Pharmacological Evaluation of Paxherbal Bitters as a Supplement for the Prevention of High-Salt and High-Fat Diet-Induced Cardiovascular Diseases

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ABSTRACT

The suggestion of WHO for an "alternative remedy" to the scourge of cardiovascular diseases which has become a seemingly unending scourge to humanity was the motivation for this study. It was therefore the aim of this study to evaluate the therapeutic potential of a locally available Paxherbal bitters as a supplement for the prevention of high-salt and high-fat diet-induced cardiovascular diseases in male *Wistar* rats. Thirty of these rats were randomly divided into six groups of five rats each, with the average weight of each group of rats being 200g. The rats were fed ad-libitum with their assigned feed and clean-tap water during the entire 6-week course of this study. Group 1 was fed the basal dietv while the other groups were fed the combined high-salt and high-fat diet. The rats in control groups 1 and 2 were given clean tap-water as a placebo, those of group 3 were given the combined drug of atorvastatin and lisinopril, while those of groups 4 to 6 were respectively administered Paxherbal bitters doses of 600mg/kg, 1,100mg/kg and 2,200mg/kg body weight of the rats'. Specialized and standardized ELISA and colorimetric assay kits were used for the biochemical indices to be determined and the manufacturer's instructions were strictly followed. The biochemical indices determined include, high sensitive C - reactive protein (HsCRP), monocyte chemotactic protein-1 (MCP-1), malondialdehyde (MDA), total antioxidant capacity (TAC), angiotensin converting enzyme (ACE), cardiac troponin-T (cTnT), lipid profile, liver and kidney function statuses, as well as the atherogenic index of plasma (AIP). The results were presented as mean ± SEM. The data were analyzed using the GraphPad Prism 8.0.2, one-way analysis of variance (ANOVA), followed by the Tukey's multiple comparisons post hoc test. A p value of less than 0.05 ($p<0.05$) was accepted as statistically significant. The result of this study reveal that Paxherbal bitters was able to prevent elevation in plasma levels of HsCRP and MCP-1. It was also able to improve the plasma total TAC and inhibit the formation MDA. It maintained the plasma ACE, cTnT and bilirubin levels and prevented the elevation of plasma total cholesterol, triglyceride, LDL-cholesterol, VLDL-cholesterol levels and elevated the HDL-cholesterol level, as well as reduce the AIP. It was also found to be as efficacious as a combination of lisinopril and atorvastatin and can therefore be said to be a good supplement for the prevention and management of cardiovascular diseases.

Key words: High-salt diet, high-fat diet, cardiovascular diseases, Paxherbal bitters, hypertension.

INTRODUCTION

The increasing deaths from cardiovascular diseases (CVD) worldwide has stimulated research in preventive measures to curb the debilitating effects of this disease [1, 2, 3]. The possible use of locally available herbal preparations (alternative medicine), precisely the newly introduced herbal bitters in the Nigerian market, in the treatment and prevention of risk factors that can lead to cardiovascular diseases (hypertension, stroke, angina, heart attack, myocardial infarction), diabetes mellitus, liver diseases, diseases of the GIT, etc. has recently come to limelight [4].

Cumulative oxidative stress has been linked to CVD and most other nonpathogenic diseases. It is therefore not surprising that herbal products with high antioxidant value that will be capable of preventing CVD's should be of interest to researchers. Research has shown that many spices and herbs especially those used to formulate herbal bitters exhibit antioxidant properties and indeed contain several secondary metabolites useful in many therapeutic applications including anti-inflammatory, hypolipidaemic, anti-hypertensive, hypoglycaemic and immuno-modulatory applications, just to mention but a few [5, 6]. The health benefits of polyherbal products like Paxherbal bitters (with 40 herbal constituents) used in this study, from scientific research findings have been attributed to the additive and synergistic effects of the complex mixture of phytochemicals in their constituent fruits and vegetables, herbs and spices [7, 8]. They have

been known to promote good health by assisting in preventing cancer and high blood pressure, stimulating the immune system, improving drug metabolism, and tissue regeneration [9].

"Herbal Bitters" the general term used to describe the bitters of this study, refers to beverages which are often alcoholic and flavoured with herbal essences that gives them a bitter or bittersweet flavour [10, 11, 12]. They are usually dark in colour and were originally valued as patent medicine for their ability to promote appetite and digestion, as well agents that act as flavouring in cocktails [13, 14]. They are produced from herb and root extracts, from the narcotic components of (primarily) tropical and subtropical plants and spices. They are made up of numerous groups of chemical compounds extracted from the herbs and roots (medicinal plants) that have the common characteristic of a bitter taste.

The Director General of the WHO in 2002, Dr Gro Harlem Brundtland, was of the opinion that "prevention is the key to lowering the global disease burden of heart attacks and strokes" [15]. WHO went further to recommend everyone at high risk of having a heart attack or stroke should be given a combination of a statin for lowering cholesterol, a low dose blood pressure lowering drug (like the ACE-inhibitor, lisinopril), and aspirin a blood thinner. They felt if this was rigorously pursued it will lead to a reduction of the incidence of cardiovascular diseases by 50%. They also advised that it be immediately implemented for people in the developed world, while "new resources" or "alternative remedy" should be found to treat people in countries where the combination is unaffordable" [16]. As envisaged, these pills are often expensive in the countries of sub-Saharan Africa and coupled with their attendant side effects, compliance to this "pill" advice by WHO has become unrealisable and unrealistic in low income countries like Nigeria.

Natural products and medicinal plants are becoming widely acceptable and used in Nigeria [17], so the use of these natural products may well be the "new resources" as suggested by WHO which countries in sub-Saharan Africa can use in combating this disease. These herbal products are cheap and easily available, and are known to contain biologically active compounds that confer on them therapeutic benefits [18]. The possibility of these herbal products being able to help prevent cardiovascular disease is therefore worth investigating. Finding a combination of these herbs (as already packaged in the present day herbal bitters) that will help combat the high prevalence of cardiovascular diseases in Nigeria is therefore not only highly desirable and expedient but should be a high priority. It is therefore important and necessary to evaluate some of these commonly consumed herbal bitters in Nigeria, to determine their efficacy in the prevention of high-salt diet induced hypertension and high-fat diet induced hypercholesterolemia/ hyperlipidaemia which are major risk factors for cardiovascular diseases.

Earlier studies carried out on the composition of herbal bitters and their biochemical effects in male albino *Wistar* rats reveal that the herbal bitters of this study and some others, contain constituents that may confer on them the potential to reduce or prevent different forms of cardiovascular diseases amongst other diseases like diabetes mellitus, peptic ulcer disease and diseases of the digestive tract. They were found to be safe for consumption and not toxic to the blood and organs of the body [19]. It is to confirm this possible "preventive property" of herbal bitters that this research seeks to investigate the therapeutic potential of Paxherbal bitters in the prevention of high-salt diet induced hypertension and high-fat diet induced hypercholesterolemia/ hyperlipidaemia and related changes they cause, which are major risk factors for cardiovascular diseases.

