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Biochemical and histological Evaluation of the Ability of Yoyo Cleanser Bitters to Prevent Cardiovascular Diseases Induced in rats by a Combined High-salt and High-Fat Diet.

*Anionye JC[1], Onyeneke EC[2] and Eze GI[3]

[1]Department of Medical Biochemistry, College of Medical Sciences, University of Benin, Benin City, Nigeria.
[2]Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.
[3]Department of Anatomy, College of Medical Sciences, University of Benin, Benin City, Nigeria.
*Corresponding Author: Dr. Anionye John Chukudi; Email: chukudi.anionye@uniben.edu;

ABSTRACT

Cardiovascular diseases have become a rising and seeming unending scourge to humanity. In line with WHO's suggestions for an alternative remedy, the aim of this study was to evaluate the therapeutic potential of Yoyo cleanser bitters as a local remedy in the prevention of cardiovascular diseases in *Wistar* rats fed a combined high-salt and high-fat diet. Thirty *Wistar* rats randomly divided into six groups of five rats each, with the average weight of each group being 200g were used. The rats were fed ad-libitum with the feed and clean-tap water during the entire 6-week course of this study. A basal diet of modified standard pelleted mash, was given daily to rats of control group 1 while the combined high-salt and high–fat diet was given to the remaining 5 groups. A combination of the drugs lisinopril and atorvastatin was also administered daily to group 3 rats, while three doses (600mg/kg, 1,100mg/kg and 2,200mg/kg of the rats' body weight) of the Yoyo cleanser bitters were also respectively administered to the rats in groups 4 – 6. Using standard methods, the weekly weight of the rats, their daily feed consumption, food efficiency, the relative organ weight of the heart and aorta, liver and kidney, and their fasting blood glucose, were determined. Histopathological evaluation of the aorta, heart, liver and kidney of the rats was also done. The data was analyzed using the GraphPad Prism 8.0.2, one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons post hoc test. A p value of less than 0.05 ($p<0.05$) was accepted as statistically significant. The results of this study reveal that Yoyo cleanser bitters prevented the development insulin resistance. The relative organ weight and histological evaluation of the organs of the rats revealed that the bitters were vasculoprotective, cardioprotective, renoprotective, hepatoprotective and were as effective as lisinopril and atorvastatin in preventing the development of features characteristic of hypertension and hypercholesterolaemia. These findings affirm Yoyo cleanser bitters as a good therapeutic supplement in the prevention and management of cardiovascular diseases.
Key words: High-salt diet, high-fat diet, cardiovascular diseases, Yoyo cleanser bitters, hypertension.

INTRODUCTION

The motivation for this research came from the desire to find an alternative to the "three-pill" suggestion by WHO to ameliorative the increasing deaths from cardiovascular diseases (CVD) worldwide [1, 2, 3]. Cardiovascular diseases have become an unending scourge to humanity. In 2002, the World Health Organisation (WHO) estimated that every year 12 million people worldwide died from cardiovascular diseases. A study published in the New England Journal of Medicine shows that the number of deaths from hypertension, stroke, heart attacks and other circulatory/cardiovascular diseases is on the increase, climbing from 12.3 million in 1990 to 17.3million in 2013 (2). This is coming on the heels of a survey that shows that hypertension rates in Nigeria jumped from 11% in 1997 to 40% in 2013 [4]. A report from [5]) revealed that several studies have shown that the death rates of cardiovascular diseases are higher in Nigeria due to late detection of cases and other risk factors such as high blood cholesterol, high blood pressure, smoking, diabetes, obesity and sedentary living.

Dr Gro Harlem Brundtland, Director General of the WHO as at 2002 opined that "Prevention is the key to lowering the global disease burden of heart attacks and strokes."(1). The 2002 WHO report went further to postulate that the burden of cardiovascular diseases will be cut by 50% if everyone at high risk of having a heart attack or

stroke was given a combination of a statin for lowering cholesterol, a low dose blood pressure lowering drug (like an ACE-inhibitor-lisinopril), and low dose aspirin (a blood thinner). Realising that this "three-pill advise" will be expensive and difficult to implement in the countries of sub-Saharan Africa, WHO advised that "new resources" that will serve as alternatives should be developed for these poor countries. 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 [5,6,7,8,9,10].

The health benefits of polyherbal products like Yoyo cleanser bitters (with 5 herbal constituents) from scientific research findings have been attributed to the additive and synergistic effects of the complex mixture of their constituent phytochemicals. Research has shown that many spices and herbs especially those used to formulate herbal bitters exhibit not just antioxidant properties, but 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. The repackaging and packaging of "unorthodox products" like the "herbal bitters" an ancient remedy for digestive problems and products like it, in an "orthodox way", has led to their resurgence and widespread acceptability [11]. Our African diet has become increasingly fortified with unhealthy fat and salt which has been implicated in the rise of cardiovascular diseases in our environment. It was therefore important and necessary to evaluate some of the commonly consumed herbal bitters in Nigeria, to determine their efficacy in the prevention of the effects of high-salt diet in the body (effects which have been shown to be similar to effects of hypertension) and the effects of high-fat diet in the body (effects which have been shown to be similar to effects of hypercholesterolemia/ hyperlipidaemia) which are major risk factors for cardiovascular diseases. It is to confirm this possible preventive property of herbal bitters that this research seeks to investigate the therapeutic potential of some of these herbal 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.

