Pharmacological Effects of Standardized Extract of *Piliostigma reticulatum* Fruit on The Central Nervous System of Swiss Albino Mice: Scientific Justification for its Use in Management of Neuropsychiatric Disorders in African Traditional Medicine

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**Abstract**

*Piliostigma reticulatum* fruit preparations are popularly used in Nigeria for therapeutic management of fatigue, stress, insomnia and as calming agent for mood. The aim of this study was to provide scientific evidence for its continuous use in folk medicine. The ethanolic fruit extract of *Piliostigma reticulatum* PRFE was standardized using reverse phase high performance liquid chromatography to establish finger print for identity and quality assurance. The oral median lethal (LD₅₀) dose was evaluated in mice. The effects of PRF diazepam and imipramine were assessed on elevated plus maze, hole- board, forced swim and open field apparatus. The extract was acutely safe at 5000 mg/kg body weight. The extract produced central depressant effect doses of 400 and 800 mg/kg in elevated plus maze, hole board and forced swim test. The results obtained suggested that *Piliostigma reticulatum* fruit extract contains bioactive components with central depressant effects that may be useful in managing neuropsychiatric disorders thus providing scientific evidence in support of its use in therapeutic application in folk medicine for neuropsychiatric disorders. The mechanism of action of the crude and identity of its active principles remains unknown.

**Citation:**

1. Introduction

*Piliostigma reticulatum* (DC) Hoehst (Syn. Bauhinia reticulate DC) (Leguminosae) is an evergreen shrub or small tree common in fallows. It is found mostly in the northern part of Nigeria (Dalziel, 1937). Tea from the leaves is given to treat cold and the bark decoction has been used traditionally to treat stomach pains and indigestion (Keay et al., 1964). The root and stem bark decoctions are used for the managing wounds, chronic ulcers, diarrhoea, toothache and gingivitis, cough and bronchitis (Dalziel, 1937).

Studies carried out by Ibewuike et al., (1997) revealed that aqueous ethanolic leaf extract of the plant possessed antibacterial activity against a wide spectrum of organism, and significantly inhibited prostaglandin synthetase enzyme (COX) in test for anti-inflammatory activity. The antibacterial and anti-
inflammatory activities of the leaf extract have been attributed to the presence of C-methylflavonols including 6,8-di-C-methyl quercetin3-methyl ether (Adesina et al., 2000; Alcaraz and Jimenez,1988; Aderogba et al., 2003), 6-Cmethylquercetin 3,7-dimethyl ether (Ibewuike et al., 1996), quercetin, quercitrin, 6-Cmethylquercetin 3-methyl ether, 16'-hydroxykauran-18-oic acid and diterpene (Ibewuike et al., 1996; Martins et al., 1997). In our earlier studies on the root extract the antidiarrhoeal (Salawu et al., 2007) and anti-ulcer (Salawu et al., 2009) effects of methanolic root extract of P. reticulatum were due to the presence of tannins. The fruits are used in Burkina Faso in various prescriptions for cough, bronchitis and headache (Kerharo and Bouquet, 1950). In northern Nigeria the fruit decoction is used as calming agent in agitated patients and as sedative for patients suffering from sleeplessness (Kerharo and Bouquet, 1950) while in southern Nigeria powdered fruit is macerated in local gin called Ogogoro for agitated individuals who finds it difficult to sleep. The aim of the current study was to evaluate the central depressant effects of the ethanolic fruit extract of Piliostigma reticulatum in mice.

2. Objective of the Experiment

The objective of the study was to provide scientific evidence in support of the use of Piliostigma reticulatum fruit extract in folk medicine for management of neuropsychiatric disorders and index of assessment of identity and quality.

3. Materials and Methods

3.1 Plant Material

P. reticulatum fruits were collected from Suleja, Niger state, Nigeria. It was identified by Mallam Muazzam of the medicinal plant research and traditional medicine (MPR&TM) department of National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja and voucher specimen (NIPRD/H/6579) was prepared and deposited at the department’s herbarium for future reference.

