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(RESEARCH ARTICLE)

Ethanol leaf extract of *Milicia excelsa* mitigates anxiety and depressive-like behaviours induced by acute restraint stress in mice

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Abstract

Milicia excelsa (Moraceae) leaf is used as tonic and anti-stress agents to reinvigorate the body among other ethnomedicinal claims in African traditional medicines, but there is lack of scientific data on its efficacy. Hence, this study investigated the anti-stress potentials of the ethanol leaf extract of *Milicia excelsa* on anxiety-, and depressive-like behaviours induced by acute restraint stress in mice. The effect of the extract on spontaneous locomotor activities of mice was also evaluated. The extract at all the doses of 50, 100 and 200 mg/kg, p.o. significantly ($p<0.05$) increased the percentage open arm entries and percentage open arm duration as well as demonstrated anti-anxiety effect as shown by the open arm avoidance index on elevated plus maze. The extract also significantly ($p<0.05$) reduced immobility time of mice in tail suspension test, indicating antidepressive-like effect. Subsequently, the extract at all the doses used in this study did not modify the spontaneous locomotor effect of the experimental mice suggesting that the observed anti-stress effect was neither due to stimulation nor sedation. This study, therefore, concluded that the extract may possess specific anti-stress effect, effective against anxiety and depression induced by acute restraint stress, thus providing scientific evidence for its suggested ethnomedicinal usage.

Keywords: *Milicia excelsa*; Locomotor activity; Acute restraint stress; Anxiety-like behaviour; Depressive-like behaviour; Anti-stress effect

1. Introduction

The present-day lifestyle has increased the physical and psychological demands, resulting in rise in various stress-related disorders like anxiety [1] [2], depression [3], cognitive dysfunction, insomnia and anorexia [4], among other disorders [5], which affect daily performance of tasks [6]. Therefore, stress plays an important role in the pathogenesis of neuropsychiatric disorders [4], which include anxiety, depression and cognitive dysfunction [7] [8]. These neuropsychiatric disorders are the commonest psychiatric diagnosis in patients attending psychiatric clinics [9]. Hence, stress-related disorders pose a concern with attendant high cost to public health [6].

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The stress-induced effects are supposed to be an outcome of altered activity of different mechanisms, such as central neurotransmitters, neurohormonal factors, particularly those linked with the pituitary-adrenal axis, and free radical generation [10]. Neurotransmitters such as norepinephrine (NE) and 5-hydroxytryptamine (5-HT) are known to be involved in the expression of behavioral disorders, in adult individuals of several species, following stress [12]. These neurotransmitters have been demonstrated to be suppressed in different regions of mice, and rat brains subjected to restraint stress [12]. More so, previous findings have shown that the currently used antidepressants act by increasing the concentration of the monoamine neurotransmitters in the brain [13].

The prevention and management of stress disorders remain a major clinical problem [14]. Although the benzodiazepine anxiolytics showed significant anti-stress activity, but have not proved effective against stress-induced adverse effects on immunity, behaviour, cognition, peptic ulcer and hypertension [15]. Furthermore, these drugs have adverse effects on the foetus during pregnancy and on the neonate during lactation [16]. Therefore, the need for an effective anti-stress agent from plant origin, against the stress-induced disorders has been suggested [17].

To this end, numerous medicinal plants such as *Adiantum capillus-veneris*, *Euphorbia maculate*, and *Zizyphus jujuba* have been evaluated and suggested to exert their beneficial effects on the central nervous system, by protecting neurons against stress-induced injury [18]. Thus, the plant kingdom may be a potential source of new chemical compounds waiting to be discovered [19].

Milicia excelsa (Welw.) C.C. Berg. popularly known as 'Iroko tree' among the Yoruba speaking tribe of South Western Nigeria or African teak, belongs to the family Moraceae. It is a large deciduous tree 30 to 50 m high occurring naturally in humid forests of West Africa [20]. Its leaf, stem bark, root, latex, and ashes are used in traditional medicine to prepare traditional remedies for the treatment of lactation failure [21], psychosis [22], general fatigue, rheumatism, female infertility, sprains and as tonic [23], among other traditional uses. The objective of this study was to examine the therapeutic potentials of the ethanol leaf extract of *Milicia excelsa* on anxiety and depressive-like behaviours induced by acute restraint stress in mice. Conducting scientific investigation on the efficacy of medicinal plants used in the management of stress in traditional medicine as reported in this study is a worthwhile contribution to the ongoing search for botanicals that could mitigate the anxiety-, and depressive-like disorders caused by increased physical and psychological demands of our present day stressful lifestyle.

