(RESEARCH ARTICLE)

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Acute toxicity (LD$_{50}$ values) and neuropharmacological profile of n-hexane fraction of *Petiveria alliacea* L. (Phytolaccaceae) in mice

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Abstract

Background: *Petiveria alliacea* L. (Phytolaccaceae) ethnomedically is known for its sedative property and beneficial treatment of other central nervous system (CNS) disorders. However, the neuropharmacological properties of the n-hexane extract of this aromatic plant leaf have not been reported, hence this study.

Objective: To determine the acute toxicity profile (LD$_{50}$) and some CNS activities of the n-hexane extract of fresh leaf of *P. alliacea* (NHEPA) in mice.

Method: The fresh leaf of *Petiveria alliacea* was collected and the n-hexane extract obtained using appropriate technique. The LD$_{50}$ was determined for both the oral and intraperitoneal (i.p.) route. All treatments were administered via i.p. route. Behavioural activity was evaluated using the novelty-induced behaviour (NIB) to assess rearing, grooming and locomotion; sedative activity was tested on ketamine-induced hypnosis while the anticonvulsant potential was assessed using chemo-convulsants. The mechanism of action was determined using amphetamine as agonist and flumazenil as an antagonist.

Results: The LD$_{50}$ values obtained for the extract was 2450 and 346 mg/kg for oral and intraperitoneal routes respectively. The extract displayed significant CNS depressant activity on all NIB parameters. NHEPA significantly prolonged sleep latency and total sleeping time at 100 and 150 mg/kg. i.p. The extract also offered some mild protection against convulsion across the model used. The probable mechanism of action may include inhibition of monoamine pathways and augmentation of the GABAA- benzodiazepine complex pathway.

Conclusion: NHEPA showed CNS depressant activity, exhibited significant sedative activity and mild anticonvulsant activity.

Keywords: *Petiveria alliacea;* N-hexane; Central nervous system; Sedative; Anticonvulsant

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1. Introduction

Plants have long played a role in human health care, whether directly or indirectly. Plant parts such as leaves, fruits, stems, bark, roots, and sometimes the entire plant are utilized directly in the treatment of various ailments [1]. In developing countries including Nigeria, medicinal plants play a critical role in the treatment of central nervous system (CNS) diseases such as insomnia and epilepsy. Traditional African healers believe that these traditional plants are more successful in treating CNS diseases, and that they are also safer, more readily available, and less expensive [2]. Epilepsy is prevalent and burdensome in Africa, particularly Nigeria, with a systematic review and meta-analysis of community-based door-to-door surveys estimating a prevalence of 8 per 1000 individuals in Nigeria [3].

Petiveria alliacea L. in the family Phytolaccaceae is a perennial subshrub with a deep, thick taproot and strong stems that can reach a height of one meter [4]. The leaves have an odour and effect similar to onions when crushed. The Yoruba speaking community in Nigeria refer to the plant as "robbu-igbo" meaning Robb of the forest named after the Robb Balm, an anti-pain topical medication. Many clinical reports and investigations show that aerial component and root extracts have considerable broad-spectrum antibacterial activity in vitro and in vivo against a wide range of bacteria, viruses, protozoa, fungus, and yeast strains [5]. *Petiveria alliacea* root extracts were found to exert behavioural, neurological, and autonomic effects on the central nervous system [6]. In mice, Gomez *et al* [7] found that isolated fractions from the roots of *Petiveria alliacea* L. (tipi) showed substantial anticonvulsant and depressive properties.

Phytochemistry studies of *P. alliacea* indicate that essential oil (Petiverina), saponinic glycosides, isoarborinol-triterpene, isoarborinol-acetate, isoarborinol-cinnamate, steroids, alkaloids, flavonoids, and tannins are among the biologically active compounds found in this plant, with qualitative and quantitative variations depending on the region of collection and harvest season [8, 9].

Petiveria alliacea had long been used for various medicinal purposes, but there is scanty report on the central nervous system activities of the n-hexane fraction of its leaf, hence this study. The aim of this study is to determine the acute toxicity profile (LD_{50}) and some central nervous system activities of the n-hexane extract of fresh leaf of *P. alliacea* in mice.

2. Material and methods

2.1. Plant collection, identification, authentication and preparation

The fresh leaf of the plant was collected from the wild on the campus of Obafemi Awolowo University (OAU), Ile-Ife. The plant was identified and authenticated by Mr. I.I Ogunlowo, the Botanist-in-charge of the Herbarium, Faculty of Pharmacy, OAU, Ile Ife and a Voucher number **FPI-2302** was issued. The fresh leaves of the plant were separated from the stalk and dried on the laboratory table for about 4 weeks. The dried leaves were powdered with a laboratory mill and 800 g of the powdered leaf was soaked in n-hexane solution, mechanically rotated intermittently to ensure good yield and then filtered after 72 h. The filtrate was concentrated to dryness in *vacuo* using a rotary evaporator at 40°C and collected into a bottle with tight-fitting cover and kept refrigerated until use. The final weight of the semi-solid extract obtained was 4.8g (0.6%w/w). The n-hexane fraction of *Petiveria alliacea* (NHEPA) was emulsified with 5% Tween 80 and diluted with distilled water to the required concentration before administration.

2.2. Laboratory materials

2.2.1. Drugs and reagents

Ketamine HCL (RotexMedica Trittan, Germany), Diazepam (Valium(R) Roche, Switzerland), pentylenetetrazole (Sigma, USA), Strychnine (Sigma, Switzerland, MSDS), Phenobarbital sodium (Sterop, Belgium), Aminophylline (Hubei Tianyao Pharma CN, China), Chlorpromazine (Ciangsu Ruinian Cianjin Pharma, Yixing, China) Normal Saline (Unique Pharm. Nig. Ltd.), Tween 80 and other reagents were of high quality grade.

2.2.2. Laboratory animals

Adults (male and female) mice for the study weighing 18-25 g were obtained from the Animal house, Department of Pharmacology, Faculty of Pharmacy, OAU, Ile-Ife. The animals were maintained under ideal conditions (12 hours light/12 hours dark cycle, temperature of 25 ± 2 °C, 35-60% humidity) and fed with standard animal pellets and water *ad libitum*. Drugs were administered intraperitoneally (*i.p*) with the exception of toxicological study in which oral route was also used. The experimental protocols followed the internationally accepted principles for Laboratory Animal Use

and Care and in compliance with National Institute of Health NIH, 1985 as being implemented by the OAU Research Committee.

