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Research Article

Toxicological Assessment of Aqueous Extract of *Phyllanthus nivosus* Leaf through *In Silico* and Experimental Studies

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Abstract

Phyllanthus nivosus leaf has been constantly used in the Eastern part of Nigeria for the treatment of malaria and other ailments, but there seem to be no available data regarding its safety. In this study, the toxicity profile of the bioactive compounds of aqueous extract of *Phyllanthus nivosus* leaf was determined through an *in silico* approach and the acute and sub-acute oral toxicity of the crude extract were evaluated in rats. 23 bioactive compounds were recognised in the aqueous extract of *Phyllanthus nivosus* leaf through Gas chromatography-mass spectroscopic (GC-MS) analysis. The *in silico* toxicity prediction identified 5(4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis[2-phenyl-; Benzofuran, 2,3-dihydro; Hydroquinone; Ethene, 1-chloro-1-fluoro-; 1-Alanine, n-propargyloxycarbonyl-ethyl ester; Benzene, 1- (bromomethyl)-3-nitro; Ethane, 1,2-dibromo; 1 - Azabicyclo [2.2.2] octane-4-ol acetate ester, 2-Ethoxypentane and Sulfurous acid, 2-propyl tetradecyl ester as carcinogenic; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; Benzene, 1- (bromomethyl)-3-nitro, Ethane, 1,2-dibromo as mutagenic; 5(4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis[2-phenyl- as hepatotoxic and Sulfurous acid, 2-propyl tetradecyl ester as immunotoxic. The toxicity classes of *P. nivosus* compounds are between 3 and 6 with their LD_{50} values ranging between 108 and 6172 mg/kg. In the acute toxicity study, 2000 mg/kg body weight of the aqueous extract of *Phyllanthus nivosus* leaf caused no apparent harmful effect in the animals. Toxic effects were however observed in the sub-acute toxicity evaluation as revealed by some alterations in the biochemical parameters of liver and kidney functions. Hence, while a single dose administration of aqueous extract of *Phyllanthus nivosus* leaf could be safe at 2000 mg/kg body weight, prolonged oral administration could lead to some toxicological consequences.

Keywords: *Phyllanthus nivosus; ProTox II; Toxicity Prediction; Gas Chromatography-Mass Spectroscopic*

Introduction

Medicinal plants and their products have been utilised worldwide from ancient periods as the main source of herbal medicine in the management and treatment of several disorders [1]. Recently, there is an increasing interest in the use of medicinal plants and their products due to their safety, accessibility, availability, beneficial and little or no side effects [2,3]. The safety profiles, chemical constituents and efficacies of these medicinal plants have little or no scientific records [1,4]. Nevertheless, several scientific works have indicated the need for caution in the use of medicinal plants. Upon administration, they can give rise to toxic effects, which may be due to overdosing, contaminations, harmful effect of the bioactive compounds, interactions, allergies and chronic uses [1,5,6]. Hence, there is a need to characterise their bioactive principles and evaluate the safety profile of these natural products in order to effectively explore their therapeutic benefits.

Phyllanthus nivosus also known as snow bush, is a plant that possess several medicinal properties. They are widely distributed in most tropical and subtropical countries and have long been used in traditional medicine to treat chronic liver diseases [7]. In Nigeria, it is used both as an ornamental plant and traditionally in the treatment of malaria, fever, headaches, toothaches and tooth infections. The stem is commonly used in the South-Eastern Nigeria as chewing sticks. The antibacterial, analgesic, anti-inflammatory and antimalarial activity of various extracts of the leaf have been reported [8-12]. However there appears to be no available records on its chemical composition and safety. In this study, the toxicity profile of the bioactive compounds of aqueous extract of *Phyllanthus nivosus* leaf was determined through an *in silico* approach and the acute and sub-acute oral toxicity of the crude extract were evaluated in rats.

Materials and Methods

Chemicals and reagents

The assay kits for total bilirubin (TB), total protein (TP), albumin (ALB), creatinine (CREA), urea, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were products of RANDOX chemicals (Randox Laboratory Limited, Cumlin, UK). All other reagents used were of analytical grade and supplied by Sigma-Aldrich Inc., St. Louis, USA.