In Adodo [20], Paxherbal [21] and Pax Herbal Centre and Research Laboratories product brochure, the uniqueness of this product is seen in its 40 herbal constituents:

- *Cymbogon citratus* (Lemon grass)
- *Aloe vera* (True aloe, Lily of the desert)
- *Rawolfia vomitoria* (Swizzle stick)
- *Sida acuta* (Broom weed)
- *Gongronema latifolium* (Utazi)
- *Zingiber officinale* (Ginger)
- *Xylopia aethiopica* (Uda)
- *Vernonia amygdalina* (Bitter leaf)
- *Tridax procumbens* (Tridax daisy, coat buttons)
- *Capsicum annuum* (Green pepper)
- *Carica papaya* seed (Pawpaw seed)
- *Glycine max* (Soya bean leaves)
- *Garcinia kola* (Bitter kola)
- *Ageratum conyzoides* (Goat weed)
- *Cajanus cajans* (Pigeon peas)
- Cocoa leaves
- Cocoa Powder
- *Bellis perennis* (Wild daisy)
- *Morinda citrifolia* (Noni)
- *Aspilia africana* (Haemorrhage plant)
- *Capsicum Spp.* (Pepper plant)
- *Citrus aurantium* (Bitter orange)

- *Alstonia boneei* (Stool wood)
- *Ocimum sanctum* (Holy basil)
- *Pennisetum pupureum* (Napier grass)
- *Tagetes erecta* (African marigold)
- *Daucus carota* peels (Carrot peels)
- *Ananas comosus* root (Pineapple root)
- *Jatropha curcas* (Physics nut)
- *Citrus limon* leaves (Lemon leaves)
- *Uraria pita* (Prishnaparni)
- *Viscum album* (Mistletoe)
- *Aloe barteri* (African aloe)
- *Citrus aurantifolia* (Lime)
- *Cassia alata* (Candlestick senna)
- *Cochlospermum religiosum* (L.) (Cotton tree/Cotton seed leaves)
- *Persea americana* (Avocado pear)
- *Eucalyptus officinalis* (Thunder protector/fever tree)
- *Musa paradisiaca* (French plantain)
- *Morinda Lucida* (Brimstone tree)

Indications or claims of the uses of Paxherbal bitters include that it promotes blood circulation, prevents kidney stones, helps in digestion and activates bile flow. It increases immunity of the body against bacterial and fungal infections. It acts 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 (preventing hyperlipidaemia/dyslipidaemia) and proper functioning of the excretory functions of the liver. It is also claimed to help in the prevention of diabetes and acceleration of body repairs, healing of wounds and toothaches. 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 its constituent herbs and their composition, there is none on its therapeutic potential (in the form it is constituted presently) to prevent the accumulation of substances or prevention of risk factors that can lead to cardiovascular diseases. This therefore made this study pertinent.

James Green in 1991 opined that the lack of bitters in our present day diet was a major cause of the increase in the ailments of our time most especially cardiovascular diseases and cancer. He called this the “bitters deficiency syndrome” [22]. It was the hope therefore of this study that the daily use of Paxherbal bitters as a supplement to our diet will ameliorate the negative consequences of our current poor diet habits and thus help prevent cardiovascular diseases.

MATERIALS AND METHODS

Materials

Test Bitters and Drugs

Paxherbal bitters was purchased from the manufacturers at the Benedictine Monastery at Ewu-Ishan in Edo State, while atorvastatin and lisinopril drugs were purchased from a reputable pharmaceutical store opposite the University of Benin Teaching Hospital (UBTH), Ugbowo Lagos Road, Benin City, Edo State, Nigeria. The bitters was bought as a liquid formulation and the reconstituted combined drug solution stored in a refrigerator throughout the period of the experiment.

Reagents/Chemicals

The chemicals and diagnostic kits used where of the finest analytical grade from Randox Lab. UK and MyBioSource.com USA, unless otherwise stated.

Methods

Care of animals

The animals of this study were obtained from the Anatomy Department, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria. They were handled in strict compliance with 1993 international guidelines on the care and use of animals in biomedical research as prescribed by the Canadian Council on Animal Care (CCAC) (21). This research also got the approval of the Research Ethics Committee (REC) on human and animal research of the College of Medical Sciences, University of Benin, with REC approval No: CMS/REC/2019/113. Before the study period commenced, the rats were allowed to acclimatize by feeding them ad-libitum with a modified standard pelleted grower’s mash and clean tap water for two weeks.

Diet formulation

The control or basal diet was a modified standard pelleted grower’s mash which was formulated following the suggestions put forward by [22] and [23]. The combined diet (combined high-salt and high-fat diet) meant to make the diet both hypertension- and hypercholesterolaemia-inducing, was made by adding a combination of both 8% NaCl and 2% cholesterol + 0.25% bile salts + 20% margarine to a proportionate quantity of the basal diet [24, 25, 26, 27]. The final composition of both the basal and combined diets are as indicated in Tables 1 and2.

Table 1: Composition of the basal diet (g/1000g) (28).

Ingredients	Quantity in basal diet (g)
Maize	260.0Z
Wheat offal	300.0
Palm kernel cake	232.0
Groundnut cake	128.0
Fish meal (65%)	12.0
Lysine	1.6
Bone meal	10.0
Limestone	52.0
Grower premix	2.4
Salt	2.0
Total	1000.0

Table 2: Composition of the combined diet (hypertension-inducing and hypercholesterolaemia-inducing diet) (g/1000g) (28)

Ingredients	Quantity in combined diet (high-salt/high-fat diet) (g)
Basal diet in Table 1	697.5
Margarine	200.0
Pure analytical grade cholesterol	20.0
Sodium taurocholate (bile-salt)	2.5
Pure analytical grade sodium chloride	80.0
Total	1000.0

Experimental design and feeding protocol

Thirty (30) male W*istar* rats weighing between 180 - 220g, was used for this study. After 14 days of their acclimatization of the animals, they were weighed and randomly divided into six (6) groups of five (5) rats each, with the weights of those in a group being 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 200.0±1.0g. The rats were fed ad-libitum during the entire course of the six weeks (42 days) study, according to the feeding protocol designed for this study. They were 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 specially designed wooden cages with a wire meshed/iron gauze flooring. This flooring was to allow the rat-excreta pass through (to prevent coprophagy). The excreta was collected unto the tiny wire-mesh covering of another wooden tray receptacle covered with a bedding material meant to hold the urine of the rats. Using an oro-gastric gavage, the animals were given orally, clean tap-water (for the control groups 1&2), the combined drug (control group 3), 3 doses of the Paxherbal bitters (groups 4 - 6)

Protocol

For the study on the prevention of the combined effects of high-salt and high-fat diet

Control groups

- Group 1: Basal diet + clean tap-water for 6 weeks (normal control)
- Group 2: Combined diet + clean tap-water for 6 weeks (-ve control)
- Group 3: Combined diet + (Lisinopril-0.14mg/kg-bw) + (Atorvastatin-0.57 mg/kg-bw) for 6 weeks (+ve control).