2.3. Acute toxicity (LD$_{50}$) studies

Acute toxicity (LD$_{50}$) effect of NHEPA was determined using Lorke's method [10] with some modifications via both oral and intraperitoneal routes. This model involved two phases for rapid determination of LD$_{50}$. In the first phase, three increasing doses (10, 100 and 1000 mg/kg) of the extract were administered intraperitoneally and orally to three different groups of mice (n=3). In the second phase, 6 dose levels 200, 300, 400, 600, 800 and 1000 mg/kg of the NHEPA were administered intraperitoneally to 6 groups of mice (n=1) and five dose levels (1000, 2000, 3000, 4000, 5000 mg/kg) of the extract were administered orally to 5 groups of mice (n=1). The animals were observed for immediate effects of the extract up to 60 minutes and the mortality within 24 hours of treatment was recorded.

The median lethal dose (LD$_{50}$) was calculated as the geometric mean of the lowest lethal dose that caused death and the highest non-lethal dose that did not cause death.

LD$_{50}$ = (A X B) $^{1/2}$ where A is maximum dose that resulted to 0% death and B is the minimum dose that resulted to 100% death. The LD$_{50}$ for each extracts was considered in the selection of the working doses for subsequent pharmacological studies.

2.4. The choice of route of administration

The oral route has been reported to be unpredictable due to effect of many factors including biodegradation of active components, poor bioavailability, effect of food substances etc. [11, 12]. The choice of *i.p.* route for neuropharmacological evaluation was also supported in previous study [13], hence, the *i.p.* route was chosen in this study. The working doses used in this study were 50, 100 and 150 mg/kg, *i.p.* which were lower than half of the LD$_{50}$ value estimated to be 346 mg/kg, *i.p.*

2.5. Neuropharmacological studies

2.5.1. General experimental design

Animals were randomly selected into 5 groups (n=6). Group I serve as the negative control and received the vehicle (5% Tween 80, 10 ml/kg) only. Test groups II–IV were treated with the NHEPA at doses of 50, 100 and 150mg/kg respectively, while group V (positive control) received the appropriate standard drugs. All treatments were by intraperitoneal (*i.p.*) route and each treatment was administered 30 min prior to test.

2.5.2. Effect of NHEPA on novelty-induced behavior (Open field test)

Novelty-induced behavioural activities (rearing, grooming and locomotion**)** was evaluated using open field model as described by Onigbogi *et al.* [14] and modified by Oyemitan *et al.* [15]. The open field consists of a rectangular arena with a hardboard floor divided by red permanent markers into 16 equal squares areas. Five groups of mice (n=5) were randomly selected. Group I was administered the vehicle (5% Tween 80, 10 ml/kg, *i.p.*). Groups II to IV were injected with different doses of NHEPA (50, 100 and 150 mg/kg, *i.p.*). Group V was injected with diazepam (1 mg/kg, *i.p.*) as positive control. All the mice were pre-treated for 30 min prior to test. Each animal was placed inside the observation cage and assessed for 20 mins for rearing (number of times the animal stands on its hind-limbs with fore limbs in the air or against the wall) for 20 mins, locomotion (number of squares crossed with all the fore and hind limbs over a specified period of time) for 20 mins and grooming (number of times the animal engages in body cleaning with paws, body and genital licking with the mouth and face washing actions) for 20 mins.

2.5.3. Mechanism of action of NHEPA using Agonist (Amphetamine) and Antagonist (Flumazenil) effect on NHEPA in Mice

Influence of NHEPA on the effect of amphetamine on novelty-induced behaviour in mice

The test was done to determine the influence of NHEPA (Vehicle; 5% Tween 80, 10 ml/kg, *i.p.,* NHEPA; 150mg/kg, *i.p.*, diazepam; 1mg/kg, *i.p.*, and chlorpromazine; 2 mg/kg, *i.p.*) on the effect of amphetamine 2 mg/kg, *i.p.* on novelty-induced behaviour with the sole aim of exploring the possible neurotransmitters or pathway through which NHEPA exerts its effects and probable mechanism of action. Seven groups of mice were randomly selected (n=6). Group I was administered with the vehicle (5% Tween 80, 10 ml/kg, *i.p.*), Group II was treated with NHEPA 150 mg/kg *i.p.* only, Group III was treated with diazepam 1 mg/kg *i.p.* only, Group IV was treated with Amphetamine 2 mg/kg, *i.p.* only, Group V was treated with Amphetamine 2 mg/kg, *i.p.* and NHEPA 150 mg/kg *i.p*, Group VI was treated with Amphetamine 2

mg/kg, *i.p.* and Diazepam 1 mg/kg *i.p.* and Group VII was treated with Amphetamine 2 mg/kg, *i.p* plus chlorpromazine (a known dopamine antagonist) 2mg/kg *i.p.* Pre-treatment with Amphetamine prior to test drugs administration was 15 mins *i.p.* Amphetamine was solubilized in distilled water while the NHEPA was solubilized in 5% Tween-80. Each animal was placed singly in the observation cage 30 mins after injection with the test drugs *i.p.* and observed for 20 mins.

Influence of flumazenil on the effect of NHEPA on novelty-induced behaviour in mice

The test was done to determine the influence of flumazenil 2 mg/kg, *i.p.* on the effect of test drugs (Vehicle; 5% Tween 80, 10 ml/kg, *i.p.,* NHEPA; 150 mg/kg, *i.p.*, diazepam; 1 mg/kg, *i.p.*, and chlorpromazine; 2 mg/kg, *i.p.*) on novelty-induced behaviour. Six groups of mice were randomly selected (n=6). Group I was administered with the vehicle (5% Tween 80, 10 ml/kg, *i.p.*), Group II was treated with NHEPA 150 mg/kg *i.p.* only, Group III was treated with diazepam 1 mg/kg *i.p.* only, Group IV was treated with flumazenil 2 mg/kg, *i.p.* only, Group V was treated with flumazenil 2 mg/kg, *i.p.* and NHEPA 150 mg/kg *i.p.* and Group VI was treated with flumazenil 2 mg/kg, *i.p.* and Diazepam 1 mg/kg *i.p.* Pre-treatment with flumazenil 2 mg/kg, *i.p.* prior to test drugs administration was 15 mins *i.p.* flumazenil was solubilized in distilled water while the NHEPA was solubilized in 5% Tween-80. Each animal was placed singly in the observation cage 30 mins after injection with the test drugs *i.p.* and observed for 20 mins.

