

Original Research Article

Neuropharmacological evaluation of the methanol leaf extract of *Phyllanthus muellerianus* (Kuntze) Exell and its ethyl acetate fraction in mice

Martha N Ofokansi^{1*}, Chinenye J Ugwah-Oguejiofor², Stella Ihim¹, Charles O Okoli¹, Peter A Akah¹

¹Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001,

²Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, PMB 2346, Sokoto, Nigeria

*For correspondence: **Email:** Martha.ofokansi@unn.edu.ng; **Tel:** +234-8037794874

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Abstract

Purpose: To investigate the neuropharmacological effects of the methanol leaf extract (ME) and fractions of *Phyllanthus muellerianus* (PM) (Phyllanthaceae) (Kuntze) Exell (PM) in mice.

Methods: Acute toxicity was carried out on the extract using standard protocol. ME was fractionated into hexane (HF), ethyl acetate (EF), and methanol (MF) fractions. Pentylentetrazol (PTZ)-induced seizure, open field (OF) and motor coordination (rotarod) tests were models employed. Mice allotted into fourteen groups of six animals each were treated orally with 100, 200, or 400 mg/kg of the extract and fractions in pentylene tetrazole (PTZ) seizure model. Seizure was induced with intraperitoneal (ip) injection of 70 mg/kg of PTZ. The positive and negative controls employed were phenobarbitone (35 mg/kg) and 5 ml/kg of 7 % Tween 80, respectively. In the OF and motor coordination tests, six groups of six mice were treated orally with ME and EF at 200 and 400 mg/kg doses. Control groups received either 5 ml/kg of 7% Tween 80 or diazepam (1 mg/kg ip) as negative and positive controls respectively

Results: In the PTZ model, only EF abolished seizures completely ($p < 0.05$), when compared with the negative control, producing 100% protection, even better than the phenobarbitone which gave 83.3% protection. In the OFT, in comparison with the control, ME at 400 mg/kg ($p < 0.05$) decreased both the number of line crossing and the number of assisted rearing similar to that produced by diazepam. EF increased both the locomotor and exploratory activities significantly ($p < 0.05$) in mice. ME at 400 mg/kg significantly ($p < 0.05$) evoked reduction in the time of fall of mice from the rotarod when compared to the control in the same way as diazepam while EF did not elicit any appreciable differences.

Conclusion: ME has anticonvulsant, sedative, and anxiolytic activities, while EF possesses anticonvulsant and anxiolytic activities devoid of sedative and cognitive impairment. The observed anticonvulsant effect was better than that produced by phenobarbitone. Thus, it may be a good lead for developing antiepileptic and other central nervous system active agents.

Keywords: Anticonvulsant, Anxiolytic, Sedation, Motor coordination, Pentylentetrazol, Open-field

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INTRODUCTION

Central nervous system (CNS) diseases/disorders are a group of neurological disorders affecting either the structure or the function of the brain and/or spinal cord. These conditions include sleep disorders, depression, anxiety, epilepsy, dementia, pain, Parkinson's disease, schizophrenia, psychosis, stroke. They are important causes of death and disability globally. CNS disorders constitute a huge burden on healthcare which has steadily been on the increase because of population expansion and ageing. It was estimated that the total incidence of CNS diseases would have risen from 12.3 to 15% in the year 2020 [1]. Additionally, most of the drugs used in the treatment of CNS diseases do not produce clinical cure and possess many side effects necessitating the need to search for new bioactive molecules. Scientists have relied on natural resources over the years for the development of new drugs to address the present health needs of man. Several plants are reported to possess neuropharmacological activities and many more researches are on-going for effective CNS disease treatment with minimum side effects. *Phyllanthus muellerianus* (PM) (Kuntze) Exell, (Phyllanthaceae) is an evergreen, monoecious, scandent shrub with numerous stems from the base. It is mostly found in tropical regions of West Africa. Preparations from the leaves are used in Nigeria for the treatment of fevers, convulsions, epilepsy, paralysis, and some neurological disorders [2]. Preliminary phytochemical studies on methanol extract (ME) revealed that glycosides, flavonoids, saponins, alkaloids terpenoids, tannins and steroids are present while ethyl acetate fraction (EF) contained the same constituents except terpenoids and steroids [3]. Previous scientific studies reported that different parts of the plant especially the leaves possess antioxidant, antibacterial antihyperlipidemic, antidiarrheal, immunomodulatory, antifungal, anti-inflammatory, and antimicrobial activities [4]. Effects of the aqueous extract of the aerial parts of the plant on pain treatment, reproductive and metabolic disorders have been reported but its neuropharmacological effect has not been reported. Methyl gallate, 3-friedelanone, 2-ethyloctylphthalate, bis 2-ethylcosylphthalate, ellagitannin, geraniin, gallic acid and kaempferol glycosides have been previously isolated from the leaves of the plant [5].

