mice

There was a significant (p<0.05) reduction in the percentage open-arm duration of the stressed mice compared to the unstressed treated control group. The ESBME at all the doses (37.5-300 mg/kg, p.o.) and diazepam (2 mg/kg i.p.) significantly (p<0.05) ameliorated the reduced percentage duration in open-arm s compared to the stressed treated control group. The result is presented in **Figure 2** (Panel B).

Table 2: Effect of ESBME on the percentage number of open arm entries, duration and anxiety status of mice on EPM. Control: 3% Tween 80 in Normal saline (10 mL/kg, p.o.), ESBME: Ethanol stem bark extract of *M. excelsa*. Each group represents Mean ± SEM (n=6). *p<0.05 (increase) and $^{\#}$p<0.05 (decrease) compared to the control treated group.mg/kg, p.o.) and diazepam (2 mg/kg i.p.) significantly (p<0.05) reversed the reduction in percentage open arm entries of the stressed treated control group.

Group	Doses (mg/kg)	% open arm entry	% open arm duration	Anxiety index
Control	10.0	24.7±2.8	21.2±2.1	77.1±1.5
ESBME	37.5	44.6±0.8*	29.7±1.1*	62.9±0.7$^{\#}$
ESBME	75.0	43.5±2.3*	23.6±1.8	66.7±0.8
ESBME	150.0	41.7±1.7*	30.9±1.9*	63.7±1.3$^{\#}$
ESBME	300.0	43.4±2.4*	30.5±2.0*	63.1±1.5$^{\#}$
Diazepam	1.0	43.4±2.4*	55.2±1.2*	48.6±1.8$^{\#}$

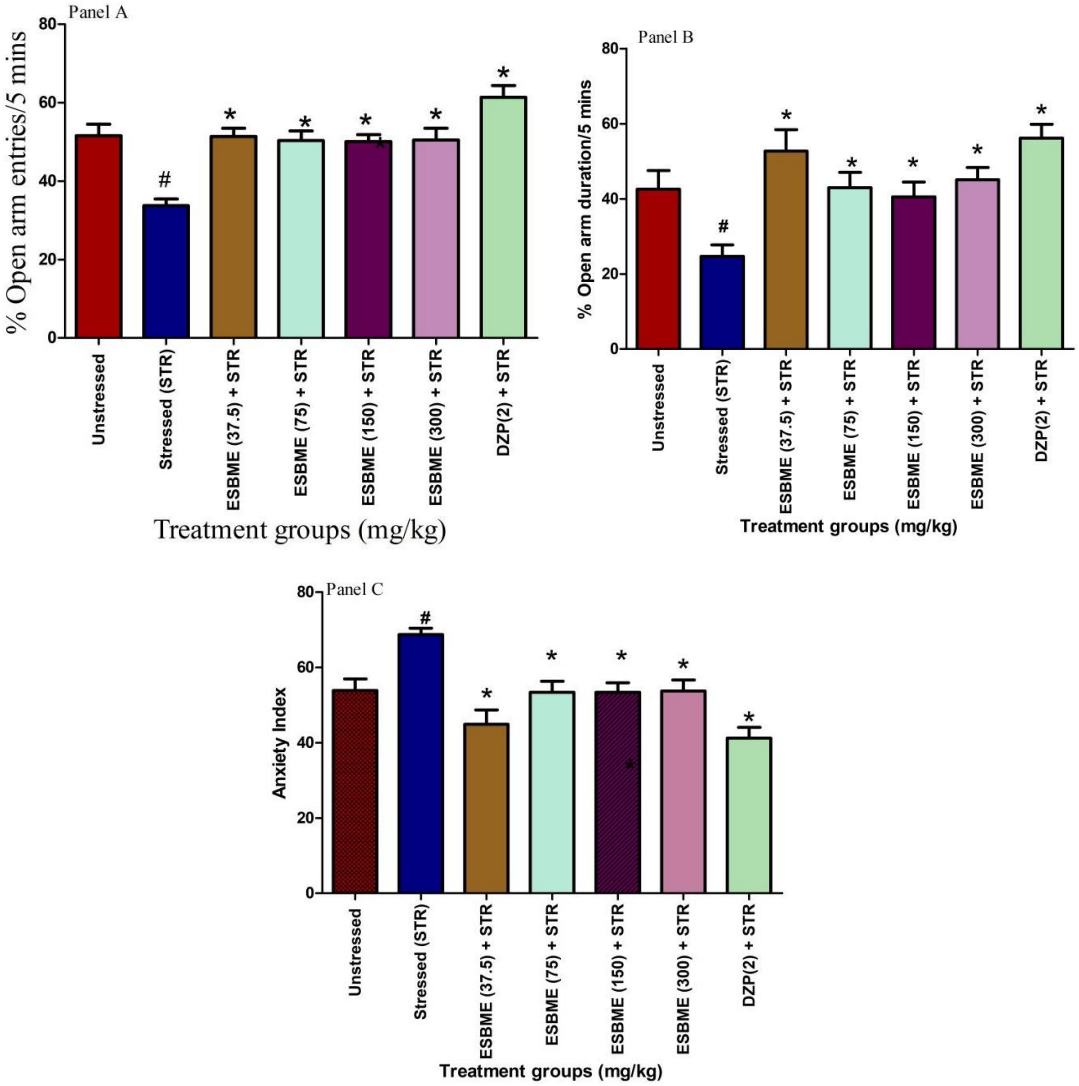

Figure 2. Effect of ESBME on anxiety-like behaviour on EPM following ARS in mice. Each bar represents Mean ± SEM (n=6). #p<0.05 and *p<0.05 compared to the UNSTR (unstressed) and STR (stressed) control group respectively.

