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In vivo Safety Evaluation of a Nigerian Polyherbal Mixture in Female Wistar Rats

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Summary: The present study evaluates the oral safety and oral toxicity reversibility of a Nigerian Polyherbal Mixture (*NPM*) in female Wistar rats. In this study, acute oral toxicity was conducted on 20 female Wistar rats using the limit dose test of Up-And-Down Procedure of the OECD Acute Oral Toxicity Testing 425 guidelines at 5000 mg/kg of *NPM*. Additionally, 40 female Wistar rats (120-150 g) were divided into 4 groups (n=10) and orally treated with 10ml/kg of distilled water, 82 mg/kg, 410 mg/kg and 2050 mg/kg of *NPM*, respectively, for 90 days. Five rats from each group were sacrificed while the remaining rats in each group were kept for another 14 days for oral toxicities reversibility test. Blood samples and vital organs were obtained for biochemical, hematological and histological changes. Results showed that acute oral toxicity testing of *NPM* caused no death in any of the three sequentially treated rats and its estimated LD_{50} value was greater than 5000 mg/kg. Chronic oral treatment with 82-2050 mg/kg *NPM* caused significant elevations in the serum urea and creatinine and full blood count parameters (except differential WBC counts). The elevated renal function parameters were corroborated by dose-related histological changes of renal tubular congestions. also caused profound thrombocytosis and histopathological changes of pulmonary interstitial widening and gastritis. In conclusion, *NPM* may not be considered safe for consumption on prolonged use and at a high dose due to its profound tendencies to cause pulmonary fibrosis, nephrotoxicity, gastritis and thrombo-embolism. However, all the biochemical and hematological but histopathological alterations induced by *NPM* were reversed 14 days after the treatment cessation.

Keywords: Oral toxicity testing, Renal and hepatic function, Histopathological assessment, Reversibility test, Nigerian Polyherbal Mixture

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INTRODUCTION

The use of herbal remedies has been widely embraced in many developed countries with Complementary and Alternative Medicine (CAM) now becoming main stream in the United Kingdom and the rest of Europe as well as in North America and Australia (Calapai, 2008; Braun *et al.*, 2010; Anquez-Traxler, 2011). Herbal Medicine is very popular and widely used in the developing as well where they offer a wide range of available and affordable alternatives to conventional drugs. In Africa, for example, up to 80% of the population depends either partly or wholly on CAM according to World Health Organization estimate (World Health Organization 2005a; 2005b). Similarly, the new health agenda in Africa and indeed Nigeria focuses on the institutionalization of traditional medicine in parallel with Orthodox Medicine into natural Health Care Scheme in order to move the health agenda forward since effective healthcare in Africa cannot be achieved through Orthodox Medicine alone unless complemented with CAM (Elujoba *et al.*, 2005).

In recent years, issues relating to increasing use of herbal products in developed countries, dependence of many people living in developing countries on plants as a major source of medicines coupled with absence or weak regulation of herbal medicines in most countries and the occurrence of high-profile safety concerns, have increased awareness of the need to monitor safety and deepen understanding of possible harmful as well as potential benefits associated with the use of herbal medicines (Rodrigues and Barnes, 2013; Ekor, 2013). Similarly, in the Nigerian health sector, one of the main concerns is the indiscriminate use of drugs including packaged polyherbal mixtures popularly sold as "bitters". Many brands of the "bitters" which have flooded the Nigerian markets and

enjoy high patronage by their consumers include *Yoyo, Alomo, Orijin, Agbo, Action, Washing and Setting, Baby-Oku, Skelewu, Man-Power, Swedish, Goko Cleanser, Ruzu, Kerewa, etc*. These "bitters" are much sought after for their acclaimed health benefits which include amongst others energizer, improved mental alertness, sexual enhancement, blood cleansing/purification, pain relief, *etc*. For example, the polyphenol contents, *in vitro* antioxidant capacity and membrane stabilizing potential of some of these herbal remedies (namely Fijk, Osomo, Alomo and Oroki) were reported (Adeyemi and Owoseni, 2015). However, there are increasing health concerns which have been reported to be associated with the consumption of some of the existing supposed "health-promoting bitters" which include chronic kidney disease (Vivekenand, 2010; Zhang *et al.*, 2015), chronic liver disease (Abdulmajid and Sergi, 2013; Amadi and Orisakwe, 2018), pancreatic and heart diseases and sudden death (Farah *et al.*, 2000; Ernst, 2002; Ekor *et al.*, 2010). Subacute animal studies of Nigerian polyherbal remedies ('*Agyanom* mixture', '*Bolex* bitters' and '*Remedia* mixture') showed that the herbal mixtures were associated with mild to severe tubular necrosis indicating the mixtures have adverse effects on the kidney and they might not be safe for human consumption (Akande *et al.*, 2010a). Similarly, subacute animal studies with 1206.5 mg/kg/day and 804.3 mg/kg/day of the Nigerian "Yoyo bitters" for 28 days was associated with organ (liver and kidney) toxicities marked by lipid peroxidation (Adeyemi *et al.*, 2012) while its recommended doses precipitated immunomodulatory activities and hemolysis in Wistar rats (Oyewo *et al.*, 2013).

Despite the wide availability and application of polyherbal remedies/mixtures to promoting human health in Nigeria and indeed other African as well as in some Western countries, there is a dearth of scientific validation of the folkloric therapeutic efficacy as well as the scientific evaluation of their safety profile (Zhang *et al.*, 2015). Therefore, the current study was designed to evaluate both the oral toxicity and reversibility profile of *NPM* in the young adult female Wistar rats, which is strongly in line with the World Health Organization set goals on determining the safety profile of medicinal plants before it can become acceptable for human use.

MATERIALS AND METHODS

Sourcing and preparation of NPM: The herbal mixture used for this study is Oroki Herbal Mixture® produced by NURD Industrial and Commercial Company. It was purchased from the Company's Head Office at No. 4 Ifelodun Street, Off Agbado Adetola Bus-Stop, Ijaye, Lagos State, Nigeria. The leaflet described its constituents as *Sorghum bicolor* (5%), *Khaya grandifoliola* bark (10%), *Cassia sieberiana* root (3%), *Staudtia stipitata* root (3%), *Alstonia congensis* bark (5%), *Ocinum basillicum* leaves (7%), *Mangifera indica* leaves (7%), *Cyathula prostrata* leaves (7%), *Securidaca longependunculata* root (5%) and *Saccharum officinarum* stem (5%).