Based on the ethnobotanical evidence indicating that PM is used to treat convulsion and other CNS disorders, this work was conceived to scientifically examine the neuropharmacological effects of the methanol leaf extract (ME) and

ethyl acetate fraction (EF) (since it was the fraction that gave the best activity in preliminary study) of *Phyllanthus muellerianus* (Kuntze) Exell in mice using different models.

EXPERIMENTAL

Collection and authentication of the study material

Fresh leaves of PM were collected in November 2014 from Ankpa Local Government Area of Kogi State, Nigeria. It was authenticated by a taxonomist, Mr. Alfred Ozioko of the International Center for Ethnobotany and Drug Development (InterCEDD) Nsukka, Nigeria. A voucher specimen (InterCEDD062014) was kept in the herbarium.

Preparation of plant material

The air-dried leaves were reduced to powder with a blender. The fine powder (2.2 kg) was extracted with methanol using a Soxhlet apparatus. ME was concentrated *in vacuo* into a semi-solid paste and thereafter dried over a water bath at 60 °C. A yield of 11.3 % w/w was realised.

Animals

Healthy ten-week-old male Sprague Dawley mice (20 - 25 g) from the animal house of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used. Standard laboratory conditions were maintained. The animals had free access to portable water. They were fed with normal animal feeds (Guinea feeds, Nigeria) with free access to drinking water. All animal tests were done in line with the guidelines of National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 - 23) [6] as well as those of University of Nigeria Ethics Committee on the use of laboratory rodents, and registered by the National Health Research Ethics Committee (NHREC) of Nigeria (approval no. NHREC/05/01/2008B). The study protocols were also approved by the Ethics Committee of the University of Nigeria.

Acute toxicity test

The oral acute toxicity (LD₅₀) of ME was estimated in mice using Lorke's method [7]. The experiment was done in two phases: In phase one, nine animals were divided into three groups of three mice each. The groups received 10, 100, and 1000 mg/kg, respectively. They were monitored intermittently for signs of acute

intoxication and/or death for 24 h. The second phase had three mice, which were given 1600, 2900, and 5000 mg/kg, respectively. They were again monitored for 24 h for death and other behavioural changes.

Fractionation of methanol extract

ME (200 g) was loaded in a silica gel (60-230 nm mesh) column (60 cm length x 7.5 cm diameter) and successively eluted with solvents of different polarity; n- hexane, ethyl acetate, and methanol. The obtained fractions were concentrated in *vacuo* to get hexane (HF), ethyl acetate (EF), and methanol fractions (MF).

High-performance liquid chromatography (HPLC) analysis

A Dionex P580 HPLC system and a photodiode array detector were used for HPLC testing of the sample (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent.

2 mg of the sample was mixed with 2 ml of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 min. 100 µL of the dissolved samples was transferred into HPLC vial containing 500 µL of HPLC grade methanol. The compounds were detected by comparing the retention times and UV spectral with an inbuilt library. Identification was based on library hit similarity of > 99 %.

Neuropharmacological evaluations

Pentylentetrazol-induced seizure test

Sprague Dawley mice were randomly divided into fourteen groups (n=6) and treated as follows: Group 1 served as normal control (vehicle) and received 5 ml/kg of 7 % v/v Tween 80 while group 2 received standard drug phenobarbitone sodium (35 mg/kg ip). The remaining groups received either 100, 200, or 400 mg/kg orally of either ME, HF, EF, and MF suspended in 7% Tween 80. Thirty minutes later, convulsion was induced in each mouse by the intraperitoneal (i.p.) administration of PTZ (70 mg/kg). The mice were placed in a transparent glass cage (20 X 20 X20 cm) and observed for the time of onset (latency) of convulsion, frequency of convulsions, duration of seizure, and mortality. The threshold seizure was an episode of a clonic spasm that

lasted for at least 30 s. Animals without threshold convulsions and no subsequent death were considered protected during the 60 minutes of observation. After this test, anticonvulsant activity was observed in only ME and EF; consequently, they were used for other neuropharmacological evaluations.