Herbal bitters are a combination of bitter herbs and other herbs with related synergistic properties. Bitter herbs are fundamental to phytotherapy's contribution to holistic medicine. They are simply herbs that have a bitter taste. The term "Herbal Bitters" refers to beverages which are often alcoholic and flavoured with herbal essence that gives them a bitter or bittersweet flavour. 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 [11,12]. Herbal bitters when consumed, act upon the bitter receptors on the tongue, thereby producing the bitter taste in the mouth. When bitter constituents are tested on the tongue, a reflex action occurs from the receptors (referred to as T2Rs) located on the taste buds. Thus taste signals are transmitted to brain. This initiates the priming of digestive function and other positive effects associated with the consumption of bitters especially after its constituent phytochemicals are absorbed into the blood stream. Increase in gastric acid is not always dependent on the oral reflex, as stomach cells may respond directly to bitter principles [13].

The product brochure of Abllat Company Nigeria Limited [14], the makers of Yoyo cleanser bitters reveal that each 200ml bottle of this product contains a proprietary blend of:

- *Aloe vera* (True aloe, Lily of the forest)
- *Acinos Arvensis* (Basil thyme)
- *Citrus aurantifolia* (Bitter orange)
- *Chenopodium murale* (Nettleleaf Goosefoot)
- *Cinamomum aromaticum* (Cassia)

Since its introduction into the Nigerian market, it has gained wide popularity and acceptance among the people. Its manufacturers claim its ingredients have a synergistic effect on the management of ailments of the digestive, circulatory, nervous, urinary and excretory systems. They also claim they help in the prevention of ulceration, prevention of hardening of tissues and that they have immunity boosting properties as well as aid in the reduction of excess body fat and a healthy weight loss. These claims have not been evaluated by NAFDAC nor have they been confirmed by any study or clinical trial.

Earlier studies carried out by [15] and [16] on Yoyo bitters revealed that: It contains phytochemical constituents and active/bitter principles that rightly put it in the class called "herbal bitters" as its aqueous and ethanolic extracts indicate that it contains in varying degrees, phytochemicals and bitter principles belonging to the following classes: Amino acids, saponins, tannins, phenols, alkaloids, glycosides, terpenoids and flavonoids.

They have a very high LD_{50} of 164,000 mg/kg and consequently also a high therapeutic index and so they are safe for consumption at the dose (600mg/kg) recommended by the manufacturers with no toxicity to the blood, liver, kidneys, heart and general body system of rats. The established effects of its constituent phytochemicals and minerals, and the inferences from the results of the in-vivo and in-vitro studies on normal *Wistar* rats, suggests that Yoyo cleanser bitters may well have some of the properties claimed by its producers, as it showed:

- ✓ In-vitro and in-vivo antioxidant capacity/activity
- ✓ And pharmacological properties ranging from in-vivo - hypolipidaemic, hypoglycaemic and immunity-boosting properties to hepatoprotective, diuretic, anti-hypertensive and reno-protective properties under normal conditions.

All these findings calls for further investigation of this herbal bitters as it seem to be a tonic that may help in the prevention of several disease-states top of which is cardiovascular disease which is the disease of interest in this present study. Apart from the studies mentioned earlier and a few studies on some of the constituent herbs in Yoyo cleanser bitters, there is paucity of scientific literature with research findings in respect of Yoyo cleanser bitters (as it is constituted presently) in terms of its therapeutic potential to prevent the accumulation of substances or prevention of the effect of some diet induced risk factors like hypertension and hypercholesterolaemia that can lead to pathological effects on the cells, organs and tissues of the cardiovascular system at the microscopic and macroscopic level, leading to cardiovascular diseases.

MATERIALS AND METHODS

Materials

Test Bitters and Drugs

The Yoyo cleanser bitters and 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 were bought as liquid formulations and it and the reconstituted drug solutions stored in a refrigerator throughout the period of the experiment. Reagents/chemicals used to measure analytes from the rats.

The Reagents used for some aspects of this study were of analytical grade and purchased by ordering them directly from the manufacturer's representative/registered outlet in Nigeria, Pyrex Laboratories, Benin City. The process for the preparation or reconstitution of some of the reagents in the kits as well as the procedure for the determination of the analytes were as described by the manufacturers in their manual insets.

METHODS

Animal for the study and their care:

All the experimental animals for this study were handled in strict compliance with 1993 international guidelines as prescribed by the Canadian Council on Animal Care (CCAC) on the care and use of animals in biomedical research (16). 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. Thirty male *Wistar* albino rats were be obtained from the Anatomy Department, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria. They were housed in a well-ventilated room, in specially designed wooden cages, in the animal house of the Department of Medical Biochemistry, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria. They were allowed a diurnal 12-hr light and dark cycle and room temperature of about 27^0C. The rats were fed ad-libitum with a modified standard pelleted grower's mash and clean tap water for two weeks to allow them time to acclimatize prior to the commencement of the study.

Diets for the study:

The control or basal diet was a modified standard pelleted grower's mash which was

formulated following the suggestions put forward by, [17] and [18] (they suggested a replacement of soy protein common in standard growers mash with other alternative protein sources as it contains phytoestrogens, genistein in particular, which has been shown to attenuate the development of hypertension in spontaneous hypertensive rats and blunting of the increase in blood pressure due to NaCl induced hypertension in other species of rats like the albino *Wistar* rat). The combined high-salt/high-fat diet (hypertension-inducing/hypercholesterolaemia-inducing diets) was made by adding to a proportionate quantity of the basal diet, 8% NaCl (to make the diet high in salt for the purpose of making the rats hypertensive) [19] and 2% cholesterol + 0.25% bile salts + 20% hydrogenated fat/margarine (to make the diet high in lipids/cholesterol for the purpose of making the rats hypercholesterolaemic) [20, 21, 22]. The final composition of both the basal and experimental diet was as indicated in Table 1 and 2.

Table 1: Composition of the basal diet (g/1000g) (23).