2. Material and methods

2.1. Plant identification and authentication

Milicia excelsa leaves were collected within the campus of the Obafemi Awolowo University (OAU) Ile-Ife Nigeria. It was identified and authenticated by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany, Faculty of Sciences, OAU, Ile-Ife and herbarium voucher number Ife 17482 was obtained.

2.2. Preparation of plant materials

The leaves of *Milicia excelsa* were air dried at room temperature. The dried leaves were pulverized and 1.0 kg of the powder was extracted with 3 litre of ethanol (70%) for 72 h. The marc was re-extracted once and the combined extract was concentrated *in vacuo* at a temperature of 40^{o}C to yield 70 g (7.0%) crude extract and coded (EME) [24].

2.3. Drugs

Diazepam (Roche, Basel, Switzerland), Tween 20, imipramine hydrochloride (Sigma Aldrich, St. Louis, Missouri, U.S.A.) and physiological saline (Unique Pharmaceutical Limited, Lagos, Nigeria) were used. EME was dissolved with 2% Tween 20 and made up to the required volume with normal saline. The drug and EME were freshly prepared on each day of the experiments.

2.4. Laboratory animals

Adult male albino mice (18–25 g) were obtained from the Animal House, Department of Pharmacology, Faculty of Pharmacy, OAU, Ile-Ife. The animals were maintained on standard animal pellets, and water *ad libitum*. The experimental procedures adopted in this study followed the approved institutional animal ethical committee guidelines, and within the context of internationally accepted principles for Laboratory Animal Use and Care (EEC Directive of 1986; 86/609/EEC) [25].

2.5. General experimental design

Male albino mice were randomly divided into six experimental groups containing 6 mice per group (n=6). Group-I (vehicle) mice received 2% Tween 20 in normal saline (10 mL/kg, p.o.) but not subjected to acute restraint stress (ARS); Group-II (stress control) mice received normal saline (10 mL/kg, p.o.) and subjected to ARS. Group-III (standard drug-treated) mice received imipramine (15 mg/kg, i.p.) for tail suspension test (TST) [26] or Diazepam (2 mg/kg, i.p.) for elevated plus maze (EPM) [27], 1 hr before subjecting to ARS. Group-IV-VI mice were treated with EME (50, 100 and 200 mg/kg, p.o.) 1 hr prior to subjecting to ARS.

2.6. Acute restraint stress (ARS) procedure

The model for restraint stress used in this study was adapted from previous reports [28, 29, 30]. The animals were randomly divided into six groups containing 6 mice per group. Stressed groups were administered with vehicle or EME and 1 hr after the treatment they were subjected to stress. Diazepam (2 mg/kg, i.p.), a reference drug was administered to a group of 6 mice, 30 minutes post treatments; each mouse in Group II-VI was then subjected to restraint stress for 7 hr by taping all its four limbs on a board after putting each mouse on its back using zinc oxide hospital adhesive tape. This restrained all physical movements without causing pain. In the period (1 hr) for the vehicle or EME or 30 minutes for diazepam or imipramine that elapsed between the treatment groups and stress procedure, mice were maintained in their home cages with free access to food and water. The mice were deprived of food and water during the entire period of exposure to stress. After 7 hr, each animal was released by unraveling the tape after moistening with acetone in order to minimize pain or discomfort. Forty minutes post-stress release, each animal was subjected to behavioural tests.

2.7. Behavioural assessment following ARS

2.7.1. Evaluation of anxiety-like behavior in ARS: elevated plus maze

EPM was used to assess the anxiety-like behaviour following ARS in mice; the EPM used was a modification of the apparatus based on Montgomery's conflict test [27]. Mice were randomized into seven groups (n=6). Each mouse was placed in the central square of the maze facing an open arm and its exploratory behaviour was monitored and recorded. During 5 minutes duration, the frequency of arm entries and the time spent on each arm (close or open arm) of EPM was recorded.

