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Evaluation of the Effects of Paxherbal Bitters on Albino Rats

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Abstract
The bitters, marketed as a "cure-all"- patent medicine, composed of forty herbal constituents was fed using an oro-gastric gavage, to different sets of male albino wistar rats, after which the acute toxicity (LD_{50}), biochemical and haematological indices as well as the subchronic toxicity and histological status of the her bal bitters on the rats were investigated using standard laboratory procedures. The bitters were well tolerated and not toxic to the rat organs and blood cells, during the 28-day duration of the study. The high acute toxicity (LD_{50}) of the bitters of 24ml/kg body weight (equivalent to 24g/kg body weight) indicates it has low-lethality. The minimal and non-significant differences ($P>0.05$) in all the indices used to assess, the liver, kidney and cardiac function statuses of the bitters-fed rats compared to same in the control rats, was indicative that the bitters preserved the functions of these organs. The increase in the CD_4^+-count and decrease in the level of the fasting blood glucose in the bitters-fed rats when compared to that of the control rats was significant ($P<0.05$). The decrease in serum triacylglycerol and LDL-cholesterol levels and the increase in HDL-cholesterol caused by the bitters was significant ($P<0.05$). The bitters-fed rats had a significant decrease ($P<0.05$) in their serum malondialdehyde (MDA) levels and a significant increase ($P<0.05$) in their serum vitamin C and E levels and activities of antioxidant enzymes namely superoxide dismutase, catalase, and glutathione peroxidase, when compared with that of the control rats. The described changes in the histopathological studies done on the tissues of the heart, kidneys, liver, pancreas, small intestine and colon of bitters-fed rats did not reveal adverse differences when compared to those of control organs. The results from this study indicate that Paxherbal bitters may be said to be - hypolipidaemic, hypoglycaemic, immunity-boosting, choleretic, hepatoprotective, antihypertensive, as well as having antioxidant properties and protective against cardiovascular diseases.

Keywords: Paxherbal bitters, haematology, serum, antioxidant, histopathology, rat

Introduction
The term "bitters" as it is used presently, is a beverage, often alcoholic, flavoured with herbal essences that gives it a bitter or bittersweet taste. It is often summarized that bitters stimulate digestive secretions and metabolism as a whole and in so doing increase appetite, relieve constipation, and generally ease the heavy glumness of sluggish digestion. But, this is really too simple and cursory a summation, and a deeper look into the actions of bitters is not only theoretically insightful but practically invaluable (1). Bitters are produced from herb and root extracts, from the narcotic components of (primarily) tropical and subtropical plants and spices. They are usually dark in colour and valued for their ability to promote appetite and digestion hence their use as patent medicine and as digestive aids and as flavouring in cocktails. Bitters are made up of numerous groups of chemical compounds extracted from the herbs and roots that have the common characteristic of a bitter taste and act to increase the vital energy centres in the body (1).

Since they act upon the bitter receptors of the mouth, thereby producing the bitter taste in the mouth, their stimulation do not produce any electrical changes on the surface of the cell. Instead, the bitter molecules bring about intracellular biochemical changes by acting on the cell membrane receptors. This facilitates an increase in calcium concentrates within the cell and signals the gustatory nerve (1, 2). This is their possible mechanism of action. As soon as the bitter taste bud is stimulated, it causes the release of the gastrointestinal hormone gastrin. A study of the common physiological actions of gastrin, reveals a close similarity with the traditional remedies of the bitters. We can therefore tally the actions of bitters with that of gastrin (1, 3).

From the Pax Herbal Centre and Research Laboratories Product Brochure (4), the following information about Paxherbal Bitters was obtained:

Pax- Herbal Bitters is locally produced in Edo State (composed of forty (40) herbal constituents) which includes: *Cymbogon citratus* (Lemon grass), *Aloe vera* (True aloe, Lily of the desert), *Gongronema latifolium* (Utazi), *Zingiber officinale* (Ginger), *Xylopia aethiopica* (Uda), *Vernonia amygdalina* (Bitter leaf), *Capsicum annuum* (Green pepper), *Carica papaya* seed (Pawpaw seed), *Glycine max* (Soya bean leaves), *Garcinia kola* (Bitter kola), *Morinda citrifolia* (Noni), *Aspilia africana* (Haemorrhage plant), *Capsicum Spp.* (Pepper plant), *Citrus aurantium* (Bitter orange), and *Musa paradisiaca* (French plantain), just to mention but a few.

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As a safeguard justification for use, the above stated plant ingredients and similar preparations using the same plant ingredients were used in most Nigerian, European, Asia, Pacific and African rural and urban areas from time immemorial to offer a wide range of remedies for control and treatment of a broad range of ailments (4).

PB is a tincture of different herbal ingredients. It promotes healthy blood circulation (prevents undue deposition of cholesterol on blood vessels during circulation), prevents kidney stones, helps in digestion, activates bile flow (and hence proper digestion and excretion of lipids), increase immunity of the body against bacterial and fungal infections. It helps in prevention of diabetes and accelerates body repairs (and healthy blood vessels), heal wounds and toothaches, among a host of other pharmacological uses (4).

These claims have not been evaluated by NAFDAC and there is paucity of scientific literature with research findings in respect of Paxherbal Bitters, though there exist some literature on some of the constituent herbs of Paxherbal bitters, but few, if any, on the final composition, effect and toxicity that may result from the product existing in the new composite form called "Paxherbal bitters"; especially as there may be cross-reactions of the individual constituents or synergistic effects from similar constituents in different herbs (40 of them!) that may cause a "toxic overdose" of that constituent. It is this and the increasing popularity of "herbal bitters" generally in Nigeria that has influenced the present "orthodox" research to determine and establish a baseline scientific data on the pharmacological effect and toxicity profile of these herbal products that are presently being marketed in several brands as a -"cure-all" patent medicine, in Nigeria.

Materials and Methods

Materials

Paxherbal Bitters was purchased from the manufacturers at the Benedictine Monastery at Ewu-Ishan in Edo State. The bitters were bought as liquid formulations and stored at room temperature (30-36^{0}c) throughout the period of the experiment.

Reagent kits and other reagents used were of standard quality and were purchased from qualified/accredited dealers/ suppliers or their manufacturers' representative in Nigeria.

All the experimental animals for all stages of this study were handled in strict compliance with international guidelines as prescribed by the Canadian Council on the Care and Use of Laboratory Animals in Biomedical Research, 1984 edition (5).

Male albino rats of the *Wistar* strain were obtained from the Anatomy Department, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria. The rats were housed in a well ventilated room in the animal house of the Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Nigeria, with the room temperature ranging from 30-36^{0}C. They were allowed the diurnal cycle, which is the recommended 12-hr light and dark cycle. The rats were fed ad-libitum with standard pelleted mash and clean tap water for an aacclimatization period of two weeks.

Acute Toxicity Study

The method of Miller and Tainter (6) as adapted by Randhawa (7) was used for the acute toxicity study. It was done in two phases.

Phase I Acute Toxicity Study: Experimental Design/Protocol

This was done using the "staircase method" for the determination of the lethal dose and dose range prior to the actual LD_{50} determination. After 14 days of acclimatization, the 10 experimental animals for the determination were divided into 5 groups of two rats each with each set of 2 rats given a dose of the bitters higher than the preceeding one to determine which dose will cause zero death and which one will cause 100% death after 72hrs of oral dose of the bitters using an oro-gastric gavage (6, 7, 8). The Animals were observed for signs of toxicity and mortality. At the end of the 3 days, for each group, the dose(s) that caused no death and the 1st dose that caused the death of the 2 rats in each sub-group was noted; these doses were used to determine the range to be used in the LD_{50} determination for the herbal bitters (6, 7, 8).

