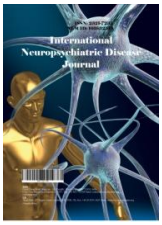

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Appraising the Neurobehavioural Toxicity Potential of Aqueous Methanol Leaf Extract of *Tapinanthus globiferus* Growing on *Azadirachta indica*

A. M. Umarudeen[1*] and M. G. Magaji[2]

[1]Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Sciences, College of Health Sciences, University of Abuja, Federal Capital Territory, Abuja, Nigeria.
[2]Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Authors AMU and MGM did the study design. Author AMU undertook the behavioural experimentation, data analysis, result interpretation and the drafting of the first manuscript. Author MGM did the manuscript proofreading. Authors AMU and MGM read and approved the final manuscript.

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ABSTRACT

The high prevalence and disease burden of anxiety disorders against the paucity and liabilities of existing anxiolytics indicates a need for the discovery of additional/new anti-anxiety agents. However, it is necessary to further screen these new/putative anxiolytic compounds/extracts to rule out the unwanted neurobehavioural toxicities inherent in the existing anti-anxiety drugs. Aqueous methanol leaf extract of *Tapinanthus globiferus* growing on *Azadirachta indica* host tree has previously demonstrated significant (p<0.05) anxiolytic effects in mice. This study, therefore, set out to counter-screen this extract for locomotion-suppressant, acute amnesic, sedative (myorelaxant) and hypnotic effects using standard mouse behavioural and biochemical paradigms. The leaf extract (150, 500 and 1500 mg/kg) did not cause significant (p>0.05) alterations in spontaneous locomotor activity, motor coordination/balance, sleep onset or duration, but dose-dependent and significant (p<0.05) increases (63.28±5.63, 65.63±4.12 and 69.18±3.69) in novel object recognition indices of

**Corresponding author: E-mail: umarudeen.monisola@uniabuja.edu.ng;*

extract-treated compared to 51.54±4.03 and 61.06±2.91 scores in diazepam- and aqua-treated mice, respectively. These findings indicate the aqueous methanol leaf extract is mostly devoid of the evaluated neurobehavioural toxicities and may possess short-term memory enhancement property in mice. These findings may justify the traditional use of *Tapinanthus globiferus* extracts for memory enhancement.

Keywords: Cognitive; effects; hypnotic; locomotor; mice; sedative; suppressant.

1. INTRODUCTION

Anxiety disorders are a highly prevalent class of mental disorders. The chronic nature of these disorders, the paucity, and the liabilities of the existing anti-anxiety drugs are among the factors significantly contributory to their high global prevalence – indicating a need for the search of additional novel and safer anti-anxiety agents [1-4]. The drawbacks of anxiety pharmacotherapy include the adverse drug reactions associated with the use of anxiolytic drugs. The most prominent and intolerable among these adverse effects are the neurobehavioural reactions, which collectively have limited the clinical usefulness of these agents [2,5,6].

For instance, the benzodiazepines are arguably the most successful class of anxiolytic drugs but currently, their use is limited mainly to only short-term anxiety states as their long-term usefulness is reportedly hampered by the emergence of sedation, addiction/physical dependence, hypnotic, cognitive and locomotor defects [6,7]. Similarly, the use of the selective serotonin/noradrenaline reuptake inhibtors (SSRIs/SNRIs) e.g., paroxetine/venlafaxine, commoly employed as the drugs of first choice for most anxiety disorders in clinical practice, has been associated with delayed efficacy onset, increased weight gain and gastro-intestinal upset. These are in addition to neurobehavioural effects such as insomnia, akathisia, agitation, sexual dysfunction and cognitive decline even on a short-term basis [8-11].

The tricyclic compounds (e.g., amitriptyline) have largely been consigned to an adjunctive role in the drug treatment of resistant anxiety disorders on account of severe adverse effects like increased anxiety, increased epileptogenesis, blurred vision, urinary retention, postural hypotension, sedation and cardiotoxicity [12,13].