Open field test (OFT)

Six groups (n= 6) of mice received 200 or 400 mg/kg orally, each of ME or EF. Control groups received either 5 ml/kg of 7 % Tween 80 or diazepam (1 mg/kg, ip). Each animal was after 30 minutes positioned at the center of the open field device and monitored for a 5-minute period using a digital camera mounted on the cage to record the mouse's operation. The open-field device was an opaque plexiglass cage (72 by 72 cm) with a 35- cm height wall. The floor was partitioned into 16 squares (18 × 18 cm) by white lines.

The following behavioural parameters were observed and recorded: line crossing, number of entrances into the central square, freeze period, rearing (number of times the animal lifts both forefeet off the floor), rearing against the wall (leaning on the walls), frequency and duration of grooming (cleaning the face and/or the body by rapidly moving the forefeet forward), urination (number of puddles or streaks of urine) and defaecation (number of faecal boli produced). In order to avoid alteration of the spontaneous behaviour of animals yet to be evaluated, the open-field was wiped carefully with 70% ethanol after each test session to remove the smell of the previously evaluated mouse. The same test was repeated on the second day to assess for habituation in the open field.

Motor coordination evaluation (rota-rod test)

The effect on motor coordination was investigated using the rotarod (rotating rod) assay. The rotarod apparatus is partitioned into five with a rotating rod (diameter 3 cm) passing through each partition. Twenty-four hours before the experiment, the rodents were made to walk on the rotarod at 20 rpm and the mice that could stay on the rotating rod longer than 3 min. were selected. On the test day, different doses (200 and 400 mg/kg) each of ME or EF were administered orally to six groups of mice (n= 6) 30 min before testing. Control groups received either 5 ml/kg of 7 % Tween 80 or diazepam (1 mg/kg, ip). On one of the five rotarod beams, each mouse was placed opposite to the direction of the beam's motion. All mice started from a stationary beam, and the rotarod was then turned

on. The rotation speed was 20 rpm. When a mouse fell into the chamber from the beam, it remained there until all other mice had either fallen off or completed a length of 3 min. on the beam. The length of time each mouse could remain on the rod was recorded. The time at which each mouse fell was recorded. After a one-minute break, they were lifted from the chamber by the tail and placed again on the beam. Each of the mice had ten trials. The average total time of stay on the rotarod for each mouse was recorded. The increase in time the animal spent on the rod was noted as an index of motor coordination/learning.

Statistical analysis

All results were shown as mean \pm standard error of mean (SEM). One-way analysis of variance (ANOVA) was used for data analysis, while multiple comparisons were done using (LSD) post hoc test. Mean difference observations were considered significant at $p < 0.05$.

RESULTS

Acute toxicity

The administration of up to 5 kg of ME to the mice produced no clinical signs of toxicity or mortality after 24 h. Therefore, in mice, the LD₅₀ of ME may be greater than 5g/kg.

HPLC fingerprinting

HPLC of ME showed 18 peaks (Fig. 1), while that of EF showed 11 peaks. The compounds were 9- α hydroxyl Pinoresinol (peak 1); Isofistularin-3 (peak 2); Epicatechin O-3,4 dimethyl gallate (peak 3); Aerophobin-1 (peak 4); Kaempferol 3-O gallate (peak 5); 3,6-O-Dimethylellagic acid (peaks 6 and 7); Naamine F (peak 8) and Septicine (peak 11) (Figure 2).

PTZ-induced seizure

The latency for the first myoclonic convulsion, tonic-clonic convulsion, and extensor phase of PTZ induced seizures in all the treatment groups were prolonged in a dose-dependent manner while EF significantly ($p < 0.05$) and completely abolished seizures at 100 and 200 mg/kg dose levels producing 100 % protection and 0 % mortality. The effect of EF at 400 mg/kg was similar to that of phenobarbitone with a prolonged onset and reduced duration of seizures (Table 1). HF and MF offered no protection to the mice and caused 100 % mortality.