Experimental groups

- Group 4: Combined diet +Paxherbal bitters (600 mg/kg-bw) for 6wks.
- Group 5: Combined diet +Paxherbal bitters (1,100 mg/kg-bw) for 6wks
- Group 6: Combined diet +Paxherbal bitters (2,200 mg/kg-bw) for 6wks

Dosage regimen for Paxherbal bitters

The manufacturer's recommendation and prior studies have established an effective dose for the herbal bitters for an adult man to be an average of 40ml daily as a single dose or in two doses [28]. In diseased conditions, physicians normally double the dosage of drugs usually given at a lower dose [29]. Considering the fact that hypertension and hypercholesterolaemia are severe disease conditions in addition to the fact that the bitters have been shown from previous studies to have a very high therapeutic index [30], it was assumed that an average of 40ml, 80ml and 160ml of the herbal bitters per day, can be consumed by an adult man, usually assigned a physiological weight of 70kg.

Appropriate calculations were done to determine the initial equivalent doses of the bitters (clean tap-water in the case of the control groups 1&2) in ml/kg mean body weight of the rats to be given to each group. As the initial mean body weights of rats in each group at the beginning of the study was about 200g, the equivalent volume [in millilitres-(ml)] of the bitters/clean tap- water that was given to the rats was calculated and this amounted for:

- ✓ 600mg/kg-bw to 0.11ml for a 200g rat; (equivalent for 40ml for a 70kg man)
- ✓ 1,100mg/kg-bw to 0.23ml for a 200g rat (equivalent for 80ml for a 70kg man) and
- ✓ 2,200mg/kg to 0.45ml for a 200g rat (equivalent for 160ml for a 70kg man)

Dosage regimen for the drugs

Following the same principles as adopted for the dosage of the herbal bitters

Dosage regimen for atorvastatin: 40mg is consumed by a 70,000g man (70kg). This amounts to 0.114mg for a 200g rat (meaning a dose of 0.114mg/200g = approx. $0.57x10^{-3}$mg/g of rat or 0.57mg/kg of rat body weight)

Dosage regimen for lisinopril: 10mg is consumed by a 70,000g man (70kg). This amounts to 0.029mg for a 200g rat. A dose of 0.029mg/200g = approx. $0.14x10^{-3}$mg/g of rat or 0.14mg/kg of rat body weight.

Dosage regimen for combined drug: The combined drug was formulated or constituted by dissolving 0.157mg of atorvastatin and 0.029mg of lisinopril in distilled water and making it up to 1ml and this was given daily to the rats fed the combined diet using an oro-gastric gavage.

Weekly body weight:The body weight of each rat was assessed using a weighing balance during the acclimatization period, before commencement of dosing (day 1), weekly during the dosing period and on the day of sacrifice (day 42) [3]. The result was used to determine the average weekly weight of the rats in each group, after which the weekly dose of the bitters to be given to the rats was determined. The weight gain/lost per day of the rats, was also determined and used in determining the food efficiency.

Relative organ weight: At the end of the 42-day experimental period, just after the rats have been weighed, anaesthetized (using chloroform anaesthesia) and blood samples collected from them, different organs namely the heart, the aorta, liver and the kidneys of the respective rats was carefully dissected out and weighed (this weight was designated as the absolute organ weight). The relative organ weight was then calculated using the formula:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$ [13].

Daily quantity of feed consumed: The crude 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. Specially designed deep feeding troughs tied securely to the sides of the cages were used to reduce the spilling of the feed. Whenever there was a spill, the weight of the spilled pelleted feed was factored into the calculation of the daily feed consumed. From the results, the average quantity consumed per day for the period of the study, was determined [6]. The results were used along with the mean weight gain/lost per day of the rats to determine their food efficiency.

Food efficiency: This was determined using the formula:

$$\text{Food efficiency} = \frac{\text{Weight gain or lost/day/rat}}{\text{Feed consumed/day/rat}} \times 100 \quad [3].$$

Clinical observations

Clinical signs that were assessed before dosing, immediately and 4hrs after dosing, include level of sedation, restlessness, changes in nature/quantity of stool and urine, and eye colour, excretion of worms, diarrhoea, haematuria, uncoordinated muscle movements [24].

Blood sample collection and preparation

The last dose of the respective diets, drugs and bitters was administered on the morning of the 42nd day which was the last day of the 6-week study. All meals were stopped by 7pm on the same day. After an overnight fast, blood samples were collected from the animals (following chloroform anaesthesia), using syringes and needles via the inferior vena cava and cardiac puncture, and put into already labelled lithium heparin bottles without undue pressure to either the arm or the plunger of the syringe. The samples will then be mixed by gentle inversion. Later they were centrifuged at 4000r/min for 10mins to obtain plasma. The plasma supernatants were separated into sterile plain bottles and used for assay of the required biochemical parameters.

Assessment of inflammation and endothelial dysfunction.

a. High sensitive C-reactive protein (HsCRP): Rat–HsCRP ELISA kit was used. This ELISA kit uses the Sandwich-ELISA principle and the procedure was as described by the manufacturers [21].
b. Determination of monocyte chemoattractant protein-1 (MCP-1): Rat MCP-1 Elisa kit was used. The MyBioSource' rat MCP-1 ELISA (Enzyme linked immunosorbent assay) kit uses the sandwich double antibody principle for the quantitative measurement of rat MCP-1 in plasma. The procedure used was as described by the manufacturers [4].

Assessment of lipid peroxidation and total antioxidant capacity

a.Determination of the plasma Malondialdehyde (MDA): Rat MDA Elisa kit was used. This ELISA kit uses the Sandwich-ELISA principle and the procedure was as described by the manufacturers [27].

b. Determination of the plasma total antioxidant capacity (TAC): Rat TAC microplate kit was used. The principle behind the microplate colometric assay kit method for measuring the "total antioxidant capacity", proceeds from its ability to give an idea of the antioxidant capacity left after the three categories of antioxidant species have finished their function and an equilibrium oxidative stress level attained [22].

Assessment of index of blood pressure change and cardiac muscle injury.

a.Determination of Angiotensin converting enzyme (ACE) activity: Rat ACE Elisa kit was used. This ELISA kit is used for the in-vitro quantitative determination of Rat ACE activity in serum, plasma and other biological fluids. It uses the sandwich-ELISA principle [10].

b. Determination of cardiac troponin T (cTnT): Rat cTnT ELISA kit was used. The cardiac troponin T (cTnT) ELISA kit applies the quantitative sandwich enzyme immunoassay technique [19].

