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Anxiolytic Mechanism(S) and Corticosterone-Attenuating Effect of Hydroalcoholic Leaf Extract of *Tapinanthus globiferus* Mistletoe Growing on *Azadirachta indica* Tree

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Authors' contributions

Both authors AMU and MGM designed the study. Author AMU carried out the experiments, analyzed the data, interpreted results and drafted the first manuscript. Author MGM did the manuscript proofreading. Both authors read and approved the final manuscript.

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ABSTRACT

Similar pharmacodynamic mechanism(s) often underlie drug actions and toxicities of anxiolytic agents and medicinal extracts. Extracts of *Tapinanthus globiferus* and related plant species have been reported with anxiolytic activities. But mechanistic evaluations on these plant extracts are few. This study investigated the anxiolytic mechanism(s), including the corticosterone-attenuating effect, of hydroalcoholic *Tapinanthus globiferus* (HATG) leaf extract harvested from *Azadirachta indica* host tree in the mouse elevated zero-maze and restraint-induced acute stress paradigms using per cent open segment time (%OST) and brain/plasma corticosterone levels as endpoints, respectively. The results show that anxiolytic activity (%OST) of 150 mg/kg HATG leaf extract was reversed by pretreatment with 5 mg/kg caffeine (HATG alone, 10.90±1.73;HATG+Caffeine,

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8.66±1.74), 2 mg/kg methysergide (MTD) (HATG alone, 98.70±14.98; HATG+MTD, 74.20±10.82) and 5 mg yohimbine (HATG alone, 120.10±10.72; HATG+Yohimine, 78.44±13.92) but not by 0.5 m/kg atropine (HATG alone, 104.60±25.31; HATG+Atropine, 105.40±11.85), 0.5 mg/kg flumazenil (HATG alone,80.27±9.69; HATG+Flumazenil, 80.75±10.19), 2 mg/kg cyproheptadine (HATG alone, 88.67±16.44; HATG+Cyproheptadine, 92.11±12.58), 0.2 mg/kg haloperidol (HATG alone, 74.11±17.33; HATG+Haloperidol, 94.00±32.54) and 5 mg/kg naloxone (HATG alone, 94.30±10.84; HATG+Naloxone, 95.30±6.86). The results also indicate HATG leaf extract (at 50, 150, 500 and 1500 mg/kg) caused largely dose-dependent and significant (p<0.05) attenuations in brain/plasma corticosterone levels (5.64±0.66/3.91±0.44,3.78±0.39/3.39±0.38, 4.26±0.34/3.22±0.18 and 2.74±0.51/2.74±0.22), respectively, in extract- compared to distilled water- (5.93±0.60/4.56±0.37) and diazepam-treated (2.34±0.19/2.44±0.29) mice subjected to restraint-induced acute stress. These findings suggest anxiolytic mechanism(s) of the extract may involve its interactions with the adenosine, non-5HT2 serotonin, alpha (α)2 receptors and the hypothalamus-pituitary-adrenal (HPA) axis. This study may constitute the first mechanistic and corticosterone modulation report on the extracts of this parasitic medicinal plant and may benefit from confirmatory radio-labelled binding assays in subsequent studies.

Keywords: Cyproheptadine; HPA axis; methanol; methysergide; mice; restraint-induced acute stress.

1. INTRODUCTION

Anxiolytic activity and adverse central nervous (CNS) drug reactions of the various anti-anxiety agents are often closely linked to their mechanism(s) of action. For instance, the incidence of sedation, ataxia, amnesia, myorelaxation and addiction liability that is seen with the benzodiazepine use and the agitation, ataxia, euphoria, dysarthria etc. observed with the anticonvulsant anxiolytics (Gabapentin, Pregabalin) are intricately related to their interactions with the GABAA receptor complex. [1,2,3,4,5,6]. Similarly, the postural hypotension, extrapyramidal effects, weight gain and sexual dysfunction reported for the anti-psychotic anxiolytics (Olanzapine, Risperidone) and insomnia, akathisia, agitation and cardiotoxicity for the serotonin reuptake inhibitors/tricyclic antidepressants are thought to result directly from their actions on specific serotonin receptors and reuptake mechanisms [7,8,9,10,11,12]. The foregoing imperatively indicates that efforts at discovering new additional anxiolytic agents should be extended to the decipherment of their probable mechanism(s) of action to gain insight to their potential therapeutic advantage or liability vis-à-vis the existing anxiolytics. Also, mechanistic probes of newly discovered anti-anxiety agents are justified on past drug discovery efforts often coming up with putative chemical compounds which not only attained anxiolytic efficacy through novel anxiety neuroreceptors e.g. adenosine, opioid, cannabinoid, glutamate, dopamine and neuropeptide receptors outside the GABAA or serotonin neuroreceptors but also nevertheless demonstrated favourable efficacy and toxicity profiles when compared with the standard anxiolytic agents [13,14,15,16,17,18].