Ingredients	Basal diet (g)
Maize	260.0
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) (23)

Ingredients	Combined Diet (High-salt/High-fat Diet) (g)
Basal diet in Table 1.1	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 *Wistar* rats weighing between 180 - 220g, were used for this study. After 14 days of acclimatization the rats 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 6 weeks study, according to the feeding protocol outlined below. Care was taken to determine the quantity of feed consumed daily. The cages, their surroundings, the receptacle tray below with its bedding, were cleaned and disinfected daily. An oro-gastric gavage was used to administer the drugs and bitters.

Protocol

Control groups

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

Experimental groups

- Group 4: Combined diet + Yoyo cleanser bitters (600 mg/kg body weight) for 6 weeks.
- Group 5: Combined diet + Yoyo cleanser bitters (1,100 mg/kg body weight) for 6 weeks.
- Group 6: Combined diet + Yoyo cleanser bitters (2,200 mg/kg body weight) for 6 weeks.

Dosage regimen for the herbal 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 40ml daily as a single dose or in two doses [23, 24]. In diseased conditions, physicians normally double the dosage of drugs usually given at a lower dose [25]. 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 [26], 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 was done to determine the initial equivalent doses of the bitters (distilled water in the case of the control groups) 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/distilled water that was given to the rats was calculated:

If 40ml was consumed by a 70,000g man (70kg) and the number of mls a 200g rat was expected to consume= Xml

$$Xml = \frac{40ml \times 200g}{70,000g} = 0.114ml \text{ (approximately } 0.11ml)$$

0.11ml for a 200g rat means a dose of 0.11ml/200g = approx. 0.6 $x10^{-3}$ml/g of rat

This is equivalent to: 0.6 ml/kg of rat or 0.6g/kg or 600mg/kg body weight of the rat.

If 600mg/kg amounts to 0.11ml that will be administered to a 200g rat, following similar calculations,

- ✓ 80ml of bitters will be equivalent to: 1,100mg/kg, which amounts to 0.23ml to be administered to a 200g rat and
- ✓ 160ml of bitters will be equivalent to: 2,200mg/kg, which amounts to 0.45ml to be administered to a 200g rat.

The rats were weighed weekly and the weight used to calculate the equivalent volume of bitters to be administered for each group of rats for that week.

Dosage regimen for the drugs

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

a.Dosage regimen for Atorvastatin: (25)

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).

b. Dosage regimen for Lisinopril: (26)

10mg is consumed by a 70,000g man (70kg)

This amounts to 0.029mg for a 200g rat means a dose of 0.029mg/200g = approx. 0.14x10^{-3}mg/g of rat or 0.14mg/kg of rat body weight.

c.Dosage regimen for the combined drug:

Following the individual drug determination above, the combined drug was thus formulated by dissolving 0.157mg of atorvastatin and 0.029mg of lisinopril in distilled water and making it up to 1ml which was given daily to the rats in the assigned group using an oro-gastric gavage.

Weekly Body Weight

The body weight of each rat was assessed using a weighing balance during the acclimatization period, once before commencement of dosing (day 1), once weekly during the dosing period, (day 7, 14, 21, 28, 35) and once on the day of sacrifice (day 42). [27].

Daily Quantity of Food 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. From the results, the average quantity consumed per day for the period of the study, by the rats, was determined. This quantity of feed consumed by each rat was assessed using a weighing balance [28]. The result was used to determine the food consumed per day per rat and this was 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:

$$\text{Food efficiency} = \frac{\text{Weight gain or lost/day/rat}}{\text{Feed Consumed/day/rat}} \times 100 \quad [28]$$

Relative Organ Weight

After the animals were anaesthetized (using chloroform anaesthesia) and blood samples collected from them, the animals were sacrificed and different organs namely the heart, aorta, liver and kidneys of the respective rats were carefully dissected out and weighed (this weight was designated as the absolute organ weight). The relative organ weight was then calculated using the formula:

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

[27].

Observations (symptoms, signs and mortality)

To access the level of acceptance and other positive or untoward effects of the diet and drugs of this study, the animals were further observed for toxic symptoms such as weakness or aggressiveness, food refusal, loss of weight, diarrhea/discharge from the anus, discharge from the eyes and ears, noisy breathing and other physiological changes including mortality, [29, 30].

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.

Blood Sample Collection and Preparation

The last dose of the respective diets, drugs and bitters was administered on the morning of the 42nd day. All meals were stopped by 7pm on the 42nd day. After an overnight fast, blood samples were collected from the animals (following chloroform anaesthesia and sacrifice/opening up of the animals), using syringes and needles via the inferior vena cava and cardiac puncture, and put into already labelled, fluoride oxalate bottles without undue pressure to either the arm or the plunger of the syringe. The samples were then mixed by gentle inversion. The sample was immediately analyzed for the fasting blood glucose.

Tissue Sample Collection and Preparation

After blood have been collected from the anaesthesized rats, the heart, aorta, liver and kidney were harvested and fixed in a 10% buffered formal-saline solution until they were needed for processing into slides for histological evaluation.

Determination of the fasting blood glucose (FBG).

The Randox Glucose Kit was used. The principle applied is the glucose oxidase method as described by Barham and Trinder [3].

Methodology for Microscopic Examination of Selected Tissues (Histology)

The heart, aorta, liver and kidney were preserved in a buffered 10% formal-saline solution and were later processed with an automatic tissue processor (Shandon Citadel Model, 2000) into tissue slides. Following dehydration and embedding, sections of the tissues, were cut at 4-5µ with a rotary microtone. Thereafter, they were de-waxed using xylene, placed on a water bath, picked out using a forceps and placed on a slide. Thereafter, staining with haematoxylin and eosin was done. Afterwhich they were then mounted using Canada balsam and a slip, and left to dry. The stained tissues were then individually

examined under a high powered field of a microscope [12].

