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Research Article

TOXICOLOGICAL EVALUATION OF THE METHANOLIC LEAF EXTRACT OF *PILIOSTIGMA RETICULATUM* (DC.) HOCHST IN MALE WISTAR RATS

***Bunmi Fayanju, Afolabi Clement Akinmoladun and Mary Tolulope Olaleye**

Phytomedicine, Biochemical Pharmacology and Toxicology Unit, Department of Biochemistry, School of Life Sciences, The Federal University of Technology, Akure, Nigeria

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Abstract

Background: The growing appropriateness of the use of herbal preparations in the treatment of countless diseases must be thoroughly accompanied by the evaluation of the safety of these remedies. Despite the availability of known antidiabetic medicine in the pharmaceutical market, diabetes and the related complications continue to be a major medical problem**. Objective:** The objective of this study was to evaluate the toxicological effects of the methanolic leaf extract of *Piliostigma reticulatum* (DC) Hochs in male Wistar rats. **Materials and Methods:** This evaluation was carried out both in vitro and in vivo. In vitro, acute toxicity test was evaluated according to Lorke's method. This method was carried out in two phases. In the first phase, the rats were grouped into three with three rats in each group and administered 10 mg/kg, 100 mg/kg and 1000 mg/kg of the extract. In the second phase, the rats were also grouped into three and administered 1600 mg/kg, 2900 mg/kg and 5000 mg/kg. The extract at a single dose of 5000 mg/kg showed no signs of toxicity nor mortality in any of the animals tested during 7 day observation period.Liver function test was also evaluated in this study. The rats were grouped into nine made up of fifteen rats each and were administered the methanolic leaf extract of *Piliostigma reticulatum* (DC) Hochst for 14 days. The serum levels of alanine and aspartate transaminases (ALT and AST), alkaline phosphatase (ALP) were carried out. **Results:** Results from the *in vitro* toxicological evaluation reveals that the LD 50 is greater than 5000 mg/kg according to Lorke's method. There was no death recorded, neither was there any convulsion, salivation or a change in the skin color recorded. Also, the liver function tests carried out revealed a significant ($p<0.0001$) increase in the activities of Alanine (ALT), Aspartate transaminases (AST) and alkaline phosphatase (ALP) enzymes compared to the negative control rats. This indicates that there was a problem with liver function. Treatment with the methanolic leaf extract of *Piliostigma reticulatum* (DC) Hochstand gilbenclamide significantly ($p<0.0001$) reduced the activities of AST, ALT and ALP and ameliorated the alteration in liver function. Treatment with extracts alone (without diabetes) did not have any significant effect on AST, ALT and ALP compared with the negative control rats. **Conclusion:** The findings in this study revealed that the methanol leaf extract of *Piliostigma reticulatum* (DC) Host does possess properties that reverse hepatocellular damage in rats. Toxicological evaluation carried out showed no mortalities or adverse clinical manifestations. The biochemical analysis also revealed that the plant is considered very safe up to 5000 mg/kg. This extract is however recommended to cure damage that may have taken place in the liver.

Keywords: Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, *Piliostigma reticulatum*.