3.6. Effects of ESBME on anxiety indices of mice on EPM following ARS

The stressed group showed a significant (p<0.05) increase in anxiety index compared to the unstressed group. However, there was a significant (p<0.05) reversal of the increased anxiety in stressed mice by the ESBME-treated and positive control mice compared to the stressed control group. The result is presented in **Figure 2** (Panel C).

3.7. Effects of oral ESBME on depression-like behavior in Tail Suspension Test (TST) following ARS in mice

There was a significant (p<0.05) increase in the duration of immobility in the stressed group when compared to the unstressed treated control group, this increase in immobility time was significantly (p<0.05) diminished by the ethanol stem bark extract of *M. excelsa* at test doses (37.5, 75 and

150 mg/kg, p.o.) and by imipramine (15 mg/kg, i.p.), a reference antidepressant agent (**Figure 3**).

The acute restraint stress (ARS) model is employed for behavioral testing to assess the molecular basis of stress-related conditions [27]. Agents possessing anxiolytic potentials may further be investigated for anti-stress effects. Consequently, this research evaluated the central nervous system (CNS) inhibitory effect of ESBME and eventually determined the anxiolytic potentiality of the extract preliminarily to its antistress potentials on stress-induced anxiety-like and depressive-like behaviors in mice.

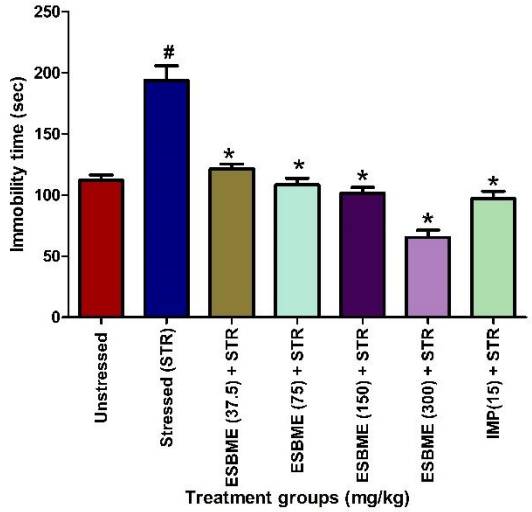

Figure 3. Effect of ESBME on the depression-like behaviour in TST following ARS in mice. Each bar represents Mean ± SEM (n=6). $^{\#}p<0.05$ and $^{*}p<0.05$ compared to the UNSTR (unstressed) and stressed (STR) control group respectively. IMP (15): Imipramine 15 mg/kg.

The dose-dependent reduction in novelty-induced rearing and locomotion following the oral administration of ESBME is indicative of the inhibitory effect on the central nervous system with an increase in the dose of the extract, which is in line with earlier studies of medicinal agents with central nervous system depressant effects [28, 29]. Also, the reduction in the novelty-induced grooming behavior brought about by the oral ingestion of ESBME is suggestive of its depressant activity [28, 29].

The increase in the number of open-arm entries and elongation of the time spent on the open-arm as well as the reduction in the anxiety index on the elevated plus maze (EPM) is indicative of anxiolytic [22, 30]. The extract may probably be acting via $GABA_A$- benzodiazepine receptor neurotransmission since EPM are sensitive to anxiolytics acting via this receptor pathway like diazepam [31].

Previous studies have demonstrated the use of hole board tests to screen antipsychotic, sedative, and antianxiety conditions in rodents [32–34]. Agents that lead to an increase in head dipping are suggested to have anxiolytic while agents that decreased head dipping are indicative of sedative potentials [35]. The increase in head dipping at the lowest tested dose in this study is suggestive of an anxiolytic potential at the dose.

Mice subjected to acute restraint stress (ARS) induced anxiety-like behavior which is in line with earlier numerous reports indicating that acute restraint stress induces anxiety-like behaviors in mice [36, 37]. However, this anxiety-like behavioral effect was diminished by ESBME as confirmed by the increase in percentage open-arm entries (**Figure 2** Panel A) and percentage open-arm duration (**Figure 2** Panel B), and anxiety index (**Figure 2** Panel C) consistent with the anti-stress effect. This result was in line with a previous report of agents that reversed the anxiety-like behavior induced by acute restraint stress and suggested to have an anti-stress effect in mice [27].

The probable neural mechanism of action of the anti-stress effects may therefore involve $GABA_A$ benzodiazepine receptor neurotransmission since $GABA_A$ benzodiazepine receptors have been implicated in stress-induced anxiety-like behavior on elevated plus maze [38].

In this study, ARS induced depression-like behavior in mice as earlier reported [24, 39]. However, this depression-like behavior was reversed by ESBME suggesting that ESBME may have an anti-stress effect (**Figure 3**). This is similar to earlier findings of medicinal agents that reversed depression-like behavior and were suggested to have an anti-stress effect [24,36,39].

The attenuation of depression-like behavior by ESBME may involve noradrenergic and/or serotonergic neurotransmission since the suppression of NE and 5-HT levels in different regions of mice and rat brains subjected to restraint stress has been differentiated in several studies on depression [24].

4. Conclusion

This study concludes that ESBME may have CNS inhibitory, anxiolytic, and anti-stress effects thereby proving scientific data for the ethnomedicinal claim of the plant. ESBME might, therefore, be a promising candidate in the management of stress-induced disorders like anxiety and depression and the development of anxiolytics.

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Conflict of interest

The authors declare to have no conflict of interest.

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