Phase II Acute Toxicity Study: Experimental Design/Protocol

This was for the determination of the LD_{50}. After 14 days of acclimatization, the 50 experimental animals for the determination of the LD_{50}, using the Miller and Tainter method (6), were weighed and divided into 5 groups of 10 rats each according to their weight range, making sure that the distribution was in such a way that the average weight per group was about 162g. Each group of 10 rats was given a dose of the bitters higher than the preceeding one following the range as determined from pre-LD_{50} determiation study (phase I). This was to determine which dose will cause death ranging from 0 to 100%, after 72hrs of oral dose of the bitters (6, 7, 8). The animals were observed for the first 2 hours, and then at the 6th, 24th, 36th, 48th, 60th and 72nd hours for any toxic symptoms. After 72hrs, the number of deceased rats were counted in each group and percentage of mortality calculated and tabulated. The percentage of dead rats for 0 and 100 was corrected before the determination of probits as shown:

Corrected % Formula for 0 and 100% Mortality (7)

For 0% dead = 100(0.25/n)

For 100% dead = 100(n-0.25)/n; where n = 10, their values were 2.5 and 97.5 respectively.

Determination of the $LD_{50:}$ The Probit values were plotted against log-doses and then the dose corresponding to probit 5, that is 50%, was extrapolated, the value identified and noted as the LD_{50}. Other calculations were made according to the method described by Miller and Tainter (6) and Randhawa (7).

Subchronic Toxicity Study

Animal for study: Sixteen (16) male albino rats of the *wistar* strain weighing between 110-210g, Average weight per group approximately 162g.

Grouping of the animals: After 14 days acclimatization, the 16 animals were weighed and divided into two (2) groups A and B, of eight (8) rats each, making sure that the weights of those in a group were representative of the weight range of all the rats, such that the average weight of all the groups at the onset of the experimental period was 162g.

Feeding regime and care of the animals: The rats were fed ad-libitum on standard pelleted mash and clean tap-water during the entire course of the 28-day study and allowed the recommended 12-hr light and dark cycle. Care was taken to determine the quantity of feed consumed daily. The rats were housed in wooden cages with a tiny-wire meshed/iron gauze flooring to allow the rat-excreta to be collected into another steel tray receptacle below covered with a bedding material. The cages, their surroundings, the receptacle tray below with its bedding, were cleaned and disinfected daily.

Experimental procedure: In addition to the feed and clean pipe-borne water, the rats in group B were given orally, the Paxherbal bitters, using an oro-gastric gavage, according to the equivalent dose (to the weight of the rats for that week) of the effective dose already prescribed for man. An equivalent volume of distilled water was given to the control group which was group A. The animals were observed for signs of toxicity and mortality.

Dosage regimen: An adult man was expected to consume on the average 40ml of herbal bitters daily. Appropriate calculations were done to determine the initial equivalent doses of the bitters (distilled water in the case of the control group) in ml/g mean body weight of the rats to be given in each group. As the initial mean weights of rats in each group at the beginning of the study was 162g, the equivalent volume [in millilitres-(ml)] of the bitters/distilled water that was given to the rats was as calculated:

If 40ml was consumed by a 70,000g man (70kg)

How many ml was a 162g rat expected to consume? (Xml)

$$\text{Xml} = \frac{40\text{ml} \times 162\text{g}}{70{,}000\text{g}} = 0.093\text{ml} \text{ (approximately 0.1ml)}$$

0.1ml for a 162g rat means a dose of 0.1ml/162g = approx. 6.2×10^{-4}ml/g of rat.

The rats were weighed weekly and the weight used to calculate the equivalent doses/volume to be administered for each group of rats for that week. The relationship between this weight and the quantity of feed consumed and appetite of the rats was also investigated.

Weekly Body Weight: The body weight of each rat was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing (day 1), once weekly during the dosing period, (day 7, 14 and 21) and once on the day of sacrifice (day 29), (8).

Weekly Quantity of Feed Consumed: The quantity of feed given to each group of rats daily was determined by subtracting the quantity of feed left the next morning from that given the day earlier. From the results the average quantity consumed weekly by the rats was determined. This quantity of feed consumed by each rat was assessed using a sensitive balance from the commencement of dosing (day 1), until the day of sacrifice (day 29). (8).

Clinical Signs and Mortality: The animals were observed for signs of weakness, increased or decreased appetite, weight loss and other physiological changes including mortality.

Clinical signs to be assessed before dosing, immediately and 4hrs after dosing, include level of sedation, restlessness, changes in nature of stool, urine and eye colour, excretion of worms, diarrhoea, haematuria, uncoordinated muscle movements, etc.

The animals will be observed for toxic symptoms such as weakness or aggressiveness, food refusal, loss of weight, diarrhoea, discharge from the eyes and ears, noisy breathing and mortality, (9, 10).

Blood Sample Collection and Preparation

Two specimen bottles were used for collection of blood from each animal. Anticoagulant bottles containing K_2 EDTA for haematological tests and lithium heparin bottles for assay of other parameters were used for initial collection of blood from all animals. The last dose of the bitters was administered on the morning of the 28^{th} day. All meals were stopped by 7pm on the 28th day. After an overnight fast and following chloroform anaesthesia and opening up of the animals, blood samples were collected from the animals using syringes and needles via the inferior vena cava and cardiac puncture, into already labelled K_2 EDTA and lithium heparin bottles without undue pressure to either the arm or the plunger of the syringe. The samples were then mixed by gentle inversion. The samples in the K_2 EDTA anticoagulant bottles were immediately sent for automated analysis for full/complete blood count and CD_4^+ T-Lymphocyte count. The samples in the lithium heparin bottles were centrifuged at 4000r/min for 10mins to obtain plasma. The plasma supernatants were then separated into sterile plain bottles and were used for assay of the required parameters.

Tissue Sample Collection and Preparation and Determination of the Relative Organ Weight

At the end of the 28-day experimental period, just after the rats have been weighed, sacrificed (using chloroform anaesthesia) and blood samples collected from them, different organs namely the heart, liver, kidneys, pancreas, small intestine and colon of the respective rats from all groups were carefully dissected out and weighed in grams (this weight was designated as the absolute organ weight). The relative organ weight was then calculated using the formula:

$$\text{Relative Organ Weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

(8).

The heart, kidney, liver, pancrease and gastrointestinal tract (duodenum and colon) of some of them were then fixed in a buffered 10% formal-saline solution. They were thereafter processed with an automatic tissue processor (Shandon Citadel Model, 2000) and following dehydration and embedding, sections of the tissues were cut at 4-5µ with a rotatory microtone. Thereafter , staining with haematoxylin and eosin was done, after which each of the stained tissues was individually examined under a high powered field of a microscope (8).

Assay of Haematological Indices:

These were determined following the instructions of the manufacturers of the automated instrument:

The full/complete blood count, was determined using a KX-21N, an automated blood cell count analyser (11), while for the CD_4^+- T-Lymphocyte count, CYFLOW SL- GREEN, an automated portable flow cytometer for the enumeration of CD_4^+- T-Lymphocyte cells in the whole blood was used **(**12, 13).

Assay of Fasting Blood Glucose:

The blood glucose was assayed using the glucose-oxidase method (14), as outlined in the glucose kit by Randox lab. UK.

Assay of Serum Lipid Profile

The parameters assayed are total cholesterol, triaclglycerol, HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol using Randox kit (Randox lab. UK) and following the standard procedures as described by the manufacturers (15, 16).

Assessing the Liver Function Status

The parameters assayed are total protein, albumin, total bilirubin, conjugated bilirubin, alanine transaminase, aspartate transaminase, alkaline phosphatase and gamma-glutamyl transferase, using Randox kit (Randox lab. UK) and following the standard procedures as described by the manufacturers (17, 18, 19, 20, 21).

Assessing the Kidney Function Status

The parameters assayed are the electrolytes-Na^+, K^+- using the Flame Photometer (22); Cl^- - using the mercurumetric (titrimetric) method (23); HCO_3^- - using the titrimetric method (24); urea- using the Berthelots reaction method (25); and creatinine- using the spectrophotometric method (26).

Assessing the Cardiac Function Status

The cardiac enzymes assessed are creatine kinase – using the UV method (27) and lactate dehydrogenase- using the UV method (28).

Assessing the Antioxidant Status and Lipid Peroxidation Effect

The parameters assessed in vivo and the methodology employed are-malondialdehyde (MDA) level (29); vitamin E(30); vitamin C (31, 32); catalase (CAT) (33); superoxide dismutase (SOD) (34); glutathione peroxidase (GPx) (35).