INTRODUCTION

Medicinal plants have been used in the treatment of various types of ailments over the past decades. The enormous diversity of plants have played crucial roles in the life of humans. Plant based products are currently being employed in the formulation of pharmaceutical preparations based on the recommendation of herbal practitioners (Valiathan, 1998). The growing appropriateness of the use of herbal preparations in the treatment of countless diseases, including diabetes, must be thoroughly accompanied by the evaluation of the safety of these remedies. The use of herbal interventions in many nations across the world is accompanied by lots of reduced regulations compared to orthodox medicines. As such, issues of toxicity are rarely addressed. However, there have been numerous reports of suspected toxicity and adverse events in the literature (Ekor *et al.,* 2010). Majority of these unpleasant and sometimes life threatening events may be as a result of overdose and presence of toxic compounds in these plant extracts. Reports of renal and hepatotoxicity of herbal preparations have been on the increase too (Liu *et al.,* 2016). Hence, there is the need for toxicological assessment of plant extracts in the light of current research. *Piliostigmareticulatum* (DC) Hochst, (Synonym: *Bauhinia reticulata*) is a leguminous plant belonging to the family Caesalpiniaceae and is widely distributed in Africa and Asia. Ethnomedically, the bark, root, pod, young stem or leaves have been used for treating leprosy, neuralgia, inflammation, smallpox, malaria, coughs, ulcer, heart pain, gingivitis, snake bite, dysentery, fever, wounds and a variety of closely related disease conditions (Babajide *et al.,* 2008; Dosso *et al.,* 2011; Pradhan and Panchawat, 2018; Kafi *et al.,* 2018). The decoction of *P. reticulatum* had been used to manage seizures, convulsion and insomnia (Bum *et al.,* 2009).Phytochemical screening of the leaf extract of the plant revealed an abundance of phenolic acids, flavonoids, tannins, alkaloids, glycosides and terpenes (Akomolafe and Adeyanju, 2013). *Piliostigmareticulatum* had been reported to possess antioxidant (Zerbo et al, 2010; Dieng et al, 2018), antimicrobial (Kafi *et al.,* 2018), antidiarrheal (Dosso *et al.,* 2011) as well anti-epileptic (Pradhan and Panchawat, 2018) activities. However, information on the protective potential of *P. reticulatum* against type 2 diabetes complications is scanty. Hence, this research seeks to investigate the influence of the methonolic leaf extract of *Piliostigmareticulatum* (DC) Hochst, in male Wistar rats.

Corresponding Author:** ***Bunmi Fayanju
Phytomedicine, Biochemical Pharmacology and Toxicology Unit, Department of Biochemistry, School of Life Sciences, The Federal University of Technology, Akure, Nigeria

MATERIALS AND METHODS

Plant material

The leaves of *Piliostigmareticulatum* (DC.)Hochst were collected in August, 2019 from Asa local government in Ilorin Kwara State Nigeria. The taxonomic identification was

authenticated by the plant department of University of Ibadan, Oyo state Nigeria with voucher number – UIH22967.

Drugs and chemicals

Distilled water, Ethanol, Hydrogen peroxide, Deoxyribose, Phosphate buffer, Trichloroacetic acid, Sodium Hydroxide (NaOH), Sodium Nitroprusside, Greiss reagent Thiobarbituric acid Methanol, $FeCl_3$, Chloroform, H_2SO_4,HCl, Wagner's reagent, Mayer's reagent, DPPH, ABTS, Trolox, Ammonia, Gallic acid, Folin-Ciocalteu reagent, Sodium carbonate, Aluminum nitrate-Potassium acetate reagent, Ethanol, Quercetin, DPPH, Ascorbic acid, EDTA, Ferrozine and α-tocopherol. All chemicals used in this experiment were of analytical grade.

Plant extraction

The leaves of *Piliostigmareticulatum* (DC) Hochstwill be collected from Afon in Asa local government Ilorin, authenticated at the hebarium unit in University of Ibadan and oven-dried at 40^{o}C. The dried leaves will be ground into powder and macerated in methanol (96%) for 48 h, with intermittent shaking and filtered through Whatman No. 1 filter paper. The filtrates will be concentrated using rotary evaporator with regular stirring to obtain methanol extract.

Acute Oral Toxicity test

Acute oral toxicity test was performed using the test procedure as per Organization for Economic Co-operation and Development (OECD) guidelines 401 using Lorke's method (OECD, 2001). This test was carried out in two phases. In the first phase, nine rats were divided into three groups of three rats each, and given 10, 100, 1000 mg/kg of the plant extract. After the administration of the plant extract, observation was made at regular interval to check for the onset of adverse effect, time to death or time to recover. The period of observation in this phase I was 24 hours. In the second phase, the animals were divided into three groups. In this phase, the dose level was either stepped up or down depending on the outcome of the result obtained from phase I. The animals were administered higher dose of 1600, 2900 and 5000 mg/kg. Toxic symptoms were observed for 24 hours as well as delayed toxic symptoms for 7 days. The lethal dose was calculated by the formula.

$$\mathbf{LD_{50}= \sqrt{(D_0 \times D100)}}$$

D_0= highest dose that gave no mortality
D_{100}= lowest dose that produced mortality

Experimental design

The plant extract was administered in a single dose by gavage using specially designed rat's oral needle. Animals were kept overnight fasting prior to drug administration by oral gavage. Following the period of fasting, animals were weighed and test substance administered orally at a dose of 10, 100 and 1000 mg/kg body weight. The administration dose was 2 ml/kg body weight of the animal for 24 hours only. Based on the body weight of the animal on the day of treatment, the quantity of the test substance was calculated. After administration of the test substance, observation was made at regular interval to check for the onset of adverse effect, time to death or time to recover. The period of observation in this phase I was24 hours. Direct observation parameters of the state of health of animals included: tremors, convulsions, salivation, diarrhea and sleep. Eyes and mucous membrane, circulatory, skin and fur, respiratory, autonomic and central nervous systems will be observed. The time of death, if any, was recorded.