(i) Open arm entries (ii) Closed arm entries

(iii) Time spent in the open arms (iv) Time spent in the close arm

The anxiety status of each mouse was calculated using the index of open arm avoidance as previously used to assess anxiety status in mice [31] as

$$\text{Anxiety index of open arm avoidance } = 100 - \frac{(\%\text{ time in open arm} + \%\text{ entries into open arm})}{2}$$

The index of open arm avoidance on EPM was used to assess the anxiety status of each mouse after ARS. This is because the anxiety index has incorporated two important parameters-the frequency of open arm entries and the time spent on the open arm as indices of anti-anxiety behaviour. If the anxiety index is at least 10 point less than the control treated group, the sample has an anti-anxiety effect (anxiolytic); conversely if the anxiety index is at least 10 points greater than the control treated group, then the sample is anxiogenic [32]. The control-treated group was the stressed control group.

2.7.2. Evaluation of depressive-like behavior in ARS: tail-suspension test

The TST adopted to evaluate the depressive-like behaviour of mice following ARS was as previously described [26]. Mice were suspended from the edge of a table 50 cm above the floor, by the adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility was recorded for the last 4 minutes, during a 6-minute test. Mice were considered to be immobile, only when they hung passively and were completely motionless.

2.7.3. Open field test (OFT)

This test utilizes behavioural changes in rodents exposed to novel environments, which is used to confirm that the observed antidepressant effect observed in this study was not due to stimulation of general motor activity. The open field test (OFT) was carried out on the dark grey floor subdivided into 16 equal parts in a wooden observation box (100 cm x 100 cm x 30 cm). Sixty minutes after oral treatment with the extract, and 30 minutes after intraperitoneal injection of diazepam (1 mg/kg). Each mouse was individually placed in the corner square of the open field apparatus. The observed parameters were the number of squares crossed (with the four paws), and the frequency of rearing elicited during a 5-minute duration [33, 34].

2.8. Statistical analysis

Results are expressed as mean ± S.E.M. The significance of difference between groups were analysed using one way analysis of variance (ANOVA), followed by post hoc analysis using the Student-Newman-Keuls test. GraphPad InStat® Biostatistics software (GraphPad Software, Inc., La Jolla, USA) was employed for the analysis. The level of significance for all tests was set at *$p<0.05$.

3. Results

3.1. Effects of EME on anxiety-like behaviour in EPM following ARS in mice

3.2. Effects of EME on percentage open arm entries on EPM following ARS in mice

There was a significant ($p<0.05$) decrease in the percentage open arm entries of the stressed mice when compared to the vehicle-treated control group. EME at all the doses used (50, 100 and 200 mg/kg, p.o.) and the standard drug diazepam 2 mg/kg i.p. significantly ($p<0.05$) increased the percentage of open arm entries when compared to the stressed treated control group. EME also showed a significant ($p<0.05$) dose-dependent decrease in the percentage of open arm entries. The result is presented in Figure 1.

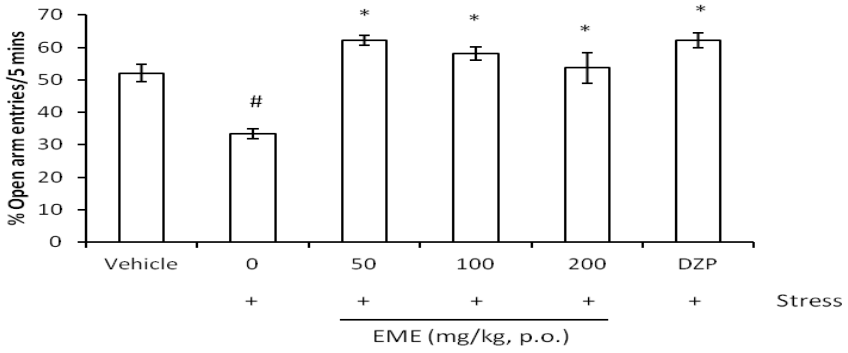

Figure 1 Effect of EME on percentage open arm entry of EPM following ARS in mice

Vehicle; 2% Tween 20 in Normal saline (10 mL/kg, p.o.), EME; Ethanol leaf extract of M. excelsa, DZP; Diazepam (2 mg/kg, i.p.). Each bar represents Mean ± SEM, ANOVA; one way analysis of variance followed by Student-Newman Keuls Test, n=6, #$p<0.05$ and *$p<0.05$ when compared to the vehicle and stressed control treated group.