Statistical Analysis

Data was subjected to appropriate statistical analysis using the students paired t-test from the computerized statistical package for the social sciences, edition 17 (SPSS 17). $P<0.05$ was considered significant. The results were expressed as Mean±SEM.

Results

Clinical Signs and Symptoms and Mortality

During the 28 days of feeding with the bitters, there was no mortality, apart from a slight increase in activity for a few minutes, (possibly from alcoholic euphoria) no other adverse clinical manifestations were observed (no sedation, no changes in nature of stool, urine and eye colour, no discharge from the eyes and ears, no haematuria, no diarrhoea and no uncoordinated muscle movements, etc). The bitters was well tolerated as it improved rather than adversely affecting the appetite of the rats; there were materials that were part of the stool suggestive of increased excretion of epithelial cells of the G.I.T which is in keeping with the claimed effect of bitters causing general toning of the G.I.T and increase in turnover of its epithelial cells that makes it possibly an ulcer preventive and healing agent.

Acute Toxicity: The LD_{50} of Paxherbal bitters was 24.00±5.10 ml/kg (expressed in other units considering the specific gravity/density of the bitters, the LD_{50} (mg/kg $x10^3$) is approximately 24.00±5.10 and the LD_{50} (g/kg) is approximately 24.00±5.10)

Subchronic Toxicity and Pharmacological/Biochemical Effects of Herbal Bitters on Rats

Table 1: Feed consumed by rats after administration of Paxherbal bitters for four weeks

Groups	Week 1	Week 2	Week 3	Week 4	4 weeks
Control	19.34±1.49[a]	17.76±0.87[a]	16.92±0.87[a]	19.38±2.10[a]	18.35±0.61[a]
Paxherbal	19.97±0.99[a]	19.32±1.31[a]	22.34±1.17[b]	19.76±2.31[a]	20.34±0.68[a]

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ significantly (P<0.05) from one another.

Though the feed consumed by the bitters-fed rats were consistently higher, apart from the 3rd week, the feed consumed were not significantly different (P>0.05) from that consumed by the control.

Table 2: Leucocyte Count , Leucocyte Differentials, CD_4 Count, and Platelet Count of rats fed with Paxherbal bitters

Groups	Leucocyte Count (x 10^3/ µL)	Lyphocyte Count (%)	Monocyte Count (%)	Neutrophil Count (%)	CD_4 Count (/µL)	Platelet Count (x 10^3/ µL)
Control	5.79±1.22[a]	65.60±2.19[a]	11.39±1.34[a]	23.43±1.90[a]	186.00±7.63[a]	72.63 ± 11.58[a]
Paxherbal	6.34±0.64[a]	70.25±4.33[a]	8.16±1.12[a]	21.66±3.94[a]	215.00±5.93[b]	81.13 ± 8.29[a]

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ significantly (P<0.05) from one another.

Though the total white blood cell count, the lymphocyte and platelet counts in rats fed Paxherbal bitters were increased compared to that of the control, statistical evaluation show that there were no significant difference (P>0.05) between them. The CD_4 lymphocyte count on the other hand was significantly (P<0.05) elevated, while the monocyte and neutrophil counts show a non-significant (P>0.05) decrease.

Table 3: Red Blood Cell (RBC) Count, Haemoglobin Concentration, Packed Cell Volume (PCV) and Red Cell Indices of rats fed with Paxherbal bitters

Groups	Red Blood Cell, RBC Count (x10^6)	Haemoglobin Concentration (g/dl)	Packed Cell Volume, PCV (%)	Mean Corpuscular Volume, MCV (fl)	Mean Corpuscular Haemoglobin, MCH (pg)	Mean Corpuscular Haemoglobin Concentration, MCHC (g/dl)
Control	6.39±0.64[a]	14.88±1.19[a]	41.56±3.80[a]	48.98±0.99[a]	19.48±0.45[a]	36.79±0.70[a]
Paxherbal	6.34±0.40[a]	14.59±1.59[a]	46.14±4.20[a]	49.26±1.69[a]	19.66±0.70[a]	37.03±0.34[a]

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ significantly (P<0.05) from one another.

Though there were differences in the RBC count, haemoglobin concentration, PCV, MCV, MCH and MCHC in rats fed Paxherbal bitters when compared to that of the control, statistical evaluation shows that these differences were not significant (P>0.05).

Table 4: The effect of Paxherbal bitters on fasting blood glucose (FBG) level of rats.

Groups	FBG (mg/dl)
Control	91.63±8.57[a]
Paxherbal	56.00±5.93[b]

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ significantly (P<0.05) from one another.

Statistical evaluation indicates that the fasting blood glucose levels in rats fed Paxherbal bitters were significantly (P<0.05) reduced compared to that of the control.

Table 5: The effect of Paxherbal bitters on lipid profile of wistar rats

Research Groups	Cholesterol (mg/dL)	Triacylglycerol (mg/dL)	HDL-Chol. (mg/dL)	LDL-Chol. (mg/dL)	VLDL-Chol. (mg/dL)
Control	98.63±3.02[a]	58.00±2.74[a]	18.88±2.14[a]	68.15±1.75[a]	11.60±0.55[a]
Paxherbal	87.25±4.50[a]	47.50±4.28[b]	29.25±0.41[b]	48.55±3.42[b]	9.45±0.87[a]

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ significantly (P<0.05) from one another.

The bitters caused a reduction in rat blood cholesterol, triacylglycerol, LDL-cholesterol and VLDL-cholesterol levels and an increase in the HDL-cholesterol when compared to that of the control. The reduction in triacylglycerol and LDL-cholesterol was statistically significant (P<0.05), same also with the elevation in HDL-cholesterol.

Table 6: liver function indices of rats administered with Paxherbal bitters

Analytes	Control	Paxherbal
Total Bilirubin (mg/dl)	0.28±0.03[a]	0.26±0.04[a]
Conjugated Bilirubin (mg/dl)	0.16±0.02[a]	0.15±0.02[a]
Aspartate Transaminase (IU/L)	28.25±2.97[a]	29.75±3.55[a]
Alanine Transaminase (IU/L)	3.88±0.30[a]	4.38±0.63[a]
Alkaline Phosphatase (IU/L)	10.13±0.61[a]	11.38±0.32[a]
Total Protein (mg/dl)	4.93±0.10[a]	5.11±0.11[a]
Albumin (mg/dl)	3.11±0.10[a]	3.11±0.07[a]
γ-Glutamyl Transpeptidase (IU/L)	2.75±0.16[a]	3.00±0.27[a]

Values are expressed as Mean±SEM. Values in the same row with different superscript letters differ significantly (P<0.05) from one another.

Though there are differences in the liver function status indices of the control and bitters-fed rats, these differences are minimal and statistical evaluation shows that there are no significant difference (P>0.05) between them.

Table 7: Kidney Function Indices of rats administered with Paxherbal bitters

Analytes	Control	Paxherbal
Na^+ (mmol/L)	137.13±2.43[a]	136.50±3.14[a]
K^+ (mmol/L)	14.05±1.08[a]	12.81±0.44[a]
Cl^-(mmol/L)	108.63±3.39[a]	115.13±2.22[a]
HCO^-_3 (mmol/L)	5.13±0.97[a]	4.13±0.23[a]
Urea (mg/dL)	35.38±2.43[a]	39.25±1.60[a]
Creatinine (mg/dL)	1.15±0.12[a]	1.03±0.07[a]

Values are expressed as Mean±SEM. The values in the same row with different superscript letters differ significantly (P<0.05) from one another.

Though there are differences in the kidney function status indices of the control and bitters-fed rats, these differences are minimal and statistical evaluation shows that there are no significant difference (P>0.05) between them.

Table 8: Cardiac Function Enzymes of the Control and Test Groups

Analytes	Control	Paxherbal
Creatine Kinase (U/L)	40.89±5.98[a]	39.84±1.10[a]
Lactate Dehydrogenase (U/L)	125.50±0.82[a]	125.75±3.30[a]

Values are expressed as Mean±SEM. The values in the same column with different superscript letters differ significantly (P<0.05) from one another.

Though there are variations in the levels of the enzymes used to assess the cardiac function status of the control and bitters-fed rats, these differences are minimal and statistical evaluation shows that there are no significant difference (P>0.05) between them.