The anticonvulsant anxiolytics (gabapentin and vigabatrin) also have had their usefulness significantly compromised by their unfavourable drug adverse reactions, including severe dizziness, blurring of vision, confusion, agitation, loss of coordination, euphoria, dysarthria and altered libido [14,15].

Also, the antipsychotic anxiolytics (Olanzapine, Risperidone), currently, have limited clinical usefulness in anxiety drug treatment due to such adverse effects as postural hypotension, extrapyramidal effects, weight gain and sexual dysfunction [16,17].

It is in this light that aqueous methanol *T. globiferus* leaf extract, having demonstrated some anxiolytic activity in previous studies is being further probed in this study for its potential to develop neurobehavioural adverse effects often associated with the existing anxiolytic agents.

Tapinanthus globiferus (A.Rich.) Thiegh. (synonym: *Tapinanthus globiferus* subsp. bangwensis (Engl. Krause) Balle., is one of the numerous *Tapinanthus* species commonly known as the African mistletoes - evergreen dioecious plants belonging to two similar families: *Viscaceae* and *Loranthaceae* [18-21]. This parasitic plant is commonly seen on Neem and other cash crop trees in most parts of Nigeria where it is called Kauchi among the Hausa-speaking and Afomo ishana, among the Yoruba-speaking ethnicities of the country. It is regarded traditionally as an all-cure plant with reported ethnomedicinal efficacy in nervous disorders, hypertension, cancer, epilepsy, and diabetes mellitus [22].

Earlier studies on the aqueous crude stem bark extract of a related species, *Tapinanthus dodoneifolius* (DC) Denser and on the crude methanol leaf extract of *Tapinanthus globiferus* have reported anxiolytic/antidepressant and antidepressant effects, respectively, in mice [23, 24]. In other studies, an aqueous residue fraction of *Tapinanthus globiferus* has demonstrated anticonvulsant effects in mice [25] and chicks [26].

The preliminary phytochemical analysis of the aqueous methanol leaf extract of *Tapinanthus globiferus* being investigated in this study using standard phytochemical methods according to Odebiyi and Sofowora, 1978 indicated the presence of tannin, saponin, terpenoids, cardiac glycosides, alkaloids, flavonoids and carbohydrate [27,28]. The Gas chromatographic-mass spectrometric (GC-MS) analysis of the same extract according to the method previously used by Karthishwaran et al., 2018 indicated the presence of Furazano [3,4-b] pyrazin5(4H)-one, 6-(1-pyrrolidinyl), isobutyl amine, N, N-dimethyl Ethanamine, Ethylenediamine, N-Ethylformamide, oxalic and acetic acids among others [29]. Also, aqueous and aqueous methanolic extracts, as well as aqueous and chloroform fractions of the same *Tapinanthus globiferus* leaves have been earlier found to exhibit significant ($p<0.05$) anxiolytic activity when compared with distilled water treatment [28,30, 31,32]. The lethal dose (LD)50 greater than 5000 mg/kg in female mice (oral) and female rat (oral) was also obtained for the same aqueous methanol *Tapinanthus globiferus* leaf extract and a 92-day daily oral administration at 150, 500 and 1500 mg/kg of the extract did not produce gross, histological and biochemical alterations in extract-treated compared to distilled water treated mice [28].

Despite the anxiolytic and other central nervous systems (CNS) effects of extracts from *Tapinanthus globiferus*, there has not been, within available literature, any scientific report on the evaluation of the neurobehavioural adverse reactions on this medicinal plant. This study, therefore, set out to evaluate the acute hypnotic, sedative (myorelaxant), cognitive and locomotion-suppressant effects of graded acute doses of aqueous methanol *T. globiferus* leaf extract in mice using standard experimental protocols.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant parts

Fresh mature leaves of *T. globiferus* growing on an *Azadirachta indica* (Neem) tree (Plate 1) located along Shuni road, Mabera, Sokoto; Sokoto State, Nigeria, were collected in March 2019. The plant was identified by Mallam Abdul Azeez Salihu (the Botanist officer) with the plant voucher bearing Specimen number: UDUH/ANS/0135 deposited at the Herbarium of the Department of Botany, Usmanu Danfodiyo University, Sokoto, Sokoto State, Nigeria.