Assessment of lipid profile

a.Determination of plasma total cholesterol: Randox Lab. kit, UK, was used. The cholesterol will be determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase [5]. The absorbance of the resulting solution is measured spectrophotometrically at a wavelength of 500nm. The intensity of the colour formed by the quinoneimine dye indicator is proportional to the concentration of cholesterol in the sample.

b.Determination of plasma HDL-cholesterol: Randox lab. kit, UK was used [6]. Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction which remains in the supernatant, is determined as already described for total cholesterol (44).

c.Determination of plasma triacylglycerol: Randox lab. kit, UK, was used [26]. The triglycerides (TG) is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (POD). The absorbance of the resulting solution will be measured spectrophotometrically at a wavelength of 500nm. The intensity of the colour formed by the quinoneimine dye indicator is proportional to the concentration of triglycerides in the sample.

d. Determination of plasma LDL-cholesterol [17]. LDL-cholesterol was calculated using the Frieldewald Equation as follows

In mg/dl: LDL-chol = total-chol – triglycerides - HDL-chol

e. Determination of plasma VLDL-cholesterol

VLDL-cholesterol was calculated using the Frieldewald's formula: In mg/dl:

$$\text{VLDL- cholesterol} = \frac{\text{triglycerides}}{5}$$

Determination of atherogenic index of plasma (AIP) [29]. The artherogenic index of plasma (AIP) was calculated using the formula: Log (TG/HDL-C)

Assessment of kidney function status

a. Determination of plasma electrolytes: sodium (Na^+), potassium (K^+), chloride (Cl^-) and bicarbonate (HCO_3^-). The plasma sodium, potassium, chloride and bicarbonate ion levels were determined using Ion Selective Electrode Device [2].

b. Determination of plasma urea: Randox lab. kit, UK, was used [9]. Urea in plasma is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction [4]. The absorbance of the resulting solution (indophenol) is measured spectrophoto-metrically at a wavelength of 546nm.

c.Determination of plasma creatinine: Randox lab. kit, UK was used [14]. Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The intensity of the complex formed is directly proportional to creatinine concentration [20]. The absorbance of the resulting solution is measured two times 2 minutes from each other spectrophotometrically at a wavelength of 492nm [11].

Assessment of liver function status

a. Determination of plasma total protein: Randox lab. kit, UK, was used [8]. Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex which will be measured spectrophotometrically at a wavelength of 546nm [14]. The intensity of the coloured complex formed is proportional to the concentration of total protein in the sample.

b. Determination of plasma albumin: Randox lab. kit, UK, was used. The measurement of serum albumin is based on its quantitative binding to the indicator 3,3,5,5- tetrabromo-m-cresol sulphonephthalein (bromocresolgreen, BCG). The albumin–BCG-complex absorbs maximally at 578nm, the level of absorbance being directly proportional to the concentration of albumin in the sample [16].

c.Determination of plasma total bilirubin: Randox lab. kit, UK, was used. The colorimetric method used was based on that described by Jendrassik and Grof, [2]. Total bilirubin was determined in the presence of caffeine, which releases albumin bound to bilirubin, by the reaction with diazotized sulphanilic acid. Both this released bilirubin and direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. The resulting solution was measured spectrophotometrically at a wavelength of 578nm [7] with the level of absorbance being indirectly proportional to the concentration of bilirubin in the sample.

d. Determination of plasma direct/conjugated and indirect/unconjugated bilirubin: The Randox lab. kit, UK, was also used [5]. The blue coloured complex formed by the reaction of the sample with diazotized sulphanilic acid is indicative of conjugated (direct) bilirubin alone, since caffeine has not been added (indirect bilirubin is only added to make up total bilirubin when caffeine breaks down the bilirubin-albumin complex to

release free bilirubin). Its absorbance is measured spectrophotometrically at 546nm.

e.Determination of plasma aspartate aminotransferase (AST), EC 2.6.1.57. Randox lab. kit, UK, was used [16]. Aspartate aminotransferase (AST) catalyses the reaction between α- oxoglutarate and L-aspartate to yield L- glutamate and oxaloacetate. AST activity is then measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-Dinitrophenylhydrazine (2, 4-DNPH). The absorbance of the resulting solution will be measured spectrophotometrically at a wavelength of 546nm [27].

f.Determination of plasma alanine aminotransferase (ALT), EC 2.6.1.2: Randox lab. kit, UK, was used [11]. Alanine aminotransferase (ALT) catalyses the reaction between α-oxoglutarate and L-alanine to yield L- glutamate and pyruate. Alanine aminotrasferase is measured by monitoring the concentration of pyrurate hydrazone formed with 2, 4-dinitrophenylhydrazine. The absorbance of the resulting solution is measured spectrophotometrically at a wavelength of 546nm [14].

g. Determination of plasma alkaline phosphatase (ALP), EC 3.1.3.1: Randox lab. kit, UK, was used [18]. This is an optimized standard method according to the recommendations of the deutsche gesellschaft fur klinische chemie (DGKC). Alkaline phosphatase (ALP) enzyme reacts with disodium para-nitrophenyl-phosphate and liberates phenol which forms a yellow coloured complex at alkaline pH. The absorbance of the resulting yellow solution is measured spectrophotometrically at a wavelength of 405nm [23]. The absorbance is again read after 1, 2, and 3 minutes at wavelength of 405nm, 1cm light path, at 25^0C.

Data Analysis

Deploying the GraphPad Prism 8.0.2 package, the data was analyzed using one-way analysis of variance (ANOVA), followed by the Tukey's multiple comparisons post hoc test. A p value of less than 0.05 ($p<0.05$) was accepted as statistically significant.

RESULTS

Table 3: High sensitive C-reactive protein (HsCRP) and monocyte chemotactic protein-1 (MCP-1) levels in the rats fed the combined diet

Groups	HsCRP (ng/ml)	MCP-1 (pg/ml)
Control	2.00 ± 0.23^{a}	5.20 ± 0.31^{a}
Combined Diet	3.60 ± 0.17^{bc}	9.70 ± 0.05^{bc}
Combined Drug Mixture	1.80 ± 0.32^{ade}	5.60 ± 0.34^{ade}
Normal Dose Paxherbal	2.20 ± 0.26^{ade}	6.10 ± 0.53^{ade}
Medium Dose Paxherbal	$1.80.\pm0.09^{ade}$	5.50 ± 0.22^{ade}
High Dose Paxherbal	2.10 ± 0.13^{ade}	$6.20.\pm0.13^{ade}$

The values are expressed as mean±SEM. Means in the same column with different superscript alphabets in the same position, differ from one another at 95% level of significance ($P<0.05$).