Tapinanthus globiferus (A.Rich.) Thiegh. (synonym:*Tapinanthus globiferus* susp. bangwensis (Engl. &K.Krause) (Balle.) that is being investigated in this study is a member of *Tapinanthus*species collectively called the African mistletoes - a broad group of medicinal plants commonly seen parasitising other trees such as *Azadirachta indica* (Neem), *Acacia nilotica*, Parkiabiglobosa (Shea Butter), Rubber, and Cocoa in different parts of Nigeria where it is called ‘’Afomo ishana’’ and ‘’Kauchii’’, respectively, in the Southwestern and Northern parts of the country [19,20,21,22]. This plant and its congeners have been credited with ethnomedicinal efficacy for diverse diseases including nervous disorders, hypertension, diabetes mellitus, fever, cancer and epilepsy [22,23,24,25].

Anti-oxidant, hypoglycemic, hypotensive, anti-inflammatory, hepatoprotective and antimicrobial effects are among the several pharmacological activities reported for extracts from *Tapinanthus globiferus* and related species [26,27,28,29]. These biological activities are viewed to be due to the presence of chlorogenic acid, caffeic acids, gallic acid, rutin and quercetin, alkaloids, saponins, cardiac glycosides, tannins, flavonoids, terpenoids and phlobabtannins already identified in different parts of these plants [30,31,32].

Previously, aqueous crude stem bark extract of a related species, *Tapinanthus dodoneifolius* (DC) Denser has exhibited anxiolytic and antidepressant effects in mice [33] and crude methanol leaf extract of *T. globiferus* has been shown to exert an antidepressant effect in mice [34], and aqueous residue fractions of *T. globiferus* have produced significant anticonvulsant effects in rodents [35,36].

Also, hydroalcoholic and aqueous leaf extracts and fractions of *T. globiferus* under investigation have demonstrated significant anxiolytic activity in rodent in-vivo protocols [37,38,39]. Despite these anxiolytic and other CNS effects of extracts obtained from this *T. globiferus*, scientific reports on the elucidation of its anxiolytic mechanism(s), including the corticosterone modulatory effect, are scarce. Hence, this study set out to determine the probable anxiolytic mechanism(s) of its hydroalcoholic extract behaviourally, by the use of the mouse elevated zero maze test and biochemically, by its corticosterone-attenuating effect in mice subjected to restraint-induced acute stress.

Rodent experimental anxiety paradigms are reputed for strong predictive translational validity for both human anxiety and pharmacological evaluation of anxiolytic activity/mechanism(s) of novel and known anxiolytic compounds owing to the similarity in the neurocircuits underlying stress response in both rodents and humans [40,41,42]. The elevated zero-maze paradigm used in the present study is well-validated for mechanistic evaluation of anxiolytic agents; combining the non-noxious, simple, inexpensive, rapid and sensitive operational principle of the commonly used elevated plus-maze test with the added advantages of absence of a confounding central square ambiguity present in the latter test and the provision of an un-interrupted runway [43,44].

The assessment of the attenuating effect of the *T. globiferus* leaf extract on the corticosterone levels of acutely stressed mice is an indirect but reliable probe of the possible interaction(s) between the extract molecules and the hypothalamus-pituitary-adrenal (HPA) axis which is primarily responsible for the neuroendocrine stress response [45,46]. Studies have also previously reported some drugs and medicinal plant extracts exerting anxiolytic action by their modulatory effect on brain and serum corticosterone levels [47,48,49].

The aim of this study, therefore, is to determine the probable mechanism(s) of anxiolytic action of hydroalcoholic *Tapinanthus globiferus* leaf extract in mice using the elevated zero-maze and corticosterone modulation paradigms.