2.1.2 Experimental animals

Apparently healthy 8-10 weeks old male Swiss Albino mice weighing 20.64±2.68 g of an outbred General purpose (GP) stock of *Mus musculus* sourced from the Nigerian Pharmaceutical Research and Drug Development centre, Abuja, Federal Capital Territory, Nigeria, were used in this study. These mice were well-suited for the toxicological investigations due to their genetic variability [31]. They were kept for a minimum of 14 days before use under a 12-hour light/dark laboratory environment with adequate ventilation, free access to drinking water and feed.

Plate 1. *Tapinanthus globiferus* leaves and fruit berries hosted on a Neem tree

2.1.3 Drugs and reagents

Aqua and diazepam injections (Roche) were sourced from reputable Pharmaceuticals nearby.

2.2 Methods

2.2.1 Plant preparation and extraction

The collected plant parts were briefly rinsed in water, air-dried, then powdered and finally stored dry in non-transparent plastic containers for subsequent use.

Two hundred and fifty (250) g of fine powder of *T. globiferus* leaves was macerated in 1 L distilled water/methanol (V/V: 30/70) for 24 hours. It was then filtered using Whatman's paper (150mm) and separately evaporated in a rotatory water bath at 45-50 degrees Celsius to yield 31.45 g (12.58%) of greenish-brown dry extract.

2.2.2 Behavioural studies

For all the behavioural experiments, a volume of 10 ml/kg of aqua and all extract doses was administered *i.p.* to the mice as recommended in [32]. Although the optimal anxiolytic dose of *Tapinanthus globiferus* leaf extract was found to be 150 mg/kg in a previous in-vivo efficacy assay [33], high graded doses (150, 500 and 1500 mg/kg) of the aqueous methanol extract likely to produce toxic effects were deliberately used.

All the mice were humanely sacrificed by chloroform euthanasia. They were thereater safely and hygienically disposed of after each experiment.

2.2.2.1 *Evaluation of the effect of acute administration of aqueous methanol T. globiferus leaf extract on locomotion*

This was carried out by exposing groups (n=10) of male mice to rectangular open-fields, each consisting of 24 equal-sized squares according to the method used by [34]. Forty-five minutes following treatment with aqua (10 ml/kg) or extract (150, 500 or 1500 mg/kg) or 1 mg/kg diazepam as used in [35], mice were each dropped at the centre of the open field and allowed to freely move around and explore it for 5 minutes. The distances traveled were calculated for each test animal.

2.2.2.2 *Evaluation of the effect of acute administration of methanol T. globiferus leaf extract on motor balance and coordination in mice*

A single horizontal bar variant of the beam (rod) walking assay of Stanley, 2005 (Plate 2) [36] as described in Deacon, 2013 [37], with minor modifications, was adopted for this evaluation in groups of mice (n=10) in two separate phases spanning two consecutive days. On the first day, the pretest screening trial was conducted on a pool of mice to select 50 healthy male mice for the main test scheduled to take place the following day by exposing each mouse to a two mm-rod of on the beam balance. The mice that stayed on or held onto the rod without falling off for 5 seconds and above were selected for the main test. Those that fell off on the first attempts were re-tried two more times. Those mice that passed the test on their second attempts were not tried the third time. The mice that succeeded on the first, second, or third attempts were all deemed to have met the selection criteria.

The beam balance consisted of a 40cm-long brass rod suspended at a height of 50 cm from the surface of a table on two wooden poles (beams) secured to two heavy wooden flat bases set 40-cm apart (Plate 2).