Table 4: Malondialdehyde (MDA) and total antioxidant capacity (TAC) of the rats fed the combined diet

Groups	MDA (ng/ml)	TAC (μmol/ml)
Control	1.10±0.05[a]	19.00±1.10[a]
Combined Diet	3.40±0.19[bc]	7.40±1.40[bc]
Combined Drug Mixture	1.10±0.13[ade]	16.00±1.40[ade]
Normal Dose Paxherbal	1.40±0.22[ade]	21.00±2.30[ade]
Medium Dose Paxherbal	1.20±0.25[ade]	26.00±2.80[ade]
High Dose Paxherbal	1.40±0.28[ade]	26.00±1.60[ade]

The values are expressed as mean±SEM. Means in the same column with different superscript alphabets in the same position, differ significantly at 95% level of significance ($p<0.05$).

Table 5: Angiotensin converting enzyme (ACE) activity and cardiac troponin-T (cTnT) level of the rats fed the combined diet

Groups	ACE (ng/ml/min)	cTnT (pg/ml)
Control	9.30±0.47[a]	1.50±0.05[a]
Combined Diet	5.70±0.19[ac]	2.90±0.03[bc]
Combined Drug	10.00±1.0[ade]	1.70±0.05[ade]
Normal Dose Paxherbal	10.00±0.91[ade]	1.40±0.13[ade]
Medium Dose Paxherbal	9.60±0.83[ade]	1.40±0.05[ade]
High Dose Paxherbal	12.00±0.37[ade]	1.30±0.09[adf]

The values are expressed as mean±SEM. Means in the same column with different superscript alphabets in the same position, differ significantly at 95% level of significance ($p<0.05$).

Table 6: Lipid profile indices of the rats fed the combined diet

Group	TOTAL CHOLESTEROL (mg/dl)	TRIGLYCERIDE (TG) (mg/dl)	HDL CHOLESTEROL (mg/dl)	LDL CHOLESTEROL (mg/dl)	VLDL CHOLESTEROL (mg/dl)	LOG TG/ HDL-C
Control	117.00±0.40^{a}	73.00±0.94^{a}	15.00±2.00^{a}	86.00±2.70^{a}	15.00±0.15^{a}	0.69±0.06^{a}
Combined Diet	177.00±6.10^{bc}	106.00±3.20^{bc}	12.00±1.40^{ac}	144.00±5.20^{bc}	21.00±0.64^{bc}	0.97±0.05^{bc}
Combined Drug	115.00±1.70^{ade}	75.00±1.60^{ade}	23.00±1.50^{ade}	77.00±2.80^{ade}	15.00±0.31^{ade}	0.51±0.03^{bde}
Normal Dose Paxherbal	120.00±5.80^{ade}	82.00±0.51^{bde}	24.00±2.10^{bde}	79.00±6.40^{ade}	17.00±0.40^{bde}	0.53±0.04^{ade}
Medium Dose Paxherbal	116.00±5.90^{ade}	77.00±1.70^{ade}	24.00±1.30^{ade}	78.00±6.10^{ade}	15.00±0.34^{ade}	0.51±0.02^{ade}
High Dose Paxherbal	120.00±6.00^{ade}	75.00±1.20^{ade}	26.00±2.10^{bde}	80.00±4.60^{ade}	15.00±0.43^{bde}	0.46±0.03^{bde}

The values are expressed as mean±SEM. Means in the same column with different superscript alphabets in the same position, differ statistically at 95% level of significance ($p<0.05$).

Table 7: Electrolytes, urea and creatinine levels of the rats fed the combined diet

Group	Potassium (mmol/l)	Sodium (mmol/l)	Chloride (mmol/l)	Bicarbonate (mmol/l)	Urea (mg/dl)	Creatinine (mg/dl)
Control	5.20±0.12^{a}	144±2.30^{a}	115±1.50^{a}	19±0.80^{a}	52±2.50^{a}	1.80±0.20^{a}
Combined Diet	5.80±0.40^{ac}	158±0.49^{bc}	123±2.00^{ac}	21±0.80^{ac}	78±6.30^{ac}	1.4±0.14^{ac}
Combined Drug	5.40±0.16^{ace}	150±0.69^{ace}	115±2.10^{ade}	20±1.30^{ace}	80±4.20^{bce}	1.8±0.14^{ace}
Normal Dose Paxherbal	5.70±0.18^{ace}	149±3.80^{ace}	118±1.20^{ace}	20±0.75^{ace}	74±5.60^{ace}	1.3±0.07^{ace}
Medium Dose Paxherbal	5.30±0.24^{ace}	154±1.40^{ace}	117±2.20^{bce}	19±1.00^{ace}	83±4.70^{bce}	1.3±0.08^{ace}
High Dose Paxherbal	5.80±0.30^{ace}	148±1.90^{ace}	120±1.30^{ace}	20±0.40^{ace}	79±9.00^{bce}	1.6±0.14^{ace}

The values are expressed as mean±SEM. Means in the same column with different superscript alphabets in the same position, differ significantly at 95% level of significancce ($p<0.05$).

Table 8: Liver function indices of the rats fed the combined diet

Groups	Total Protein (g/l)	Albumin (g/l)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Total Bilirubin (mg/dl)	Indirect Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)
Control	100±2.60[a]	35±2.90[a]	48±9.00[a]	143±5.40[a]	43±6.20[a]	0.72±0.09[a]	0.54±0.07[a]	0.17±0.03[a]
Combined Diet	92±8.70[ac]	28±2.80[ac]	54±10.00[ac]	183±22.00[ac]	35±3.3[ac]	0.43±0.05[bc]	0.31±0.03[bc]	0.13±0.03[ac]
Combined Drug	98±3.10[ace]	37±2.60[ace]	67±8.40[ace]	165±2.20[ace]	36±2.20[ace]	0.83±0.06[ade]	0.62±0.03[ade]	0.22±0.03[ace]
Normal Dose Paxherbal	95±5.90[ace]	33±2.50[ace]	59±8.80[ace]	133±3.90[ade]	40±4.20[ace]	0.50±0.02[acf]	0.38±0.02[ace]	0.12±0.01[acf]
Medium Dose Paxherbal	101±3.80[ace]	32±3.20[ace]	70±2.50[ace]	125±14.00[ade]	32±3.50[ace]	0.52±0.02[acf]	0.39±0.03[ace]	0.13±0.01[ace]
High Dose Paxherbal	100±3.90[ace]	32±0.46[ace]	41±5.80[ace]	147±4.60[ace]	40±4.80[ace]	0.56±0.04[ace]	0.40±0.04[ace]	0.15±0.01[ace]

The values are expressed as mean±SEM. Means in the same column with different superscript alphabets in the same position, differ significantly at 95% level of significance ($p<0.05$).