2. MATERIALS AND METHODS

2.1 Drugs and Reagents

Diazepam and atropine injections (Roche), flumazenil, naloxone, caffeine and haloperidol injections (Ranbaxy Pharmaceuticals), cyproheptadine tablets (Fidson, Nigeria Ltd), yohimbine (Sigma Aldrich) and methysergide (Sigma Aldrich) were sourced from the Department of Pharmacology & Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Enzyme-Linked Immunosorbent Assay kits for corticosterone concentration determination was purchased from Koon Coon Biotech Co. limited, Shanghai (ref: CK-bio15948).

2.2 Experimental Animals

Male Swiss Albino mice obtained from the animal house of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria were used for the study. They were kept in home cages (10-15 per cage) under good laboratory practices with free access to food and water under 12-hour dark/light environmental conditions for 2 weeks before the behavioural experimentation.

2.3 Plant Extract

Fresh leaves of *T. globiferus* growing on *Azadirachta indica* tree located along Shuni road, Mabera, Sokoto; Sokoto State, Nigeria, were collected in March 2019. They were then briskly washed, dried under a shade and ground into a powder. Two hundred and fifty grams (250 g) of the fine powder was soaked and allowed to macerate in 1 L of 70% methanol for 24 hours, then filtered using Whatman's paper (150 mm) and evaporated in a rotatory water bath at 45-50 degree Celsius to yield 31.45 g of brownish-green paste.

2.4 Behavioural Studies

Determination of anxiolytic mechanism(s) of hydroalcoholic *T. globiferus* extract was done by the reversal, or not, of the anxiolytic activity (per

cent open segment time, % OST)in groups (n=8 or 10) of mice exposed to the elevated zero-maze 45-minute following the administration of the extract (150 mg/kg) (optimum anxiolytic dose) which was pretreated 15-minute earlier with a sham injection of distilled water or the antagonists to the different receptors putatively involved in this activity. Thus, the involvement of GABA-A receptor was evaluated by pretreatment with flumazenil (0.5 mg/kg) according to the method previously used in [50], adenosine (A1/A2) receptors by pretreatment with caffeine (5 mg/kg) according to [51], central muscarinic receptors involvement by atropine (0.5 mg/kg) pretreatment as in [52] and opioid (μ) receptors involvement evaluated by pretreatment with naloxone (5 mg/kg)according to [53]. Others neuroreceptors evaluated for included pan-serotonin receptors by pretreatment with MTD, 2 mg/kg according to the procedure previously adopted in [54], 5-HT2 serotonin receptor involvement by cyproheptadine (CHTD, 2 mg/kg) pretreatment as in [55] with slight modifications, dopamine (D2) receptor involvement by pretreatment with haloperidol (HLD, 0.1 mg/kg) as in [56] with minor modifications, and involvement of alpha- (α)-2 adrenergic receptors by yohimbine (1 mg/kg) pretreatment as in [56].

The modulatory effect of the plant extract on plasma/brain corticosterone was determined in groups (n=8) of male mice subjected to immobilization-induced acute stress according to a method previously used in [47] with slight modifications. Briefly, mice were randomly selected into groups one of which was treated with distilled water (10 ml/kg), extract (50, 150, 500, 1500 mg/kg) or diazepam (1 mg/kg), intraperitoneally. Forty-five minutes later, they were each subjected to acute stress by being restrained within a slit PVC plastic pipe (5.0 X2.5 cm) strapped to a flat surface. At the end of the test period, each mouse was sacrificed by cervical dislocation. The blood and brain were harvested for further processing for the determination of their corticosterone concentrations by an ELIZA-based procedure.

3. RESULTS

3.1 Determination of Mechanism(s) of the Anxiolytic Activity of Hydroalcoholic *T. globiferus* Leaf Extract

Anxiolytic activity of the leaf extract (150 mg/kg) in the experimental mice was reversed by caffeine, yohimbine and MTD but not by flumazenil, naloxone, haloperidol, cyproheptadine and atropine pretreatments (Table 1).

Compared to distilled water-treated controls, both serum and brain corticosterone levels were dose-dependently attenuated by acute administration of the leaf extract, the highest doses of which significantly (p<0.05) achieved a corticosterone attenuating effect comparable with that of 1 mg/kg diazepam dose (Table 2).