3.2 Preparation of Fruit Extract

The fruits of P. reticulatum were cleaned, cut into pieces, air-dried and crushed into a fine powder. The powdered fruit (200 g) was cold macerated with 1.0 L of 70 % ethanol for 24 h with constant shaking using a GFL shaker. The resultant mixture was filtered using Whatman filter paper No. 1 (Cat. No. 100125) and the filtrate concentrated by rotary evaporation at 45° C. The filtrate was dried on steam bath yielding 8.66% on dry weight basis. The dried sample was stored in specimen bottle and kept in refrigerator until required for use. Drugs and extract were administered orally throughout the study via stainless steel oral cannula.

3.3 Phytochemical Analysis

Screening for phytochemical constituents of PRFE was done using standard methods (Harborne, 1998; and Sofowora, 2008).

3.4 High Performance Liquid Chromatography Analysis

The chromatographic system includes Shimadzu HPLC system consisting of Ultra-Fast LC-20AB prominence equipped with SIL-20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector; column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, VP-ODS 5µm and dimensions (150 x 4.6 mm). The chromatographic conditions included mobile phase: solvent A: 0.2% v/v formic acid; solvent B: acetonitrile; mode: linear gradient; flow rate 0.6 ml/min; injection volume 20 µl of 250 µg/ml solution of PRFE in ethanol; detection UV 254 nm; reference standard, Rutin (Fluka, Germany) 50 µg/ml in methanol was used as internal standard as reported by Bienvenu et al., 2002 with some modifications. The HPLC operating conditions were programmed to give the following: at 0.01 min, solvent B: 20%; at 5 min, solvent B: 30%; at 15 min, solvent B: 60%; at 20 min, solvent B: 20%. Column oven temperature was 400°C. The total run time was 20 minutes. Analysis was carried out for rutin only, PRFE only, old PRFE with rutin and freshly prepared PRFE with rutin to check for stability of PRFE.

3.5 Animals

Adult Swiss albino mice (18–20 g) of either sex, obtained from Animal Facility Centre (AFC) of National Institute for Pharmaceutical Research and Development (NIPRD) were used for this study. The rats were housed in transparent plastic cages padded with wood shavings, under standard conditions of temperature, relative humidity and light/dark cycles (12/12 h). They were fed with pelletized feeds obtained from Feeds cap limited Ibadan and water ad libitum. Rats were approved for use by the AFC committee after reviewing the protocol. We certify that all experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use
of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All efforts were made to minimise the number of mice used and their suffering.

3.6 Acute toxicity study
Oral acute toxicity study of *Piliostigma reticulatum* (PR) ethanolic fruit extract was carried out according to Lorke’s (1983) method modified by Salawu et al., (2009). Groups of three mice were orally administered with single doses of PR (10, 100 and 1000 mg/kg) in the first phase of the study. In the second phase of the study three groups of three mice per group were given single doses of PR (1600, 2900 and 5000 mg/kg). The mice were observed for gross behavioral, neurologic, autonomic effects and mortality at 30, 60 min and at each 2 h intervals up to 8 h and then once a day for 7 days.

3.7 Treatments
Saline, PR (200–800 mg/kg), or drugs (positive controls) were administered by oral route (gavage) using a tuberculin syringe fitted with oral cannula (0.1 cm × 4 cm). All treatments were administered to mice at a volume of 10 ml/kg.

3.8 Drugs
As controls of behavioral tests, the following drugs were used: diazepam 2.5 mg/kg (Roche pharmaceuticals) and imipramine hydrochloride 15 mg/kg (Sigma-Aldrich, MO, EUA), which were dissolved in normal saline (NaCl 0.9%). The doses of the reference standards were based on previous reports (Lolli et al., 2007; Ergün et al., 2008).