3.2.1. Effects of EME on percentage open arm duration on EPM following ARS in mice

There was a significant ($p<0.05$) decrease in the percentage open arm duration of the stressed control mice when compared to the vehicle-treated control group on EPM. EME at all the doses used (50, 100 and 200 mg/kg, p.o.) and the standard drug diazepam 2 mg/kg i.p. significantly ($p<0.05$) increased the percentage duration on open arms when compared to the stressed treated control group. EME also showed a significant ($p<0.05$) dose-dependent decrease in the percentage duration on the open arm. The result is presented in Figure 2.

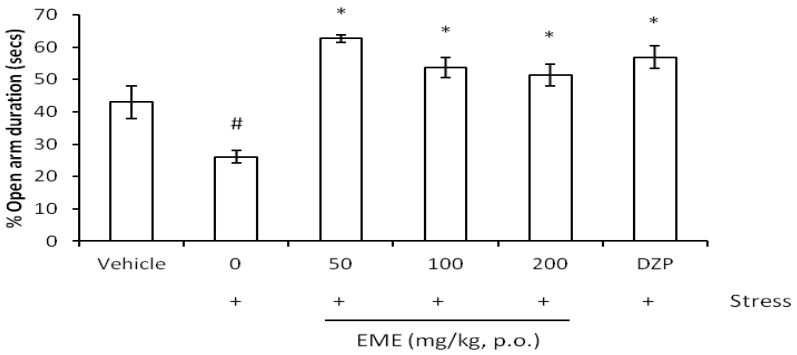

Figure 2 Effect of EME on percentage open arm duration on EPM following ARS in mice

Vehicle; 2% Tween 20 in Normal saline (10 mL/kg, p.o.), EME; Ethanol leaf extract of M. excelsa, DZP; Diazepam (2 mg/kg, i.p.). Each bar represents Mean ± SEM, ANOVA; one way analysis of variance followed by Student-Newman Keuls Test, n=6, #$p<0.05$ and *$p<0.05$ when compared to the vehicle and stressed control treated group.

3.2.2. Effects of EME on open arm avoidance index (OAAI) in EPM following ARS in mice

The stressed control group showed anxiety index of 69.00 ± 1.71 compared to the vehicle-treated group which showed anxiety index of 53.42 ± 3.00. EME at all the doses used (50, 100 and 200 mg/kg, p.o.) showed anxiety indices of 37.56 ± 0.63, 44.08 ± 2.37 and 47.48 ± 3.35 respectively and diazepam 2 mg/kg, i.p. (a reference anti-anxiety drug) showed anxiety index of 40.49 ± 2.36. The anxiety indices of the extract treated groups at all the doses used and the vehicle-treated control were 10 point less than the control treated group (stressed group). The result is presented in Figure 3.

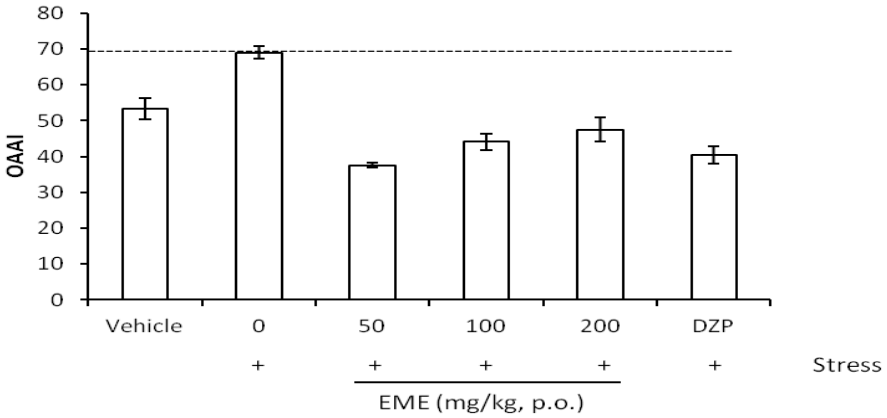

Figure 3 Effect of EME on open arm avoidance index (OAAI) on EPM following ARS

OAAI; Open Arm Avoidance Index, EPM; Elevated Plus Maze, Control; 2% Tween 20 in Normal saline (10 mL/kg, p.o.), DZP; Diazepam (2 mg/kg, i.p.), EME; Ethanol leaf extract of M. excelsa, Each bar represents Mean ± SEM. If the anxiety index is at least 10 point less than the control treated group, the sample has anti-anxiety effect (anxiolytic), conversely if the anxiety index is at least 10 points greater than the control treated group, then the sample is anxiogenic.