DISCUSSION

Considering the fact that the World Health Organisation (WHO) has been routing for local preventive measures to the debilitating cardiovascular diseases in sub-Saharan Africa, this study was designed to evaluate the therapeutic potential of Paxherbal bitters in preventing cardiovascular diseases in male albino *Wistar* rats fed a combined high-salt and high-fat diet. This was motivated by the fact that high blood pressure from high salt diet and hypercholesterolaemia from a high fat diet are major causative/risk factors for cardiovascular diseases. The need therefore to ascertain if Paxherbal herbal bitters can ameliorate the inflammation, endothelial dysfunction, oxidative stress, changes in lipid levels and dysfunction/changes in some organs, normally associated with a high-salt (hypertension-inducing) and a high-fat (hypercholesterolaemia-inducing) diet, which in themselves are predisposing factors to cardiovascular diseases, will be beneficial in the fight against the scourge of these cardiovascular diseases. This study also was designed to compare the effect of Paxherbal herbal bitters, to a combination of the anti-hypertensive drug (Lisinopril, an ACE-inhibitor) and the anti-hypercholesterolaemic drug (Atorvastatin, one of the Statins) to ascertain their relative efficacy.

The result in Tables 3 indicate that the combined diet caused a significant increase ($p<0.05$) in HsCRP and MCP-1 levels in their individual negative control group of rats, when compared with the level of this same analytes in the normal control rats. Generally, the combined drug and the Paxherbal bitters prevented this increase significantly in their respective groups ($p<0.05$) when compared with their respective negative control groups. The effect of the herbal bitters was not dose-dependent but was significantly as potent ($p<0.05$) as the combined drug mixture. HsCRP and MCP-1 are markers of inflammation and endothelial dysfunction and their elevation in this study is in keeping with the inflammatory effect and dysfunction in endothelial function already established for high-salt and high-fat diets [1]. Hypercholesterolaemia, hypertension and insulin resistance caused by a high-salt and/or high-fat diet individually and collectively contribute to endothelial dysfunction and inflammation in the vascular wall. They also cause increased lipoprotein oxidation, smooth muscle cell proliferation, extracellular matrix deposition, cell adhesion and thrombus formation. These are all processes involved in the development of atherosclerosis and ischaemic heart disease [11]. C-reactive protein (CRP), are acute phase proteins, involved in innate immune responses and has roles that include activating the complement system and enhancing phagocytosis [24]. Currently, HsCRP is recognized as the inflammatory marker with the strongest association with hypertension [27]. Its level is positively associated with systolic blood pressure, pulse pressure and the incidence of hypertension [4]. Thus HsCRP and high blood

pressure since they are already recognized as independent determinants of cardiovascular risk, confers an additional predictive value for cardiovascular outcomes when they exist together [21]. HsCRP is now known to be elevated also in hyperlipidaemia and that hyperlipidaemia further induces elevation of HsCRP (56). It has also been ascertained that the use of statins and fenofibrate to tackle hypercholesterolaemia and hypertriglyceridaemia, always also led to a lowered HsCRP level, in patients such disorders. [14]. Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family and as its name implies, it is a potent chemotactic factor that recruits circulating monocytes to sites of inflammation [19]. Its marked elevation is not only associated with the onset of atherosclerosis, it has also been shown to be elevated in hypercholesterolaemic and hypertensive patients compared with normal controls [11]. Over the years, MCP-1 has come to be accepted as a major factor in the development of atherosclerosis through its promotion of monocyte/macrophage accumulation in atherosclerotic plaques, leading in turn to chronic inflammation, smooth muscle cell proliferation, and plaque instability [23].

The prevention of the elevation of plasma HsCRP and MCP-1 in this study by the Paxherbal bitters at the same potency level attributed to atorvastatin and lisinopril in combination, is therefore a positive finding. This is so because in not allowing the high-salt and high-fat diet to exert their negative inflammatory effect and consequential endothelial dysfunction that favour cardiovascular diseases, they prevent the onset of such diseases. These findings confirms the earlier claims of the possible anti-inflammatory properties of this herbal bitters [10, 18,]. The bitters were able to achieve this likely because of the plants they contain which have anti-inflammatory properties. Example of such plants are: *Aloe vera, Vernonia amygdalina*, *Gangronema latifolium*, *Cymbogon citratus*, *Garcinia kola, Chenobium murale* and *Cinnamonum cassia* [10, 11, and 18], just to mention but a few. The anti-inflammatory properties of these plants, stem from their phytochemical constituents which likely act synergistically to bring down or prevent a rise in the blood levels of CRP and MCP-1 [6]. Earlier studies indicate that the bitters of this study have appreciable amounts of alkaloids, tannins, flavonoids, phenols, saponins and cyanogenic glycosides which are phytochemicals known to have anti-inflammatory properties [10, 6]. Their flavonoid content alone can as well explain their anti-inflammatory property and ability to prevent endothelial dysfunction. Examples of flavonoids derivable from the constituents that make up the bitters include quercitrin, quercetin, kaempferol [16]. Flavonoids, like quercetin, are polyphenolic compounds, which have been suggested to possess a prime role in the treatment and prevention of hypertension [24]. Flavonoids are regarded as having anti-inflammatory (inhibit inflammatory metabolites and granulation tissue formation), anti-allergic (inhibit histamine release), and anti-oxidant effects. [16]. [18] went a step further to reveal the possible mechanisms via which flavonoids like quercetin exhibits its antihypertensive effects to decrease the prevalence of cardiovascular diseases. Such mechanisms include reduction in oxidative stress, inhibition of angiotensin converting enzyme (ACE) activity, reduction in inflammation and improving of the endothelial function [4]. Paxherbal bitters containing these flavonoids may likely also be imparting their effects by following the same mechanisms of action. The anti-inflammatory, antioxidant and anti-hypertensive properties of Paxherbal bitters is also likely due to some of its identified constituent biological compounds whose properties have also been identified. Such compounds identified from the research done by Elufioye and Mada, [4] include:

- ✓ Terpinen-4-ol (4.54%, known to be anti-inflammatory, anti-bacterial and anti-fungal),
- ✓ 2, 4- Shogaol (15.16%, known to be anti-tussive, anti-inflammatory, anti-hypertensive and a memory enhancer),
- ✓ Caryophyllene (9.33%, known to be anti-inflammatory and anti-cancer),
- ✓ Isospathulenol (2.14%, known to be immune-inhibitory and anti-inflammatory),
- ✓ Cyclohexanemethanol (5.45%, known to be anti-inflammatory and antiviral) and
- ✓ p-Heptylacetophenone (9.435%, known to be anti-allergic and anti-inflammatory).