Plant collection and preparation of extracts

Fresh samples of *P. nivosus* leaf were collected in Jos, Plateau State, Nigeria. The plant leaf was botanically authenticated at the Herbarium unit of the Department of Plant Science and Technology, University of Jos, Jos, Plateau State, Nigeria, where the voucher specimen was deposited. The leaves of *P. nivosus* were washed with distilled water and shade dried to a constant weight. The dried plant was pulverized using an electric blender and the pulverized plant (400g) was macerated in 400 ml of distilled water for 48 hours at room temperature with intermittent shaking at eight hours intervals. The mixture was filtrated using Whatman No. 1 filter paper followed by evaporation of the filtrate in an oven at 40^0C to obtain the extract.

Gas chromatography-mass spectroscopic (GC-MS) analysis

The Gas Chromatography-Mass spectroscopy (GC-MS) analysis of the aqueous extract *P. nivosus* leaf was performed using a GC-MS (QP 2010 Plus Model, Shimadzu, Japan) analyser. For GC-MS detection, injector temperature was set at 250°C, the oven temperature was programmed at 60^0C and the pressure was set at 100.2 KPa. The ion source and interface temperature were fixed at 200 and 250^0C respectively. The column and purge flow were 1.61 and 5.6 mL/min respectively, while the total flow time was 39.4 mL/min. At 10°C/min, 2 ml of water solution of the samples was manually injected in the split less mode, with a split ratio of 20:0. Total running time of GC-MS was for 11 minutes. The relative percentage of each extract constituents were expressed as a percentage with peak area normalization. Interpretation of mass spectrum of plant extracts were conducted using the database of National Institute of Standard and Technology (NIST) library having more than 62,000 spectral patterns.

In silico toxicity prediction

The oral acute toxicity class, LD_{50}, hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity and immunotoxicity potentials of the compounds were predicted using the ProTox-II online server. The canonical smiles of the compounds obtained from the PubChem database were uploaded to the ProTox-II online server. The acute toxicity class together with the different toxicity endpoints are calculated for an input compound based on chemical similarities to toxic compounds and trained machine learning models.

Experimental animals

Wister rats (120 - 150g) of both sexes were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, University of Jos. They were kept in the animal transit room with adequate ventilation. They were maintained on standard animal feed and were allowed access to water *ad libitum*. An approval was obtained from the Animal Ethics Committee, University of Jos with the number F17 - 00379. The institutional animal ethical guidelines were strictly observed. The animals were allowed to acclimatize for 2 weeks before the commencement of the study.

Oral acute toxicity study

The acute oral toxicity study was carried according to OECD guidelines 423 (Paragraph 23), which involves the use of only three animals [13,14] per group. The experimental animals were fasted for 12 hours after which their body weights were measured. Aqueous extract of *P. nivosus* at the dose of 2000 mg/kg body weight was orally administered to the rats. The animals were regularly observed for general signs of toxicity and behavioural alterations after administration for the first 24 hours with a unique attention given during the first 4 hours. These observations were followed up daily for a period of 14 days.

Sub-acute toxicity study

Sub-acute oral toxicity was carried out according to OECD guidelines (No. 407). The experimental animals were randomly distributed into three groups of three rats each. The first group of rats were treated with equal volumes of vehicle while the other 2 groups were administered with 500 and 1000 mg/kg body weight of *P. nivosus* daily for 21 days. The administration of the extract was carried out using the oral gavage.

Blood collection and serum preparation

The animals were sacrificed under diethyl ether anaesthesia on day 22. The rats were fasted overnight (12 hours fasting) and blood samples were collected through jugular vein puncture into plain sample bottles. The blood samples were allowed to stand for 30 minutes and centrifuged for 10 minutes at 1000g in order to separate blood cells from the serum. The serum samples collected were transferred into clean plain sample bottles, labelled and stored in a -4^{0}C freezer before being used for biochemical analyses.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using statistical package for social sciences (SPSS, version 21) by subjecting data to one-way analysis of variance followed by Duncan multiple range test for multiple comparisons between the control and the treatment groups. Differences were considered significant at p value less than 0.05 ($p < 0.05$).

Results

Gas chromatography-mass spectroscopic (GC-MS) analysis

Figure 1 shows the GC-MS chromatogram of the aqueous extract of *P. nivosus* leaf. The analysis shows the presence of 23 phyto-compounds. Table 1 shows the chemical profile of the identified bioactive compound including the molecular formula, retention time, peak area (%), molecular weight and structural formula of the compounds. From the identified compounds, glycerin has the lowest retention time of 3.642 minutes while sulphurous acid has the highest retention time of 13.475 minutes. However, 4-benzyloxy-2-methoxymethoxyphenol and 1-azabicyclo [2,2,2] octane-4-ol acetate (ester) has the lowest peak area of 0.06% while stearic acid has the highest peak area of 26.65%.