They were grouped as follows – Phase 1 (24 hours)

Group 1: Normal rats + 10 mg/kg of methanolic fraction of *P. Reticulatum* extract (n=3)
Group 2: Normal rats + 100 mg/kg of methanolic fraction of *P. Reticulatum* extract
Group 3 Normal rats + 1000 mg/kg of methanolic fraction of *P. Reticulatum* extract
Phase II: (7 days)
Group 1: Normal rats + 1600 mg/kg of methanolic fraction of *P. Reticulatum* extract (n=3)
Group 2: Normal rats + 2900 mg/kg of methanolic fraction of *P. Reticulatum* extract
Group 3 Normal rats + 5000 mg/kg of methanolic fraction of *P. Reticulatum* extract

Liver Toxicity Tests

Liver function tests are a helpful screening tool which is an effective modality to detect hepatic dysfunction. Since the liver performs a variety of functions, no single test is sufficient to provide a complete estimate of the function of the liver.

The following liver function assays will be carried out:

1) Alanine amino transferase
2) Aspartate amino transferase
3) Alkaline phosphatase

Determination of Plasma Alanine Amino Transferase (ALT)

Assay of alanine aminotransferase (ALT) were carried out using the procedure provided by the RANDOX KIT Manufacturer as described by Reitman and Frankel (1957).

Principle

Alanine amino transferasecatalyzes the transfer of L-amino groups from L-alanine to α-oxoglutarate, a reaction which produces L-glutamate and pyruvate. The unstable pyruvate is then complexed with 2, 4-dinitrophenylhydrazine (DNPH) to produce an intensely coloured hydrazone on the addition of NaOH. This coloured complex absorbs radiation at 530-550nm. The ALT is measured by monitoring the concentration of pyruvate hydrazone formed with DNPH. The equation for the reaction is given below:

$$\text{α-oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L -glutamate} + \text{pyruvate}$$

Reagent Composition

1. **Buffer R1**; Phosphate buffer (100mmol/l, pH 7.4), L-alanine (200mmol/l), α-oxoglutarate (2.0mol/l)
2. **R2**; 2, 4-dinitrophenylhydrazine (2.0mmol/l)
3. **0.4mol/l NaOH;** 1.6g of NaOH was dissolved in distilled water and the solution was made up to 100 ml using same.

Procedure

0.5ml of R1 were added to a test tube already containing 0.1ml of plasma sample and the mixture incubated at 37^{o}C for 30 minutes. 0.5ml of R2 were added and the solution incubated again at 20^{o}C for 20 minutes. Finally, 5ml of NaOH were added. The solution were allowed to stand for 5 minutes at room temperature and the absorbance read at 546nm. The activity of ALT in the plasma was determined from the standard curve for ALT activity obtained from the kit.

Determination of Plasma Aspartate Amino Transferase (AST) Activity

Activity of AST was assayed using the procedure provided by the RANDOX KIT Manufacturer which followed the principle described by Reitman and Frankel (1957).

Principle

The aspartate amino transferasecatalyzes the transfer of L-amino groups from L-aspartate to α-oxoglutarate, a reaction that produces L-glutamate and oxaloacetate. The unstable oxaloacetate is then complexed with 2, 4-dinitrophenylhydrazine (DNPH) to produce an intensely coloured hydrazone on the addition of NaOH. This coloured complex absorbs radiation at 530-550nm. The AST activity was measured by monitoring the concentration of oxaloacetate hydrazone formed with DNPH. The equation for the reaction is given below:

α-ketoglutarate + L-aspartate $\xrightarrow{\text{Aspartate aminotransferase}}$ L-glutamate + Oxaloacetate

Reagent Composition for AST includes:

1. **Buffer (R1)**; Phosphate buffer (100 mmol/l, pH 7.4), L-aspartate (200 mmol/l), α-oxoglutarate (2.0 mol/l)
2. **Reagent (R2)**; 2, 4-dinitrophenylhydrazine (2.0mmol/l)
3. **0.4mol/l NaOH;** 1.6g of NaOH was dissolved in distilled water and the solution was made up to 100 ml using same

Procedure

0.1ml of plasma sample was mixed with phosphate buffer (100mmol/L, pH 7.4), L-aspartate (100mmol/L), and α-oxoglutarate (2mmol/L) and the mixture incubated for exactly 30min at 37^{o}C. 0.5ml of 2, 4-dinitrphenylhydrazine (2mmol/L) was added to the reaction mixture and allowed to stand for another 20min at 25^{0}C. Then, 5.0ml of NaOH (0.4mol/L) was added and the absorbance read against the reagent blank after 5 min at 546nm. The activity of AST in the plasma was determined from the standard curve for ALT activity obtained from the kit.

Alkaline Phosphatase

Alkaline phosphatase activity is determined by measuring the rate of conversion of p-nitro-phenylphosphate (pNPP) in the presence of 2-amino-2-methyl-1-propanol (AMP) at pH 10.4.

pNPP + AMP $\xrightarrow[Mg^{2+}]{ALP}$ pNP + AMP-PO_4

The rate of change in absorbance was due to the formation of pNP which is measured bichromatically at 410/480 nm and is directly proportional to the ALP.

STATISTICAL ANALYSES

Results were analyzed using appropriate analysis of variance (ANOVA) followed by Turkey multiple comparison tests. In all the tests, $P<0.05$ was taken as criterion for statistical significance. The statistical software used to analyze the data was Graph Pad Prism 6.01 (Graph Pad Software Inc., CA, USA).

RESULTS

Toxicological testing of methanolic leaf extract of *Piliostigmareticulatum*

No death was recorded and animals showed no signs of neurological deficits nor negative change in the state of health. Skin color and urination appears normal in all the doses used which signify that PR did not cause or have side effects on liver and kidney functions. Animals did not show any sign of toxicity after administration of the extract.

Table 1: Oral LD_{50} of methanolic leaf extract of *Piliostigmareticulatum* in Wistar rat

S/N	No of animals	Dosage (mg/kg)	Mortality (x/N)
Phase 1	3	10	0/3
	3	100	0/3
	3	1000	0/3
Phase 2	3	1600	0/3
	3	2900	0/3
	3	5000	0/3

x- No of dead animal(s); N- No of animals in the group; NS- No Symptoms

Effect of methanolic leaf extract of *Piliostigmareticulatum* on serum AST, ALT and ASP of male Wistar rats

The effect of PR treatment on serum level of AST, ALT and ALP in the rats is presented in Table 3. There was significant ($p<0.0001$) increase in the level of these liver function parameters in the positive control rats compared with negative control rats. This indicates that there was a problem with liver function. Treatment with PR and gilbenclamide significantly ($p<0.0001$) reduced the level of AST, ALT and ALP and ameliorated the alteration in liver function. Treatment with extracts alone did not have any significant effect on AST, ALT and ALP compared with the negative control rats.

DISCUSSION

Since time immemorial, medicinal plants have represented an important means of providing pharmacological intervention and health-care product for the treatment, management and control of variety of disease. Ethno medicinal interventions has been a symbol for accepted therapeutic system suggested to people for their primary health care in the developing countries because of their less harmful effects and low cost of therapy, most especially in Africa (Aliyu *et al.,* 2015). Appropriate dose monitoring in administration of herbal medicine is a serious problem which could result in toxicity. Acute toxicity testing measures the adverse effects that occur within a short time administration of single dose of a chemical. Acute toxicity study is performed principally in rodents and provides information on the hazards likely to arise from short-term exposure and is usually an initial step in the evaluation of the toxic characteristics of a substance for both health and environmental effects (Rispin *et al.,* 2002).