Five liters of *NPM* was concentrated to complete dryness in an aerated oven preset at 40℃. The solid residue left behind was scrapped, weighed on a Mettler weighing balance and kept in air- and water-proof container before it was stored in a refrigerator at -4℃. The percentage yield was 56.94 ± 8.4%.

Preliminary qualitative phytochemical analysis of NPM: Preliminary qualitative phytochemical analysis to confirm the presence or absence of secondary metabolites such as flavonoids, alkaloids, saponin, tannin, phlobatinnins, terpenoids, glycosides and anthraquinones in *NPM* were conducted using standard procedure as described by Sofowora (1993) and adopted by Adeneye *et al.* (2006).

Experimental animals: Sixty healthy and nulliparous female Wistar Albino female rats (120- 150 g) were obtained from Bayo's Animal Farm, Sango-Otta, Ogun State, after an ethical approval for the study was obtained. The rats were housed in the Animal House of the Lagos State University College of Medicine, Ikeja, Lagos State under controlled conditions with a 12 hour light/12 hour dark schedule and fed with commercially available rat pelleted diet (Animal Care Feeds, Ibadan, Oyo State) and water *ad libitum* throughout the period of the experiment. The rats were acclimatized for 14 days. Thereafter, twenty rats were randomly selected from the rat population for the acute oral toxicity studies using the Up-and-Down Procedure of the OECD/OCDE Test Guidelines on Acute Oral Toxicity under a computer-guided Statistical Programme- AOT425 StatPgm, version 1.0 as described by Adeneye *et al.* (2006).

The remaining forty rats were randomly divided into 4 groups of 10 rats such that the weight differences within and between groups do not exceed ±20% of the average body weight of the rat population. The rats which were housed in standard metallic cages were divided into four major groups and two sub-groups. The cage beddings and water bottles were cleaned on a daily basis and the experimental animals were handled in accordance with Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993) and international guidelines on the Handling and Care of Experimental Animals (United States National Institutes for Health, 1985). The whole experiment lasted for 104 days (90 days for subchronic toxicity testing and 14 days for oral toxicity reversibility testing).

Acute oral toxicity study of NPM using limit dose test of Up and Down Procedure in Wistar rats: The Acute oral toxicity study was conducted using the limit dose test of up and down procedure according to

OECD/OCDE Test Guidelines on Acute Oral Toxicity under a computer-guided Statistical Programme-AOT425 StatPgm, version 1.0 as adopted by Adeneye *et al.* (2006), at a limit dose of 5000 mg/kg body weight/oral route and default of Sigma at 0.5. A total of 3 female young adult Wistar rats were systemically selected out of a population of 10 Wistar rats (8-12 weeks old) by systematic randomization techniques. The population sample was selected such that the weight differences do not exceed ±10% of the mean initial weight of the sample population. The rats were fasted of rat feed overnight prior to dosing on each occasion. A rat was picked at a time, weighed and dosed with equivalent 5000 mg/kg body weight of the *NPM* extract. After the extract administration, each rat was observed for the first 5 minutes after oral administration for signs of possible regurgitation and then kept in a cage for observation. Each was watched for every 15 min in the first 4 hour after dosing, then every 30 minutes for the successive 6 hours and then daily for the successive 38 hours for the short-term outcome and the remaining 12 days for the long-term possible lethal outcome.

Behavioral manifestations of acute oral toxicity were also observed such as restlessness, hyperactivity, dullness and general morphological changes. All observations were systematically recorded with individual records being maintained for each rat.

Subchronic oral toxicity studies of NPM in Wistar rats: The young adult nulliparous female Wistar rats were randomly divided into four major groups consisting of twelve rats each such that the weight differences within and between groups do not exceed ±20% of the average body weight and were all orally treated on daily basis for 90 days. Graded oral doses {82 mg/kg/day (sub-therapeutic), 410 mg/kg/day (therapeutic) and 2025 mg/kg/day (supra-therapeutic)} of the *NPM* extract administered to the Wistar rats for a period of 90 days were determined from the preliminary earlier conducted. The rats were randomly divided and allotted to Groups I-IV consisting of ten female Wistar rats per group. The allotment was done such that the average body weight within groups and between groups does not exceed 20% of each other. Group by group oral treatments of rats are as follows:

Group I: 10 ml/kg/day of distilled water
Group II: 82 mg/kg/day of *NPM* extract dissolved in distilled water
Group III: 410 mg/kg/day of *NPM* extract dissolved in distilled water
Group IV: 2025 mg/kg/day of *NPM* extract dissolved in distilled water

Measurement of body weight of treated rats: The weights of all the rats were taken using electronic Mettler weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland) on days 1, 30, 60, and 90 respectively.

Animal euthanasia and blood sample collection: A day prior to termination of the first phase of the experiment on day 90, the rats were fasted of feed except for potable drinking water which was still freely available. The rats were humanely sacrificed under deep inhaled diethyl ether anesthesia. Blood samples were collected directly from the rat heart chamber with a 21 G needle mounted on a 5 ml syringe plunger (Unique Pharmaceuticals, Sango Otta, Ogun State, Nigeria). 5 ml of blood sample for full blood count and biochemical (liver and renal functions) was collected into 10 ml capacity EDTA-treated blood sample bottles. The blood sample for the biochemical analysis was centrifuged with Uniscope Laboratory Centrifuge (Model SM 112, Surgifriend Medicals, England) at 3500 rpm for 10 minutes to separate the plasma from the blood cells. The plasma were carefully separated into new, well labeled, corresponding plain sample bottles at room temperature 23-26 ºC. The plasma obtained were used to analyze for the possible toxic effect of the *NPM* on the liver (using liver function test parameters such as ALT, AST, ALP, TP, ALB, TC and HDL) and kidney (renal function parameters such as electrolytes-sodium, potassium, calcium, chloride; urea and creatinine).

Measurement of organ weight and calculation of relative organ weight of rats: The rats were dissected and vital organs such as the lung, spleen, stomach, heart, liver, and kidneys were identified and dissected out *en bloc*. The organs were then rinsed of blood and weighed with electronic Mettler weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland). The weight of each organ relative to the 100 g of body weight of the rats from which the organ was harvested was calculated as: {Organ weight (g) ÷ body weight of rat (g) x100}

Determination of plasma renal function parameters: Plasma creatinine and blood urea were assayed using Randox Diagnostic kits (Randox Laboratories Ltd., Crumlin, U.K.) by standard quantitative methods (Peake and Whiting, 2006; Salazar, 2014). Plasma levels of sodium, potassium, chloride, calcium, bicarbonate and phosphate were determined using the ISE 6000 BYY SFRI spectrophotometer using the procedure earlier described by Adeneye *et al.* (2006).