Table 9: The Effect on Lipid Peroxidation (MDA) and Antioxidant Status of rats administered with Paxherbal bitters

Groups	Malondialdehyde (MDA) (U/mg protein x10^{-4})	Vitamin C (g/100ml)	Vitamin E (mMoles)	Superoxide Dismutase (SOD) (U/mg protein x10^{-2})	Catalase (CAT) (U/mg protein)	Glutathione Peroxidase(GPx) (U/ml)
Control	2.98±0.29[a]	0.87±0.07[a]	0.79±0.05[a]	3.90±0.52[a]	0.35±0.08[a]	0.53±0.08[a]
Paxherbal	1.85±0.16[b]	1.78±0.09[b]	4.06±0.32[b]	5.19±0.48[a]	3.56±0.77[b]	3.03±0.57[b]

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ significantly (P<0.05) from one another.

The parameters used to measure the level of lipid peroxidation and antioxidant status show general statistically significant (P<0.05) increase in their level or activity in the Paxherbal bitters-fed rats when compared with the control, with the exception of SOD whose increase was not statistically significant (P>0.05).

Histological Studies of Rats Fed with Paxherbal Bitters

The Heart:

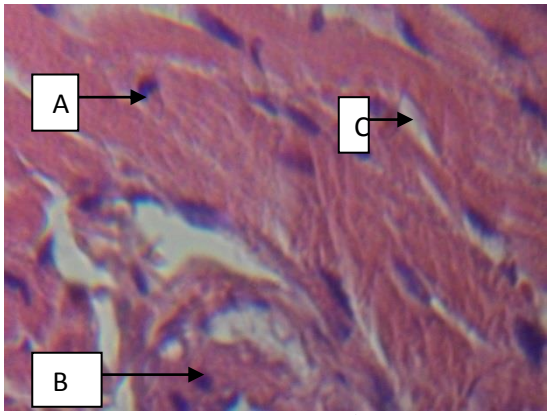

Plate 1a: Control: Rat Heart composed of myocardium A, pierced by coronary vessel B and separated by interstitium C (H&E x 40)

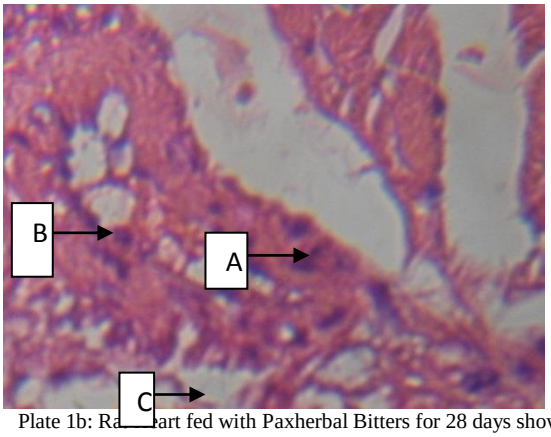

Plate 1b: Rat Heart fed with Paxherbal Bitters for 28 days showing mild vascular hypertrophy A, moderate intimal interruptions B and tissue separation C (H&E x 40)

The effect of Paxherbal bitters on the heart comprised mild tissue separation and mild interruption of the coronary vessel intima.

The Kidneys

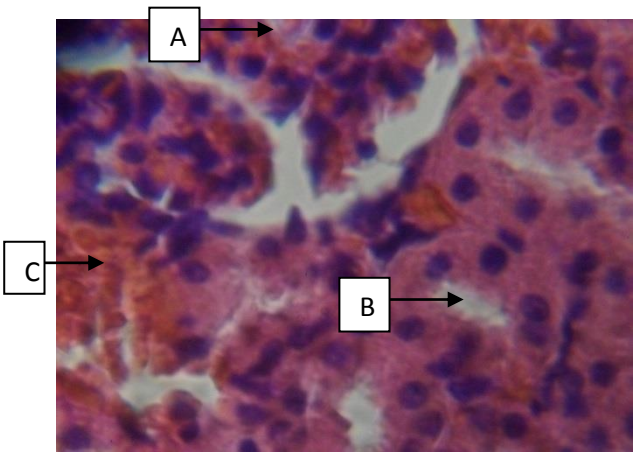

Plate 2a: Control: Rat Kidney composed of cortical glomerulus A and tubules B separated by interstitium C (H&E x 40)

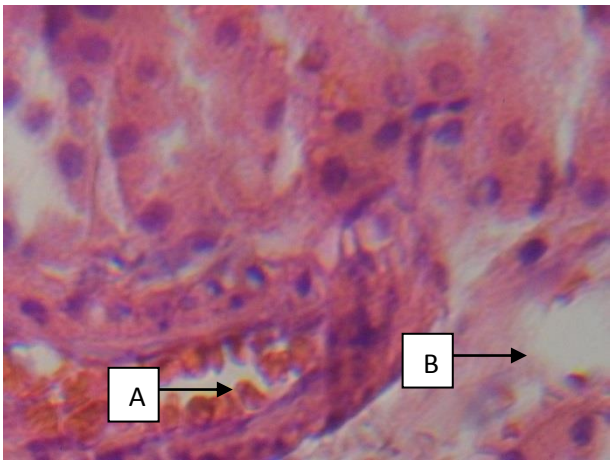

Plate 2b: Rat Kidney fed with Paxherbal Bitters for 28 days showing mild interstitial vascular congestion, hypertrophy, intimal interruption A and mild tissue separation B (H&E x 40)

Paxherbal bitters produced mild interstitial vascular congestion, tissue separation and haemorrhage

The Liver

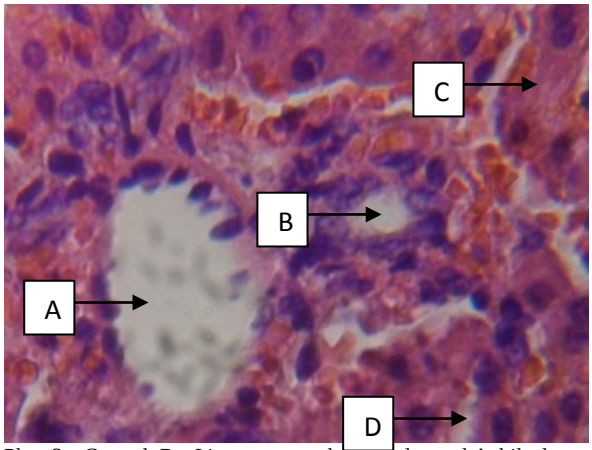

Plate 3a: Control: Rat Liver composed of portal vessel A, bile duct and hepatocytes C separated by sinusoids D (H&E x 40)

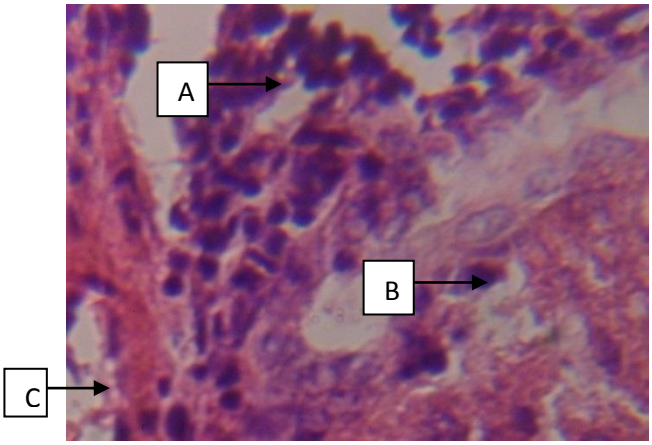

Plate 3b: Rat Liver fed with Paxherbal Bitters for 28 days showing mild periportal infiltrates of chronic inflammatory cells A, mild kupffer cell activation B and mild vascular hypertrophy and intimal interruption C (H&E x 40)

Paxherbal bitters produced mild periportal lymphocyte infiltration, activated kupffer cells and mild portal vessel intimal interruption.

