Anxiolytic Mechanism(s) and Corticosterone-attenuating Effect of Hydroalcoholic Leaf Extract of *Tapinanthus globiferus* Mistletoe growing on *Azadirachta indica* Tree

ABSTRACT

Similar pharmacodynamic mechanism(s) often underlie the efficacy and toxicities of anxiolytic drugs 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) and 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. Anxiolytic activity (%OST) of 150 mg/kg HATG leaf extract was reversed by 5 mg/kg caffeine, 2 mg/kg methysergide (MTD) and 5 mg yohimbine but not by 0.5 m/kg atropine, 0.5 mg/kg flumazenil, 2 mg/kg cyproheptadine, 0.2 mg/kg haloperidol and 5 mg/kg naloxone pretreatments. HATG leaf extract (50, 150, 500, 1500 mg/kg) also dose-dependently and significantly (p<0.05) attenuated acute restraint-induced rise in both brain and plasma corticosterone levels in the experimental animals. 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 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

1.0 INTRODUCTION

Anxiolytic activity and adverse central nervous (CNS) drug reactions of 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 intolerable adverse effects of postural hypotension, extrapyramidal effects, weight gain and sexual dysfunction reported for the anti-psychotic anxiolytics (Olanzapine, Risperidone) and of insomnia, akathisia, agitation and cardiotoxicity for the serotonin reuptake inhibitors/tricyclic antidepressants are thought to result directly from their actions on specific serotonin reuptake and receptor systems [7, 8, 9, 10, 11, 12]. The foregoing imperatively indicates efforts at discovering new additional anxiolytic agents should be extended

to the decipherment of their probable mechanism(s) of action to gain insight to the potential therapeutic advantage or liability inherent in their mechanism(s) of action over the existing anxiolytics. Hence, this study set out to investigate the probable mechanism(s) of action of anxiolytic activity earlier reported for *Tapinanthus globiferus* hydroalcoholic leaf extract and its fractions using rodent in-vivo protocols [13, 14, 15].

It is worthy to note some of the past drug discovery efforts have come up with putative chemical compounds which attained anxiolytic efficacy through novel neuronal pathways e.g. adenosinergic [16], opioidergic [17], cannabinoidergic [18], glutamatergic]16], dopaminergic [20] and neuropeptidergic [21] outside the GABAAergic or serotoninergic neurotransmission but which nevertheless demonstrated favourable efficacy and toxicity profiles when compared with the standard anxiolytic agents. This scenario also points to the necessity to probe anxiolytic mechanism(s) of putative new agents beyond these traditional anxiety pathways.

Although anxiolytic/antidepressant activity has been reported for extracts of *Tapinanthus globiferus* and related plant species [22, 23]; and anxiolytic action linked to corticosterone modulation has also been reported for some other non-*Tapinanthus* medicinal plants [24, 25] in animal studies, there has not been, within available literature, any report on the scientific elucidation of the anxiolytic mechanism(s) of the leaf extract of this plant by the use of in-*vivo* behavioural and biochemical (corticosterone modulation) endpoints.

Thie 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. While the former test is reliable and well-validated for anxiolytic activity evaluation and viewed to be an advancement over the popular elevated plus-maze paradigm [26, 27], the latter is a reliable test for both stress-induced alterations in, and the attenuating effect of plant extracts on, corticosterone levels [28].

2.0 Materials and Methods

Drugs and reagents

Drugs such as 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).

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.

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 (250g) 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.

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 preceded 15-minute earlier by pretreatment with 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 [29], adenosine (A1/A2) receptors by pretreatment with caffeine (5 mg/kg) according to [30], central muscarinic receptors involvement by atropine (0.5 mg/kg) pretreatment as in [31] and opioid (μ) receptors involvement evaluated by pretreatment with naloxone (5 mg/kg) according to [32]. Others neuroreceptors evaluated for included pan-serotonin receptors by pretreatment with MTD, 2 mg/kg according to the procedure previously adopted in [33], 5-HT2 serotonin receptor involvement by

cyproheptadine (CHTD, 2 mg/kg) pretreatment as in [34] with slight modifications, dopamine (D2) receptor involvement by pretreatment with haloperidol (HLD, 0.1 mg/kg) as in [35] - with minor modifications, and involvement of alpha- (α)-2 adrenergic receptors by yohimbine (1 mg/kg) pretreatment as in [35].