2.5.4. Sedative activity

Ketamine (100 mg/kg, *i.p.*) was used to induce sleep in mice [16, 17]. Mice were divided into five groups (n=6). Group 1 was pre-treated with the vehicle (5% Tween 80, 0.1 ml/kg, *i.p.*), groups 2-4 were administered with different doses of NHEPA (50, 100 and 150 mg/kg, *i.p.*) and group 5 received diazepam (1 mg/kg) as positive control [15, 18]. Mice in all the groups were pretreated with the above test drugs 30 mins prior to administration of ketamine (100 mg/kg, *i.p.*). The animals were observed for interval between the administration of ketamine until loss of righting reflex which was recorded as onset of sleep or sleep latency (SL), while the time from the loss, to the regaining of the righting reflex was recorded as duration of sleep or total sleeping time (TST). Maximum sleeping time of 120 mins was adopted for all animals that slept over 2 hrs.

2.5.5. Effect of NHEPA on chemically induced convulsion.

The anticonvulsant activity of the NHEPA was investigated via intraperitoneal route using chemo-convulsion models.

Strychnine (STR) - induced convulsion

Strychnine (4 mg/kg, *i.p.*) was used to induce tonic-clonic convulsions [15, 19]. Mice of either sex were divided into five groups (n=6). Group 1 was pre-treated with the vehicle (5% Tween 80, 0.1 ml/kg, *i.p.*), groups 2-4 were administered with different doses of NHEPA (50, 100 and 150 mg/kg, *i.p.*) and group 5 received diazepam (5 mg/kg, *i.p.*) as positive control. All the above were administered 30 mins before strychnine 4 mg/kg, *i.p.* was given. Onsets to forelimb clonic and tonic seizures were recorded. Mice that did not convulse 30 mins after strychnine administration were considered protected [20].

Pentylenetetrazole (PTZ)-induced convulsion

Pentylenetetrazole (85 mg/kg*, i.p.*) was administered as a convulsant agent to induce tonic clonic seizure [21]. Mice of either sex were divided into five groups (n=6). Group 1 was pre-treated with the vehicle (5% Tween 80, 0.1 ml/kg, *i.p.*), groups 2-4 were administered with different doses of NHEPA (50, 100 and 150 mg/kg, *i.p.*) and group 5 received diazepam (1 mg/kg, i.p.) as positive control. All the above were administered 30 mins before 85mg/kg*, i.p.* of PTZ was given [15, 22]. Onsets to forelimb clonic and tonic seizures were recorded. Mice that survived beyond 30 mins after PTZ injection will be considered protected in this model [23].

Aminophylline-induced convulsion tests

Aminophylline (300 mg/kg, *i.p*) was admnistered as a convulsant agent to induce tonic clonic [24, 25]. Mice of either sex were divided into five groups (n=6). Group 1 was pre-treated with the vehicle (5% Tween 80, 0.1 ml/kg, *i.p.*), groups 2-4 were administered with different doses of NHEPA (50, 100 and 150 mg/kg, *i.p.*) and group 5 received phenobarbitone (30 mg/kg, *i.p*) as positive control. All the above were administered 30 mins before 300 mg/kg, *i.p.* aminophylline was given [25]. Each mouse in each group will be individually monitored for latency of convulsions. Mice that survived beyond 30 mins after aminophylline injection will be considered protected in this model.

2.6. Statistical analysis

The results were expressed as Mean ± SEM and analysed using one-way analysis of variance (ANOVA) followed by post hoc test using Dunnett's comparison test. The results of the influence of agonist and antagonist on the effect of NHEPA were analyzed with ANOVA, followed by Student–Newman-Keuls post hoc test. Student-Newman-Keuls test. Graph pad Instat R version 3.0. 10.0 (UK) copyright © 2013 by Graph Pad Software Inc. was used to analyse the one-way ANOVA result while graph pad prism version 5.0. 3.0 (US) copyright © 2010 was used to plot graphs. The level of significance was set at 95% confidence interval at $p < 0.05$ for all treatment carried out compared to control groups.

3. Results

## 3.1. Acute toxicity (LD_{50}) studies

In the oral route, the lowest dose of NHEPA that produced mortality was 3000 mg/kg while the highest dose without mortality was 2000 mg/kg. In the intraperitoneal route, the lowest dose of NHEPA that produced mortality was at 1000 mg/kg while the highest dose without mortality was at 100 mg/kg. According to Lorke (1983), the LD_{50} values were calculated to be 2450 and 346 mg/kg for the oral and intraperitoneal routes, respectively.

Table 1 Acute toxicity (LD_{50}) profile of NHEPA in mice

Dose (mg / kg)	Death pattern after 24 hours	
	Intraperitoneal route (*i.p*)	Per oral route (*p.o*)
PHASE 1 (n=3)		
10	0/3	0/3
100	0/3	0/3
1000	3/3	0/3
PHASE 2=1		
200	0/1	
300	0/1	
400	1/1	
600	1/1	
800	1/1	
1000		0/1
2000		0/1
3000		1/1
4000		1/1
5000		1/1
LD_{50}	$(300 \times 400)^{1/2}$ =346 mg/kg, *i.p*	$(2000 \times 3000)^{1/2}$ =2450 mg/kg, *p. o*