The Pancreas

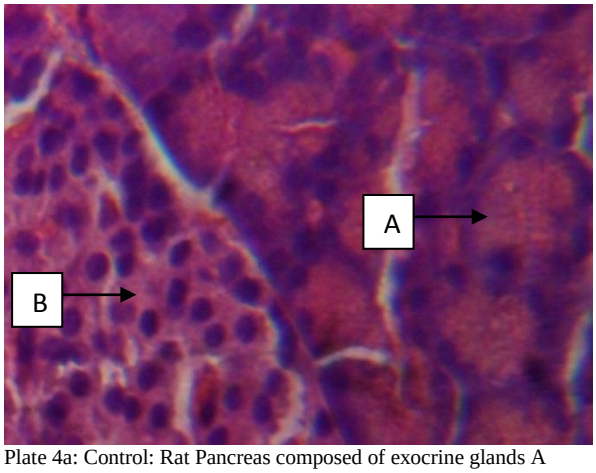

Plate 4a: Control: Rat Pancreas composed of exocrine glands A islet and endocrine islet of Langerhans B (H&E x 40)

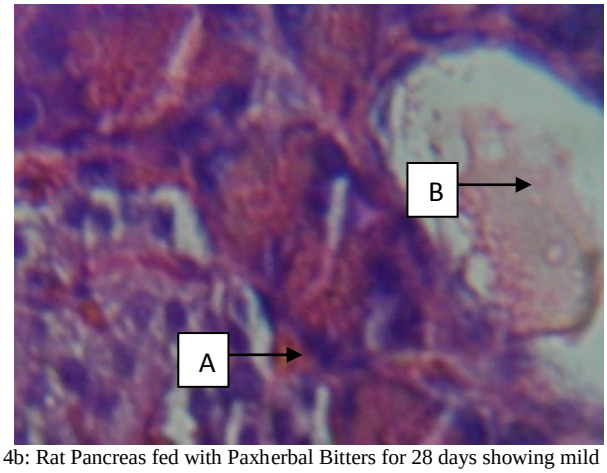

Plate 4b: Rat Pancreas fed with Paxherbal Bitters for 28 days showing mild vascular congestion A and moderate ductal luminal protein cast B (H&E x 40)

Paxherbal bitters produced mild islet vascular congestion, mild interlobar infiltrates of chronic inflammatory cells and moderate ductal luminal protein casts as well as mild vascular intimal interruption, focal infiltrates of islet chronic inflammatory cells, severe activation of lymphoid aggregates, mild vascular dilation and intimal interruption.

The Small Intestine

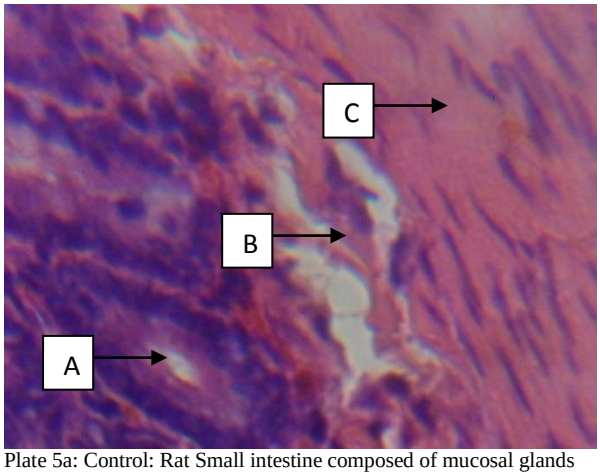

Plate 5a: Control: Rat Small intestine composed of mucosal glands A, sub-mucosa B and muscularis propria C (H&E x 40)

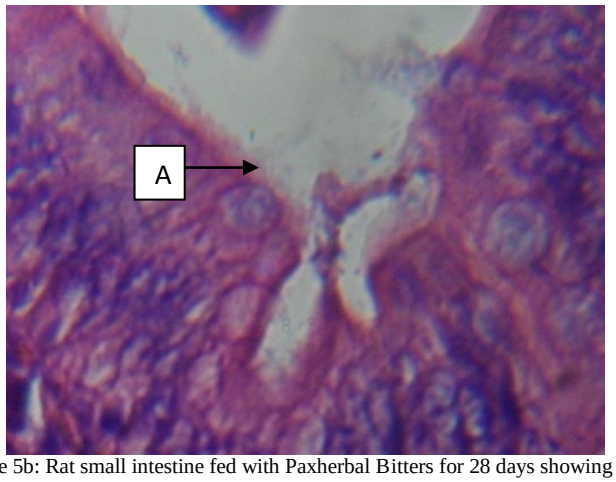

Plate 5b: Rat small intestine fed with Paxherbal Bitters for 28 days showing fairly regular mucosal lining A (H&E x 40)

Paxherbal bitters showed unremarkable but well-structured mucosal lining and glands.

The Colon

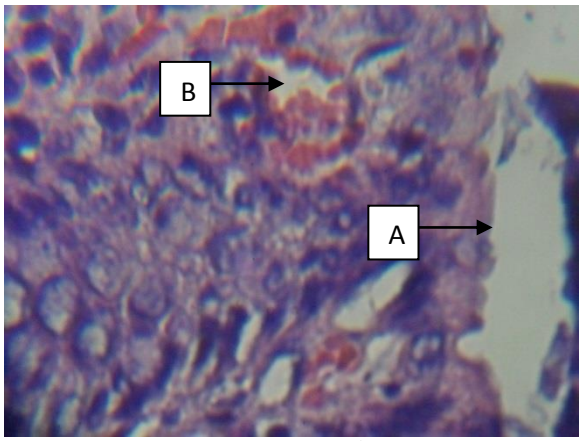

Plate 6a: Control: Rat Colon composed of mucosal lining and glands A as well as lamina propria B (H&E x 40)

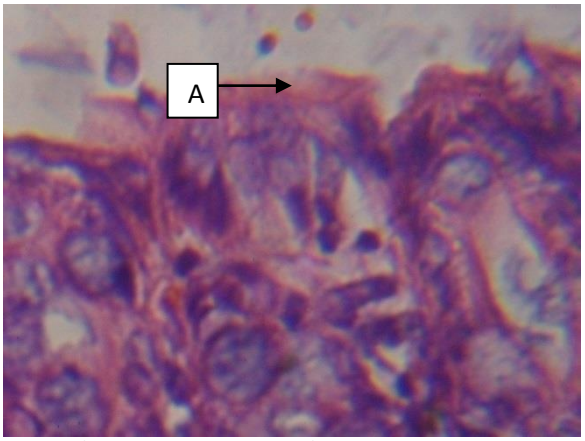

Plate 6b: Rat Colon fed with Paxherbal Bitters for 28 days showing fairly regular mucosal lining A (H&E x 40)

Paxherbal bitters produced unremarkable mucosal lining and glands

Discussion

It is often summarized that bitters stimulate digestive secretions and the metabolism as a whole and in so doing increase appetite, relieve constipation, and generally ease the heavy glumness of sluggish digestion. But, this is really too simple

and cursory a summation, and a deeper look into the actions of bitters is not only theoretically insightful but practically invaluable, especially as some plant products have been known to be toxic to the human system (1, 36). Their composition as they are presently constituted have never been ascertained neither has their numerous pharmacological claims being subjected to proper scientific scruitiny yet the use of bitters is getting popular, hence this study.

The LD_{50} of the bitters of this study is high (24,000mg/kg body weight), meaning that one may have to consume them more than 10 times their normal therapeutic dose before one gets a lethal/toxic effect. The fear that Paxherbal bitters may be toxic may not be unconnected with the alcohol level (44.34±1.13%), leading to a possible contribution of alcohol toxicity to herbal bitters toxicity. Research and endeavours are geared towards discovery of new therapeutic agents or newer and richer sources of known drugs of natural origin, and the basic goal of such drug discovery efforts always hinges on developing new products with enhanced therapeutic benefits, that is, higher efficacy and low toxicity profile (37). The result of the LD_{50} of Paxherbal bitters indicates it has a low toxicity profile and this is in agreement with the findings of Mendie (38) who investigated a similar bitters- Yoyo bitters, where he came to a conclusion that it may have a very low lethality in humans at the recommended dose as he found its LD_{50} to be very high.