Briefly, on the trial day, the test was started by gently holding the test animal by the tail and placing it on the flat Table roughly midway between the lower ends of the wooden beams perpendicular to the suspended steel rod. With a gentle but firm pool at the tail, the animal was then subjected to a quick backward sliding movement for about 20 cm and then rapidly lifted in a way its forepaws would grab the 2-mm steel rod just as the tail was simultaneously being released. Each mouse was observed for its balance on the beam rod and was deemed to meet selection criteria if it stayed on or held onto the rods for 5 seconds or longer.

On the second day of the test, the mice that met the selection criteria were randomised into 5 groups (n=10) and were each exposed, as earlier described for the 2-mm rod, to a 4-mm rod on the beam balance 30 minutes following intraperitoneal treatments with aqua (10 ml/kg), extract (150, 500 or 1500 mg/kg) or diazepam (2 mg/kg) as used in [35]. The time spent on the beam balance by each mouse was determined by the use of a watch timer.

Scoring for the times spent by the mice staying on/holding onto the rod was done as described by Deacon, 2013 [37] with slight modifications, as follows:

Falling off between 1-5 sec = 1, between 6-10 sec = 2, between 11-20 sec = 3 and between 21-30 sec = 4. Staying on top of the rod for/longer than 30 sec = 5; holding onto the rod by placing one or both forepaws on it without falling for 30 sec = 5, and climbing onto the top of the rod with all 4 paws on it at any time within 30 seconds = 5. Reaching any of the vertical support beams at any time within test duration = 5. The scores were collated and recorded for each mouse group.

2.2.2.3 Evaluation of the hypnotic effect of acute administration of aqueous methanol T. globiferus leaf extract

The hypnotic potential of aqueous methanol *T. globiferus* leaf extract was determined by a diazepam-induced sleep test according to the method of Rakotonirina et al. (2001). Randomised groups I-IV (n=10) of male mice were each given *i.p.* injection of aqua (10 ml/kg) or extract (150, 500 or 1500 mg/kg) followed 30 minutes later by *i.p.* administration of diazepam (25 mg/kg) as used in [35]. The onset of sleep was determined as the time lapse between last drug administration and the total loss of straightening reflex. In contrast, sleep duration was taken as the time lapse between the total loss and full return of the straightening reflex in the test animals.

2.2.2.4 Evaluation of the cognitive effect of acute administration of aqueous methanol T. globiferus leaf extract

Novel object recognition test (NORT) was used to evaluate the effect of acute administration of the leaf extract on the short-term memory in mice according to the method previously used by Hashemi-Firouzi et al. [38].

Briefly, the first day of the 2-day test was mainly to habituate test animals to the test environment and tool. For this habituation exercise, the mice were each made to spend 10 minutes in an open field test device and allowed to freely explore the environment. The following day, each mouse was again re-introduced into the test arena, but this time with two similar white plastic bottles placed at the opposite corners of the open field. The mice were allowed to freely explore the test environment for 10 minutes - with only those which explored both objects for a minimum of 20 seconds of the test period deemed to have passed the test, and therefore selected for the main test.

Plate 2. Beam balance for motor coordination and balance

Successful mice were after that randomised into groups (n=10), one of which was administered *i.p.* injections of aqua (10 ml/kg), extract (150, 500 or 1500 mg/kg) or diazepam (1 mg/kg) as used in [39]. Forty minutes post-treatment and 4 hours post-retention, they were each subjected to a 5-minute recognition test again, but this time around with one of the white plastic bottles replaced with a green bottle They were allowed to freely explore their environment and the times spent exploring the objects were recorded.

Recognition or preference index (d3) = [b/e2] X 100. Where b = time spent by the mice exploring the new object and e = time spent by the mice on both new and old objects.

2.3 Statistical Analysis

IBM SPSS version 2.0 was used for data analysis. All data from the experiments were entered as means ± S.E.M. and analysed using analysis of variance (ANOVA) followed by Turkey post hoc test. *P*-values less than 0.05 were considered significant.