3.2. Effect of NHEPA on novelty induced behavior

1.2. The NHEPA (50, 100 and 150 mg/kg, i.p.) significantly decreased rearing [$p < 0.01$; $F_{(4, 25)} = 17.34$], locomotor [$p < 0.01$; $F_{(4, 25)} = 16.2$] and grooming [$p < 0.01$; $F_{(4, 25)} = 26.39$] activities compared to vehicle. Diazepam (1 mg/kg, i.p.) also produced significant decrease in rearing, locomotion and grooming. The NHEPA dose-dependently suppressed exploratory behaviour significantly (Figure 1A, 1B and 1C).
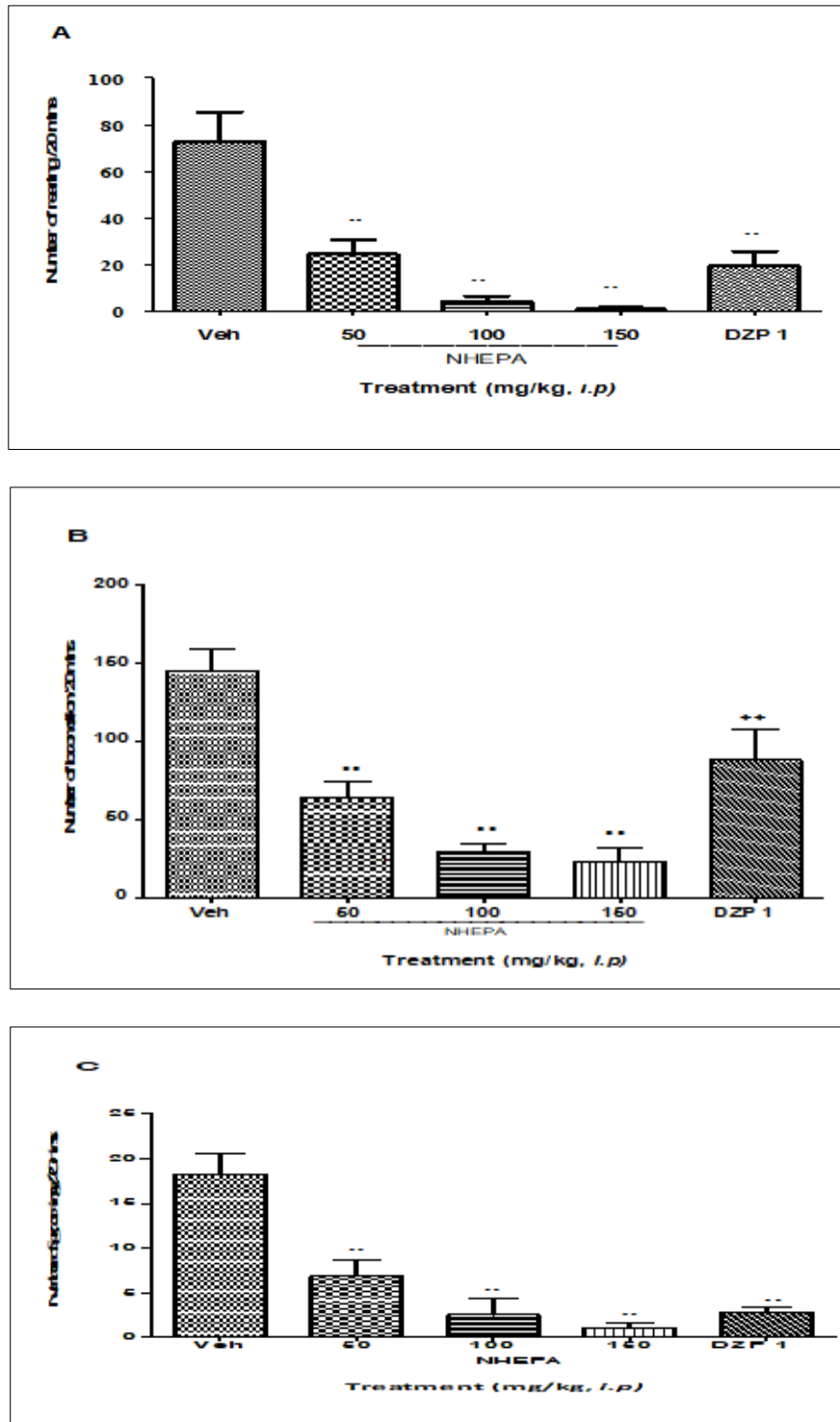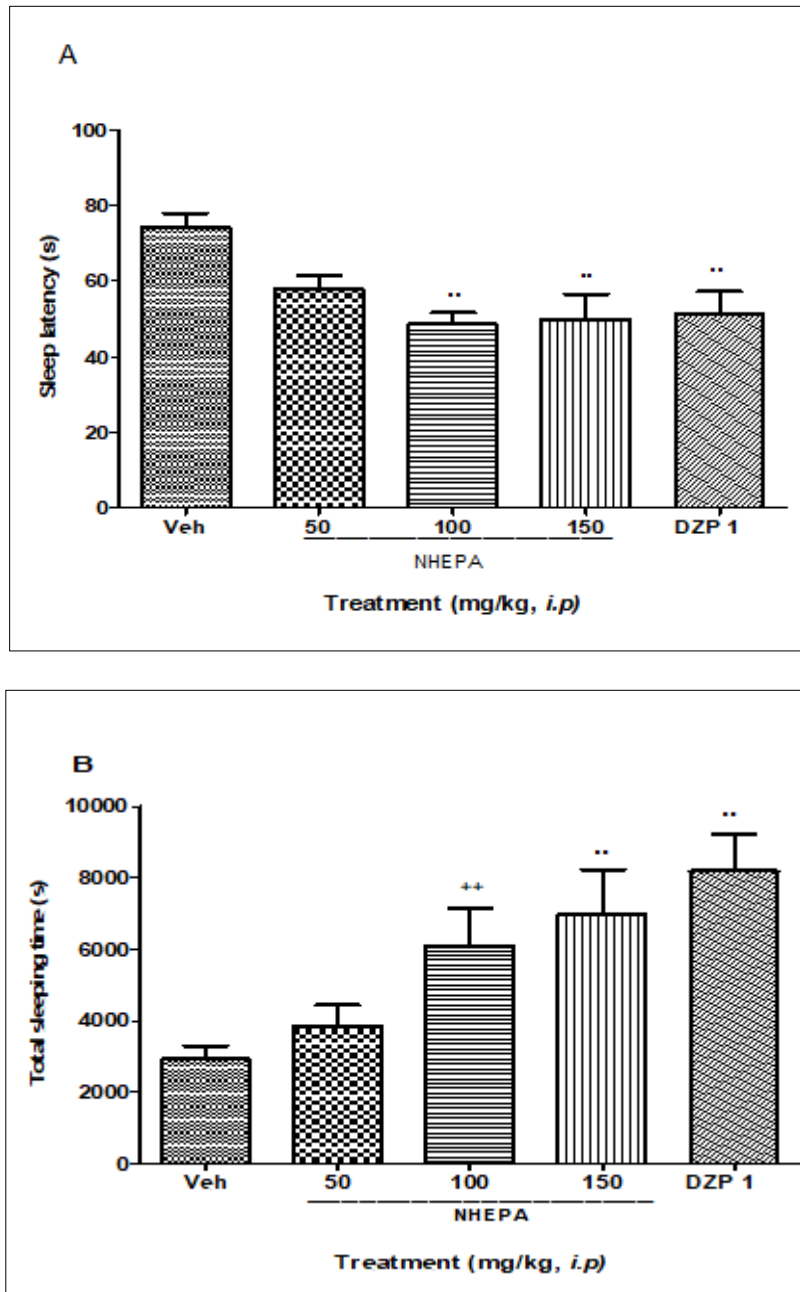
Bars represent mean values with standard error of the mean (n = 6); VEH, DZP 1 and NHEPA represent vehicle (5% Tween 80, 10 ml/10 g, i.p.), diazepam (1 mg/kg, i.p.) and n-hexane fraction of *Petiveria alliacea* respectively; .. p< 0.01 statistically significant compared to vehicle (ANOVA, Dunnett's test); ++ p< 0.05 statistically significant compared to vehicle (ANOVA, Dunnett's test).