The results reflected in tables 1 show that the feed consumed per day is higher in rats fed Paxherbal bitters, when compared with that of the control; statistical evaluation however shows that there was no significant difference ($P>0.05$) in the mean of the feed consumed. These changes however give the impression that the bitters were generally well tolerated leading to an increase in appetite with more food being consumed and giving credence to the claim that some bitters increase appetite (1). The findings from this study is also in agreement with the earlier reports of Aniagu and colleagues (8) and as with Nature Cure bitters, our results are suggestive that the bitters did not possibly cause any drastic alterations in the carbohydrate, protein or fat metabolism in such a way as to prevent a weight gain expected of animals that are continually supplied with food and water *ad libitum,* (8). The weight gain/change seen in Paxherbal bitters-fed rats are not significantly different ($P>0.05$) from the weight gain/change seen in the control rats, so it can be said to be a healthy weight gain that is expected of animals that are continually supplied with food and water *ad libitum.*

If a herb or herbal tonic is toxic, this can be reflected in a reduction in some or all of the haematological parameters measured in a full/complete blood count because of direct toxicty to or lysis of the cells in the blood. If however it is non toxic or actually nourishing and immunity boosting, this will reflect in the maintainance or increase in levels of some of the haematological parameters and cells especially those implicated as imparting immunity, though this increase will not be as high as the increase seen in a pathological state (8, 39, 40). The cells implicated as contributing especially to natural immunity are maintained at normal levels or raised to normal levels or a little above normal levels by herbs. Herbs have been shown to be more involved in imparting natural immunity than acquired immunity, though it can enhance acquired immunity when necessary (8, 39, 40). The results from this study as shown in tables 8 and 9 indicate that the herbal bitters did not exhibit any form of haematological toxicity, as statistical evaluation did not show any significant difference ($P>0.05$) between the values of the haematological parameters studied in the rats fed herbal bitters compared to the control. However the leucocytosis (though not significant at $P\leq0.05$) and lymphocytosis (though not significant at $P\leq0.05$) seen in rats fed Paxherbal bitters, and the significant ($P<0.05$) increase in the CD_4 lymphocyte count in rats fed the Paxherbal bitters (Table 2) could be an attestation to the claim that bitters improve body immunity as it may be arising from the fact that the bitters may contain biologically active principles that have the ability to boost the immune system through increasing the population of defensive white blood cells (8). The CD_4 T-lymphocyte came to limelight or common knowledge of lay-folk in relation to immunity, with the advent of the HIV/AIDS epidemic. The CD_4^+ helper T lymphocytes secrete cytokines which stimulate B lymphocytes to develop into antibody-producing plasma cells. They also help to activate CD_8^+ cytotoxic T lymphocytes, participate in delayed hypersensitivity reactions (with macrophages), and modulate cellular immune responses. CD_4^+ helper T cells respond to (recognize) antigen bound to MCH (major histocompatibility complex) Class II protein. Cells bearing MHC Class II molecules are B lymphocytes and macrophages. CD_4^+ helper cells are drastically reduced in number in HIV infection and their return to higher values in HIV infection is indicative of improved immunity (41). Their increase therefore in rats fed with bitters may as well indicate improved immunity. Platelet count in rats fed Paxherbal bitters was elevated (Table 2) and the lack of significance of this increase compared to that of control makes it impossible to say if this increase gives credence to the claim that bitters aid blood clotting and wound healing; if it does this, it may be as a result of its phytochemical constituents and not because it causes increased platelets.

The manufacturers of the bitters used in this study made claims ranging from assertions that bitters help to increase the activity of the pancreas by bringing sugar level under control, assist in the elimination of sugar, to the assertion that it helps in the prevention of diabetes. The results from this study as shown in table 4 seem to provide some form of evidence to this claim as the fasting blood glucose in rats fed the bitters were significantly ($P<0.05$) reduced compared to that of the control. According to Saidu and colleagues (42), the medicinal properties of plants used by traditional medical practitioners including their hypoglycaemic properties may be due to one or more of the many arrays of phytochemical constituents of these plants. These phytochemicals include complex carbohydrates, alkaloids, flavonoids, tannins, glycopeptides, peptides and amines, terpenoids, cyanogens, steroids, lipids, coumarins, sulphur compounds and inorganic ions, just to mention but a few, most of which are contained in the bitters used for this study.

The results of the study of the lipid profile of the rats fed with the bitters compared with that of the control (Table 5) reveal that generally, the bitters relatively have hypo-cholesterolaemic and hypo-triacylglycerolaemic effects, while decreasing the LDL-cholesterol, VLDL-cholesterol levels and increasing the HDL-cholesterol levels. This result seems to give credence to the claim by the bitters manufacturers that they have hypo-lipidaemic effect. Bitters act on both the

pancreas and liver/gall bladder, helping to promote the production and release of the pancreatic enzyme lipase and bile, which ensure good digestion of fats and oils and proper functioning of the excretory functions of the liver thereby confering on it hypolipidaemic properties. It acts as a liver tonic, being hepatoprotective and enhancing its functions. A healthy flow of bile helps rid the liver of waste products, prevents the formation of gallstones, and emulsifies lipids, which the pancreatic enzymes then breakdown along with proteins and carbohydrates for absorption in the small intestine. The results from this study on the serum lipid profile give positive evidence that the herbal bitters have the potential of being a lipid-lowering supplement/drug in mixed hyperlipidaemic states. There is evidence that a salient relationship exists between high serum cholesterol levels and the incidence of atherosclerosis and cardiovascular diseases (8), the observed hypocholesterolaemic effect of these herbal bitters is therefore a desired positive effect.

Liver cell damage is characterised by a rise in some plasma enzymes (AST, ALT, LDH etc). From the results of this study the AST activity was consistently higher than that of ALT, which is to be expected, since body cells contain more AST than ALT, this is in agreement with the findings of Aniagu and colleagues (8). But since AST is more intracellular than ALT which is localised primarily in the cytosol of hepatocytes, ALT is a more sensitive marker of hepatocellular damage than AST. Thus the minimal and non- significant differences ($P>0.05$) in the AST and ALT levels in the bitters-fed rats compared to that of the control from this study is indicative that the bitters did not cause any hepatocellular damage to the liver of the rats (43).

The minimal and non-significant differences ($P>0.05$) in the ALP, total bilirubin and conjugated bilirubin levels in the bitters-fed rats compared to that of the control from this study is indicative that the bitters did not cause any form of cholestasis, excessive haemolysis, nor did it impair the capacity of the liver to excrete bilirubin. Cholestatic liver disease is characterised by an elevation in the plasma level of alkaline phosphatase (ALP), while hyperbilirubinaemia is seen in conditions causing excessive haemolysis and hepatic liver diseases that impair the excretion of bilirubin (43).

The minimal and non-significant differences ($P>0.05$) in the serum albumin and total protein levels in the bitters-fed rats compared to that of the control from this study is also indicative that the bitters did not cause any dysfunction in the synthetic function of the liver (43).

Increased synthesis of gamma-glutamyl transpeptidase in the liver resulting from microsomal enzyme induction by drugs such as alcohol (in chronic drinkers) produces increased plasma levels (43). The minimal increase seen in the level of gamma-glutamyl transpeptidase in plasma of the bitters-fed rats from this study (Table 6) may as well be as a result of their "high" alcoholic content; this increase however did not result in a level of gamma-glutamyl transpeptidase that is significantly different from that of the control, and so this increase is not associated with any hepatocellular damage (43).

The reduced levels of sodium and creatinine (though not significant) as seen from this study, probably indicate that the bitters did not interfere with the renal capacity to excrete these metabolites. The lack of significant difference between the ions of the control and bitters-fed groups used in assessing the kidney function status may also be a reflection of the preserved renal integrity of the treated rats (8). Hence the bitters can be said not to have a reno-toxic effect on the kidneys of the rats as they preserved their renal integrity and did not affect their capacity to excrete these ions.

Cardiac cell/muscle damage is characterised by a combination of a rise in some plasma enzymes (creatine kinase, LDH etc). Results obtained from this study showed no significant increase ($P>0.05$) in the activities of either creatine kinase nor LDH; infact the creatine kinase level was consistently lower in the bitters-fed rats compared to the control suggesting some form of cardio-protectivity. This is not just a reflection of the preserved cardiac integrity of the treated rats but indicative that the bitters did not cause any cardio-cellular damage to the heart of the rats (43). Hence, the bitters can be said not to have a cardio-toxic effect on the heart as they preserved cardiac integrity.