Tables 4 reveal that the combined diet caused a significant increase ($p<0.05$) in malondialdehyde (MDA) and a significant decrease ($p<0.05$) in the total antioxidant capacity (TAC) levels in the negative control group of rats when compared with the normal control rats. Generally, the combined drug and the bitters significantly prevented this respective increase and decrease ($p<0.05$) in the rats of their respective groups when compared to the negative control groups. The effect of the bitters was not dose-dependent in reducing MDA but was dose dependent in increasing TAC. This means that with an increase

in the consumption of the herbal bitters, there is an increase in the total antioxidant capacity in the rats. In addition, the ability of the bitters to increase the TAC level of the rats was significantly more potent ($p<0.05$) than that of the combined drugs. The results of this study aligns with earlier studies that postulated the possible antioxidant properties of Paxherbal bitters [3, 10, 18 and 12]. High-fat diet causing hypercholesterolaemia and its ability to induce oxidative stress is important in the pathogenesis of cardiovascular diseases because of the contribution of oxidative stress to the formation of atheromatous plaques resulting in atherosclerosis that is fully implicated as a major cause of cardiovascular diseases [5]. The high-salt diet also contributes to oxidative stress which translates to a predisposition to cardiovascular diseases. High-salt diet causes oxidative stress by activating the renin-angiotensin-aldosterone-system (RAAS) and increasing the level of angiotensin II [15]. Apart from the angiotensin II causing vasoconstriction, which leads to an elevation of the blood pressure, it causes oxidative stress by activating endothelial NADPH-oxidase to produce reactive oxygen species (ROS) [10]. The ROS then causes an impairment in the ability of NO to cause vascular relaxation, resulting in endothelial cell dysfunction. This eventually causes an increase in total peripheral resistance with a subsequent increase in blood pressure (hypertension).

Oxidative stress leads to the generation of MDA *in-vivo* via the peroxidation of polyunsaturated fatty acids [4]. MDA interacts with proteins and is itself potentially atherogenic. Hence its elevation can precipitate a negative cardiovascular event [24]. MDA's reaction with lysine residues generates lysine – lysine cross-links which have been identified in apolipoprotein B (apoB) fractions of oxidized low density lipoprotein (OxLDL), and have been postulated to impair the interaction between OxLDL and macrophages and thereby to promote atherosclerosis [16]. It therefore is a positive finding of this study that Paxherbal bitters were able to prevent the high salt and high fat from causing the oxidative stress that would have led to the rise of MDA and a decrease in the total antioxidant capacity of the system. It is doubly impressive that they had the ability to increase this total antioxidant capacity over and above that of the drugs of this study and what could be said to be the normal level in the control rats. The bitters were likely able to achieve this because of its constituent antioxidant plants and antioxidant phytochemical constituents. Example of such plants are: *Xylopia aethiopica, Aloe vera, Vernonia amygdalina*, *Gangronema latifolium* , *Cymbogon citratus*, *Garcinia kola*, *Chenobium murale*, *Zingiber officinale*, *Capsicum annum*, *Garlic allium, Theobroma cacao* [10, 18]. Example of constituent phytochemicals in Paxherbal bitters with antioxidant and other related properties include:

- ✓ Isoborneol (0.61%, known to be antioxidant and neuroprotective),
- ✓ 2, 4-Ditertbutylphenol (4.61%, known to be antioxidant and antifungal), and
- ✓ Shogaol (15.16%, known to be anti-tussive, antioxidant [6].

The results reflected in tables Table 5 for angiotensin converting enzyme (ACE) activity and cardiac troponin – T (cTnT) level reveal that the combined diets caused a significant increase ($p<0.05$) in cTnT level and a decrease in ACE activity of the rats in their respective groups, when compared with their normal controls. Generally, the combined drug and Paxherbal bitters prevented this respective increase and decrease significantly ($p<0.05$) in their respective groups when compared with their negative control. The effect of the bitters was not dose-dependent in terms of their action on ACE and cTnT, and they were significantly more potent ($p<0.05$) in preventing cardiac injury at higher doses than the combined drug. This reduction in plasma ACE level is commonly found in hypertensive and hypercholesterolaemic conditions possibly because the major ACE activity that supports hypertension in these conditions is renal-tissue and vascular-bed based (68, 69 and 70), and the little or normal quantity of ACE in the blood is further used up, and reduced when they are mobilized to contribute their quota to sustaining the hypertensive state [3, 12 and 17]. This normally happens after the high-salt/high-fat triggered activation of RAAS needs ACE to mediate the release and production of angiotensin II to sustain the hypertensive state. Paxherbal bitters ability to be able to resist this reduction in plasma ACE level leaving it at levels as found in the normal control rats, stems from the fact that they block the activation of RAAS by the high-salt and high-fat diet in tissues. This ensures that the ACE in the blood no longer needs to be consumed in the conversion of angiotensin I to angiotensin II. The ability of Paxherbal bitters to prevent the fall in plasma ACE level by inhibiting the activation RAAS in tissues, is therefore a positive finding of this study as it is evidence of their anti-hypertensive property or anti-hypertensive ability. The anti-hypertensive ability of this bitters is also not far-

fetched considering its numerous plant/herbs and their phytochemical constituents postulated as having anti-hypertensive properties. It most likely exerts this anti-hypertensive effect by the synergistic action of its constituent phytochemicals which prevent the activation of RAAS or inhibit the action of angiotensin II. The result of this study confirms earlier studies of [3] and [10] which postulated the possibility that Paxherbal bitters contain phytochemicals that confer not just antihypertensive but cardiovasculo-protective properties on them. The results also complements the work of [4] and [6] whose studies reveal that extracts of herbs, like those found in Paxherbal bitters, are rich in flavonoids, phenolic acid and other phytochemicals. Their study asserts that these phytochemicals are useful in the treatment of hypertension,hypercholesterolaemia,cardiovascular diseases, liver cirrhosis and hepatitis [15, 19]. The smooth muscles of the heart also undergo oxidative stress and inflammation induced by a high-salt and a high-fat diet and the strain of hypertension on the heart leads to ventricular hypertrophy or cardiac hypertrophy. It also causes some form of cardiac injury which can progress further into more dangerous cardiovascular injury and cardiac tissue death. Marginally elevated concentrations of cardiac troponin T (cTnT) are associated with future adverse cardiac events [5]. An elevated plasma cardiac troponin level indicates cardiac injury and in the presence of corroborating clinical signs and symptoms, it is now the accepted gold standard for the diagnosis of myocardial infarction [17]. Cardiac troponin T (cTnT) along with troponin I (cTnI) are cardiac regulatory proteins that control the calcium mediated interaction between actin and myosin [7]. Hence, the result of this study that shows that Paxherbal bitters can prevent an elevation in cTnT that can arise from the toxic effect of a high-salt and high-fat diet on the myocardium, is a positive therapeutic finding. This result confirms earlier claims from studies by [3], [10], [18] and [16]. They suggested a possible cardio-protective property of these bitters owing to their herbal phytochemical constituents especially those coming from their flavonoids, phenols and hydrolysable tannins content.