Determination of plasma liver function parameters: Samples of the clear plasma obtained for were assayed for the following liver function parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, triglyceride, total cholesterol and cholesterol fractions [high density lipoprotein

cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), and very low density lipoprotein cholesterol (VLDL-c)], total (TB) and conjugated bilirubin (CB). Serum ALT, AST and ALP were measured using the enzyme kinetic method described by Peake *et al.* (1988) and Huang *et al.* (2006). Other biochemical determinations include triglyceride, total cholesterol and cholesterol fractions using method of Fossati and Principe (1982). The total protein was estimated by Biuret method (Okutucu *et al.*, 2007) while that of albumin was determined by the method described by Rees *et al.* (2012). The total bilirubin and the conjugated bilirubin were determined by Westwood method (1982).

Hematological Assays: Blood samples were collected directly from the heart chamber from anaesthetized rats with 12 G needle mounted on a 5 ml syringe plunger (Unique Pharmaceuticals, Sango-Otta, Ogun State, Nigeria) and collected into EDTA-treated bottles for full blood count on Automated Haematology System (Sysmex Haematology-Coagulation Systems®, Model KX-21N, Sysmex Incorporation, Kobe, Japan). Full blood parameters measured include red cell count (RBC), packed cell volume (PCV), haemoglobin concentration (Hb), platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total leucocyte count (TLC), and differential neutrophils (%Neut.), differential lymphocytes (%lymph) and differential granulocytes (%Gran.).

Histopathological studies: After the dissected vital organs were rinsed and weighed, the organs were preserved in 10% formo-saline before they were completely dehydrated in absolute (100%) ethanol. The organs were then embedded in routine paraffin blocks. From the embedded paraffin blocks, 4-5 μm thick sections of each tissue was prepared and stained with haematoxylin-eosin. These were examined under a photomicroscope (Model N-400ME, CEL-TECH Diagnostics, Hamburg, Germany) connected with a host computer. Sections were illuminated with white light from a 12V halogen lamp (100 W) after filtering with a 520nm monochromatic filter (Thanabhorn et al., 2006). The prepared slides were examined for possible associated histological lesions.

Oral toxicity reversibility test of NPM: At the end of the chronic oral toxicity study period, all the animals are sacrificed humanely under anaesthesia with the exception of six randomly selected rats from each of the treatment and control groups. These were left untreated with the *NPM* extract but had free access to water and feed for 14 days. The rats were then fasted overnight and on the 15th day, all the remaining rats were sacrificed and had their blood samples collected for biochemical, haematological and histopathological assessment as described for chronic toxicity study. The reversibility study is aimed at evaluating if the biochemical, haematological and histopathological alterations induced in the course of the chronic oral toxicity study would become reversible with withdrawal of the oral extract treatment or not after 14 days (Adeneye *et al.*, 2010). Autopsy is performed on all animals and vital organs weighed and examined for gross and histological lesions (Ibrahim *et al.*, 2018).

Data Analysis: All data were expressed as mean ± S.D. for body weight and relative organ weights while data for biochemical and hematological assays were expressed as mean ± S.E.M. Significant differences among the group were determined by One-way analysis of variance (ANOVA) and *post hoc* test determined by Newman-Keuls test using GraphPad Prism 5 software. Results were considered to be significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

RESULTS

Percentage Yield of NPM extract: Complete drying of *NPM* resulted in dark brown, sweet-smelling and sticky solid residue with an average yield of 56.94 ± 8.4%.

Preliminary Qualitative Phytochemical Analysis of NPM: Preliminary phytochemical analysis of *NPM* showed the presence of phenols, flavonoid, saponin, terpenoids, cardiac glycosides, steroid glycosides and anthraquinones while tannin, alkaloids and phlobatannin were absent.

Acute Oral Toxicity Studies of NPM Using the Limit Dose Test of Up and Down Procedure: Single oral treatment of rats with 5000 mg/kg body weight of *NPM* produced no lethality within the short- and long-term outcome of the limit dose test of Up-and-Down Procedure (Table 1). However, the resulting behavioral toxicity signs observed included irritation, bilateral narrowing of the eyelids and abnormal posture (which was characterized by tugging of the head in-between the hind-limbs) and feed refusal. However, the software-generated LD_{50} value calculated to be greater than 5000 mg/kg body weight/oral route.

Table 1.
Sequence and Results of Limit Dose test of Up and Down Procedure of Acute Oral Toxicity of *NPM* in treated female nulliparous Wistar rats

Test sequence	Animal ID	Dose (mg/kg)	Short-term result (48 h)	Long-term result (12 days)
1	01	5000	Survival	Survival
2	02	5000	Survival	Survival
3	03	5000	Survival	Survival

Table 2.
Effect of subchronic oral treatments with 82-2050 mg/kg of *NPM* on the average body weight (bwt) of treated rats on 1st, 30th, 60th and 90th day of treatment

Group	Day 1	Day 30	Day 60	Day 90
I	123.2 ± 9.1	135.0 ± 9.1	151.4 ± 7.2	172.1 ± 3.38
II	105.6 ± 17.0	135.0 ± 19.0	139.00 ± 18.3	167.5 ± 7.1
III	127.1 ± 24.2	118.4 ± 11.4	131.1 ± 11.3	170.9 ± 12.5
IV	125.8 ± 10.3	131.0 ± 10.7	149.2 ± 9.4	187.7 ± 22.8

Results are expressed as mean ± SD. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*

Table 3.
Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on relative weights of liver (RLW), kidney (RKW), lung (LGW), spleen (RSW), heart (RHW) and stomach (RSTW) in treated rats for 90 days

Organ	Groups			
	I	II	III	IV
RLW	4.58 ± 0.95	4.03 ± 0.23	4.01 ± 0.35	4.21 ± 0.87
RKW	1.13 ± 0.06	0.80 ± 0.06	0.80 ± 0.09	0.74 ± 0.17
LGW	1.93 ± 0.71	1.18 ± 0.10a	1.16 ± 0.10a-	0.91 ± 0.16b-
RSW	0.68 ± 0.47	0.53 ± 0.14	0.48 ± 0.09	0.42 ± 0.13
RHW	0.45 ± 0.08	0.43 ± 0.08	0.47 ± 0.06	0.38 ± 0.13
RSTW	2.95 ± 0.40	1.70 ± 0.37b	1.37 ± 0.24c	2.60 ± 0.80a