Table 2. Toxicity determination of the methanolic leaf extract of *Piliostigmareticulatum* (PR)

Response	Doses				
	10 mg/kg	100 mg/kg	1000 mg/kg	2900 mg/kg	5000 mg/kg
Alertness	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent
Skin colour and urination	Normal	Normal	Normal	Normal	Normal
Tremor and convulsions	Absent	Absent	Absent	Absent	Absent
Hyperactivity	Absent	Absent	Absent	Absent	Absent
Grooming	Absent	Absent	Absent	Absent	Absent
Death	Absent	Absent	Absent	Absent	Absent

Table 3: Effect of methanolic leaf extract of *Piliostigmareticulatumon* serum biochemical indices

Groups	AST	ALT	ALP
Negative control	99.62 ± 0.66	34.31 ± 0.54	41.00 ± 1.38
Positive control	192.42 ± 0.61$^{\#\#\#}$	98.78 ± 0.51$^{\#\#\#\#}$	73.73 ± 0.96$^{\#\#\#}$
Diabetic rats + 200 mg/kg PR	140.43 ± 4.61***	60.13 ± 0.97***	42.78 ± 3.52****
Diabetic rats + 400 mg/kg PR	118.44 ± 2.16****	40.53± 6.07****	50.86± 4.47****
Diabetic rats + 600 mg/kg PR	123.76± 5.09****	57.14 ± 3.57****	69.92 ± 1.59**
Diabetic rats + Glibenclamide	122.67 ± 1.91****	58.16 ± 2.06****	49.64 ± 2.95****
Normal rats + 200 mg/kg PR	121.98 ± 2.79****	44.10 ± 1.13****	48.49 ± 1.48****
Normal rats + 400 mg/kg PR	125.46 ± 1.14****	42.19 ± 1.16****	53.62 ± 1.47****
Normal rats + 600 mg/kg PR	130.62 ± 0.96****	55.58 ± 1.64****	56.77 ± 1.47****

Results are expressed as mean ± SD (n = 15). Columns having different superscript are significantly different. Significance with Tukey's test following one-way ANOVA is indicated as $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p<0.001$ vs Negative control. **p < 0.01, ****p < 0.0001 vs positive control. AST: alanine aspartate; ALT: alanine aminotransferase; ALP: alanine phosphatase; negative control: rats only receive normal saline; positive control: STZ-induced diabetic rats; PR: methanolic leaf extract of *Piliostigmareticulatum*

The commonly used term to described acute toxicity testing is median lethal dose, LD_{50}, which is the dose that is acutely lethal to 50% of the animals that the chemical substance was administered under controlled laboratory condition through routes like oral, dermal, inhalation or intravenous. Determination of the test examines the relationship between dose and the most extreme response, death. The more toxic or potent the substance, the lower the LD_{50} and the smaller the dose needed to cause death (Raj *et al.,* 2013). In this study, the range of doses of PR used on Wistar rats (10-5000 mg/kg b.wgt) showed there was no mortality observed and rats did not show any sign of toxicity, including no sign of neurobehavioral defect such as alertness, hyperactivity, tremor and convulsions nor negative change in the state of health such as skin color, urination, salivation and grooming. This signifies that there was no multiple organ toxicity associated with oral administration of PR. Report from other workers report that substance with LD_{50} values greater than 5,000 mg/kg are practically non-toxic (Lorke, 1983). Therefore, according to Lorke (1983), PR is considered safe. Different organs are highly affected by diabetes with liver being one of the most important enzymes (Ahmadieh and Azar, 2014). It has been reported that hyperglycemia-induced oxidative stress and the subsequent disturbance in carbohydrate, protein and lipid metabolisms are the most important causes of liver damage in diabetes complication (Mohamed *et al.,* 2016). In this study, elevated activity of serum ASL, ALT and ALP was observed in STZ-induced animals. ASL, ALT and ALP are well-known enzyme in liver cells and elevation of these enzymes in the serum signifies liver damage (Obafemi*et al.,* 2017). Therefore, in this study, treatment with PR attenuated the increased serum activity of these hepatic enzymes and may be due to the protective action of PR possibly via its antioxidant effect in reversing liver damage.

Conclusion

The findings in this study revealed that the methanol leaf extract of *Piliostigmareticulatum* (DC) Host does possess properties that reverse hepatocellular damage in rats. Toxicological evaluation carried out showed no mortalities or adverse clinical manifestations. The biochemical analysis also revealed that the plant is considered very safe up to 5000 mg/kg. This study provides valuable scientific credence for the safety and efficacy of *Piliostigmareticulatum* (DC) Host in traditional herbal medicine. Isolation of the active principles responsible for the biological activities will provide useful compounds for its potential use in drug discovery.

Conflicts of Interest: The authors have no conflicts of interest to declare

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