Data Analysis

The results were presented as Mean±SEM. The data were analyzed using the GraphPad Prism 8.02, 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: The weight, feed consumed and food efficiency of the rats fed the combined diet

Groups	Mean of Weight Gained or Lost Per Day/Rat (g)	Mean of Feed Consumed Per Day/Rat (g)	Food Efficiency (%)
Control	0.89 ± 0.22^{a}	22.00 ± 0.00^{a}	1.20 ± 1.10^{a}
Combined Diet	-0.25 ± 0.04^{bc}	15.00 ± 0.00^{a}	-1.70 ± 0.25^{bc}
Combined Drug	-0.49 ± 0.12^{bce}	15.00 ± 0.00^{a}	-3.20 ± 0.82^{bce}
Normal Dose Yoyo	-0.13 ± 0.00^{bce}	16.00 ± 0.00^{a}	-0.79 ± 0.02^{bce}
Medium Dose Yoyo	-0.30 ± 0.12^{bce}	18.00 ± 0.00^{a}	-1.70±0.6bce
High Dose Yoyo	-0.26 ± 0.07^{bce}	16.00 ± 0.00^{a}	-1.66 ± 0.46^{bce}

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

Table 4: The relative organ weight of the rats fed the combined diet

Groups	Heart and Aorta (%)	Kidney (%)	Liver (%)
Control	0.42 ± 0.03^{a}	0.40 ± 0.04^{a}	2.60 ± 0.26^{a}
Combined Diet	0.74 ± 0.02^{bc}	0.66 ± 0.05^{bc}	4.90 ± 0.23^{bc}
Combined Drug	0.55 ± 0.05^{ade}	0.52 ± 0.03^{ace}	4.30 ± 0.20^{bce}
Normal Dose Yoyo	0.46 ± 0.03^{ade}	0.56 ± 0.04^{bce}	3.70 ± 0.14^{bde}
Medium Dose Yoyo	0.49 ± 0.04^{ade}	0.58 ± 0.03^{bce}	4.10 ± 0.15^{bce}
High Dose Yoyo	0.43 ± 0.02^{ade}	0.57 ± 0.02^{bce}	4.00 ± 0.29^{bce}

The results are presented 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: Fasting blood glucose of the rats fed the combined diet

Groups	Fasting Blood Glucose (mg/dl)
Control	57.00±3.60[a]
Combined Diet	120.00±5.50[bc]
Combined Drug Mixture	85.00±1.20[bde]
Normal Dose Yoyo	88.00±3.8[bde]
Medium Dose Yoyo	84.00±1.40[bde]
High Dose Yoyo	75.00±2.60[bde]

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

Results of the histological analysis done on the rats fed combined high-salt and high-fat diet

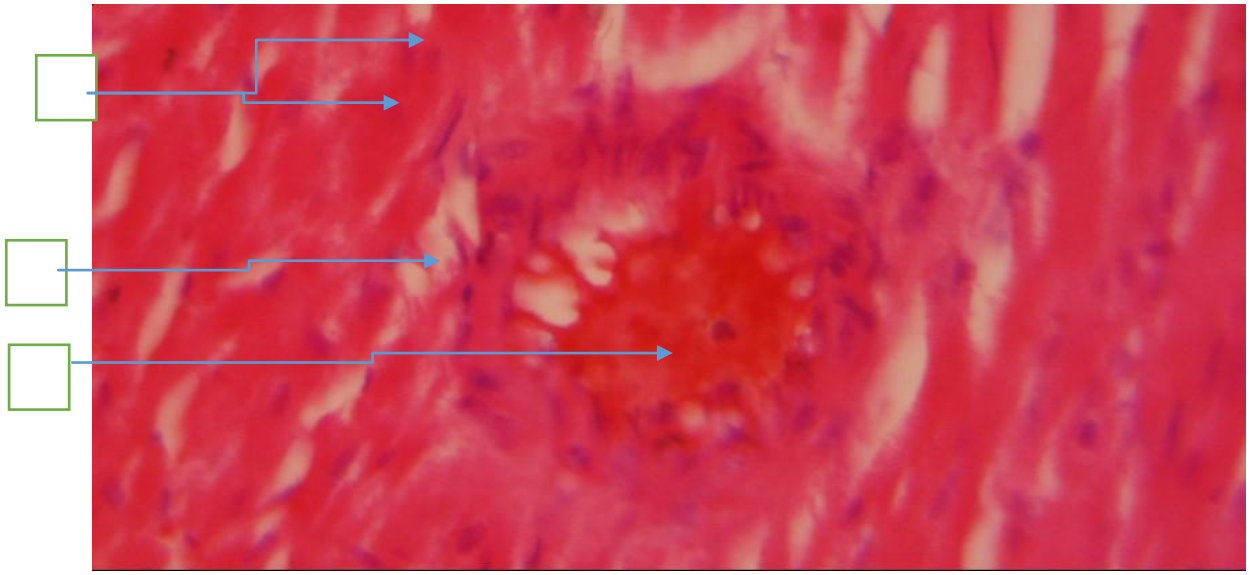

Plate 1. Heart of Control rat for the combined diet groups, showing, normal A: myocardial fibres, B, interstitial space and C: coronary artery (H&E x 400)