Results are expressed as mean ± S.D. a-, b- and c- represent significant reductions at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, when compared to control (Group I) values. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*

Table 4.
Effect of a 14-days oral toxicity reversibility test of 82-2050 mg/kg/day of *NPM* on relative weights of liver (RLW), kidney (RKW), lung (LGW), spleen (RSW), heart (RHW) and stomach (RSTW) in treated rats for 90 days

Organ	Groups			
	I	II	III	IV
RLW	3.64 ± 0.33	4.70 ± 0.68	3.99 ± 0.28	4.37 ± 1.18
RKW	1.18 ± 0.18	0.96 ± 0.04	1.00 ± 0.04	0.95 ± 0.08
LGW	1.12 ± 0.06	1.35 ± 0.04	1.25 ± 0.08	0.94 ± 0.08
RSW	0.76 ± 0.30	0.66 ± 0.11	0.60 ± 0.09	0.59 ± 0.17
RHW	0.73 ± 0.27	0.71 ± 0.16	0.75 ± 0.06	0.52 ± 0.05
RSTW	2.56 ± 0.79	3.91 ± 0.72	5.04 ± 0.34	3.39 ± 0.97

Results are expressed as mean ± S.D. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*

Effect of subchronic oral treatment with 82-2050 mg/kg/day of NPM on the average body weights (g) in treated rats: Single, daily oral treatment of rats with 82-2050 mg/kg of *NPM* for 90 days did not result in any significant ($p>0.05$) changes in the average body weight in any of the treatment groups on days 1, 30, 60 and 90 of the treatment when compared to that of the control group (Table 2).

Effect of subchronic oral treatment with 82-2050 mg/kg/day of NPM on the relative weights of liver (RLW), kidney (RKW), lung (LGW), spleen (RSW), heart (RHW) and stomach (STW) of treated rats: Single, daily oral treatment of rats with 82-2050 mg/kg of *NPM* for 90 days did not result in any significant ($p>0.05$) changes in the relative weights of liver, kidney, spleen and heart but caused significant dose-dependent reductions ($p<0.05$, $p<0.01$) in the relative lung weight and non-dose dependent significant reductions ($p<0.05$, $p<0.01$, $p<0.001$) in the relative stomach weight when compared to the control (Group I) values (Table 3).

Effect of oral toxicity reversibility of NPM on the relative weights of liver (RLW), kidney (RKW), lung (LGW), spleen (RSW), heart (RHW) and stomach (STW) of treated rats: Table 4 showed effect of oral toxicity reversibility of *NPM* on the relative weights of liver, kidney, lung, spleen, heart and stomach of rats after 14 days of ceasing the oral administration of *NPM*.

Effect of sub-chronic oral treatment with 82-2050 mg/kg/day of NPM on the plasma liver enzymes (ALT, AST and ALP), albumin (ALB) and total protein (TPR) of treated rats: Single, daily oral treatment of rats with 82-2050 mg/kg of *NPM* for 90 days did not result in any significant ($p>0.05$) alterations in the plasma levels of liver enzymes (ALT, AST and ALP), albumin and total

Table 5.
Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on the plasma liver enzymes (ALT, AST and ALP), albumin (ALB) and total protein (TPR) of treated rats

Groups	ALP (U/l)	ALT (U/l)	AST (U/l)	ALB (g/l)	TPR (g/l)
I	80.47±7.54	46.67±6.18	170.50±16.50	45.87±01.00	75.30±01.15
II	107.70±11.35	30.27±3.21	167.50±17.27	42.45±01.16	71.72±04.05
III	83.97 ±7.26	49.45±7.63	148.60±08.72	43.63±01.42	76.77±02.05
IV	92.23±8.86	37.60±5.51	182.00±34.43	43.77±01.66	76.22±02.54

Result expressed as mean ± SEM. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of NPM; III= 410 mg/kg/day of NPM; IV= 2050 mg/kg/day of NPM

Table 6.
Effect of 14-days oral toxicity reversibility test of 82-2050 mg/kg/day of *NPM* on the plasma liver enzymes (ALT, AST and ALP), albumin (ALB) and total protein (TPR) of treated rats

Groups	ALP (U/l)	ALT (U/l)	AST (U/l)	ALB (g/l)	TPR (g/l)
I	79.28±07.76	18.33±00.82	32.49±02.03	43.80±00.80	80.30±00.80
II	91.60±06.83	17.86±00.69	32.90±01.98	44.00±01.40	81.30±01.80
III	93.10 ±06.50	18.50±01.23	31.60±01.48	42.00±01.80	83.80±01.50
IV	86.55±06.69	18.17±01.46	31.91±01.22	41.30±00.50	85.30±00.70

Result expressed as mean ± SEM. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of NPM; III= 410 mg/kg/day of NPM; IV= 2050 mg/kg/day of NPM

Table 7.
Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on the plasma total cholesterol (TC), triglyceride (TG) and total bilirubin (TB) of treated rats

Treatment Groups	total cholesterol (mg/dl)	triglycerides (mg/dl)	total bilirubin (mg/dl)
I	72.59 ± 01.69	97.85 ± 07.32	00.67 ± 00.08
II	70.97 ± 03.77	87.20 ± 04.81	00.90 ± 00.26
III	67.55 ± 01.55	64.07 ± 03.99$^{\#}$	00.67 ± 00.10
IV	59.64 ± 01.17$^{\#}$	49.38 ± 02.00$^{\$}$	00.80 ± 00.90

Result expressed as mean ± SEM. # and $ represent significant decreases at p<0.001 and p<0.0001, respectively, when compared to the control (Group I) values. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of NPM; III= 410 mg/kg/day of NPM; IV= 2050 mg/kg/day of NPM

Table 8.
Effect of 14-days oral toxicity reversibility test of 82-2050 mg/kg/day of *NPM* on the plasma total cholesterol (TC), triglyceride (TG) and total bilirubin (TB) of treated rats

Treatment Groups	total cholesterol (mg/dl)	triglycerides (mg/dl)	total bilirubin (mg/dl)
I	91.58 ± 03.17	117.20 ± 14.87	00.54 ± 00.04
II	89.72 ± 03.45	90.17 ± 04.90	00.50 ± 00.05
III	87.21 ± 02.23	93.34 ± 05.64	00.55 ± 00.08
IV	97.11 ± 08.32	110.70 ± 10.83	00.53 ± 00.20

Result expressed as mean ± SEM. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of NPM; III= 410 mg/kg/day of NPM; IV= 2050 mg/kg/day of NPM

protein on the 90th day of the treatment when compared to the control (Group I) values (Table 5).