2.9 Behavioral Tests
All the behavioral procedures were carried out between 8:00 hand 12:00 h a.m. in a temperature controlled room (23±1 °C). The mice were grouped such that each group consisted of equal number of males and females separately housed.

3.9.1 Elevated plus Maze
The elevated plus maze is an anxiety paradigm based on the rodent’s natural aversion to a novel and potentially dangerous environment represented by the open and elevated spaces (Lister, 1987). The elevated plus maze apparatus is a plus (+) shaped wooden structure, consisting of two open arms (40 cm × 5 cm × 10 cm) and two enclosed arms (40 cm × 5 cm × 10 cm) extended from a central platform (10 cm × 10 cm). The maze was elevated 50 cm from the room floor. Mice were habituated to the testing room under dim light for at least 1 h before the test and then randomly divided into five groups of control (10 ml normal saline/kg body weight orally, extract (200, 400 and 800 mg/kg body weight orally)-and diazepam (2.5 mg/kg body weight p.o)-treated groups. One hour later, each mouse was placed at the center of the maze, facing one of the open arms and allowed to explore the maze freely for a 5 min testing period. The time spent and frequencies of entries in open and enclosed arms were recorded. The maze was thoroughly cleaned between tests with a tissue paper moistened with 70% ethanol.

3.9.2 Hole-board test
The hole board test procedure for exploratory behaviour used was as described by Ozturk et al., (1996), with some modifications. The apparatus was composed of a wooden box (60 cm by 30 cm) with 16 equidistant holes (1 cm diameter, 2 cm depth). The apparatus was positioned 50 cm above the ground, in a dimly illuminated room. Adult mice of either sex were randomly divided into five groups of five mice per group. Three groups received graded doses of the extract (200-800 mg/kg, p.o.). One group received diazepam (2.5 mg/kg, p.o) and the remaining group received normal saline to serve as control. One hour later, each mouse was placed in the centre of the hole-board and allowed to freely explore the apparatus for 5 min. The number of head dips into the holes was counted recorded (Wolfman et al., 1994). A head dip was scored if both eyes disappeared into the hole. An agent that decreases this parameter reveals a sedative behaviour (File and Pellow, 1985) while anxiolytics have been shown to increase the number of head dips (Takeda et al., 1998).

3.9.3 Forced swim test (FST)
The FST was performed according to the methods described by Porsolt et al., (1977) and modified by Ben et al., (2012). In brief, mice were individually placed in 25 cm glass beaker (10 cm diameter) containing water at 23±1 °C. Each rat was placed in a transparent glass cylinder (height 46 cm, diameter 20 cm) containing water at 25 °C to a depth of 30 cm; and was forced to swim for 15 min (pretest session). The water depth of 30 cm allowed the rats to swim or float without their hind limbs touching the bottom of the tank (Detke and Lucki, 1996; Kirby and Lukic, 1997). The rats were removed and allowed to dry in a heated enclosure (32 °C) before being returned to their home cages after the 15 min pretest swim in water. The rats were randomly divided into various treatment groups. Duration of immobility was recorded during a 6-min
swimming test. A mouse was judged to be immobile when it floated and its hind-limbs were immobile, and only small movement of the forepaws was made to keep its head above water.

3.9.4 Open field
The Open Field (OF) test was carried out using the method described by Herrera-Ruiz et al., 2008). The OF apparatus consists of a transparent glass box (45×45 cm). The floor was divided by lines drawn into 9 equally sized squares (Royce, 1977). Twenty five mice were randomized into five groups of mice each. Three groups received graded doses of the extract (200-800 mg/kg, p.o.). Another group received diazepam (2.5 mg/kg, i.p.) while the last group received 5 ml normal saline/kg body weight orally and served as negative control. One hour after extract and thirty minutes after diazepam administration, each mouse was placed in the center of the apparatus. The frequency of horizontal movement (locomotion) and vertical movement (rearing) were recorded manually for 5 min period. The apparatus was thoroughly cleaned between tests with a tissue paper moistened with 70% ethanol.