3. RESULTS

3.1 The Effect of Acute Administrations of Aqueous Methanol *T. globiferus* Leaf Extract on Spontaneous Locomotion in Mice

Acute administrations of aqueous methanol *T. globiferus* leaf extract did not cause any significant (p>0.05) change in the spontaneous locomotor activities of the extract-treated compared with aqua-treated mice. The diazepam-treated mice traveled the shortest in the open-field test (Table 1).

3.2 The Effect of Acute Administrations of Aqueous Methanol *T. globiferus* Leaf Extract on Motor Coordination and Balance

Acute treatment with the extract at all tested doses, including the highest dose of 1500 mg/kg with the mean score of (4.60±0.27), caused no significant (p>0.05), but diazepam treatment (1.90±0.42) caused a significant (*p*<0.05) reduction in the mean scores in the mice compared to aqua treatment (5.00±0.00) (Table 2).

3.3 The Hypnotic Effect of Acute Administrations of Aqueous Methanol *T. globiferus* Leaf Extract

Acute treatments with high graded doses of the leaf extract did not significantly (p>0.05) alter diazepam sleep induction or duration of the extract-treated mice compared to the aqua group (Table 3).

3.4 The Cognitive Effect of Acute Administrations of Aqueous Methanol *T. globiferus* Leaf Extract

Acute doses of the extract exhibited dose-dependent increases in the recognition indices of extract-treated mice – with the highest dose producing a significant (*p*<0.05) recognition index when compared to aqua- and diazepam-treated mouse groups (Table 4).

4. DISCUSSION

The neurobehavioural end-points evaluated in this study i.e. spontaneous locomotor activity suppression, cognitive, sedative (myorelaxant) and hypnotic effects are all gamma-aminobutyric acid (GABA)A receptor-linked CNS benzodiazepine effects [40], which, in addition to tolerance and addiction liabilities, have limited the long-term clinical benefits accruable from this otherwise effective anxiolytic class.

Spontaneous locomotor (SLA) and exploratory activities are natural rodent behaviours when exposed to a novel environment [41]. Inhibition of SLA is an undesirable adverse effect of the benzodiazepines resulting from their activation of the GABAA α1-mediated sedation and α2-linked myorelaxation [42]. In this study, while the diazepam-treated mice exhibited reduced horizontal SLA, the extract-treated did not differ significantly (*p*>0.05) from the aqua-treated controls on this activity – thus, suggesting extract administration did not cause any significant SLA suppression in the experimental animals.

Motor coordination and balance is another rodent behavioural parameter that has been shown to vary according to their degree of sedation and myorelaxation [43]. And the Beam walking assay adopted in this study, has been reported to be a reliable test to measure the sedative effect of drugs on motor coordination in rodents [42]. This assay has also been shown to exhibit greater

Table 1. The effect of acute administration of aqueous methanol *Tapinanthus globiferus* leaf extract (AMTG) on locomotion in mice exposed to the open-field test

Treatment groups	Mean distances traveled (cm)
Distilled water (10 ml/kg)	689.40±102.41
Diazepam (1 mg/kg)	478.80±107.24
AMTG (150 mg/kg)	769.00±144.t2
AMTG (500 mg/kg)	521.90±120.83
AMTG (1500 mg/kg)	549.60±71.39

*Data were entered as mean ± S.E.M. * Statistically significant (p < 0.05)*

Table 2. The effect of acute administration of graded doses of aqueous methanol *T. globiferus* leaf extract (AMTG) on motor coordination and balance in mice

Treatment groups	Mean performances
Distilled water(10 ml/kg)	5.00±0.00*
Diazepam (2 mg/kg)	1.90±0.42
AMTG (150 mg/kg)	5.00±0.00*
AMTG (500 mg/kg)	4.60±0.27*
AMTG (1500 mg/kg)	4.60±0.27*

*Data were entered as mean ± S.E.M. * Statistically significant (p < 0.05)*

Table 3. The effect of acute administration of graded doses of aqueous methanol *T. globiferus* leaf extract (AMTG) on mean sleep latencies and durations in mice