3.3. Effects of EME on depressive-like behaviour in TST following ARS in mice

There was a significant ($p<0.05$) increase in duration of immobility in stressed group when compared to the vehicle-treated control group, this increase in immobility time was significantly ($p<0.05$) mitigated by EME at all the doses used (50, 100 and 200 mg/kg, p.o.) and by imipramine (15 mg/kg, i.p.), a reference antidepressant agent. The result is presented in Figure 4.

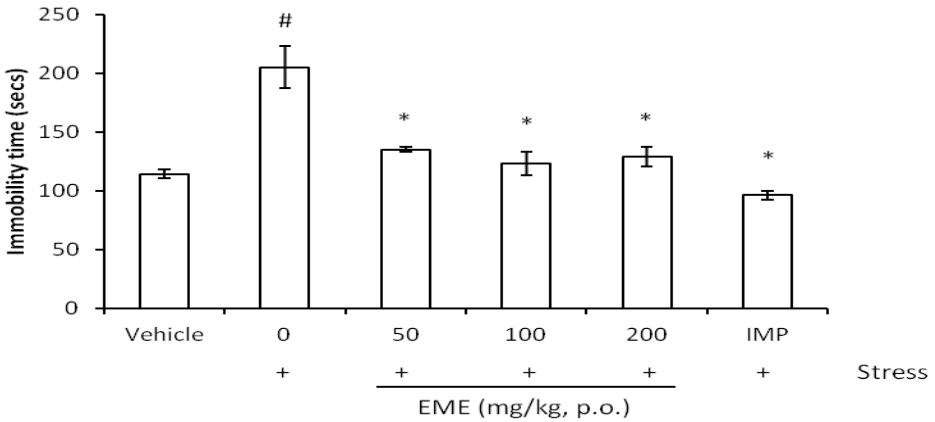

Figure 4 Effect of EME on depressive-like behaviour in TST following ARS in mice

Vehicle; 2% Tween 20 in Normal saline (10 mL/kg, p.o.), EME; Ethanol leaf extract of *M. excelsa,* IMP; Imipramine (15 mg/kg, i.p.). Each bar represents Mean ± SEM, ANOVA; one way analysis of variance followed by Student-Newman Keuls Test, n=6, #$p<0.05$ and *$p<0.05$ when compared to the unstressed and stressed control treated group.

3.4. Results of EME on locomotor activity: open field test

EME at doses of 50, 100 and 200 mg/kg, per oral and DZP (1 mg/kg, i.p.), did not show any significant effects on locomotor activity in mice, as assessed by the OFT (Table 1). However, diazepam (1 mg/kg, i.p.) significantly ($p<0.05$) reduced rearing behaviour in mice compared to the vehicle treated control group.

Table 1 Effects of diazepam and EME on behaviour of mice in open field paradigm

Group	Doses (mg/kg)	Number of square crossed	Frequency of rearing	Total
Control	10	95.2 ± 4.3	28.7 ± 2.2	123.8 ± 4.1
EME	50	91.3 ± 2.7	28.5 ± 1.4	119.8 ± 2.4
EME	100	89.0 ± 4.8	32.3 ± 1.3	121.3 ± 4.2
EME	200	92.3 ± 3.2	32.3 ± 1.8	124.7 ± 3.6
Diazepam	1	93.3 ± 3.4	17.2 ± 3.1*	110.5 ± 5.0

Each group represents Mean ± SEM (n=6). *$p<0.05$ compared to the vehicle treated control group.

4. Discussion

The acute restraint stress (ARS) is a widely used behavioural model to study the molecular basis of stress-related issues [35]. In this study, anxiety- and depressive-like behaviours were induced in the stress control mice following ARS, the effect of which was mitigated by ethanol leaf extract of *Milicia excelsa* (EME) suggesting that EME may have anti-stress effects in mice.

Previous report has demonstrated the acute toxicity (LD_{50}) of ethanol leaf extract of *Milicia excelsa* to be $\geq$ 5000 mg/kg, per oral and suggested its safety in mice [24]. Thus, lower doses of 50, 100 and 200 mg/kg, per oral were used in this study.