Effect of 14-days oral toxicity reversibility test of NPM on the plasma liver enzymes (ALT, AST and ALP), albumin (ALB) and total protein (TPR) of treated rats: Similar non-significant ($p>0.05$) alterations in the plasma liver enzymes, albumin and total protein levels were also seen in the 14-days oral reversibility test with *NPM* (Table 6)

Effect of subchronic oral treatment with 82-2050 mg/kg/day of NPM on the plasma liver total cholesterol (TC), triglyceride (TG) and total bilirubin (TB) of treated rats: Single, daily oral treatment of rats with 82-2050 mg/kg of *NPM* for 90 days caused significant decreases ($p<0.001$, $p<0.0001$) in the plasma total cholesterol and triglyceride levels when compared to the control (Group I) values (Table 7). However, oral treatment with *NPM* for 90 days did not cause any significant ($p>0.05$) alterations in the plasma total bilirubin levels in the treated rats when compared to control values (Table 7).

Effect of 14-days oral toxicity reversibility test of NPM on the plasma liver total cholesterol (TC), triglyceride (TG) and total bilirubin (TB) of treated rats: Withdrawal of oral treatment with 82-2050 mg/kg/day of *NPM* for 14 days resulted in the reversal of the earlier significant reductions ($p<0.001$ and $p<0.0001$) in the plasma total cholesterol and triglyceride with non-significant alteration ($p>0.05$) in the plasma albumin levels (Table 8).

Table 9.
Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on the plasma electrolytes (Na_{+}, K_{+}, Cl_{-} and HCO_{3-}) of treated rats

Groups	Na^{+}(mmol/l)	K^{+} (mmol/l)	Cl^{-}(mmol/l)	HCO_3^{-}(mmol/l)
I	145.80 ± 04.55	06.27 ± 00.17	95.90 ± 01.77	10.60 ± 02.65
II	147.10 ± 02.48	06.87 ± 00.22	94.60 ± 01.55	10.90 ± 00.95
III	137.90 ± 02.73	08.41 ± 00.15	93.63 ± 00.97	11.23 ± 00.21
IV	122.50 ± 02.67^{f-}	09.65 ± 00.11^{c+}	92.20 ± 00.94^{d-}	16.30 ± 00.86

Result expressed as mean ± SEM. $c+$ represents a significant increase at $p<0.001$ while $d-$ and $f-$ represent significant decreases at $p<0.05$ and $p<0.0001$, respectively, when compared to the control (Group I) values. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*

Table 10.
Effect of 14-days oral toxicity reversibility test of 82-2050 mg/kg/day of *NPM* on the plasma electrolytes (Na_{+}, K_{+}, Cl_{-} and HCO_{3-}) of treated rats

Groups	Na^{+}(mmol/l)	K^{+} (mmol/l)	Cl^{-}(mmol/l)	HCO_3^{-}(mmol/l)
I	139.40 ± 05.48	06.93 ± 00.87	99.73 ± 03.36	10.00 ± 00.35
II	140.00 ± 02.01	06.18 ± 00.86	96.98 ± 01.18	12.78 ± 01.09
III	150.20 ± 02.11	05.66 ± 00.69	96.99 ± 02.01	10.43 ± 01.35
IV	142.70 ± 02.12	05.12 ± 00.13	102.30 ± 01.87	12.00 ± 01.65

Result expressed as mean ± SEM. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*

Table 11. Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on the plasma urea and creatinine of treated rats

Groups	Creatinine (mg/dl)	Urea (mg/dl)
I	0.40 ± 00.26	51.28 ± 3.15
II	0.58 ± 00.24	58.03 ± 0.83
III	0.70 ± 00.10^{a+}	65.85 ± 2.65^{b+}
IV	0.88 ± 00.37^{b+}	74.35 ± 02.37^{c+}

Results are expressed as mean ± SEM. $a+$, $b+$ and $c+$ represent significant increases at p<0.05, p<0.01 and p<0.001, respectively, when compared to the control (Group I) values

Table 12. Effect of 14-days of oral toxicity reversibility of 82-2050 mg/kg/day of *NPM* on the plasma urea and creatinine of treated rats

Groups	Creatinine (mg/dl)	Urea (mg/dl)
I	01.14 ± 00.03	33.13 ± 02.37
II	01.17 ± 00.04	36.49 ± 02.37
III	01.11 ± 00.03	39.24 ± 01.84
IV	01.24 ± 00.08	37.09 ± 02.92

Results are expressed as mean ± SEM. I = 10 ml/kg of distilled water; II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*

Effect of subchronic oral treatment with 82-2050 mg/kg/day of NPM on the plasma electrolytes (Na_{+}, K_{+}, Cl_{-} and HCO_{3-}) of treated rats : Single daily oral treatment of rats with 82-2050 mg/kg of *NPM* for 90 days caused significant dose related decreases (p<0.05, p<0.01 and p<0.001) in the plasma levels of Na_{+} and Cl_{-} and a significant dose related increases in the plasma K_{+} while it had no significant alterations in the plasma bicarbonate levels when compared to the control (Group I) values (Table 9).

Effect of 14-days oral toxicity reversibility of NPM on the plasma electrolytes (Na_{+}, K_{+}, Cl_{-} and HCO_{3}) of treated rats: Upon withdrawal of oral treatment with *NPM* for 14 days, there was a non-significant (p>0.05) alterations in the plasma levels of Na_{+}, K_{+}, Cl_{-} and HCO_{3-} when compared to control (Group I) values to levels comparable to the control (Group I) values (Table 10).

Effect of subchronic oral treatment with 82-2050 mg/kg/day of NPM on the plasma urea and creatinine of treated rats: Single, daily oral treatment of rats with 82-2050 mg/kg of *NPM* for 90 days caused significant dose related increases (p<0.05, p<0.01 and p<0.001) in the plasma levels of urea and creatinine when compared to the control (Group I) values) (Table 11).

Effect of 14-days oral toxicity reversibility test of NPM on the plasma urea and creatinine of treated rats: Withdrawal of oral treatment with *NPM* for 14 days was associated with non-significant alterations in the plasma levels of urea and creatinine when compared to the control (Group I) values (Table 12)

Effect of subchronic oral treatment with 82-2050 mg/kg/day of NPM on full blood count parameters of treated rats: Single, daily oral treatment of rats with 40-1000 mg/kg of *NPM* for 90 days caused a significant (p<0.05) dose related increases in RBC, HGB, HCT, MCH, MCHC, PLT and WBC when compared to the control values (Table 6) while causing non-significant (p>0.05) alterations in the white blood cell differentials (% LYM, % EOS, % MON, %BAS and %NEU) when compared to the control values (Table 13).