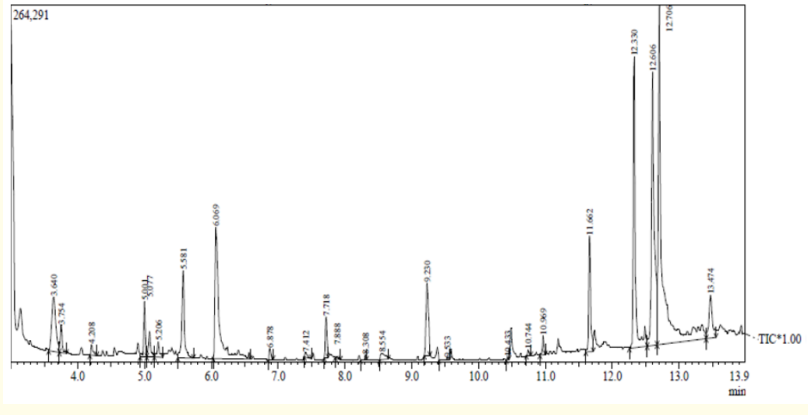
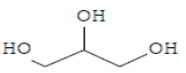
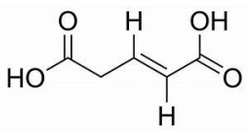
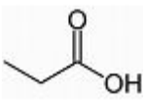
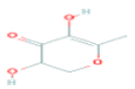
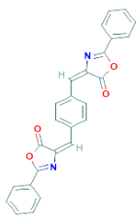
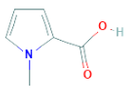
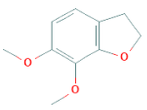
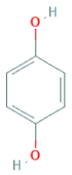
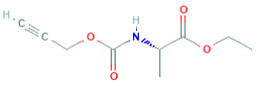
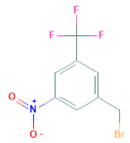


Figure 1: *Gas chromatography-mass spectrometry chromatogram of aqueous extract of P. nivosus leaf.*

Peak	IUPAC Name	Molecular Formula	Retention Time (minute)	Peak Area (%)	Peak Height (%)	Molecular Weight	Structural Formula
1	Glycerine	$C_3H_8O_3$	3.640	4.95	3.33	92	
2	Glutaconic anhydride	$C_5H_4O_3$	3.754	1.19	1.67	114.10	
3	Propanoic acid	$C_7H_{14}O_3$	4.208	0.48	0.65	74.08	
4	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	5.001	2.00	3.39	144.12	
5	5(4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis[2-phenyl-	$C_{26}H_{16}N_2O_4$	5.077	1.33	1.54	420.4	
6	N-Methylpyrrole-2-carboxylic acid	$C_6H_7NO_2$	5.206	0.90	0.94	125.13	
7	Benzofuran, 2,3-dihydro	$C_{10}H_{12}O_3$	5.581	4.85	5.30	120.15	
8	Hydroquinone	$C_6H_6O_2$	6.069	11.54	8.01	110.15	
9	Ethene, 1-chloro-1-fluoro-	C_2H_2ClF	6.878	0.44	0.67	80.49	
10	1-Alanine, n-propargyloxycarbonyl-ethyl ester	$C_9H_{13}NO_4$	7.412	0.23	0.28	199.2	
11	Benzene, 1-(bromomethyl)-3-nitro	$C_8H_5BrF_3NO_2$	7.718	1.60	2.49	284.03	