Figure 1 Effect of NHEPA on novelty-induced rearing (A), locomotion (B) and grooming (C) in mice

3.3. Effect of NHEPA on ketamine-induced hypnosis

The NHEPA (100 and 150 mg/kg, i.p.) significantly [$F_{(4, 25)}$ = 4.778, P < 0.01] reduced sleep latency (SL) in a dose dependent manner compared to vehicle. However, no significant reduction in SL was seen with NHEPA at a dose (50 mg/kg. i.p) compared with the vehicle. The NHEPA at 100 and 150 mg/kg. i.p. caused a significant [p<0.05 and <0.01 respectively] increase in the total sleeping time induced by ketamine (100 mg/kg, i.p.) compared to the vehicle (5%

Tween-80). The standard drug, diazepam 1 mg/kg, i.p. significantly ($p<0.01$) reduced SL and also significantly ($p<0.05$) prolonged the TST compared to vehicle (Figure 2 A and B).

Bars represent mean values with a standard error of the mean (n = 6); VEH, DZP 1 and NHEPA represent vehicle (5% Tween 80, 10 ml/10 g, i.p.), diazepam (1 mg/kg, i.p.) and n-hexane fraction of *Petiveria alliacea* respectively; .. p< 0.01 statistically significant compared to vehicle (ANOVA, Dunnett's test); ++ p< 0.05 statistically significant compared to vehicle (ANOVA, Dunnett's test).

Figure 2 Effects of NHEPA on ketamine-induced sleep latency (A) and ketamine-induced total sleeping time (B) in mice

3.4. Effect of NHEPA on chemically induced convulsion tests

The results of chemo-convulsion tests are summarized in Table 1. Mice in the vehicle group were not protected in all the models. The NHEPA (100 and 150 mg/kg, i.p.) offered 16.7% protections compared to diazepam (1 mg/kg, i.p.) which offered 100% protection against PTZ-induced convulsion. In the aminophylline-induced convulsion test, the NHEPA at 50, 100 and 150 mg/kg, i.p., offered 33.33%, 33.33% and 16.67% protections respectively, while phenobarbital (30 mg/kg, i.p.) gave a 66.67% protection .Strychnine induced convulsion test produced severe tonic-

clonic convulsion in all the treated groups. The NHEPA doses (50, 100, and 150 mg/kg, i.p) and diazepam (5 mg/kg, i.p.) offered no protection against strychnine-induced convulsion. The results are shown in Tables 2,3,4)

Table 2 Effect of NHEPA on pentylenetetrazole-induced convulsion in mice

Treatment *i.p.* (n=6)	Convulsion Latency (CL) (Mean±SEM) (S)	Time of Death (S) (Mean±SEM)	% protection
VEH 0.1 ml/10 g	67.17±5.72	310.00±47.54	0.00
NHEPA 50 mg/kg	107.17±11.40	410.00±233.11	0.00
NHEPA 100 mg/kg	367..67±286.61	580.00±267.28	16.67
NHEPA 150 mg/kg	355.67±288.92	870.00±295.06	16.67
DZP 1 mg/kg	1800.00±0.00**	1800.00±0.00**	100.00

VEH, NHEPA and DZP represent vehicle (5% Tween-80), n-hexane extract of *Petiveria alliacea* and diazepam respectively; **p<0.01 statistically significant compared to the vehicle (ANOVA, Dunnett's test).

Table 3 Effect of NHEPA on aminophylline-induced convulsion in mice

Treatment *i.p.* (n=6)	Convulsion Latency (CL) (Mean±SEM) (S)	Time of Death (S) (Mean±SEM)	% protection
VEH 0.1 ml/10 g	548.33±50.87	800.00±120.66	0.00
NHEPA 50 mg/kg	987.33±262.06	1060.00±250.12	33.33
NHEPA 100 mg/kg	1027.33±267.87	1050.00±259.11	33.33
NHEPA 150 mg/kg	911.33±187.03	960.00±176.64	16.67
PHEN 30 mg/kg	1487.00±160.59**	1570.00±147.31**	66.67

VEH, NHEPA and PHEN represent vehicle (5% Tween-80), n-hexane extract of *Petiveria alliacea* and phenobarbital respectively; **p<0.05 statistically significant compared to the vehicle (ANOVA, Dunnett's test).

Table 4 Effect of NHEPA on strychnine-induced convulsion in mice

| Treatment *i.p.* (n=6)	Convulsion Latency (CL) (Mean±SEM) (S)	Time of Death (S) (Mean±SEM)	% protection
VEH 0.1 ml/10 g	150.17±16.82	180.00±21.91	0.00
NHEPA 50 mg/kg	200.67±88.82	225.00±89.95	0.00
NHEPA 100 mg/kg	141.17±4.59	161.67±8.33	0.00
NHEPA 150 mg/kg	177.67±42.60	285.00±90.91	0.00
DZP 5 mg/kg	356.67±92.84	385.00±89.95	0.00

VEH, NHEPA and DZP represent vehicle (5% Tween-80), n-hexane extract of *Petiveria alliacea* and diazepam respectively

3.5. Mechanism of action of NHEPA using Agonist and Antagonist Model in Mice

3.5.1. Influence of NHEPA on the effect of amphetamine on novelty-induced behaviour in mice (Agonist model)
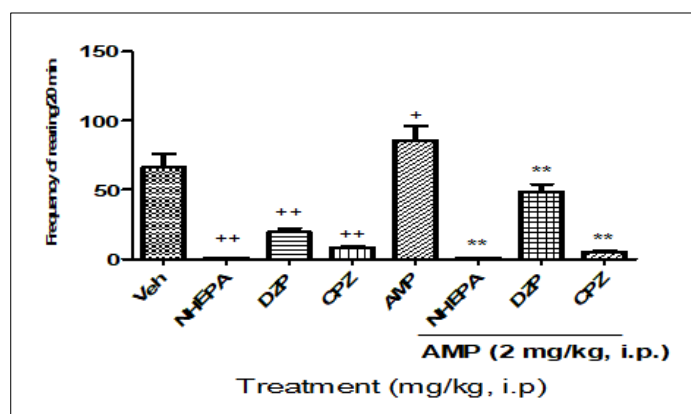
NHEPA (150 mg/kg i.p.), diazepam (1 mg/kg, i.p.) and chlorpromazine (2 mg/kg, i.p.) alone significantly decreased locomotion and rearing activity as compared to the vehicle alone. Amphetamine (2 mg/kg, i.p) caused significant increase in locomotion and rearing (0.01 and 0.05 respectively) compared with the vehicle alone. Amphetamine (2 mg/kg, i.p.) plus NHEPA (150 mg/kg i.p.) as well as amphetamine (2 mg/kg, i.p) plus chlorpromazine (2 mg/kg, i.p.)