Effect of 14-days oral toxicity reversibility of NPM on full blood count parameters of treated rats: Withdrawal of oral treatment with *NPM* for 14 days was associated with significant ($p<0.0001$) dose related reductions in %eosinophil and %basophil differentials and significant increases in %lymphocyte differentials when compared to the control (Group I) values (Table 14)

Histopathological studies of the effect of subchronic oral treatment with 82-2050 mg/kg/day and oral toxicity reversibility of NPM on vital body organs of treated rats: The effect of the subchronic oral treatment with 82 mg/kg/day, 410 mg/kg/day and 2050 mg/kg/day of *NPM* on some selected vital body organs in the treated rats are depicted in Figures 1-6 depicting different histological lesions induced by different doses of *NPM* with which the different groups of rats were treated.

Table 13.
Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on the full blood count parameters of treated rats

Parameters	I	II	III	IV
RBC	07.15±00.15	07.67±00.31	07.80±00.27	07.83±0.05^{a+}
HGB	13.36±00.29	14.26±00.26	14.40±00.41	14.80±0.34^{a+}
HCT	44.08±00.55	46.02±00.54^{a+}	46.94±00.33^{b+}	48.12±0.52^{c+}
MCV	71.42±02.13	74.66±01.23	75.00±01.56	76.60±0.45
MCH	26.98±00.51	20.25±00.43	20.66±00.19	21.16±0.18^{a+}
MCHC	26.98±00.57	30.12±00.35^{b+}	31.32±00.45^{c+}	32.38±0.41^{c+}
PLT	640.80±25.62	651.80±48.60	800.00±32.62	855.80±65.60^{a+}
WBC	06.54±00.32	06.98±01.04	08.37±00.47	09.54±0.18^{a+}
%LYM	45.00±00.97	55.10±07.63	54.72±02.36	54.22±05.85
%EOS	03.92±02.19	01.37±00.86	02.70±01.07	02.12±0.54
%BAS	00.42±00.15	01.47±00.94	01.15±00.58	00.27±0.08
%NEUT	40.94±03.21	33.02±05.23	38.58±01.70	46.22±02.53

$^{a+}$, $^{b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, when compared to control (Group I) values. I = control (10 ml/kg of distilled water); II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*; WBC: White Blood Cell; RBC: Red Blood Cell; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Cell Volume; MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; PLT: Platelets; LYM: Lymphocytes; NEUT: Neutrophil; BAS: Basophil; EOS: Eosinophil

Table 14.
Effect of subchronic oral treatment with 82-2050 mg/kg/day of *NPM* on the full blood count parameters of treated rats.

Parameters	I	II	III	IV
RBC	07.15±00.15	07.67±00.31	07.80±00.27	07.83±0.05^{a+}
HGB	14.05±00.52	13.73±00.12	14.38±00.26	14.21±00.21
HCT	48.47±00.99	45.78±01.12	48.88±01.03	47.45±00.67
MCV	66.52±00.22	64.03±00.74	65.70±01.21	65.00±01.03
MCH	19.28±00.47	19.04±00.26	19.25±00.26	19.38±00.41
MCHC	28.95±00.65	30.32±00.91	28.93±00.24	29.52±00.67
PLT	808.40±48.06	639.20±17.19	564.50±56.18	571.80±38.12
WBC	06.71±00.47	05.52±00.06	06.08±00.79	06.13±00.63
%LYM	39.82±03.00	69.73±00.78^{c+}	44.03±02.94	73.50±01.14^{c+}
%EOS	04.67±00.23	00.77±00.80^{f-}	00.92±00.10^{f-}	00.80±0.09^{f-}
%BAS	00.52±00.04	00.12±00.05^{f-}	00.22±00.05^{f-}	00.10±0.03^{f-}
%NEUT	47.79±03.41	24.51±00.50^{f-}	47.30±03.91	19.10±00.56^{f-}

$^{a+}$, $^{b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, when compared to control (Group I) values. I = control (10 ml/kg of distilled water); II = 82 mg/kg/day of *NPM*; III= 410 mg/kg/day of *NPM*; IV= 2050 mg/kg/day of *NPM*; WBC: White Blood Cell; RBC: Red Blood Cell; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Cell Volume; MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; PLT: Platelets; LYM: Lymphocytes; NEUT: Neutrophil; BAS: Basophil; EOS: Eosinophil

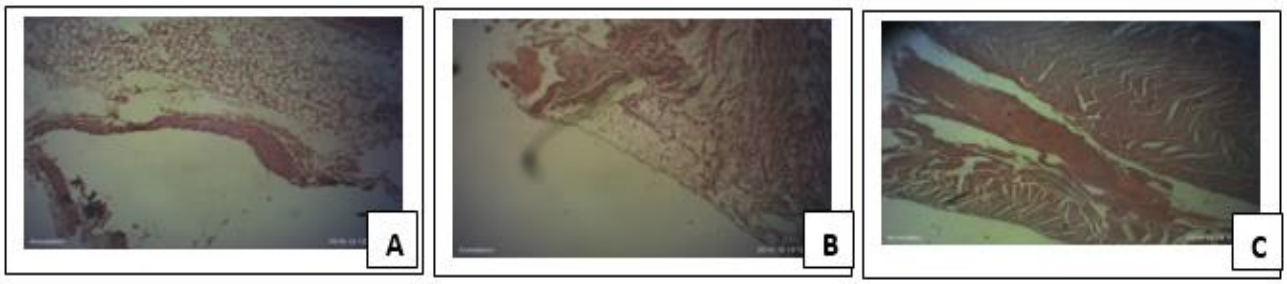

Figure 1.
Photomicrograph of heart from rats treated with: (A) distilled water, (B) 2050 mg/kg/day of *NPM* showing mild vascular congestion and reduced pericardial fatty tissue, and (C) 2050 mg/kg/day of *NPM* showing reduced pericardial fatty tissue and moderate vascular congestion 14 days post-withdrawal of *NPM*. H & E, X100.