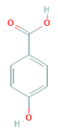
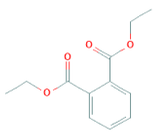
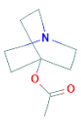
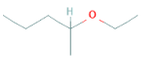
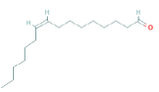
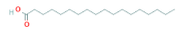
12	2-Butanol, 4-bromo-3,3,4,4-tetrafluoro	$C_4H_5BrF_4O$	8.308	0.13	0.35	224.98	
13	Benzoic acid, 4-hydroxy	$C_7H_6O_3$	8.554	0.71	0.40	138.12	
14	Diethyl Phthalate	$C_{12}H_{14}O_4$	9.230	4.01	4.41	222.24	
15	Ethane, 1,2-di-bromo	$C_2H_2Br_2Cl_2$	9.533	0.06	0.13	256.75	
16	1 – Azabicy-clo[2.2.2]octane-4-ol acetate ester	$C_9H_{15}NO_2$	10.433	0.07	0.16	169.22	
17	2-Ethoxypentane	$C_7H_{16}O$	10.744	0.18	0.34	116.20	
18	1,14-tetradecane-diol	$C_{14}H_{30}O_2$	10.969	0.66	0.18	230.39	
19	1-(+)-Ascorbic acid 2,6-dihexa-decanoate	$C_{38}H_{68}O_8$	11.662	4.78	7.04	652.9	
20	n-Nonadecanol – 1	$C_{19}H_{40}O$	12.330	12.72	17.73	284.5	
21	Cis-9-hexadecenal	$C_{16}H_{30}O$	12.606	17.23	16.65	238.41	
22	Octadecanoic acid (stearic acid)	$C_{18}H_{36}O_2$	12.706	26.65	20.65	284.5	
23	Sulfurous acid, 2-propyl tetra-decyl ester	$C_{17}H_{36}O_3S$	13.474	3.25	2.62	320.5	

Table 1: *Chemical profile of aqueous extract of P. nivosus leaf.*
IUPAC: International Union of Pure and Applied Chemistry.

Toxicity profile of the bioactive compounds of aqueous extract *P. nivosus* leaf

Table 2 shows the toxicity potentials of compounds identified in the aqueous extract *P. nivosus* leaf. The toxicity classes of the compounds range between 3 (toxic if swallowed at $50 < LD_{50} \leq 300$) and 6 (non-toxic at $LD_{50} > 5000$) and their LD_{50} values are between 108 and 6172 mg/kg. 5(4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis [2-phenyl-; Benzofuran, 2,3-dihydro; Hydroquinone; Ethene, 1-chloro-1-fluoro-; 1-Alanine, n-propargyloxycarbonyl-ethyl ester; Benzene, 1- (bromomethyl)-3-nitro; Ethane, 1,2-dibromo; 1 – Azabicyclo [2.2.2] octane-4-ol acetate ester, 2-Ethoxypentane and Sulfurous acid, 2-propyl tetradecyl ester have carcinogenic potentials. 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; Benzene, 1- (bromomethyl)-3-nitro, Ethane, 1,2-dibromo predicted to be mutagenic; 5 (4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis [2-phenyl- is predicted to be hepatotoxic while Sulfurous acid, 2-propyl tetradecyl ester is likely to be immunotoxic.

Compound	LD_{50} mg/kg	Toxicity Class	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Glycerine	4090	5	-	-	-	-	-
Glutaconic anhydride	5000	5	-	-	-	-	-
Propanoic acid	300	3	-	-	-	-	-
4H-Pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl	595	4	-	-	-	+	-
5(4H)-Oxazolone,4, 4'-(1,4-Phenylenedimethylidyne) bis[2-phenyl-	4000	5	+	+	-	-	-
N-Methylpyrrole-2-carboxylic acid	1550	4	-	-	-	-	-
Benzofuran, 2,3-dihydro	1743	4	-	+	-	-	-
Hydroquinone	225	3	-	+	-	-	-
Ethene, 1-chloro-1-fluoro-	500	4	-	+	-	-	-
1-Alanine, n-propargyloxycarbonyl-ethyl ester	3000	5	-	+	-	-	-
Benzene, 1-(bromomethyl)-3-nitro	2440	5	-	+	-	+	-
2-Butanol, 4-bromo-3,3,4,4-tetrafluoro	1000	4	-	-	-	-	-
Benzoic acid, 4-hydroxy	2200	5	-	-	-	-	-
Diethyl Phthalate	6172	6	-	-	-	-	-
Ethane, 1,2-dibromo	108	3	-	+	-	+	-
1 – Azabicyclo[2.2.2]octane-4-ol acetate ester	2750	5	-	+	-	-	-

2-Ethoxypentane	1870	4	-	+	-	-	-
1,14-tetradecane-diol	1000	4	-	-	-	-	-
1-(+)-Ascorbic acid 2,6-dihexadecano-ate	2500	6	-	-	-	-	-
n-Nonadecanol – 1	1000	4	-	-	-	-	-
Cis-9-hexadecenal	5000	5	-	-	-	-	-
Octadecanoic acid (stearic acid)	900	4	-	-	-	-	-
Sulfurous acid, 2-propyl tetradecyl ester	1950	4	-	+	+	-	-

Table 2: *Toxicity profile of the bioactive compounds of P. nivosus.*
Active (+), Inactive (-).