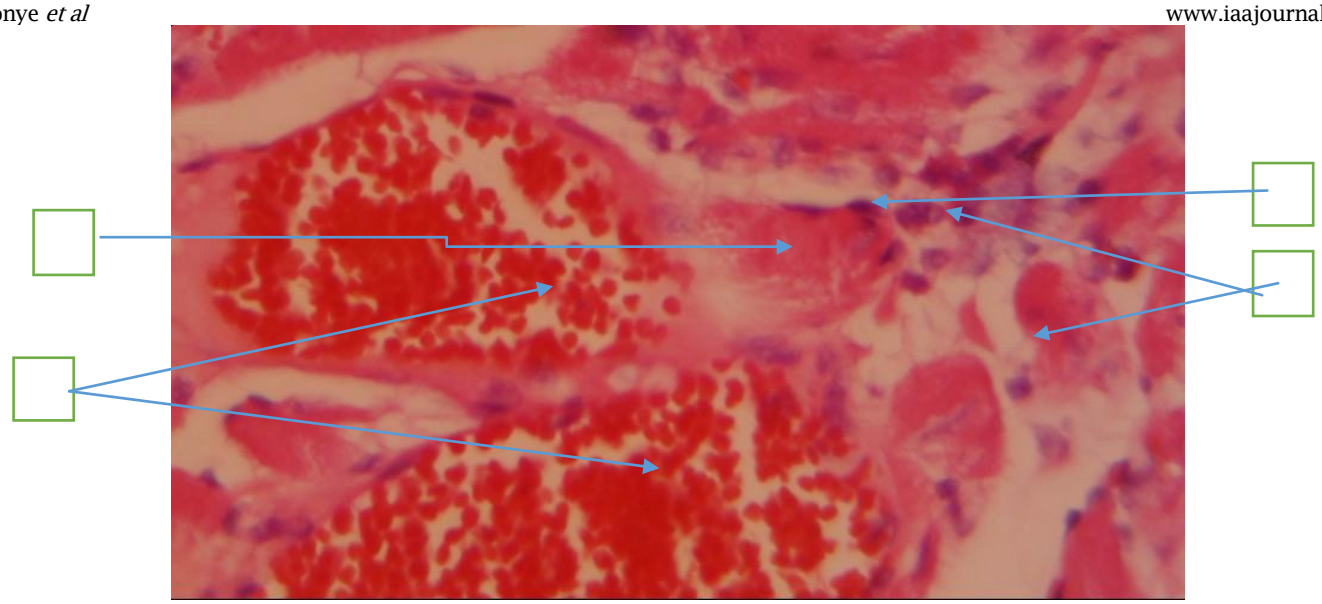

Plate 2. Heart of rat fed a combined diet of high-fat and high-salt, showing A: vascular stenosis, B: perivascular inflammatory infiltrates, C: focal myocardial degeneration and D: vascular congestion (H&E x 400)

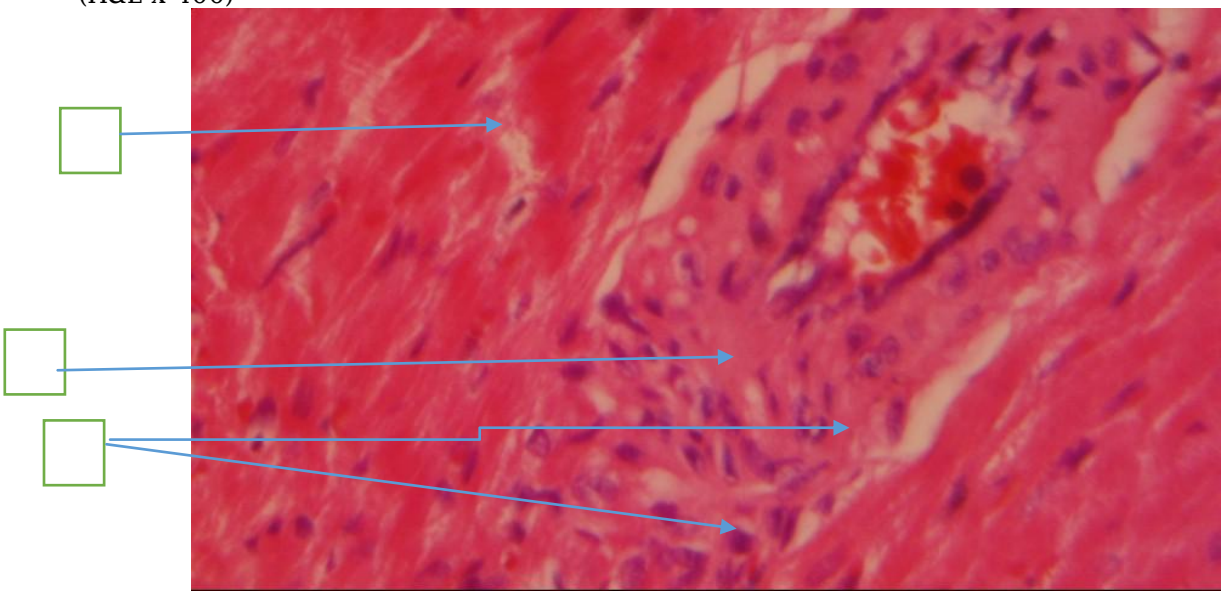

Plate 3. Heart of rat given the combined diet plus Atorvastatin. A: normal myocardial fibres, B: asymmetric hypertrophy and C: perivascular inflammatory infiltrates (H&E x 400).