caused a significant ($p<0.01$) reduction in the locomotion and rearing activity as compared with amphetamine only (reversal of amphetamine-induced hyperlocomotor and hyper-rearing activity). Amphetamine (2 mg/kg, i.p) plus diazepam (1 mg/kg, i.p.) showed no significant reduction in locomotion but a significant ($p<0.01$) reduction in rearing activity was observed compared to mice administered with amphetamine (2 mg/kg, i.p.) only. The result is represented in Figures 3A and 3B.
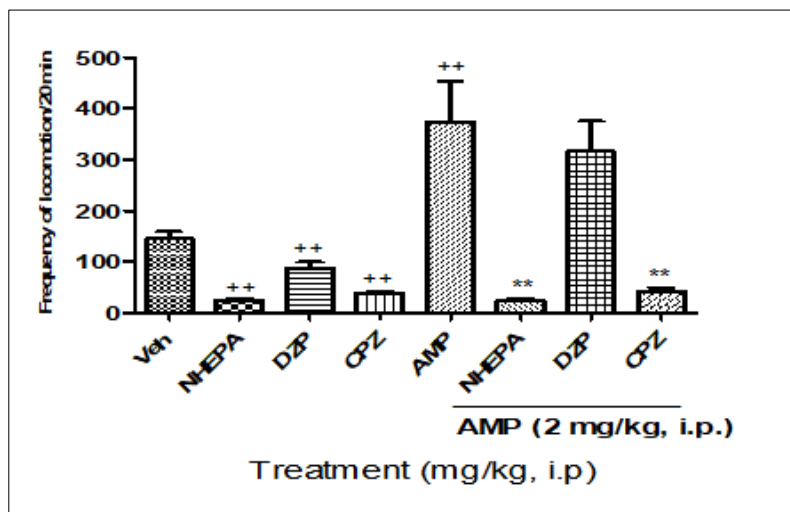
Bars represent mean values with a standard error of the mean (n = 6); VEH, DZP, NHEPA, AMP and CPZ represent vehicle (5% Tween 80, 10 ml/10 g, i.p.), diazepam (1 mg/kg, i.p.), n-hexane fraction of *Petivera alliacea* (150 mg/kg, i.p.), amphetamine (2 mg/kg, i.p.) and chlorpromazine (2 mg/kg, i.p.) respectively; + $p< 0.05$ statistically significant compared to vehicle; ++ $p< 0.01$ statistically significant compared to vehicle; ** $p<0.01$ statistically significant compared to amphetamine

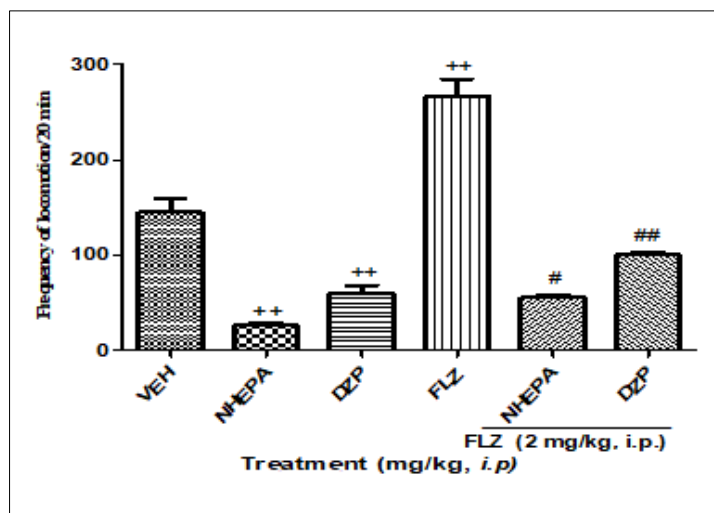**Figure 3A** Influence of NHEPA on the effect of amphetamine on locomotion

Bars represent Mean values with a standard error of the mean (n = 6); VEH, DZP, NHEPA, AMP and CPZ represent vehicle (5% Tween 80, 10 ml/10 g, i.p.), diazepam (1 mg/kg, i.p.), n-hexane fraction of *Petivera alliacea* (150 mg/kg, i.p.), amphetamine (2 mg/kg, i.p.) and chlorpromazine (2 mg/kg, i.p.) respectively; + $p< 0.05$ statistically significant compared to vehicle; ** $p< 0.01$ statistically significant compared to vehicle; ## $p<0.01$ statistically significant compared to amphetamine

**Figure 3B** Influence of NHEPA on the effect of amphetamine on rearing

3.5.2. Influence of flumazenil on the effect of NHEPA on novelty-induced behaviour in mice (Antagonist model)

NHEPA (150 mg/kg i.p.) and diazepam (1 mg/kg, i.p.) alone significantly ($p<0.01$) decrease locomotion and rearing as compared to the vehicle alone. Flumazenil (2 mg/kg, i.p.) alone showed a significant ($p<0.01$) increase in locomotor and rearing activity compared to the vehicle. Flumazenil (2 mg/kg, i.p.) plus NHEPA (150 mg/kg i.p.) showed a significant ($p<0.05$) increase in locomotor activity but insignificant increase in rearing activity compared to NHEPA alone. Likewise, flumazenil (2 mg/kg, i.p.) plus diazepam (1 mg/kg, i.p.) significantly ($p<0.01$) reversed the inhibitory effect of diazepam on locomotion and rearing. The result is represented in Figures 4A and 4B.