The elevated plus maze (EPM) is considered to be an etiologically valid animal model of anxiety which uses natural stimuli like fear of a novel open space, and fear of balancing on a relatively narrow raised platform that can induce anxiety in mice [36]. However, report has shown that measurement of anxiety states post ARS induction made the animals to show further anxious behaviour on EPM [37]. In the present study, ARS precipitated anxiety-like behaviour as observed by the reduction in the percentage number of open arm entries and percentage duration in open arms as well as increased anxiety index as recorded from the index of open arm avoidance suggesting induction of anxiety-like behaviours in stress control mice compared to the vehicle-treated group on EPM. However, administration of EME and diazepam (a reference anti-stress agent) reversed these parameters on EPM indicating that EME may have an anti-stress effect like diazepam, effective against anxiety-like behaviour induced by ARS in mice. This finding is in accordance with the previous study that acute restraint stress in rodents precipitates anxiety-like behaviour which could be attenuated by medicinal plants [8]. Although the mechanism of anti-stress effect of EME on anxiety-like behaviour on EPM was not delineated in this study but it could be suggested that EME might be acting like diazepam via GABAA-benzodiazepine receptor, since drugs like diazepam act via GABAA-benzodiazepine receptor-Cl- channel complex and this could be corroborated by earlier report that GABAA-benzodiazepine receptor-Cl2 channel complex is involved in the attenuation of stress-induced anxiety-like behaviour by Euphorbia hirta [38].

Numerous earlier reports have demonstrated that rodents (mice and rats) exposed to restraint stress, in different duration of stressful events, exhibited depressive like-behaviour, evidenced by increased immobility time in TST [39, 40]. In this study, ARS increased the immobility time of stress control mice in the TST, indicating depressive-like behaviour. This result is in consonance with earlier finding, which demonstrated that ARS induced depressive-like behaviour, as evidenced by increased immobility time in TST [30]. However, pre-treatment with EME and imipramine reversed the depressive-like behaviour in TST induced by ARS suggesting that EME may have anti-stress effect as it was effective against depressive-like behaviour induced by ARS in mice. This finding is in conformity with previous studies that reported acute restraint stress in rodents precipitates depressive-like behaviour which could be attenuated by medicinal substances [8] [30].

Similarly, the mechanism of EME on stress-induced depressive-like behaviour in TST was not investigated, but it could be speculated that EME like imipramine, might be acting via norepinephrine (NE)/or serotonergic (5-HT) pathways, to elicit the observed anti-depressive effect in this model. Since suppression of NE and 5-HT levels in different regions of mice and rat brains subjected to restraint stress has been clearly differentiated in several studies on depression [12].

In addition to the speculated mechanism of anti-stress effect of EME above, EME could also be acting via strengthening the antioxidant defensive system since ARS is a well-known method to produce oxidative damage by causing derangement in the antioxidant defense mechanism [41]. Increased oxidative damage and weak antioxidant defense mechanisms have been reported to be implicated in anxiety and depression [29]. Likewise, it could also be suggested, that EME might be acting to counter the stress-induced increase in blood corticosterone levels. Also countering the increase in serum triglycerides levels and hyperglycemia is a possibility, since stress response is characterised by the activation of the hypothalamic-pituitary-adrenal axis, resulting in the increase in these biochemical parameters [37].

Compounds altering locomotor activity may give false-positive/negative effects in behavioural tests [42]. EME at all the doses used in this study did not alter the locomotor activities of the mice, suggesting that the observed anti-stress effect was not due to spontaneous motor effect. Thus, the anti-stress effect of EME is specific. This is in line with the previous reports, that alteration of locomotor behaviour may give false-positive/negative effects in behavioural tests [33, 42].

5. Conclusion

This study concludes that EME may have anti-stress effects, effective against stress induced anxiety and depression in ARS model in mice. EME might therefore, be a promising candidate in the management of stress induced anxiety- and

depressive-like behaviours. However, further studies may be warranted to isolate the active ingredient(s) responsible for the observed pharmacological effects in EME and subsequently the elucidation of its probable anti-stress mechanism of action using biochemical and pharmacological parameters.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The study was carried out under approval of the Obafemi Awolowo University Ethical Committee on use and care of laboratory animals. The approval derived from the implementation of the EEC Directive of 1986; 86/609/EEC as implemented by the Postgraduate College, Obafemi Awolowo University vide the registration number PHP 11/12/H/2766 issued to AKINPELU Lateef Abiola in 2012.

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