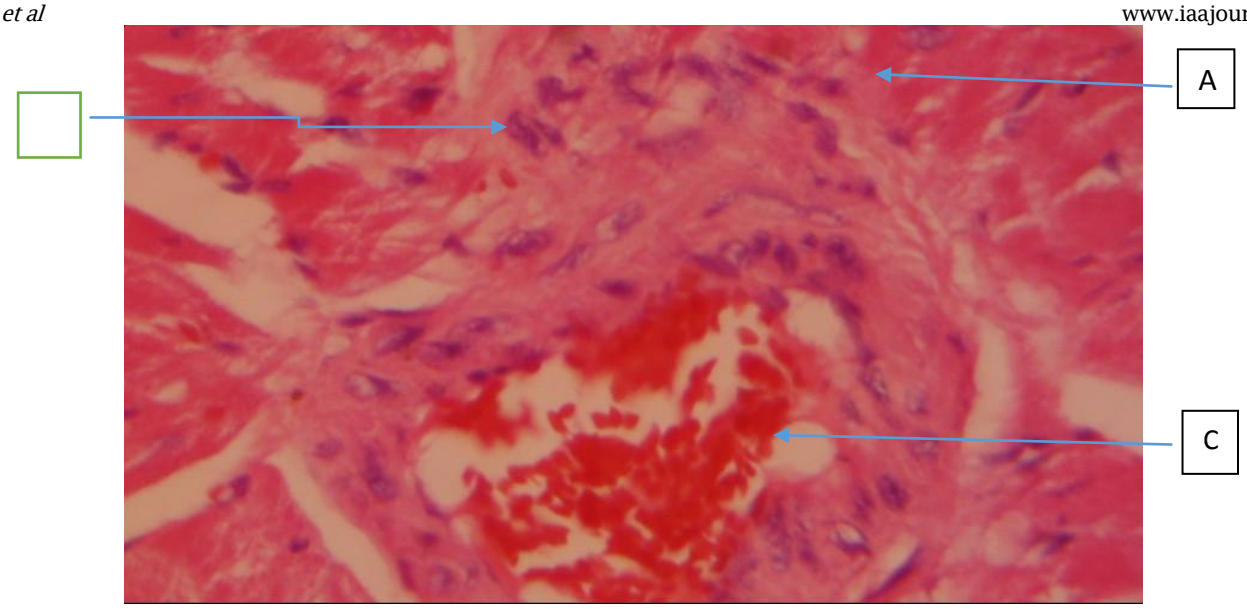

Plate 4. Heart of rat given the combined diet plus 600mg/kg Yoyo bitters. A: normal myocardial fibres, B: perivascular inflammatory infiltrates and C: near normal vasculature with active congestion and resolving patchy vascular ulceration (H&E x 400)

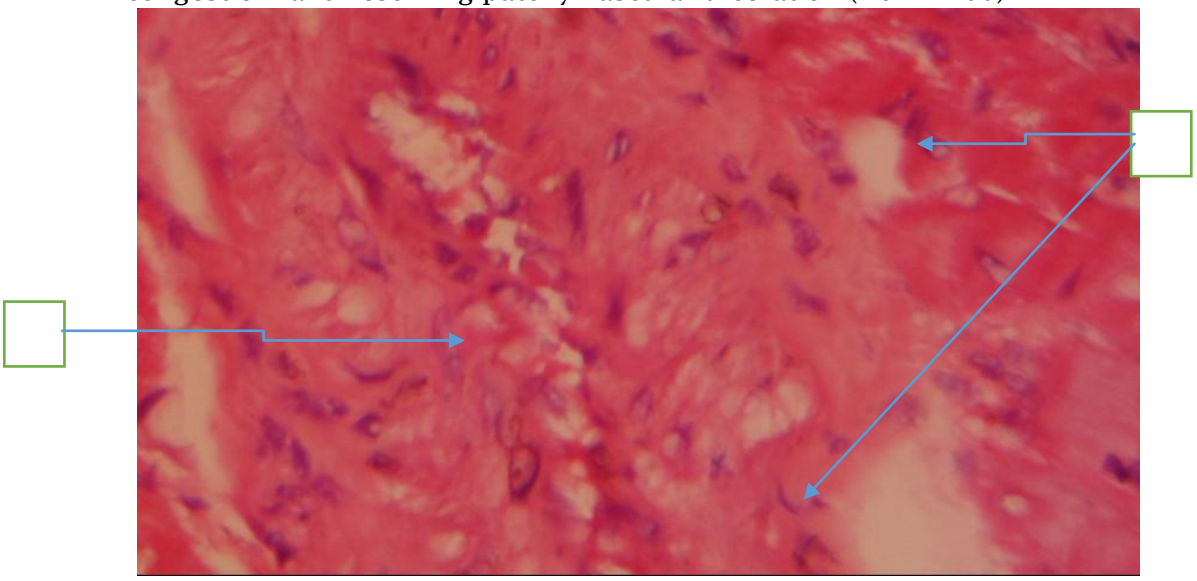

Plate 5. Heart of rat given the combined diet plus 1,100mg/kg Yoyo bitters. A: normal myocytes and areas of focal myocardial degeneration and B: unresolved vascular constriction/severe vascular stenosis (H&E x 400)