Bars represent mean values with a standard error of the mean (n = 6); VEH, DZM, NHEPA and FLZ represent vehicle (5% Tween 80, 10 ml/10 g, i.p.), diazepam (1 mg/kg, i.p.), n-hexane fraction of *Petivera alliacea* (150 mg/kg, i.p.) and flumazenil (2 mg/kg, i.p.) respectively; ++ p< 0.01 statistically significant compared to vehicle; # p< 0.05 statistically significant compared to NHEPA only; ## p<0.01 statistically significant compared to diazepam

Figure 4A Influence of flumazenil on the effect of NHEPA on locomotion
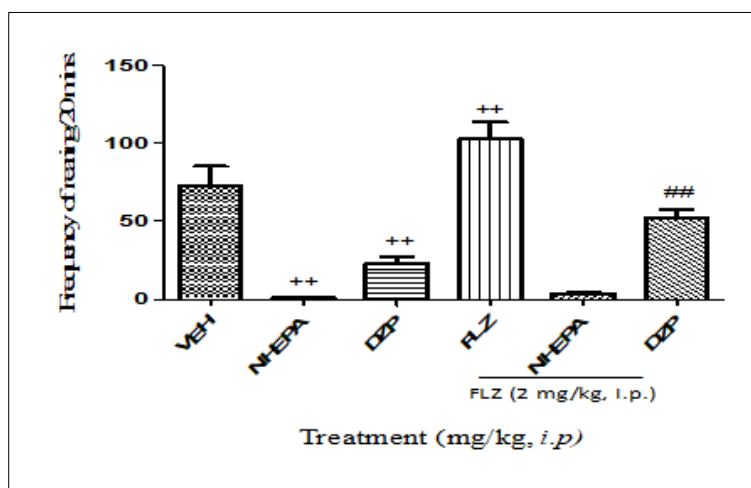
Bars represent mean values with a standard error of the mean (n = 6); VEH, DZM, NHEPA and FLZ represent vehicle (5% Tween 80, 10 ml/10 g, i.p.), diazepam (1 mg/kg, i.p.), n-hexane fraction of Petivera alliacea (150 mg/kg, i.p.) and flumazenil (2 mg/kg, i.p.) respectively; ++ p< 0.01 statistically significant compared to vehicle; ## p<0.01 statistically significant compared to diazepam

Figure 4b Influence of flumazenil on the effect of NHEPA on rearing

4. Discussion

The LD_{50} values obtained for the extract were 2450 and 346 mg/kg for oral and intraperitoneal routes respectively (Table 1), an indication that drugs administered through the intraperitoneal route are more toxic than through the oral route [17]. The higher value of LD_{50} via the oral route of administration is majorly due to hepatic first-pass metabolism of the drug by cytochrome P-450 enzymes when absorbed and delivered through portal circulation [26]. In addition, the acidic content of the stomach and other gastrointestinal tract enzymes lowers absorption and bioavailability [26]. All the above factors lead to reduced toxicity. The intraperitoneal route however gives faster and more consistent and reproducible results [13] hence, it was used in this study. The above result showed the extract is slightly toxic orally and moderately toxic intraparenterally [27, 28].

Different extracts of *Petiveria alliacea* had given diverse results after toxicological tests [29], however, the plant is generally considered to exhibit low toxicity when given via oral route [30]. In this study, the LD_{50} value (2450 mg/kg)

obtained for the n-hexane extract via the oral route of NHEPA was found to be similar to the LD_{50} value >2000 mg/kg of aqueous extract of *Petiveria alliacea* obtained by Garcia-Gonzelez et al. which supported the mild toxicity nature of the plant [31]. Gomes et al reported that female Swiss mice experience low toxicity (due to reduce locomotion and passive behaviour) with intraperitoneal administration of the hydroalcoholic extract of *P. alliacea* roots at 100–400 mg/kg [7]. However, an acute toxicity study has not been conducted on the n-hexane fraction of the leaf of this plant.

The effect of the NHEPA on NIB (locomotion, rearing and grooming) was found to be inhibitory at all the doses (50, 100 and 150 mg/kg, i.p) (Figures 3.1-3.3) and in a dose-dependent manner compared to the vehicle. Diazepam (1 mg/kg, i.p.) also expectedly decreased NIB parameters [32]. The novelty-induced behavioural (NIB) responses are well known to be regulated by varied neurotransmitters including serotonin (5-HT), gamma-aminobutyric acid (GABA), acetylcholine (Ach), dopamine, opioids and norepinephrine [33, 34]. More specifically, rearing is modulated by dopaminergic neurotransmission and cholinergic pathway while locomotion is mediated mainly by dopamine with the interplay of other neurotransmitters [32, 33, 35]. The low frequency of excitatory behaviours (rearing and locomotion) in this study indicates depression or inhibitory effect [17].

Results showed that NHEPA (100 and 150 mg/kg. i.p.) reduced the sleep latency and total sleeping time induced by ketamine (100 mg/kg, i.p.) significantly in a dose-dependent manner compared to the vehicle. Reduction of sleep latency and prolongation of total sleeping time indicate sedative activity and this is in agreement with the inhibitory activity effect as demonstrated in the novelty-induced behaviour [36]. Sedative agents exert their central nervous system inhibitory effect via potentiation of GABA inhibitory effect by binding to GABAA receptors like benzodiazepines or antagonizing the effect of glutamate by blocking glutamate receptors such as N-methyl-D-aspartate (NMDA), AMPA, Kainate, glycine or metabotropic receptors [32]. Other neurotransmitters involved in sedation include adenosine, acetylcholine, biogenic amines (serotonin, histamine, dopamine, norepinephrine), opiods and peptides (hypocretin, leptin and ghrelin) [37]. Ketamine was used in this study to induce hypnosis. Ketamine is an antagonist of the N-Methyl-D-Aspartate (NMDA) receptor, an excitatory receptor that plays a key role in seizures as well as causing sedation via its GABAA receptors potentiation [38]. This study also showed the sleep latencies and total sleeping time of NHEPA at 100 mg/kg and 150 mg/kg i.p. were comparable to the standard hypnotic agent, diazepam (1 mg/kg, i.p.) used in this study. The result therefore established that the extract exhibit similar hypnotic property to diazepam. The hydro-alcoholic root extract of *Petiveria alliacea* L. has also been observed to have a similar effect of reducing phenobarbitone-induced sleep latency time and prolonging total sleeping time [7] similar to the effect of N-Hexane leaf extract in this study.

The assessment of the anticonvulsant effect of the extract was carried out using three animal models; PTZ-, aminophylline- and strychnine-induced convulsions models. In the PTZ model, It was observed that there was an insignificant increment in the anticonvulsant effect of NHEPA at all the three dose levels (50, 100, 150 mg/kg i.p.) in both the onset of convulsion and the time of death compared to the vehicle. In addition, NHEPA (100 and 150 mg/kg. i.p.) both only offered negligible protection of 16.7% each against PTZ-induced convulsion (Table 1) in mice compared to 100% protection offered by the standard anticonvulsant, diazepam (1 mg/kg, i.p.). PTZ is a known convulsant agent which inhibits benzodiazepine (BDZ) site of the GABA receptor channel complex [39]. The mild protections offered by NHEPA against PTZ-induced convulsion further suggested that the extract possibly has some level of interaction with the GABAA-benzodiazepines receptor complex in the CNS [40, 41]. A previous study showed that all the fractions of the roots (hydro-alcoholic, n-hexane and aqueous extracts) of *P. alliacea* increased the latency to the first convulsion and the lethal time of the PTZ-induced convulsions test in the animals [7].