Acute oral toxicity effects of *P. nivosus* leaf in rats

No animal death was observed during and after treatment with 2000 mg/kg body weight of aqueous extract of *P. nivosus* leaf and no obvious sign of toxicity was detected throughout the 14-day period of observation. Hence, the acute lethal dose (LD_{50}) of aqueous extract of *P. nivosus* leaf in both male and female rats could be estimated to be more than 2000 mg/kg.

Sub-acute oral toxicity effects of *P. nivosus* on male and female rats

In the sub-acute toxicity study carried out for 21 days, the effects of the aqueous extract of *P. nivosus* leaf on some biochemical indices in male and female rats are as follows:

Kidney function indices

Table 3 shows the kidney function indices of male rats administered with aqueous extract of *P. nivosus* leaf. The levels of urea, creatinine and sodium were all significantly ($p < 0.05$) reduced at all doses when compared to the control group. However, the level of potassium and chloride were inconsistently changed when compared to the control group.

	Urea (g/dl)	**Creatinine (mg/dL)**	**Serum NA^+**	**Serum K^+**	**Serum Cl^-**
Control	11.0 ± 1.1	92.4 ± 0.9	91.0 ± 2.0	8.3 ± 0.7	63.7 ± 0.4
PN_{500}	7.6 ± 0.5*	70.2 ± 1.1*	70.8 ± 1.2*	11.5 ± 0.7*	58.9 ± 0.3*
PN_{1000}	6.5 ± 1.3*	81.3 ± 1.5*	80.1 ± 2.2*	6.0 ± 0.8*	65.7 ± 0.3*

Table 3: *Kidney marker indices of male rats in the sub-acute toxicity study of the aqueous extract of P. nivosus leaf.*
Values were presented as mean of three replicates ± SD.
**Significantly different at ($p < 0.05$) compared with the control. PN: P. nivosus.*

Table 4 shows the kidney function indices of female rats administered with aqueous extract of *P. nivosus* leaf. The levels of creatinine and potassium were significantly ($p < 0.05$) decreased at all doses compared with the control group. The levels of urea and chloride were inconsistently altered at 500 mg/kg while no significant ($p > 0.05$) alteration was observed at 1000 mg/kg. The levels of sodium were significantly ($p < 0.05$) decreased at 500 and 1000 mg/kg.

	Urea (g/dl)	Creatinine (mg/dL)	Serum NA^+	Serum K^+	Serum Cl^-
Control	7.6 ± 0.8	92.4 ± 0.6	70.8 ± 0.3	13.4 ± 0.6	51.0 ± 3.3
PN_{500}	5.6 ± 0.4*	63.0 ± 2.0*	60.6 ± 1.6*	3.7 ± 0.1*	58.9 ± 0.2*
PN_{1000}	6.4 ± 0.6	52.0 ± 2.0*	68.0 ± 1.7*	5.0 ± 0.3*	51.0 ± 1.1

Table 4: *Kidney marker indices of female rats in the sub-acute toxicity study of the aqueous extract of P. nivosus leaf. Values were presented as mean of three replicates ± SD. *Significantly different at (p < 0.05) compared with the control. PN: P. nivosus.*

Liver function indices

Table 5 shows the liver function indices of male rats administered with aqueous extract of *P. nivosus* leaf. At doses of 500 and 1000 mg/kg, activities of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were inconsistently altered compared to the control group. The activities of alanine aminotransferase (ALT) were not altered at 1000 mg/kg but at 500 mg/kg, there was a significant alteration relative to the control group. The levels of total bilirubin (TB) were significantly increased at 500 and 1000 mg/kg compared to the control group. The levels of total protein (TP) were not altered at 1000 mg/kg while at 500 mg/kg, TP level was significantly ($p < 0.05$) reduced compared to the control group. Contrarily, the level of ALB was not altered when compared to the control group.