These findings indicate the observed anxiolytic activity of the leaf extract may involve its interactions with the adenosine, non-5HT2 serotonin and α-2 adrenergic receptors and HPA axis but may not involve the GABA-A, serotonin subtype 2 (5-HT2), dopamine subtype 2 (D2), central muscarinic and opioid receptors.

4. DISCUSSION

The results of the investigation of the anxiolytic mechanism(s) of action of hydroalcoholic *Tapinanthus globiferus* leaf extract from both the behavioural and biochemical assays indicate its anxiolytic activity may involve the interactions of its molecules with the adenosine, non-5HT2 serotonin and alpha (α)2 adrenergic receptors and the hypothalamus-pituitary-adrenal (HPA) axis but may not involve its interactions with the GABA-A, serotonin subtype 2 (5-HT2), dopamine subtype 2 (D2), central muscarinic and opioid receptors. This study may be the first report of an extract from *Tapinanthus globiferus* growing on *Azadirachta indica* exerting an anxiolytic action probably through its modulatory interactions with these CNS neurotransmitter systems and the HPA axis.

Adenosine is a universal nucleoside in the CNS that is involved in the regulation of neural excitability, the function of several ion channels and release of other neurotransmitters via its G-protein-coupled receptors [57,58]. The important contribution of adenosinergic neurotransmission to anxiety pathogenesis is illustrated by studies showing genetic adenosine A-2A receptor deficiency and single nucleotide polymorphisms in the A-2A receptor gene being associated with increased anxiogenesis in mice [59,60]. The finding of a possible role for adenosine neurotransmission in the anxiolytic activity of HATG leaf extract agrees with some previous studies implicating adenosine and its receptors in the anxiolytic activity of extracts of Arillus of *Euphoria longana* [61], *Ziziphus spinosa* and *Magnolia officinalis* [62].

Table 1. Determination of probable anxiolytic mechanism(s) of hydroalcoholic *tapinanthus globiferus* leaf extract based on itsanxiolytic activity reversal (per cent open segment time) in mice pretreated with various central nervous system antagonists

Treatment groups	GABAA receptor	Adenosine receptor	Muscarinic receptor	Pan-serotonin receptors	5HT2 serotonin receptor	Dopamine (D2) receptor	Opioid(μ) receptor	Alpha (α)2adrenoceptors
Distil. water	39.80±8.20	4.00±1.34	45.70±11.34	39.20±4.88	47.67±12.70	27.55±4.94	44.00±4.35	46.10±7.28
CNS receptor antagonist	Flumazenil 24.70±4.27	Caffeine 8.10±2.18	Atropine 64.70±8.25	Methysergide 44.60±10.15	Cyproheptadine 27.56±4.94	Haloperidol 47.66±12.70	Naloxone 44.80±6.14	Yohimbine 59.00±7.89
HATG	80.27±9.69*	10.90±1.73*	104.60±25.31*	98.70±14.98*	88.67±16.44*	74.11±17.33*	94.30±10.84*	120.10±10.72*
HATG+CNS receptor antagonist	80.75±10.19*	8.66±1.74*	105.40±11.85*	74.20±10.82	92.11±12.58*	94.00±32.54*	95.30±6.86*	78.44±13.92

*Data were entered as mean ± S.E.M. of mice (n=10 or 8) and analysed using the One-way ANOVA. *Statistically significant (p<0.05). HATG = 70% methanol T. globiferus, + = pretreated with.CNS = central nervous system, Distil. = distilled*

Table 2. Effect of acute doses of hydroalcoholic *tapinanthus globiferus* leaf extract on serum and brain corticosterone levels in mice

Sample	Serum corticosterone concentrations (ng/ml)	Brain corticosterone concentrations (ng/ml)
Distilled water (10 ml/kg)	5.93±0.60	4.56±0.37
Diazepam (1 mg/kg)	2.34±0.19*	2.44±0.29*
HATG (50 mg/kg)	5.64±0.66	3.91±0.44
HATG (150 mg/kg)	3.78±0.39*	3.39±0.38
HATG (500 mg/kg)	4.26±0.34	3.22±0.18*
HATG (1500 mg/kg)	2.74±0.51*	2.74±0.22*

*Values were expressed as mean ± S.E.M of mice (n =8, 7, 6) and analysed using the One-way ANOVA. *Statistically significant (p ≤0.05). HATG, 70% methanol T.globiferus leaf extract*