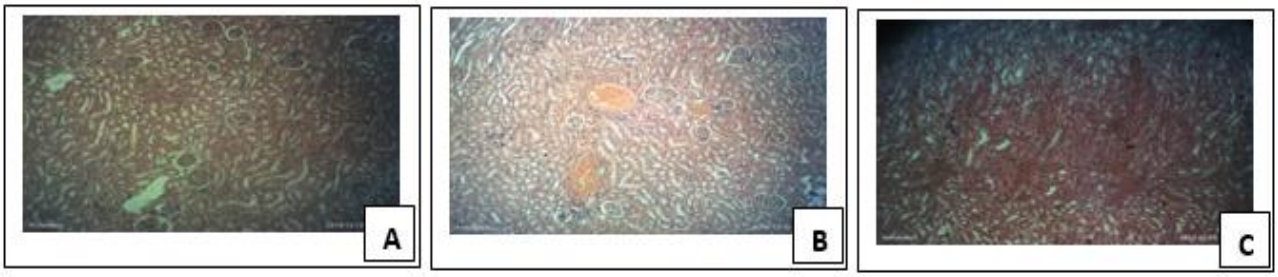

Figure 2.
Photomicrograph of Kidney from rats treated with: (A) 10 ml/kg/day of distilled water for 90 days showing normal glomeruli and renal tubules, (B) 2050 mg/kg/day of *NPM* for 90 days showing remarkable renal vascular congestion and interstitial hemorrhage, and (C) 2050 mg/kg/day of *NPM* for 90 days showing marked renal vascular congestion and interstitial hemorrhage 14-days post-withdrawal of *NPM*. H & E, X100.

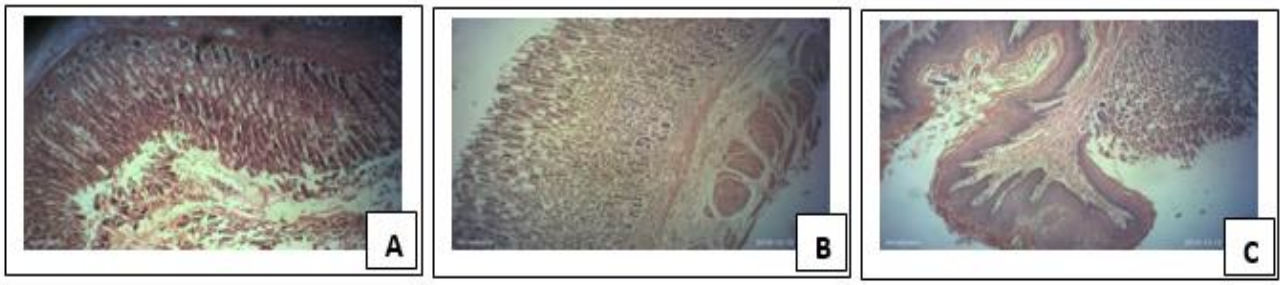

Figure 3.
Photomicrograph of stomach from rats treated with: (A) 10 ml/kg/day of distilled water for 90 days showing normal gastric architecture, (B) 2050 mg/kg/day of *NPM* for 90 days showing some degree of mucosal erosion with infiltration of lamina propria with mild inflammatory cells mostly neutrophils and eosinophils and, (C) 2050 mg/kg/day of *NPM* for 90 days showing some degree of mucosal erosion with infiltration of lamina propria with inflammatory cells mostly neutrophils and eosinophils 14-days post-withdrawal of the herbal mixture. H & E, X400.

Figure 4. Photomicrograph of splenic tissue from rats treated with: (A) 10 ml/kg/day of distilled water for 90 days showing normal splenic architecture, (B) 2050 mg/kg/day of *NPM* for 90 days showing distortion of the lymphoid architecture and distension of the splenic sinuses. and, (C) 2050 mg/kg/day of *NPM* for 90 days still showing distortion of the lymphoid architecture and distension of the splenic sinuses 14-days post-withdrawal of *NPM*. H & E, X400.

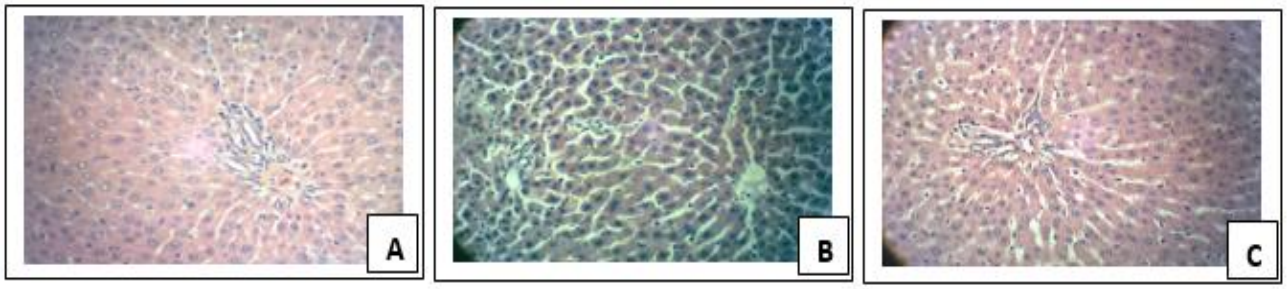

Figure 5. Photomicrograph of liver from rats treated with: (A) 10 ml/kg/day of distilled water for 90 days showing normal hepatic architecture, (B) 2050 mg/kg/day of *NPM* for 90 days showing moderately congested hepatic vessels and sinusoids but normal portal triads and, (C) 2050 mg/kg/day of *NPM* for 90 days showing slightly congested hepatic vessels and sinusoids but normal portal triad and hepatocytes indicating some degree of recovery. H & E, X400.

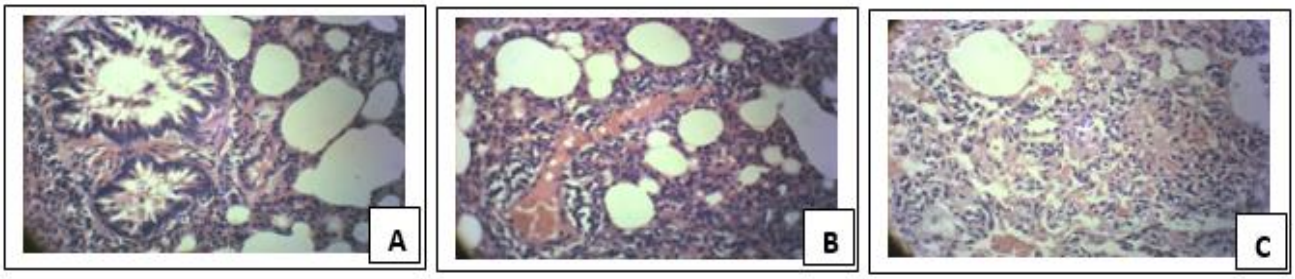

Figure 6. Photomicrograph of lung section from rats treated with: (A) 10 ml/kg/day of distilled water for 90 days showing normal lung tissue architecture, (B) 2050 mg/kg/day of *NPM* for 90 days showing marked congested pulmonary blood vessels and normal alveoli and, (C) 2050 mg/kg/day of *NPM* for 90 days showing persistent congested pulmonary blood vessels but normal alveoli 14-days post-withdrawal of the *NPM*. H & E, X400.