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 [28] 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.

3.0 Results

Determination of mechanism(s) of 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 (Table1).

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.

Table 1: Determination of probable mechanism(s) of anxiolytic activity of hydroalcoholic *T. globiferus* leaf extract using a reversal of anxiolytic activity (per cent open segment time) of 150 mg/kg leaf extract dose in mice pretreated with various CNS antagonists

Treatment groups	$GABA_A$ receptor	Adenosine receptor	Muscarinic receptor	Pan-serotonin receptors	5HT2 serotonin receptor	Dopamine (D2) receptor	Opioid(μ) receptor	Alpha (α)2 adrenoceptors
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 *T. 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 ($p \leq 0.05$). HATG, 70% methanol *T. globiferus* leaf extract.

4.0 Discussion

This study investigated the probable mechanism (s) of anxiolytic activity of HATG leaf extract by the use of the mouse elevated zero-maze test and physio-biochemical assay using perturbation of the mouse serum corticosterone levels by restraint-induced acute stress. The elevated zero-maze paradigm is well validated for detecting both anxiolytic activity and mechanism (s) of action of known and putative anxiolytic agents [26, 36]. Also, studies have reported modulation of plasma corticosterone levels by known and putative anxiolytic agents as a reliable but indirect assessment of a possible interaction between anxiolytic agents and the hypothalamus-pituitary-adrenal (HPA) axis [25, 35].

The results of both the behavioural and biochemical assays indicate the anxiolytic activity of the leaf extract of HATG may involve its interactions with the adenosine, non-5HT2 serotonin and alpha (α)2 adrenergic receptors and the hypothalamus-pituitary-adrenal (HPA) axis but may not involve 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 modulates neural excitability, the function of several ion channels and release of other neurotransmitters via its G-protein-coupled receptors [37, 38]. 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 [39, 40]. 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* [41], *Ziziphus spinosa* and *Magnolia officinalis* [42].

The inference that serotonin (non-5HT$_2$) receptors may be involved in the anxiolytic mechanism(s) of HATG leaf extract is premised on an initial reversal of its anxiolytic activity by methysergide pretreatment (pan-serotonin receptor blockade), followed by a failure of reversal of

the same anxiolytic activity 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 [43, 44, 45]. 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 total 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). 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 anxiolytic 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 [34, 35, 46]. 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 [47, 48]. Studies have also previously reported some medicinal plant extracts and drugs exert anxiolytic action by their modulatory effect on brain and serum corticosterone levels [24, 25, 28]. Thus, the dose-dependent attenuations of serum and brain corticosterone levels by the extract of this study may be a pointer to its probable interaction 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.

5.0 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)2 adrenergic, 5HT1A serotonergic neurotransmissions and the HPA axis activity. Radio-labelled receptor binding assays will be useful to confirm the involvement of these neurotransmitter systems in the anxiolytic mechanism (s) of the leaf extract.

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Both serum and brain corticosterone levels were dose-dependently attenuated by acute methanol T. globiferus leaf extract treatments with the highest extract's doses significantly (p

Levels of glucocorticoids (cortisol/corticosterone) in the body have been shown to increase proportionally to the degree of dysregulation directly

The finding of MTG leaf extract largely dose-dependently attenuating serum and brain corticosterone levels with the highest extract dose (1500 mg/kg) producing significant (pattenuating effects (compared with distilled water treatment) that approximated those by 1.0 mg/kg diazepam.

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 activity (Aguilera, 1998; Tsigos and Chrousos, 2002; Papadimitriou and Priftis, 2009; Glover et al., 2010). Studies have also previously reported a number of medicinal plant extracts and known drugs to exert anxiolytic action by their modulatory effect on brain and serum corticosterone levels (Sheikh et al., 2007, Hlavacova et al., 2010; Shi et al., 2014). Thus, the dose-dependent attenuations of serum and brain corticosterone levels by the extract of this study may be a pointer to its probable interaction with the HPA axis in a fashion similar to previous reports by (Pramanik et al., 2011; Barua et al., 2016)