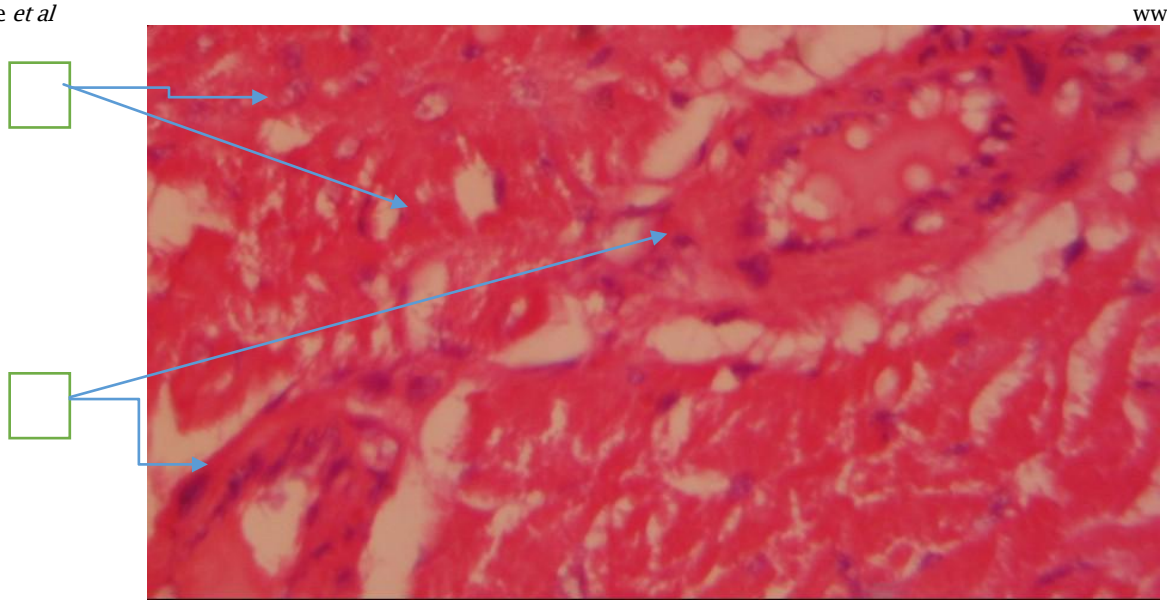

Plate 6. Heart of rat given the combined diet plus 2,200mg/kg Yoyo bitters. A: normal myocardial fibres and B: some unresolved vascular distortions (H&E x 400)

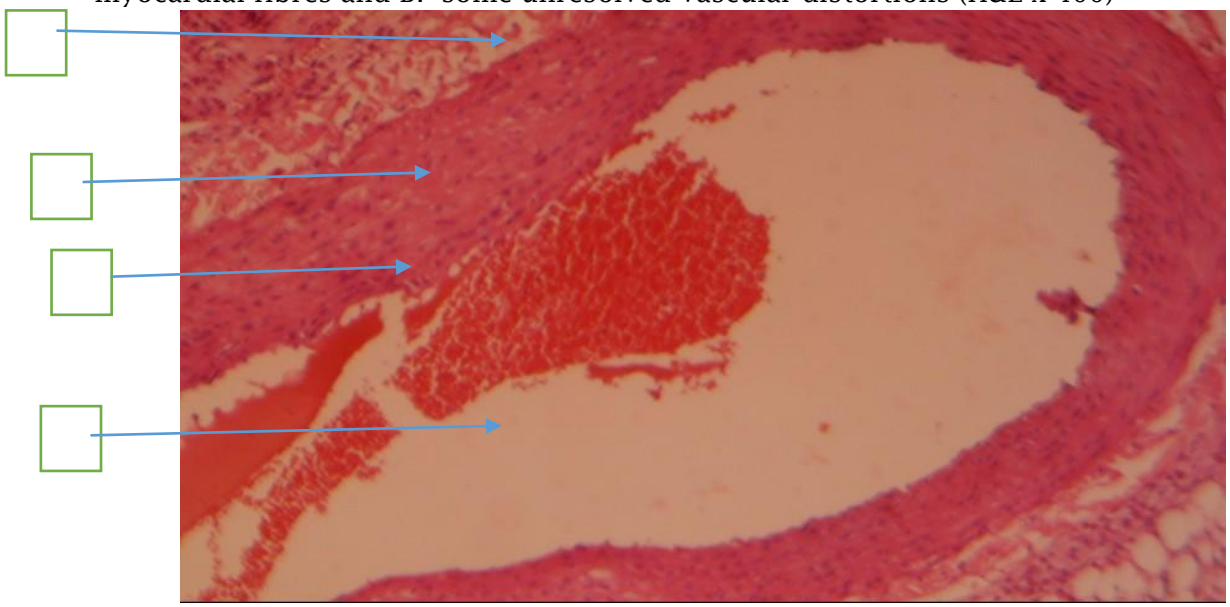

Plate 7. Aorta of Control rat for the combined diet groups. A: Tunica adventitia, B: media, C: intima and D: lumen (H&E x 100)

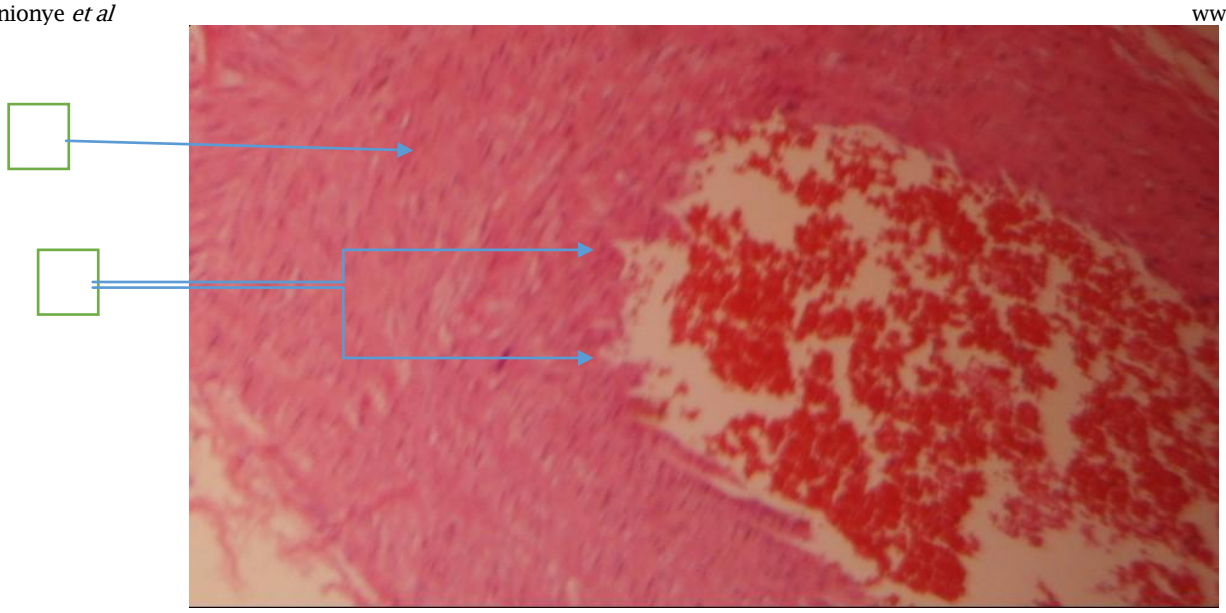

Plate 8. Aorta of rat fed the combined diet. A: Marked asymmetric media hypertrophy and B: intimal erosion (H&E x 100)