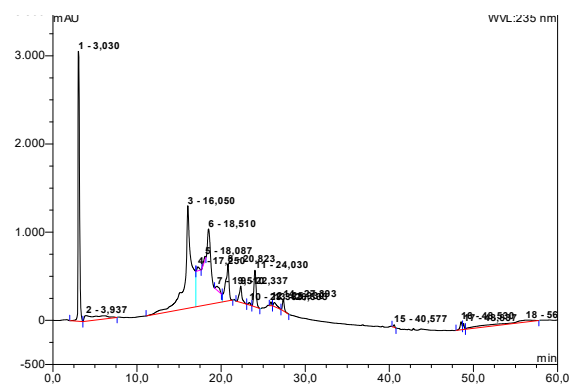


Figure 1: Chromatographic profile by HPLC of the crude methanol extract of *Phyllanthus muellerianus* showing 18 peaks

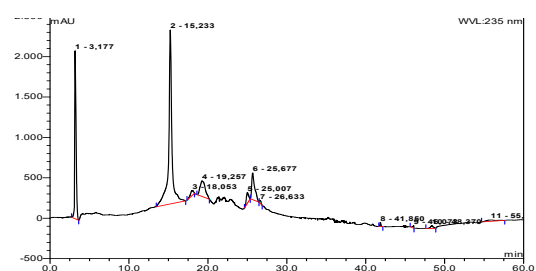


Figure 2: HPLC fingerprints of ethyl acetate fraction of *Phyllanthus muellerianus* showing 11 peaks; 9- α hydroxyl Pinoresinol (peak 1); Isofistularin-3 (peak 2); Epicatechin O-3,4 dimethyl gallate (peak 3); Aerophobin-1 (peak 4); Kaempferol 3-O gallate (peak 5); 3,6-O-Dimethylellagic acid (peaks 6 and 7); Naamine F (peak 8); Septicine (peak 11).

Effects of ME and EF on locomotor and exploratory activities

ME at 400 mg/kg evoked a significant reduction ($p < 0.05$) in the number of line crossing in the open field test while EF at 200 mg/kg caused a significant ($p < 0.05$) increase in line crossing on day 1 of the experiment (Table 2). Number of centre square crossing was significantly increased at 400 mg/kg of EF. In the exploratory activity determination, ME (200 mg/kg) produced a significant ($p < 0.05$) increase in the number of rearings in air and the assisted rearings; however, there was a decrease in the number of assisted rearing at 400 mg/kg. On the other hand, EF produced a significant ($p < 0.05$) increase in the number of rearings and assisted rearings at 400 mg/kg.

Diazepam, like ME (400 mg/kg), produced a significant ($p < 0.05$) decrease in the number of line crossing and assisted rearings when compared to the control (Table 2).

Table 1: Effect of methanol extract and fractions of *Phyllanthus muellerianus* on PTZ-induced convulsion

Treatment	Dose (mg/kg)	Onset of seizures (min)	Duration of seizures (min)	Mortality	Protection (%)
Control (7% Tween 80)	5 ml/kg	1.1 ± 0.3	0.9 ± 0.3	6/6	0
Phenobarbitone	35	13.3 ± 1.4*	0.1 ± 0.4*	0/6	83.3
ME	100	1.0 ± 0.2	9.0 ± 4.5*	6/6	0
	200	1.6 ± 0.1	7.0 ± 0.1*	5/6	0
	400	1.6 ± 0.2	12.5 ± 4.6*	4/6	16.67
HF	100	1.2 ± 0.2	2.0 ± 0.5	5/6	0
	200	1.6 ± 0.2	4.0 ± 1.8	6/6	0
	400	1.8 ± 0.4	4.2 ± 1.2	6/6	0
EF	100	-*	0.0 ± 0.0*	0/6	100
	200	-*	0.0 ± 0.0*	0/6	100
	400	12.8 ± 0.6*	0.1 ± 0.0*	0/6	83.3
MF	100	1.8 ± 0.2	4.8 ± 1.9	6/6	0
	200	2.1 ± 0.7	1.9 ± 0.5	6/6	0
	400	2.3 ± 0.6*	6.6 ± 2.8*	6/6	0

n = 6; ME = Methanol extract; HF = n- hexane fraction; EF = ethyl acetate fraction; MF = methanol fraction; - = no onset of seizure; *p < 0.05 compared to the control

On day 2, ME (400 mg/kg) showed a reduction in line crossing and a significant ($p < 0.05$) increase in the number of rearings in air at both 200 and 400 mg/kg. At 200 mg/kg, ME significantly ($p < 0.05$) increased the number of assisted rearings. The EF (200 mg/kg) elicited an increase in the number of line crossing and significant ($p < 0.05$) increase in centre square crossing, unlike in day 1. In contrast, at 400 mg/kg, it significantly ($p < 0.05$) increased the number of rearings in air and assisted rearings (Table 2).