In the strychnine-induced convulsions model, none of the tested doses of NHEPA (50, 100 and 150 mg/kg, i.p.), as well as diazepam (5 mg/kg i.p.) offered any protection. Strychnine causes convulsion by selectively and competitively antagonizing the inhibitory action of all glycine receptors at the brainstem and spinal cord [17]. Glycine is an important inhibitory transmitter of the motor neurons and interneurons in the CNS and its inhibition results in excitation of the central nervous system and increasing spinal reflexes [17, 42]. This result indicated that the extract does not have any appreciable effect on the glycinergic mechanism, hence no significant anticonvulsant activity in strychnine-induced convulsions model.

In the aminophylline-induced convulsions model, the results revealed the vehicle offered no protection but NHEPA at 50, 100 and 150 mg/kg i.p. offered 33.33%, 33.33% and 16.7% protection respectively while phenobarbital (30 mg/kg, i.p.) offered 66.67% protection. Aminophylline is a methylxanthine derivative with the varied proposed mechanisms of actions including phosphodiesterase-3 inhibition, adenosine antagonism, calcium flux modulation and catecholamine release [24, 43]. It is a potent central nervous system stimulant and its narrow therapeutic index with reasonable high toxicity potential makes it a good agent to induce a seizure in animals [24]. One or more of the above mechanism of action in addition to accumulation of reactive oxygen species in the CNS precipitating neurotoxicity as well as inhibition of cerebral 5-nucleotidase activity have been implicated in its convulsiogenic effect [25, 44]. NHEPA partially protects

the animals against aminophylline-induced convulsion and death, hence may also have some minimal influence on the aforementioned pathway. There is also a possibility it may possess some anti-oxidant activity against the oxidative stress induced by free radicals released by aminophylline in the CNS. However, this hypothesis will require further study to be substantiated or otherwise.

As a result of the observed inhibitory effect of NHEPA on NIB parameters in this study, the neurochemical pathways mainly involved in CNS depression were explored using amphetamine (CNS stimulants, 2 mg/kg, i.p.) and flumazenil (GABAA-Benzodiazepine receptor antagonist, 2 mg/kg, i.p.) to determine the probable mechanism of action of the extract [32]. Administration of NHEPA (150 mg/kg, i.p) after pretreatment with amphetamine (2 mg/kg, i.p.) reversed the effects of the amphetamine on rearing and locomotor behaviours, thus suggesting the extract may mediate its CNS inhibitory effect through inhibition of any or a combination of the excitatory pathway through which amphetamine exert its pharmacological action [32, 45].

Amphetamine enhances dopaminergic, adrenergic and to a lesser extent the serotonergic pathway via various mechanisms [45]. Amphetamine enters the presynaptic nerve vesicles and causes displacement of monoamine neurotransmitters (dopamine, norepinephrine and serotonin) into the neuronal cytoplasm via inhibition of vesicular monoamine transporter 2 (VMAT2) and disruption of the electrochemical gradients necessary for vesicular transporter function (Wise and Bozarth, 1987; Nickell et al., 2014). Subsequently, the dopamine can then be released into the extracellular space by outward transport by the monoamine transporters (DAT, NET, and SERT) [45, 46]. In addition to the above, amphetamine also inhibits the reuptake of released monoamine neurotransmitters by the plasma membrane monoamine transporters resulting in an increased level of extracellular monoamines [45, 47]. Amphetamine-stimulated hyperactivity behaviour is mediated by the potentiation of the aforementioned excitatory neurotransmission (mainly dopamine) by amphetamine and this effect was significantly reversed by NHEPA in this study. Hence, it could be inferred that the inhibitory effect of NHEPA on NIB parameters may be due to the interaction between the extract and the aforementioned excitatory receptors. Chlorpromazine (2 mg/kg i.p.), is a drug well known for its anti-dopaminergic, antiadrenergic and anticholinergic properties [48, 49]. This drug expectedly reverses the amphetamine-induced CNS hyperactivity on all NIB parameters in a similar fashion to NHEPA. This result further reinforces that the observed inhibitory property of NHEPA is probably mediated via the monoamine pathway inhibition. Lastly, although diazepam (1 mg/kg i.p.), a known GABAA antagonist has no significant effect on the amphetamine-induced hyper locomotor behaviour, it was observed to significantly reversed amphetamine-induced hyper-rearing activity. It has been previously reported that benzodiazepines has some inhibitory effect on dopaminergic transmission via GABAergic mediated suppression of striatal dopaminergic activity and blocking cyclic AMP production which releases dopamine in the nucleus accumbens [50, 51]. Hence, the above reasons may justify this observation.

To determine the involvement of GABA-benzodiazepine receptors in the observed inhibitory effect of the extract, flumazenil (2 mg/kg i.p), a specific GABA-benzodiazepine antagonist, was administered 15 minutes before administration of NHEPA (150 mg/kg, i.p). It was observed that flumazenil plus NHEPA (150 mg/kg, i.p) showed a significant increase in locomotor activity compared to NHEPA alone. This observation suggests that there is a significant interaction between some compounds in the extract and the GABA-benzodiazepine receptors. Therefore it can be inferred that the ability of flumazenil to significantly reverse the inhibitory effect of the extract on locomotion suggest that the GABAergic neurochemical pathway play a significant role in the CNS inhibitory effect of the extract. Flumazenil as expected significantly reversed the inhibitory effect of diazepam (1 mg/kg, i.p.) on locomotor and rearing behaviour.

5. Conclusion

The present study revealed that the n-hexane extract of *Petiveria alliacea* was slightly toxic orally and moderately toxic parenterally; displayed significant central nervous system depressant activity; exhibited significant sedative activity and mild anticonvulsant activity and possibly act via CNS monoamine pathway or augmentation of the GABAA-benzodiazepine complex pathway. The various CNS effects demonstrated by the extract in this study established the pharmacological basis for the ethnomedicinal use of the plant in the management of insomnia and epilepsy.

Compliance with ethical standards

Disclosure of conflict of interest

The authors report no conflicts of interest in this work.

Statement of ethical approval

Ethical approval was obtained from the Health Research and Ethics Committee, Institute of Public Health, OAU, Ile-Ife (HREC006).

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