3.5 Statistical analysis
All data were expressed as the mean ±standard error of mean (SEM). Statistical analysis was carried out using one-way analysis of variance (ANOVA). Any significant difference between means was assessed by student’s t-test at 95% level of significance.

4. Results

4.1 Phytochemical analysis
The phytochemical tests carried out on PRFE indicated the presence of alkaloids and tannins. Anthraquinones, saponins and cardiac glycosides were not detected (Table 3.1)

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Test/Reagents</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Dragendorff’s reagent and Meyer’s reagent</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Borntrager’s test</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>Lieberman’s test, Keller-Kiliani test</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>Frothing test</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>Ferric chloride reagent, Hydrochloric acid reagent</td>
<td>+</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td></td>
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</tbody>
</table>

Figure 3.1: HPLC chromatogram of PRFE

Figure 3.2: HPLC chromatogram of Rutin

Figure 3.3: HPLC chromatogram of PRFE-fresh with Rutin

Figure 3.4: HPLC chromatogram of PRFE-old with Rutin
4.2 Acute toxicity study
The oral administration of *Piliostigma reticulatum* ethanolic fruit extract at the doses of up to 5 000 mg/kg did not produce any clinical symptom of toxicity and mortality. Therefore, the oral median lethal dose (LD₅₀) of *Piliostigma reticulatum* ethanolic fruit extract was estimated to be greater than 5000 mg/kg.

4.3 Effect on EPM
*Piliostigma reticulatum* fruit extract (PRFE) at 200 mg/kg significantly (*F*₄,₂₅=17, *p*<0.05) decreased the time spent in the closed arm of elevated plus maze. The effect of the extract at 400 and 800 mg/kg on time spent in closed arm of elevated plus maze were not different from that of the control. Diazepam significantly (*p*<0.0001) decreased time spent on closed arm of the elevated plus maze at 2.5 mg /kg body weight. PRFE significantly (*F*₄,₂₅=20, *p*<0.0001) increase the time spent in the open arm at 200 mg/kg while its effect at 400 and 800 were not different from the control. Diazepam significantly increased time spent in the open arm of the elevated plus maze (Fig 3.5 and 3.6).

4.4 Effect on exploratory behaviour
The extract (400 and 800 mg/kg) and diazepam (2.5 mg/kg) significantly (*F*₄,₂₀=36, *p*<0.0001) reduced frequency of head dip of mice on hole board (Fig 3.7).

4.5 Effect on onset of immobility in Forced Swim Test
The extract (200, 400 and 800 mg/kg) significantly (*F*₄,₂₅=140, *p*<0.0001) reduced while imipramine increased the onset of immobility in mice (Fig 3.8).

4.6 Effect on duration of immobility in Forced Swim Test
The extract (200, 400 and 800 mg/kg) significantly (*F*₄,₂₅=220, *p*<0.0001) increased while imipramine increased duration of immobility in forced swim test by mice (Fig 3.9).

4.7 Effect on locomotion
The extract had no effect (*F*₄,₁₆= 0.14) on locomotor activity of mice on the open field (fig 3.10).

5. Discussion
In the present study, the extract of *Piliostigma reticulatum* fruit extract (PRFE) was standardized against Rutin and its behavioural effects were investigated. A finger print was obtained that can be used to ascertain the identity and stability of the extract over time.
Figure 3.8: Effect of oral administration of *Pilostigma reticulatum* fruit extract significantly (F₄, ₂₅=140, p<0.0001) reduced onset of immobility while 15 mg imipramine /kg prolonged onset of immobility.

Figure 3.9: Effect of Methanol fruit of *Pilostigma reticulatum* on duration of immobility. The extract significantly (F₄, ₂₃=220, p<0.0001, n=6) increased while 15 mg imipramine/kg reduced duration of immobility of mice in FST.