Effect of ME and EF on grooming behaviour

No significant change was seen in the frequency of grooming in all the groups on day 1. However, on day 2, increases in grooming frequency were seen in all the groups, including the control, but

EF at both 200 and 400 mg/kg produced significant ($p < 0.05$) effects. The increase in frequency seen at EF (400 mg/kg) on day 2 was significantly more than that observed on day 1 (Figure 3). In comparisons with the control, ME at both 200 and 400 mg/kg elicited a significant ($p < 0.05$) reduction in the duration of grooming on both day 1 and 2 (Figure 3). EF evoked a significant ($p < 0.05$) decrease in the duration of grooming only at 400 mg/kg on day 1 (Figure 3).

Effect of ME and EF on total duration of freezes

ME and EF at all doses produced increases in freezing duration with 400 mg/kg having a significant ($p < 0.05$) effect when compared to the control on day 1 of the experiment.

Table 2: Effect of *Phyllanthus muellerianus* on mice in the open field

Treatment period	Treatment	Dose (mg/kg)	Locomotor activity		Exploratory activity	
			No of line crossings	No of Centre square crossings	No of rearings in air	No of assisted rearing
Day 1	ME	200	33.0 ± 13.8	0.0 ± 0.00	1.8 ± 1.2*	9.2 ± 3.78
		400	15.0 ± 10.2*	0.2 ± 0.20	0.2 ± 0.2	3.6 ± 2.03*
	EF	200	59.0 ± 22.2*	0.0 ± 0.00	0.2 ± 0.2	9.2 ± 4.21
		400	38.8 ± 27.4	1.0 ± 1.00*	1.6 ± 1.4*	11.8 ± .97*
	Diazepam	1	7.3 ± 0.50*	0.0 ± 0.00	0.0 ± 0.0	3.7 ± 2.03*
	control	5 ml/kg	36.6 ± 12.2	0.0 ± 0.00	0.0 ± 0.0	7.8 ± 3.2
Day 2	ME	200	25.0 ± 10.3	0.2 ± 0.2	1.8 ± 0.8*	13.4 ± 5.7*
		400	17.8 ± 7.7*	0.2 ± 0.2	2.8 ± 1.2**	8.2 ± 3.8
	EF	200	44.0 ± 16.0	2.0 ± 1.1*†	0.2 ± 0.2	12.4 ± 4.4*
		400	60.2 ± 11.4*	0.2 ± 0.2	1.4 ± 0.5*	16.6 ± 1.2*
	Diazepam	1	8.9 ± 4.6*	0.2 ± 0.2	0.2 ± 0.2	4.8 ± 2.3
	control	5 ml/kg	38.6 ± 17.3	0.2 ± 0.2	0.6 ± 0.4	8.2 ± 2.9

*P < 0.05 compared to the control, +p < 0.05 compared to day 1, (ANOVA; LSD post hoc); ME = Methanol extract; EF = ethyl acetate fraction

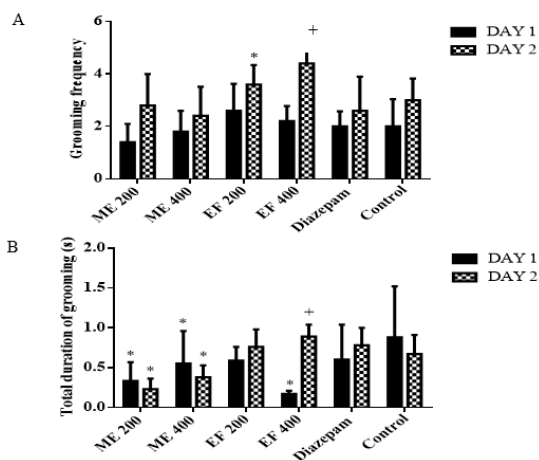


Figure 3: Effect of the extract of *Phyllanthus muellerianus* on grooming frequency (A) and duration of grooming (B) in the Open-field, $n = 6$; $*p < 0.05$ compared to the control, $+P < 0.05$ compared to day 1, ME = Methanol extract; EF = Ethyl acetate fraction

On day 2, there were significant ($p < 0.05$) decreases in freezing duration in all the groups when compared to day 1. Only ME at 400 mg/kg showed a significant ($p < 0.05$) increase in freeze on day 2 when compared to the control (Figure 4). The effect of ME at 400 mg/kg was comparable to that of diazepam.