	AST (U/I)	ALT (U/I)	ALP (U/I)	TB (mg/dL)	TP (g/dL)	ALB (g/dL)
Control	39.0 ± 2.0	32.0 ± 3.0	69.4 ± 0.7	0.7 ± 0.2	71.5 ± 3.5	50.5 ± 2.8
PN_{500}	43.0 ± 2.0*	27.0 ± 2.0*	97.3 ± 2.7*	1.6 ± 0.2*	62.4 ± 7.2	47.9 ± 1.7
PN_{1000}	52.0 ± 1.6*	35.0 ± 2.0	60.1 ± 3.6*	1.4 ± 0.1*	78.4 ± 1.2	44.5 ± 4.9

Table 5: *Liver marker indices of male rats in the sub-acute toxicity study of the aqueous extract of P. nivosus leaf. Values were presented as mean of three replicates ± SD. *Significantly different at (p < 0.05) compared with the control. AST: Aspartate Transaminase; ALT: Alanine Transaminase; ALP: Alkaline Phosphatase; TB: Total Bilirubin; TP: Total Protein; ALB: Albumin, PN: P. nivosus.*

Table 6 shows the liver function indices of female rats administered with aqueous extract of *P. nivosus* leaf. The activities of AST and ALP were inconsistently changed at all doses as compared to the control group. The activities of ALT was significantly ($p < 0.05$) decreased at doses of 500 and 1000 mg/kg relative to the control group. The levels of TB were inconsistently altered at 500 and 1000 mg/kg relative to the control group. In a different fashion, the level of albumin (ALB) was significantly ($p < 0.05$) decreased at 500 and 1000 mg/kg compared to the control group. However, the level of TP was significantly ($p < 0.05$) reduced at all doses relative to the control group.

	AST (U/I)	ALT (U/I)	ALP (U/I)	TB (mg/dL)	TP (g/dL)	ALB (g/dL)
Control	67.0 ± 1.0	23.0 ± 2.0	120.1 ± 2.2	1.0 ± 0.1	78.5 ± 0.3	50.1 ± 2.1
PN_{500}	89.0 ± 4.4*	9.0 ± 3.5*	187.9 ± 4.6*	0.4 ± 0.1*	64.0 ± 1.1*	39.7 ± 2.1*
PN_{1000}	52.0 ± 1.0*	21.0 ± 2.7	175.1 ± 6.1*	1.2 ± 0.1*	72.8 ± 1.1*	47.2 ± 2.2

Table 6: *Liver marker indices of female rats in the sub-acute toxicity study of the aqueous extract of P. nivosus leaf. Values were presented as mean of three replicates ± SD. *Significantly different at (p < 0.05) compared with the control. AST: Aspartate Transaminase; ALT: Alanine Transaminase; ALP: Alkaline Phosphatase; TB: Total Bilirubin; TP: Total Protein; ALB: Albumin; PN: P. nivosus.*

Discussion

Apart from the several ethnobotanical survey of *P. nivosus* indicating its applications in traditional medicine, the *in vitro* and *in vivo* antiplasmodial activity of the plant have been demonstrated in our previous reports [10,11]. It is therefore important that the safety status of the natural herb be investigated to serve as a guide for the management of its usage in traditional preparations. *In silico* toxicity prediction is an efficient, fast and cost-effective approach for the evaluation of potential drug candidates including natural products [15]. This could also be followed by the use of animal models to validate the safety and possible harmful effects of these natural products in humans [16]. The identification of potential carcinogenic, mutagenic, hepatotoxic and immunotoxic agents in the aqueous extract of *P. nivosus* leaf in an indication of the probable toxicity risk associated with indiscriminate use of the medicinal plant, even though the predicted toxic effects are for the individual compounds and not necessarily in combination with other constituents of the plant. The *in silico* study also provided the acute oral toxicity classes and LD_{50} values of the compounds when swallowed individually. The toxicity classes of the compounds range between 3 (toxic if swallowed at $50 < LD_{50} \leq 300$) and 6 (non-toxic at $LD_{50} > 5000$) with their LD_{50} values ranging between 108 and 6172 mg/kg. Hence, the plant should be used within the lowest possible dosage to ensure safety while maximising the therapeutic benefits.

The additive effects of all the bioactive compounds of the crude extract of *P. nivosus* was assessed through the *in vivo* study. The administration of 2000 mg/kg of *P. nivosus* as a single dose did not cause any observable adverse effects or mortality on the rats throughout the 14-day observation period which suggests that the lethal dose could be greater than 2000 mg/kg. This could be attributed to synergistic activities between the compounds which could neutralise the potential activities of the toxic components.