Figure 3.10: Effect of single administration of PRFE on locomotion on open field.

The extract of *Pilostigma reticulatum* fruit had no effect on locomotion on the open field (F₃, ₁₆=0.66, p=0.14).

No signsof toxicity or deaths were observed 7 days after oral administration of PRFE up to a dose of 5000 mg/kg body weight. The high safety margin of the extract may explain the continuous widespread use of the plant preparations in folk medicinal management of behavioural disorders and other ailments (Salawu et al., 2009). A single oral administration of PRFE produced Anxiolytic effect at low dose and anxiogenic effect at higher doses (400 and 800 mg/kg) in the elevated plus maze, sedative effect on hole board, depressant effect indicated by shortened latency to immobility and prolonged duration of immobility in Forced Swim Test. The extract however had no effect on locomotion.

The Elevated plus Maze (EPM) has been classically used to evaluate Anxiolytic and anxiogenic effects of drugs (Lister, 1987). In the present work the treatment of mice with diazepam, a benzodiazepine Anxiolytic drug, lead to a significant increase in the time spent in the open arm of the EPM. The extract at 200 mg/kg body weight showed Anxiolytic like effect by increasing time spent in the open arm and reducing time spent in the closed arm of EPM. But at doses of 400 and 800 mg/kg the extract produced anxiogenic like effect characterized by increased time spent in the closed arm and reduced time in open arm of the EPM. This observation is consistent with the effects of typical sedative - anxiolytics like diazepam that produce Anxiolytic-like effect at low dose and sedative effect at higher doses.

The hole-board test is a measure of exploratory behaviour (Crawley, 1985) characterized by the frequency of head dip and time spent dipping head in hole board. The effect of any agent that decreases the frequency of head dip in the hole-board is best described as sedative (File and Pellow, 1985) while Anxiolytics have been shown to increase the number of head dips in the hole-board test (Takeda et al., 1998). The extract dramatically and dose-dependently diminished the exploratory behaviour in mice, thereby suggesting that the extract possessed sedative activity rather in addition to Anxiolytic effect. The observation in hole board test may explain why mice treated with PRFE favours the closed arm of the EPM.

In the forced swim test the extract shortened the onset of immobility and prolonged duration of immobility. However, the standard drug imipramine, a prototype antidepressant prolonged onset and shortened duration of immobility.

Central nervous system depressants and sedatives are known to prolong the duration of immobility in FST while antidepressants psychostimulants, euphoric shortened the duration of immobility in FST. The Forced Swim Test (FST) has been widely employed to...
evaluate the effects of CNS depressants, antidepressants, sedative-hypnotics, psychostimulants, euphorics, nootropics and adaptogens. The works of Porsolt et al., (1978) has shown that the immobility seen in rodents during swimming reflects behavioural despair as seen in human depression. FST has been used extensively to evaluate the anti-stress effect of natural products from medicinal plants in mice and rats (Ozturk et al., 1995; Maity et al., 2000). The sedative effect of PRFE was further illustrated by the shortened onset and prolonged duration of immobility in FST. The effect of the extract on the FST may therefore be described as sedative or as central depressant.

In order to ascertain that the effect of the extract was centrally mediated and not due to its adverse effect on skeletal muscle, its effect was evaluated on locomotion of mice in the open field. The absence of effect on locomotion of mice in the open field showed that the extract behavioural effects were not due to neuromuscular blockade but centrally mediated.

It may therefore be concluded, that the PRFE contains psychoactive principles that behave as anxiolytics at low dose and as sedative and or central depressant at higher doses. These effects may be due to the presence of tannins and alkaloids in PRFE. We suggest that these properties may be explored in the management of neuropsychiatric disorders characterized by anxiety, psychological agitation and insomnia.

**Author's Contribution**

All author listed have contributed equally to the research that resulted in this manuscript and have declared no conflict of interest.

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