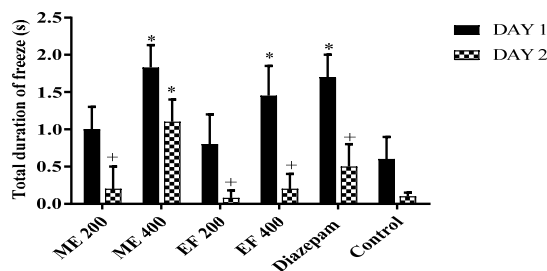


Figure 4: Effect of *Phyllanthus muellerianus* on total duration of freezing ($n = 6$). $*P < 0.05$ compared to the control, $+P < 0.05$ compared to day, ME = methanol extract; EF = ethyl acetate fraction

Effect of ME and EF on some autonomic activities (urination and defecation)

On day 1, all the treated groups produced significant ($p < 0.05$) reductions in the number of urine streaks. However, on day 2, there was no significant difference in the number of urine streaks when compared to the control. A significant increase ($p < 0.05$) in the number of faecal boli was seen with ME at 400 mg/kg and EF at 200 mg/kg. On day 2, a significant ($p < 0.05$) difference over day 1 was observed with ME 400 mg/kg and EF at both doses (Figure 5).

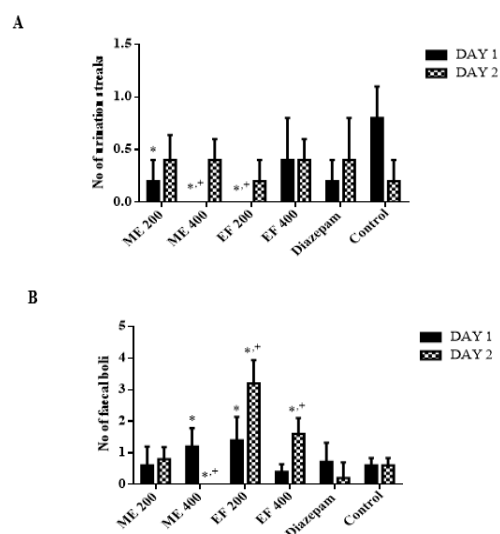


Figure 5: Effect of the extract of *Phyllanthus muellerianus* on some autonomic activities ($n = 6$). (A) urination streaks; (B) faecal boli. $*p < 0.05$ compared to the control, $+p < 0.05$ compared to day 1, ME = methanol extract; EF = ethyl acetate fraction

Effect of ME and EF on motor coordination

A significant difference ($p < 0.05$) was seen only with ME (400 mg/kg), which reduced the time of fall of the mice from the rotarod in the same way as diazepam (Figure 6).

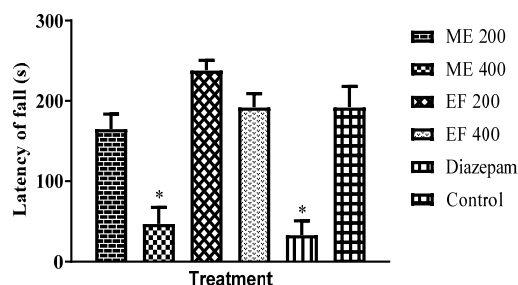


Figure 6: Effect of extract of *Phyllanthus muellerianus* on motor coordination. $*p < 0.05$ compared to the control, ME = methanol extract; EF = ethyl acetate fraction

DISCUSSION

This study evaluated the effects of the leaf extracts of PM on the CNS using different neuropharmacological models in mice. Although seizures can be controlled with currently available anticonvulsant drugs, they do not produce a cure, and they also cause significant side effects that most patients find intolerable. This has sparked interest in the search for novel therapies from medicinal plants for the treatment

of epilepsy. Anxiety and anxiety-like disorders are neurological disorders in which fear is a prominent symptom often leading to depression [8]. The anxiolytic drugs employed to treat these conditions are also not without side effects; notable among them is sedation. For these reasons, the effect of PM on the central nervous system was evaluated in mice with a focus on anxiety, convulsion, and motor coordination.

The evaluation of the acute toxicity could be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics. In the present study, the oral LD_{50} was more than 5000 mg/kg, probably indicating that the extract is safe and may not cause acute intoxication [9].