DISCUSSION

Polyherbal combinations are generally believed to provide holistic treatment of human diseases even when the safety profile of such a herbal mixture remains unknown (Sharma *et al.*, 2020). In this present study, the acute and subchronic oral toxicity studies of *NPM* in young nulliparous Wistar rats using anthropometric, biochemical, hematological and histopathological parameters as measured endpoints were conducted using standard scientific procedures.

Single oral treatment of rats with 5000 mg/kg body weight of *NPM* produced no death within the short- and long-term outcomes of the limit dose test of Up and Down Procedure. However, the behavioral toxicity observed include restlessness/agitation, abnormal body posture, generalized body tremor, feed and water refusal within 24 hours post-treatment which gradually subsided after 24 hours post-treatment with full recovery attained by the treated rats 48 hours post-treatment. Thus, *NPM* can be considered orally safe on acute or short-term oral exposure.

Subchronic oral treatment with 82, 410 and 2050 mg/kg/day of *NPM* over the period of 90 days showed that *NPM* did not cause profound alterations in the serum levels of liver enzymes and other liver function parameters indicating safety profile of *NPM* in the treated rats even on prolonged oral exposure. Liver is known to be the main organ of detoxification for most drugs and alterations in its enzyme markers are considered strong indicators of toxicity profile of a drug (Woodman, 1996; David and Hamilton, 2010). Thus, the fact that *NPM* caused no significant alterations in these hepatic enzyme markers suggests that *NPM* is not injurious to the liver since liver injury (hepatotoxicity) is marked by profound elevations in the serum levels of ALT, AST, ALP and at times reduced serum total protein and albumin levels (Giannini *et al.*, 2005; Arika *et al.*, 2016). However, on the renal function parameters measured which included plasma urea and creatinine levels, oral treatments with 82-2050 mg/kg/day of *NPM* for 90 days induced profound elevations in these measured parameters. These findings are suggestive of the potential nephrotoxic effect of prolonged oral exposure to *NPM*, although results of the histopathology of the kidneys of *NPM*-treated rats were corroborative of these biochemical findings showing dose-dependent renal vascular congestions. Alterations in the plasma levels, particularly, profound elevations in these renal function parameters are induced by drugs with nephrotoxic potentials. The mere fact that *NPM* profoundly elevated the plasma levels of the measured renal function parameter coupled with the histopathogical report of associated vascular congestions are strong indications that *NPM* may have a deleterious effect on the renal function upon prolonged exposure to it. However, the nephrotoxic potential of *NPM* could be attributed to the presence of *Cassia sieberiana* which has

previously been reported to have caused significant elevation in the serum creatinine and urea concentration in Wistar rats treated with 20, 60 and 180 mg/kg of the aqueous stem bark extract of *Cassia sieberiana* for 6 weeks (Obidah *et al.,* 2009). Similarly, histopathological reports showed that *NPM* may also have deleterious effects on the heart tissues causing dose-dependent vascular congestions on the heart tissue; on the lung tissues causing interstitial distortion as well as on the stomach causing dose-dependent gastritis in the treated rats. These were also reflected on the relative organ weights especially on the lungs and the stomach where prolonged oral treatment with 82-2050 mg/kg/day of *NPM* induced profound non-dose dependent reductions in the relative organ weights of lungs and stomach of treated rats.

On the hematological parameters, *NPM* significantly improved the full blood counts except for the differential white cell counts which were not significantly altered by prolonged oral treatment of rats with 82-2050 mg/kg/day for 90 days. However, these improvements could be attributed to the presence of *Sorghum bicolor* which has been widely reported to have pronounced hematopoietic effect due to the abundant polyphenols (particularly flavonoids contents (Ogwumike, 2002; Akande *et al.*, 2010b; Benson *et al.*, 2013) although the presence of other constituent plants may also have contributed to the improved hematological profile as recorded in this study. Another worthy observation is the tendency towards hypercoagulability with 2080 mg/kg/day of the herbal mixture which was strongly indicated by thrombocytosis (significant elevation in the platelet counts in the blood) which is closely regulated by the kidney- and liver-producing thrombopoeitin (Hitchcock and Kaushansky, 2014). Literature has shown a strong and direct correlation between thrombocytosis and vascular thromboembolism and stroke resulting from increased platelet aggregation (Rinder *et al.*, 1998; Khorshed *et al.*, 2007; Chu *et al.*, 2010; Koupenova *et al.*, 2017).

Another significant finding of this study is that *NPM* caused non-significant alterations (be it loss or gain) the average body weights and relative organ weight of the treated rats (except that of stomach and lungs which were significantly reduced although in non-dose dependently). These findings may be related to the absence of tannins and phlobatannin in the *NPM* which have been reported to induce weight loss in extract/polyherbal formula abundantly rich in this secondary metabolite due to their appetite inhibiting effect and anemic effect (Chung *et al.*, 1998; Amesa *et al.*, 2018; Valenti *et al.*, 2019). However, in the oral toxicity reversibility studies, all of the measured biochemical (liver and renal function parameters) and hematological changes induced by chronic oral treatment with 82-2050 mg/kg/day of *NPM* were reversed upon stoppage of the oral treatment (reversibility test) of the herbal mixture for 14 days but the "tell-tale" signs of histological lesions in the heart, kidneys, stomach and spleen were still remarkable despite withdrawal of the herbal mixture.

In conclusion, *NPM* although widely consumed to traditionally relieve pains associated with gastrointestinal disorders such as rectal prolapse and hemorrhoids, menstrual and waist pain, it may not be considered safe for consumption on long term use as it showed tendency to be nephrotoxic and cause gastritis and deleterious effects on other body organs likes the lungs and spleen on prolonged oral exposure, although, our studies showed that its prolonged oral consumption caused improved hematological profile in the treated rats. While *NPM* may modulate biochemical and hematological balance in the system, patients with occult or underlying renal diseases or reduced renal function should exercise caution in its use as it may result into full blown renal failure.

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