The inference that serotonin (non-5HT2) receptors may be involved in the anxiolytic mechanism(s) of HATG leaf extract is premised on an initial reversal of its activity by methysergide pretreatment (pan-serotonin receptor blockade), followed by a failure of reversal of the same by cyproheptadine pretreatment (5HT2 blockade). This suggests that 5HT2 receptor subtypes A, B, and C are not likely involved in the anxiolytic activity of HATG leaf extract. Studies have shown that the serotonin receptor subtypes with significant roles in anxiety neurotransmission and the most abundant CNS serotonin receptors are 5HT1A, 5HT2A and 5HT2C [63,64,65]. If these receptors are agreed to be the most abundant and the only significantly involved in anxiety neurotransmission of all the serotonin receptor subtypes in the brain; and in this study, 5HT2A and 5HT2C have been shown not to contribute to the anxiolytic activity of the extract by the demonstration of cyproheptadine (a selective 5-HT2 blocker) failing to reverse/reduce its anxiolytic activity. It is, therefore, reasonable to attribute the portion of the overall anxiolytic activity of HATG leaf extract due to the serotonin receptors which was not blocked by cyproheptadine pretreatment to 5HT1A. Thus, 5HT1A neurotransmitter system may be involved in its anxiolytic mechanism (s). In that case, this finding will be in agreement with previous studies whereby quercitrin from *Albizia julibrissin* and essential oil from *Citrus aurantium* L. were found to exert their anxiolytic activities through agonist action on 5-HT1A receptor [66,67]. However, confirmatory studies involving the use of selective 5HT1A full agonist e.g. 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (8-OH-DPAT) or partial agonist e.g. buspirone, and antagonists e.g. NAN-190, WAY-135 or pindolol with appropriate receptor-ligand binding assays will be useful in fine-tuning these findings and generating more specific results.

The likelihood of the involvement of alpha (α)2 adrenergic neurotransmitters in the anxiolytic activity of HATG leaf is based on the partial reversal of its activity by pretreatment with yohimbine - a selective α2 adrenoceptor antagonist. Similar findings have been reported for known and putative anxiolytic agents whose mechanism (s) of action was shown to involve alpha (α)2 adrenergic neurotransmission [55,56,68]. Again, it is desirable to further probe this finding by relevant competitive radio-ligand displacement studies involving yohimbine and HATG molecules on the α-2 adrenoceptor.

Our findings also show HATG leaf extract largely dose-dependently attenuated acute stress-induced rise in serum and brain corticosterone levels in the experimental animals in such a manner that the highest extract dose (1500 mg/kg) produced a significant (p<0.05) attenuating effect that was comparable to diazepam (1 mg/kg) treatment.

Research has shown the HPA axis, in both animals and humans, is induced into hyperactivity on exposure to stressful or anxiogenic stimuli and that acute stress-induced corticosterone release is largely under the control of the HPA axis whose activity is in turn regulated by the corticotrophin-releasing factor [69,70]. Studies have also previously reported some medicinal plant extracts and drugs exert anxiolytic action by their modulatory effect on brain and serum corticosterone levels [47,48,49]. Thus, the dose-dependent attenuations of serum and brain corticosterone levels by the extract of this study is similar to the findings of these earlier studies and may be a pointer to the probable interaction *Tapianthus globiferus* leaf extract with the HPA axis. However, further studies based on behavioural and ligand-binding assays between selective corticotropin-releasing factor receptor subtype-1 (CRF-1) agonists e.g. stressin or

bovine cortagine and CRF-1 antagonists e.g. antalarmin or CP-154.526 will be useful to specifically confirm the involvement of the corticotropin neurotransmission in the mechanism(s) of anxiolytic activity of HATG leaf extracts.

5. CONCLUSION

Anxiolytic activity of hydroalcoholic leaf extract of *Tapinanthus globiferus* growing on *Azadirachta indica* tree may involve its modulation of the adenosinergic, the alpha (α)2adrenergic, 5HT1A serotonergic neurotransmissions and the HPA axis activity. These findings may represent the first scientific report on this medicinal plant exerting its anxiolytic activity through these neurotransmitter systems. Radio-labelled receptor binding assays will be useful to confirm the involvement of these receptors in the anxiolytic mechanism (s) of the leaf extract.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethics committee approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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