To evaluate the CNS effect of PM, its anticonvulsant effect in mice was investigated using PTZ model. PTZ produces convulsion by non-competitively antagonizing gamma-aminobutyric acid (GABA)-benzodiazepines receptor complex [10], particularly the post-synaptic GABA_A receptor mediated chloride (Cl⁻) channel conductance by diminishing benzodiazepine site. Myoclonic and absence seizures in humans can be controlled by agents that protected against tonic-clonic seizures in mice caused by PTZ [11]. Our study showed that EF completely abolished seizure and gave 100% protection even better than phenobarbitone, the standard drug. The duration of seizure before death in ME at all doses was significantly prolonged compared to the control. ME also offered some protection to the mice at the highest dose. Therefore, the activity of ME and EF on PTZ induced seizure model suggests that the plant may possess anticonvulsant potential. The EF gave better protection than the ME and other fractions, possibly suggesting the presence of some antagonistic constituents in the crude extract. This activity is likely mediated by enhancing GABA-mediated inhibition in the brain since PTZ antagonizes GABA to bring about convulsion. More studies are, however, needed to confirm the anticonvulsant mechanism of this plant.

The sedative and anxiolytic effects of ME and EF were assessed by observing the spontaneous locomotor and exploratory activities of the mice in the open field. This test reflects the conflict between the mice's tendency to explore a new arena and their inborn fear of the central area of a novel open field. Exposure to a novel environment is associated with emotional disturbances and anxiety. An anxious animal exhibits reduced locomotion accompanied by periodic immobility or freeze; it also stays more at the peripheral areas, near the walls of the field

with reduction in exploration (rearings). An increase in movement depicts CNS excitability, while its decrease is indicative of sedation and calmness resulting from CNS depression [12]. In our study, ME, like diazepam, decreased the number of line crossing which suggests decrease in the curiosity of the mice to explore the new environment (decrease in locomotor activity). This decrease in locomotor activity suggests that the plant may possess sedative and CNS depressant activity. Diazepam and other benzodiazepines cause suppression in exploratory activities due to their sedative properties [1]. Even though ME caused sedation at the higher dose like diazepam, it elicited an increase in exploratory activities at the lower dose, indicating that the extract may also possess anxiolytic activity. Magaji *et al*, reported a significant rise in exploratory activities with diazepam, which is in opposition with the result of this study [13]. This variance may be a result of different doses employed. While 1 mg/kg of diazepam was used in this study, the authors used 0.5mg/kg. These biphasic effects of diazepam at different doses are well documented [14]. It is interesting to note that EF caused increases in both locomotor and exploratory activities. A similar result was reported where lamotrigine, a drug known for its therapeutic use in reducing behavioural disturbance and alleviating psychiatric disorders, increased both locomotor and exploratory activities in open field test [15]. In clinical practice, EF may be beneficial in treating epilepsy because of its ability to abolish seizure without compromising mood.

In a novel environment, it is expected that mice display grooming behaviour. Decrease in grooming behaviour signifies reduced stress. Even though the frequency of grooming was unaffected by ME and EF in this study, the duration was significantly reduced. This is consistent with drugs with anxiolytic-like properties [16]. In our study, the frequency of grooming in EF was increased after 24 h possibly due to habituation.

The control animals, though they exhibited the least duration of freeze, showed little or no centre square crossing and rearing behaviours (which were significantly increased in ME and EF). These reactions meant lack of exploration and depicted fear and anxiety. The reduced duration of freeze in control animals without exploration further promotes the anxious state of the control animals, while a significant increase in freeze duration seen with ME may result from sedation and not anxiety since its effect on exploratory activities increased significantly. This result

suggests that the extract of PM may possess sedative and anxiolytic effects.

Furthermore, in anxiety, augmented autonomic activity leading to increased defaecation and urination may also be present [17]. Higher locomotion, reduced defecation, and urination could confirm some anxiolytic activities. All the treatment groups, compared to the control, significantly reduced urination streaks, with some recording no urination at all, confirming the anxiolytic effect of the plant since anxious animals tend to urinate more frequently. The validity of increase in defaecation as an appropriate anxiety measure has been questioned [17], and the result of this study seems to support the view that a decrease in this factor is not a reliable index in measuring the anxiolytic effects of drugs. This is because from the study, (except ME at 400 mg/kg), a significant increase in defaecation was observed in the groups that exhibited mostly anxiolytic activities even on repeated exposure when compared to the control animals. Moreover, the antidiarrhea effect of the extract has been reported, suggesting that the increased defaecation in the extract group was not a result of the laxative effect of the extract [18].