Treatment groups	Sleep onset (seconds)	Sleep duration (minutes)
Distilled water(10 ml/kg)	131.80±16.23	255.78±30.99
AMTG (150 mg/kg)	136.40±25.22	357.10±29.50
AMTG (500 mg/kg)	103.10±15.08	343.80±34.56
AMTG (1500 mg/kg)	138.80±22.57	321.30±4.68

*Data were entered as mean ± S.E.M. * Statistically significant (p < 0.05)*

Table 4. The cognitive effect of acute administration of graded doses of aqueous methanol *T. globiferus* leaf extract (AMTG) in mice on the novel object recognition test

Experimental groups	Mean recognition index (%)
Distilled water (10 ml/kg)	61.06±2.91
AMTG (150 mg/kg)	63.28±5.63
AMTG (500 mg/kg)	65.63±4.12
AMTG (1500 mg/kg)	69.18±3.69 *
Diazepam (1 mg/kg)	51.54±4.03

*Data were entered as mean ± S.E.M. * Statistically significant (p < 0.05)*

sensitivity and stronger predictive ability of clinically sedative doses of benzodiazepines than, and so viewed as an improvement over, the rotarod test that has been hitherto conventionally used for the assessment of this parameter [36].

Again, the results from this assay indicate there was no significant ($p>0.05$) difference amongst the aqua- and extract-treated mice – as there was no manifestation of suppression of motor coordination and balance across the graded extract dose levels. However, there was a significant ($p<0.05$) suppression of this parameter in the 2 mg/kg diazepam-treated group which exhibited a low mean performance score of 1.90±0.42 compared to 4.60±0.27 score by the mice treated with the highest (1500 mg/kg) extract dose and 5.00±0.00 score by the aqua-treated mice.

These findings indicate that the aqueous methanol *Tapinanthus globiferus* leaf extract is not likely to have a depressant effect on locomotion, motor coordination and balance. These findings are similar to those obtained from a related study whereby extracts of *Tapinanthus globiferus* from different host trees were found not to have any significant suppressant effect on

these same behavioural parameters [24,44]. Besides, the African mistletoes (*Tapinanthus globiferus* and related species) have been previously reported to ameliorate cholesterol-induced motor deficits in mice [45]. This is in clear contrast to the overt locomotor suppressant effect of diazepam (a classical benzodiazepine anxiolytic) reported in previous animal studies [46,47].

Hypnosis is an extension of GABAA α1-mediated sedation, often seen as another unwanted effect associated with benzodiazepine use [42]. The results of the diazepam sleeping time test indicate the leaf extract at all doses used only caused an insignificant (p>0.05) shortening of sleep onset at the second-highest dose and a marginal dose-independent potentiation of sleep duration in the experimental animals. It is worthy to note *Tapinanthus globiferus* investigated in this study is one of the numerous species of the African mistletoes traditionally used to treat insomnia [48]. Hypnotic activity may be an asset to its anti-insomnia effect. Already, hypnotic effects in chicks and mice have been previously demonstrated for an aqueous residue fraction of *Tapinanthus globiferus* harvested from Ficus gum [49].

The results of the cognitive impact of the extract show a dose-dependent enhancement rather than inhibition of object recognition in the experimental animals with the highest (1500 mg/kg) extract dose, causing a significant (p<0.05) increase in recognition index compared to diazepam and aqua treatments. Similar short-term memory enhancement by *Tapinanthus globiferus* and related mistletoes has been previously reported in animal studies [50,51]. This positive cognitive effect is thought to be related to the anti-oxidant, brain-derived neurotrophic factor-enhancing, and neuroprotective activity of the *Tapinanthus* species [52,53]. These findings may justify the traditional use of this plant for memory enhancement [54,55].

5. CONCLUSION

This study has shown aqueous methanol *Tapinanthus globiferus* leaf extract is largely devoid of the benzodiazepine-related neurobehavioural adverse effects and may enhance short-term memory in mice. There is a need, however, to determine the presence or lack of tolerance and addition liabilities in this medicinal plant in a subsequent study.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal ethics committee approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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