Oxidative stress represents an imbalance in production and clearance of reactive oxygen species/free radicals in biological systems (44). Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage certain components of the cell, including protein, lipid and DNA; hence in humans, oxidative stress has been identified as one of the causal factors in many diseases (45). Reactive oxygen species may be beneficial as they are used by the immune system as a way to attract and kill pathogens (45). Excessive oxidative stress particularly at unwanted places (e.g vascular lining, blood brain barrier) can therefore lead to damages that can result in a wide range of diseases.

Result obtained from this study indicate that the MDA levels in the bitters-fed rats were significantly ($P<0.05$) reduced when compared to the control. Malondialdehyde (MDA) is a product of lipid peroxidation that can be easily measured and the results from this study show the herbal bitters prevented lipid peroxidation of the membranes of tissues and cells in the rats; this is an antioxidant effect, indicating that the bitters have antioxidant capacity. This aligns with the known fact that antioxidant constituents can delay or inhibit the oxidation of lipids and other compounds by inhibiting the propagation of oxidation chain reaction (32, 46).

Vitamin C and Vitamin E levels of the bitters-fed rats were significantly elevated ($P<0.05$) when compared to the control. This can be said to be as a result of the bitters adding to and preserving the immediate use of these vitamins in the rats as its inherent antioxidant capacity act as firstline antioxidants as well as protects the rats from excessive use of its indigenious antioxidants (32, 47). Herbaceous plants and species like those used as constituents of Paxherbal bitters are harmless sources for obtaining natural antioxidants. Primarily, their antioxidant effect is due to phenolic compounds such as phenolic acid, flavonoids and phenolic diterpenes and their mode of action as antioxidant compounds is due to their redox reaction properties which can absorb and neutralize free radicals by quenching singlet and triplet oxygen

(32, 47). Vitamins C (ascorbates) and E are known powerful antioxidants that have also the antioxidant ability to prevent the formation of carcinogenic substances.

Superoxide dismutase, catalase and glutathione peroxidase activities of the rats fed Paxherbal bitters were increased compared to the activities in the control rats. This further confirms the antioxidant improving capacity of the herbal bitters and further gives credence to the claim that bitters can be anti-carcinogenic as their antioxidant constituents and antioxidant capacities have been associated with anticancer properties. The potential of the phytochemicals of the plant materials found in this bitters have large scale pharmacological and biological implications, for example, its antioxidant constituents (hydrolysable tannins, phenolic acid and flavonoids etc.) have been proven to be effective in the care of the heart and protection from coronary heart diseases and cancer, as well as for their anti-carcinogenic and anti-mutagenic effects (32, 48) while its antioxidant capacity from its varieties of herbaceous vegetables are protective against various diseases, particularly cardiovascular diseases.

The Paxherbal bitters produced mild histological changes in the selected tissues studied which appear consistent with little variation when compared to normal tissues; these changes comprise vascular events, epithelial, immune and ductal luminal changes in the pancreas. The epithelial changes involved mild deepening in basophilia (hyperchromicity) of the nuclei of cells lining the mucosal epithelium and the colon.

The immune changes involved the intense activation of mucosal associated lymphoid tissues (M.A.L.T) in the small intestine. The activation of the local immune system of lymphoid aggregates in the pancreas is very intense too and mild and focal infiltrates of lymphocytes were observed in the portal zone of the liver in rats fed this bitters. There was in addition activation of the sinusoidal kupffer cells, being mild with Paxherbal bitters.

However, some abnormal changes were observed mainly in the blood vessels of all the tissues, where there were mild intimal interruptions; this was not significant since it was also noted in the intima of the control. In the pancreas there were varied levels of dilation of the lumen of the interlobar ducts, with the presence of luminal protein casts. Also, there was mild chronic inflammation, with Paxherbal bitters-fed rats having islet cell chronic inflammation; all these abnormal morphological changes are associated with chronic pancreatic inflammation in alcohol ingestion. Though alcohol may also produce vascular intimal interruption, the fact that these intimal interruptions were found in the control may just mean that they are artefactual and so not a significant morphological change occasioned by the bitters.

The conclusive histological findings from this study however indicate that at the prescribed dosage of the Paxherbal bitters, it did not produce toxic injuries to the treated rats. The abnormal effects of intimal injury, pancreatic inflammation and protein casts in ductal lumen may be caused by alcohol, which is used as a base in this tonic; these effects were however minimal and at a level the immune system was able to handle. The feature of vascular congestion indicates more blood flow to the tissues and hence better oxygenation of the tissues and excretion of waste products, while the activation of the local immune system of lymphoid aggregates and increase in the population of lymphocytes, kupffer cells and chronic inflammatory cells and hence a general increase in tissue defensive white blood cells are features of beneficial effects and more in keeping with the claim that bitters cause a boosting of the immune system. The claim of easy epithelial reconstitution and hence protection against ulcer about bitters may well be explained by the additional mild hyperplastic, hyperchromatic and pleomorphic cells in the well-structured mucosal linings and submucosal glands of the intestine and colon of the bitters-fed rats. Moreover there are no significant difference ($P>0.05$) between all the relative organ weights of the organs in the bitters-fed rats and that of the control, so conclusively it can be said that Paxherbal bitters have no toxic effects on the heart, kidney, liver, pancreas, small intestine and colon of the experimental animals.

Conclusion

The results of this study showed that Paxherbal bitters was safe for consumption, as the acute toxicity (LD_{50}) of the bitters is relatively high LD_{50} with low-lethality at doses it is likely to be consumed. The biochemical and haematological indices obtained from this study with inferences derived from some of the already established reports gave some evidence that the Paxherbal bitters may be said to have the following pharmacological properties- hypolipidaemic/hypocholesterolaemic, hypoglycaemic, stimulant and immunity-boosting/immuno-modulatory, choleretic/hepatoprotective and antihepatotoxic, invivo and invitro antioxidant capacity and by extension anticarcinogenic/antineoplastic/ antimutagenic/antitumour, as well as antihypertensive properties and the ability to protect against/prevent coronary artery disease and cardiovascular diseases. The conclusive biochemical and histopathological findings from this study indicated that at the prescribed dosage of the bitters, it did not cause toxic injuries to the cells, tissues, organs and systems of the treated rats.

References

1. Hoffmann, D (ed.): Healthy Digestion: A Natural Approach to Relieving Indigestion, Gas, Heartburn, Constipation, Colitis & More, (1st edn). Storey Publishing, LLC, Massachusetts. pp. 1- 123. 2000.
2. Vaughan, JG and Geissler, C (eds.): The New Oxford Book of Food Plants: A Guide to the Fruit, Vegetables, Herbs and Spices of the World, (1st edn.), Oxford University Press, London. pp. 1-239. 1997.
3. Hoffmann, D (ed.): Medical Herbalism: The Science and Practice of Herbal Medicine, (1st edn). Healing Arts Press, Vermont. pp. 1-672. 2003.
4. Pax Herbal Centre: Paxherbal Bitters. Pax Herbal Centre and Research Laboratories Brochure. Ewu – Esan, Edo, Nigeria. http://www.paxherbals.net. Accessed June 22, 2014.