The sub-acute toxicity study showed the effects of prolonged administration of the plant extract on some biochemical parameters of liver and kidney functions in rats at 500 and 1000 mg/kg. The liver and kidneys are typically the target organs of many harmful substances due to their vital roles in the excretion and detoxification processes [17]. These organs are highly important in toxicity studies as a result to their sensitivity to foreign chemicals and their ability to give a clue on the mechanism of toxicity [17]. The activity of liver enzymes in the blood is directly related to the extent of the tissue damage and it is indicative of cellular leakage and loss of functional integrity of the membranes of liver cells. Alterations in the membrane permeability and cell death triggered by the lipid peroxidation and inflammatory mediators, increases the serum levels of liver-specific enzymes. Increased levels of enzymes such as ALT, AST and ALP are indicative of loss of the functional integrity of the hepatocyte cell membranes and of liver lesions [18,19]. In this study, there was an inconsistent alteration (increase and decrease) in some of the activities of these enzymes in both male and female rats. Increase in the activities of these enzymes may be due to the stimulating effect of the extract on the enzymes as a result of the stress inflicted on the tissues [20-22]. Decrease in their activities may also be due to the abrogation of the enzymes in the cells [23]. Bilirubin level is a vital index for diseased liver and it is used to assess the conjugation, binding and excretory ability of the liver [24]. From this study, the level of Total Bilirubin was reduced in the male rats while it was elevated in the female rats. This increase may be as a result of the inability of the injured liver to excrete bilirubin [25].

Total protein (TP) is made up of globulin and albumin and it is an index of balance between protein catabolism and anabolism [26]. However, albumin can be an index of liver biosynthetic function [27]. From this study, there was a significant decrease in the level of TP in the female rats and the level of ALB was significantly decreased in both male and female rats (Table 5 and 6). These alterations could be associated with liver damage [28,29] and this may be attributed to the presence of the 5(4H)-oxazolone, 4,4'-(1,4-phenylenedimethylidyne) bis [2-pheny-] which, according to the *in silico* toxicity prediction, is a potential hepatotoxic agent.

Impaired concentration of urea and creatinine in the serum are indicative of potential renal dysfunction [30]. From this study, the level of urea and creatinine were significantly decreased in both male and female rats compared to the control rats. This may be due to the potential of the extract to stimulate the clearance function of the kidney [31]. Serum electrolytes which include potassium, sodium and chloride are also essential clinical index for estimating renal function [32]. From this study, the level of sodium in the male rats was significantly decreased while the levels of potassium and chloride were altered inconsistently when compared to the control rats. Similarly, the levels of sodium and potassium in the female rats were significantly decreased while the level of chloride was altered inconsistently as compared to the control rats. Increase in electrolytes like sodium and chloride may be due to defects in the excretory function of renal organs [33].

Conclusion

Twenty-three bioactive compounds identified in the aqueous extract of *P. nivosus* leaf through GC-MS analysis were subjected to *in silico* toxicity prediction. 5(4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis[2-phenyl-; Benzofuran, 2,3-dihydro; Hydroquinone; Ethene, 1-chloro-1-fluoro-; 1-Alanine, n-propargyloxycarbonyl-ethyl ester; Benzene, 1- (bromomethyl)-3-nitro; Ethane, 1,2-dibromo; 1 - Azabicyclo [2.2.2] octane-4-ol acetate ester, 2-Ethoxypentane and Sulfurous acid, 2-propyl tetradecyl ester were predicted to be carcinogenic; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; Benzene, 1- (bromomethyl)-3-nitro, Ethane, 1,2-dibromo were identified as mutagenic; 5 (4H)-Oxazolone,4,4'-(1,4-Phenylenedimethylidyne) bis[2-phenyl- as hepatotoxic and Sulfurous acid, 2-propyl tetradecyl ester as immunotoxic. The toxicity classes of *P. nivosus* compounds are between 3 and 6 and their LD_{50} values range between 108 and 6172 mg/kg. A single dose administration of 2000 mg/kg of the extract showed no apparent adverse effect in rats while 21-day repeated doses showed alterations in the liver and kidney function indices at 500 and 1000 mg/kg. Hence, prolonged oral administration of *P. nivosus* leaf extract could lead to some toxicological consequences.

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