Taking a look at the results on the lipid profile presented in Tables 6, it can be seen that the combined diet respectively, caused a statistically significant increase ($p<0.05$) in plasma total cholesterol, triglyceride, LDL- and VLDL-cholesterol levels in the rats of their respective negative control group, when compared to their normal control. This increases were retarded significantly ($p<0.05$) by the combined drug and the bitters. The combined diet also reduced significantly ($p<0.05$) the HDL-cholesterol level of the rats in their negative control group, compared to their respective normal controls. The combined drug and the bitters however not only significantly ($p<0.05$) prevented the reduction of the HDL-cholesterol level, they also significantly ($p<0.05$) elevated the HDL-cholesterol level when compared to their respective normal and negative controls. The combined diet significantly increased ($p<0.05$) the atherogenicity potential in the rats by increasing their atherogenic index of plasma, when compared to that of their respective normal controls. The combined drugs as well as the bitters not only significantly prevent this increase in the atherogenic index of plasma in the rats of their respective groups, they also reduced them significantly below ($p<0.05$) the value, recorded for their negative and normal control rats. The bitters were also found to be as effective as the combined drug, in maintaining a good lipid profile. The results of the study on the lipid profile of the rats administered the bitters in addition to the experimental diet compared with that of the negative control rats administered only the experimental diet, reveal that generally, the bitters relatively have hypo-cholesterolaemic and hypo-triacylglycerolaemic effects, while decreasing the LDL-cholesterol, VLDL-cholesterol, atherogenic index of plasma and increasing the HDL-cholesterol levels. This result gives credence to the claim by the bitters manufacturers that they have hypo-lipidaemic effect by their ability to stimulate the pancreas and liver/gall bladder [3,10, 18, 8, 7], and in so doing 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 [3,10, 18, 7, 9]. It has also been postulated that the bitters use their phytochemical constituents (like their saponins and flavonoids) and acting like the statins, prevent the endogenous biosynthesis of cholesterol, while using their soluble fibre contents to form complex with lipids, reducing their absorption. Saponins in particular have been said to have cholesterol binding properties [3, 10, 18, and 19]. The results of this study on the plasma lipid profile give positive evidence that the herbal bitters have the ability of reducing the atherogenic potential and being a lipid-lowering supplement in mixed hyperlipidaemic states. As earlier explained, there is evidence that a salient

relationship exists between high serum cholesterol levels and increased atherogenicity and the incidence of atherosclerosis. The ability of these bitters to put the blood cholesterol in check, hence preventing atherosclerosis ultimately helps in the prevention of cardiovascular diseases [30]. The observed hypocholesterolaemic effect of these herbal bitters and their ability to reduce the atherogenic index of plasma are therefore desired positive effects.

Table 7 shows that the plasma sodium and urea levels were the only indices affected in the rats fed the combined diet. Their levels were significantly elevated ($p<0.05$) in the negative control rats when compared with that of the normal control rats. The combined drug and the bitters resisted the diet-induced elevation of plasma sodium in the experimental rats, but failed to do same for urea, as the urea levels remained significantly higher ($p<0.05$) than that recorded for the normal control rats. Considering the fact that other indices for measuring kidney function remained unchanged, the elevated urea experienced was most likely not due to irreversible kidney damage. The result of the study by [29], suggests that the elevated urea observed, may be due to dehydration occasioned by increased loss of fluids following the effort by the kidney to maintain homeostasis in a high-salt environment [24]. This results in a dehydrated plasma, hence the levels of urea appear elevated. Not being a pathological elevation, the bitters have nothing to resist, so they seem inactive. The lack of significant difference between the analytes of the control and bitters groups is a reflection of the preserved renal integrity of the treated rats [12, 11]. Hence, the bitters can be said not to be reno-toxic to the kidneys as they seem rather reno-protective. This confirms the earlier studies by [18], [8, 9], [3], [10] and [10], ascribing a possible reno-protective and non-toxic effect to the bitters of this study.

The result on the liver function indices of the animals of this study as reflected in Tables 8 indicate that the combined diet did not significantly affect ($p>0.05$) the total protein, albumin, and the direct/conjugated bilirubin levels and the alkaline phosphatase, AST and ALT activities. The combined drug and bitters resisted any changes and maintained the status quo of the levels of these analytes in the treated rats when compared with their respective negative and normal controls. There was however a significant decrease ($p<0.05$) in the total bilirubin and the indirect bilirubin in the rats fed the combined diets when compared to that of their normal control. The combined drug and the bitters retarded or resisted this decrease in total and indirect bilirubin. The combined drug had the same potency were more effective ($p<0.05$) than Paxherbal bitters in resisting this decrease in bilirubin. The minimal and non-significant differences ($p>0.05$) in the majority of liver analytes in the treated and control rats is indicative that the diets and the bitters did not cause any major or significant ($p>0.05$) damage to the liver of the treated rats. Hence they can be said to be hepatoprotective [2]. This means that apart from there being no severe liver cell damage, there was no form of cholestasis, no excessive haemolysis, neither was there any impairment in the excretory and synthetic functions of the liver (82). The significant decrease ($p<0.05$) in the total and indirect bilirubin levels in the rats fed the combined diet compared to the bitters fed rats is however a curious finding. The fact that the bitters resisted this decrease and kept the total and indirect bilirubin levels at the same level with that of the normal control rats is also of significant note. Bilirubin has been discovered to have the property of being a chain breaking antioxidant [13]. The only plausible explanation that can be given at this juncture for the decrease of bilirubin, is that the bilirubin having antioxidant properties, is reduced because it was consumed and cleared by macrophages after it has been modified by oxidants produced essentially by the negative effects of the high-salt and high-fat content of the diet. It is of interest because low circulating total bilirubin levels from decreased indirect bilirubin levels are considered a risk factor for or have been associated with cardiovascular diseases [13]. It is therefore a positive finding of this study that the action of the bitters in preventing the reduction in bilirubin levels and maintaining it at normal levels, helps to protect against the development or progression of cardiovascular diseases.

CONCLUSION

This study set out to evaluate the therapeutic potential of Paxherbal bitters in preventing cardiovascular diseases in male albino *Wistar* rats. To achieve this, conditions that precipitate diseases like hypertension and hypercholesterolaemia were simulated in rats, using a combined high-salt and high-fat diet, which are in themselves high risk factors for cardiovascular diseases. If it is true that the lack of bitters in our diet has resulted in a 'bitter deficiency syndrome" which makes us prone to diseases, a positive result of the effect of a daily

supplementation of bitters like Paxherbal bitters in our diet will be a welcome development. The results of this study revealed that Paxherbal bitters have similar efficacy as the combined effect of the lisinopril and atorvastatin drug in preventing the negative effects of hypertension and hypercholesterolaemia. The therapeutic action of Paxherbal bitters from this study reveal that they were able to prevent inflammatory processes, oxidative stress and endothelial dysfunction *in-vivo*. They were also able to prevent the development of dyslipidaemia and hypercholesterolaemia, as well as the onset of hypertension and reduced the atherogenic index of plasma. These findings, most especially as the positive factors against cardiovascular diseases were enhanced while the negative ones were subdued in rat, puts the bitters of this study in a good stead to be the supplement of choice in the prevention and management of cardiovascular diseases in man if these findings can be extrapolated to man.

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