5. Canadian Council of Animal Care, 1984 edn.: Guide to the Handling and use of Experimental Animals. Ottawa. Canada; (Vol. 2) http://www.ccac.ca/en/CCAC - Programs/Guidelines-policies/Guides/English/V2-84/Chx 1 X 1. html . Accessed 15th Jan 2013.
6. Miller, LC and Tainter, ML: Estimation of LD_{50} and its error by means of log-probit graph paper. *Proceedings of the Society of Experimental Biology and Medicine.* **57:** 261-264. 1944.
7. Randhawa, MA: Calculation of LD_{50} values from the method of Miller and Tainter, (1944). *Journal of Ayub Medical College Abbottabad,* **21(3)**: 184-185. 2009
8. Aniagu, SO, Nwinyi, FC, Akumka, DD, Ajoku, GA, Dzarma, S, Izebe, KS, Ditse, M, Nwaneri, PEC, Wambebe, C and Gamaniel, K: Toxicity studies in rats fed nature cure bitters. *African Journal of Biotechnology*, **4(1)**: 72-78. 2005
9. Fielding, D and Metheron, G (eds): Rabbits. The Tropical Agriculturalist (1st edn). CTA. Macmillan Education Ltd. Macmillan Publishers London, UK. pp. 16–17. 1991.
10. Vijayalakshmi, T, Muthulakshmi, V and Sachdanandam, P: Toxic studies on biochemical parameters carried out in rats with Serankottai nei, a siddha drug–milk extract of *Semecarpus anacardium* nut. *Journal of Ethnopharmacology* **69** (1): 9–15. 2000.
11. Sysmex: Sysmex KX-21N Operators Manual. Sysmex America, Incorporated. USA. pp. 1-20. 2003.
12. Cyflow: Service manual for service technicians. Sysmex Partec GmbH, Görlitz,Germany. pp:1-20. 2004.
13. Cassens, U, Göhde, W, Kuling, G, Gröning, A, Schlenke, P, Lehman, LG, Traoré, Y, Servais, J, Henin, Y, Reichelt, D and Greve, B: Simplified volumetric flow cytometry allows feasible and accurate determination of CD_4^+ T-lymphocytes in immunodeficient patients worldwide. *Antiviral Therapy* **9 (3)**: 395-405. 2004.
14. Barham, D and Trinder, P: An improved colour reagent for the determination of blood glucose by the oxidase system. *Analyst*, **97:** 142-145. 1972.
15. Trinder, P: Assay principles for serum lipids. *Annals of Clinical Biochemistry.* **6**:24-27. 1969.
16. Friedewald, WT, Levy, RI and Fredrickson, DS: Estimation of the concentration of low- density lipoprotein cholesterol in plasma, without the use of preparative centrifuge. *Clinical. Chemistry.* **18**, 499-502. 1972.
17. Jendrassik, L and Grof, P: Colorimetric method for the determination of bilirubin. *Biochemistry Zeitschrift*. **297**:81-89. 1938.
18. Reitman, S and Frankel, S: A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *American Journal of Clinical Pathology* **28 (1)**:56-63. 1957.
19. Szasz, G: A kinetic photometric method for serum gamma-glutamyl transpeptidase. *Clinical Chemistry*. **22:** 124-136. 1969.
20. Doumas, BT, Watson, WA and Biggs, HG: Albumin standards and determination of serum albumin with bromocresol green. *Clinica Chimica Acta.* **31:**87-96.1971.
21. DGKC: Standard method for the determination of alkaline phosphatase activity (Recommendation of the German Society for Clinical Chemistry). *Journal of Clinical Chemistry and Clinical Biochemistry.* **10**:182-183. 1972.
22. Tietz, NW, Pruden, LE and Andersen, S Electrolytes In: Tietz, N. W. (ed.), Fundamentals of Clinical Chemistry, (2nd edn.), W.B. Saunders Co. Philadelphia USA: pp. 721-738. 1996.
23. Schales, O and Schales, SS: A simple and accurate method for the determination of chloride in biological fluids. *The Journal of Biological Chemistry*, **140**: 879-884. 1941
24. Davidson, I and Henry, JB (eds): Clinical Diagnosis by Laboratory Method, (1st edn). ELBS New York. pp. 340-500. 1979
25. Weatherburn, MW: Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry.* **39 (8):** 971-974. 1967.
26. Bartels, H and Bohmer, M: Serum creatinine determination without protein precipitation. *Clinica Chimica Acta*. **37:**193-197. 1972
27. DGKC: Standard method for the determination of creatine kinase activity (Recommendation of the German Society for Clinical Chemistry). *Journal of Clinical Chemistry and Clinical Biochemistry.* **15**: 255-260. 1977.
28. DGKC: Determination of plasma lactate dehydrogenase (Recommendation of the German Society for Clinical Chemistry). *Journal of Clinical Chemistry and Clinical Biochemistry.* **8 (6)**: 658-660. 1970.
29. Varshney, R and Kale, RK: Effect of Calmodulin antagonist on radiation induced lipid peroxidation in microsomes. *International Journal Radiation Biology.* **58**:733-743. 1990.
30. Rutkowski, M, Grzegorczyk, K and Paradowski, MT: Kolorymetryczna metoda oznaczania całkowitej witaminy E w osoczu krwi – modyfikacja własna metody Tsena [Colorimetric method of blood plasma total vitamin E determination – the own modification of Tsena method]. *Diagnostic Laboratory*, **41:**375-376. 2005. [in Polish]
31. Bajaj, KL and Kaur, G: Spectrophotometric determination of L. ascorbic acid in vegetable and fruits. *Analyst*, **106:** 117-118. 1981.
32. Hussain, I, Ullah, R, Khurram, M, Ullah, N, Baseer, A, Khan, FA, Khattak, MR, Zahoor, M, Khan, J and Khan, N: Phytochemical analysis of selected medicinal plants. *African Journal of Biotechnology*, **10(38):** 7487-7492. 2011.

33. Cohen, G, Dembier, D and Marcus, J: Measurement of catalase activity in tissue extract. *Analytical Biochemistry*. **34**:30-38. 1970.
34. Misra, HP and Fridovich, I: The role of superoxide dismutase anion in the autooxidation of epinephrine and simple assay for superoxide dismutase. *Journal of Biology and Chemistry*, **247**:3170-3175. 1972.
35. Sato M, Ramarathnam, N, Suzuki, Y, Ohkubo, T, Takeuchi, M and Ochi, H: Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources. *Journal of Agricultural and Food Chemistry* **44**: 34-41. 1978.
36. Mcdonald, J: Blessed bitters. pp. 141-154. http:// www.herbcraft.org/bitters.pdf. Accessed Feb. 20th 2014.
37. Gamaniel, KS: Toxicity from medicinal plants and their products. *Nigeria Journal of Natural Product Medicine*. **4**: 04-09. 2000.
38. Mendie, UE (ed.): Yoyo Bitters Clinical Report. Abllat Nigeria Company Limited. Lagos, Nigeria. pp: 1-5. 2009.
39. Glatzel, vonH and Hackenberg, K: Rontgenologische untersuchungen der wirkungen von bittermitteln auf die verdauunogsorgane. *Planta Medica*; **15**: 223-232. 1967.
40. Garg, SK, Shah, MAA, Garg, KM, Farooqui, MM and Sabir, M: Antilymphocytic and immunosuppressive effects of *Lantana camara* leaves in rats. *Indian Journal of Experimental Biology.* **35**; 1315-1318. 1997.
41. Cheesbrough, M (ed.): *District Laboratory Practice in Tropical Countries Part 2* (2nd edn update), Cambridge University Press, Capetown, South Africa. pp. 1-320. 2010.
42. Saidu, Y, Bilbis, LS, Lawal, M, Isezuo, SA, Hassan, SW and Abbas, AY: Acute and sub-chronic toxicity studies of crude aqueous extract of *Albizzia chevalieri* harms (Leguminosae). *Asian Journal of Biochemistry* **2(4)**:224-236. 2007.
43. Nsirim, N (ed.): Clinical Biochemistry for Students of Pathology, (1st edn.). Longman Nigeria Plc., Lagos Nigeria. pp. 42-126. 1999.
44. Lennon, SV, Martin, SJ and Cotter, TG: Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Proliferation*, **24 (2):** 203–14. 1991.
45. Amer, J, Ghoti, H, Rachmilewitz, E, Koren, A, Levin, C and Fibach, E: Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. *British Journal of Haematology*, **132 (1):** 108–113. 2006.
46. Kim, SY, Kim JH, Kim, SK, Oh MJ and Jung MY: Antioxidant activities of selected oriental herb extracts. *Journal of the American Oil Chemists' Society.* **71:** 633-640. 1994.
47. Krauss, RM and Howard B: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation*, **102**: 2284-2299. 2000.
48. Loliger J: *The Use of Antioxidants in Food.* In: O. I. Aruoma, & B.Halliwell (eds.), Free Radicals and Food Additives. pp. 129-150. 1991.