It should be noted that over time (on repeated exposure, i.e., on day 2), habituation occurred with most anxiety-related behaviours decreasing (i.e., increase in locomotion and decrease in freezing duration) in all the groups, including the control animals. Exploratory activities also increased. Habituation means that there was no cognitive deficit. The mice were able to remember that they were exposed to the open-field arena previously [19]. Anxiety/fear in a new environment should naturally decrease with time as the animals get accustomed to the environment. This is consistent with a time-dependent reduction in fear and not as a result of drug treatment. The treated animals exhibited habituation, suggesting that the extract did not cause memory impairment, as is the case with most psychotropic drugs. Additional studies are, however, required to ascertain the effect this extract has on memory.

The effects of ME and EF on motor coordination were assessed using the rotarod test. Rotarod test is used to assess muscle relaxation and motor incoordination produced by a substance, especially psychotropic drug. When there are motor coordination deficits or losses, as seen in many neurological disorders, a sudden and sharp loss of connectivity between the CNS and the muscles of the body will occur. This test reveals that on a rotating rod, equilibrium can be

retained by animals with normal motor efficiency. This apparatus also allows for the measurement of drug-induced increases in performance. In this study, ME at a higher dose recorded a significant ($p < 0.05$) reduction in performance of the mice on the rotarod, and the effect is comparable to that of diazepam. This further supports the sedative effect of the extract. On the other hand, EF tend to increase performance of the mice on the rotarod thereby strengthening the assertion that EF possesses anticonvulsant and anxiolytic effect without dysfunction of motor coordination. This result is in consonance with the previous work that reported the anticonvulsant, anxiolytic and non-sedating actions of imidazenil (an imidobenzodiazepine) [20].

Previously ME and EF tested positive for glycosides, alkaloids, flavonoids, steroids, saponins, and steroids [3]. This plant likely owes its CNS effects to these constituents. CNS depressant, antidepressant, and anxiolytic actions of some plants containing triterpenoids, saponins, tannins, flavonoids, phenols, and alkaloids have been reported [21]. Notably amongst these are flavonoids which bind the benzodiazepine site of GABA receptor with high affinity, while PTZ induces seizure by blocking benzodiazepine site at GABA receptors [22]. Therefore, it is possible that the flavonoids in the extract occupied this site to elicit its anti-PTZ seizure effect.

HPLC fingerprinting of EF revealed the presence of 9 α hydroxyl pinoselinol, isofistularin-3, epicatechin O-3,4 dimethyl gallate, aerophobin-1, kaempferol 3-O gallate, 3,6-O-dimethylellagic acid, naamine F and septicine. These compounds have been identified in this plant for the first time. Antiproliferative effect of aerophobin-1 and anti-inflammatory activity of septicine have been previously determined [23]. Antioxidant and hepatoprotective effect of epicatechin O-3,4 dimethyl gallate have been reported while kaempferol has been demonstrated to reduce oxidative stress [24]. These compounds may be responsible for the anticonvulsant and anxiolytic effects of EF. Moreover, seizures induced by PTZ have been reported to respond to antioxidant compounds [2, 21]. More research is ongoing in our lab to isolate and test the compound(s) eliciting the activities present in *P. muellerianus*.

CONCLUSION

Based on the results, methanol leaf extract of *P. muellerianus* may have sedative, anxiolytic and anticonvulsant activities, while its ethyl acetate fraction possesses anticonvulsant and anxiolytic

activity but devoid of sedative effect and cognitive impairment. Taken together, these have brought to light the possibility of developing agonists with anticonvulsant and anxiolytic activities devoid of sedative effect associated with most non-selective GABA agonists like diazepam. The sedative constituent(s) in ME may be explored as a lead in the development of agents for insomnia. Our future work is geared towards isolating the bioactive principle(s) present and the exact mechanism(s) of its action.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with the study

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Charles Okoli conceived and designed the study, Martha Ofokansi collected and analysed the data, Stella Ihim and Chinenye Ugwah-Oguejiofor wrote the manuscript, Peter Akah supervised the work. All authors read and approved the manuscript for publication.

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