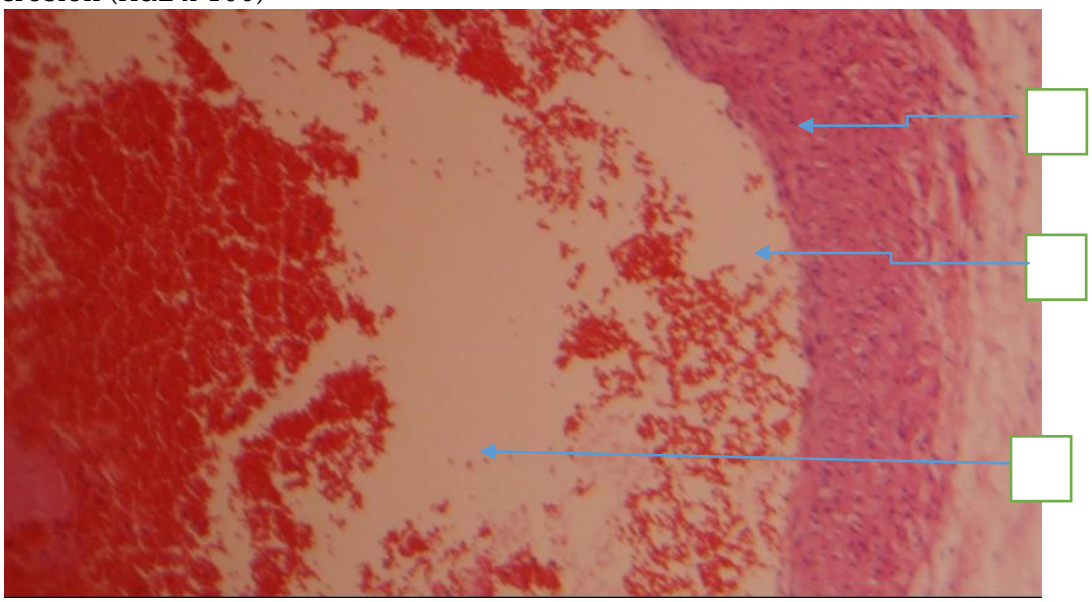

Plate 9. Aorta of rat given the combined diet plus Atorvastatin. A: normal media and B: normal intima and C: luminal dilatation (H&E x 100)

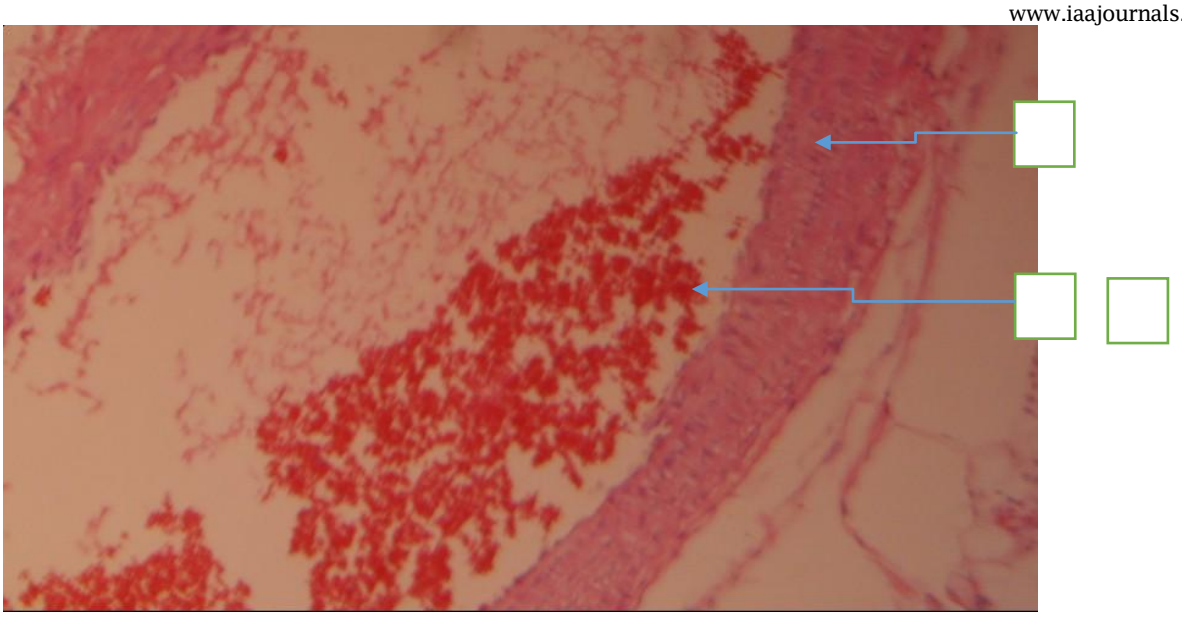

Plate 10. Rat given combined diet plus 600mg Yoyo bitters. A: normal tunica media and B: normal intimal microstructure (H&E x 100)

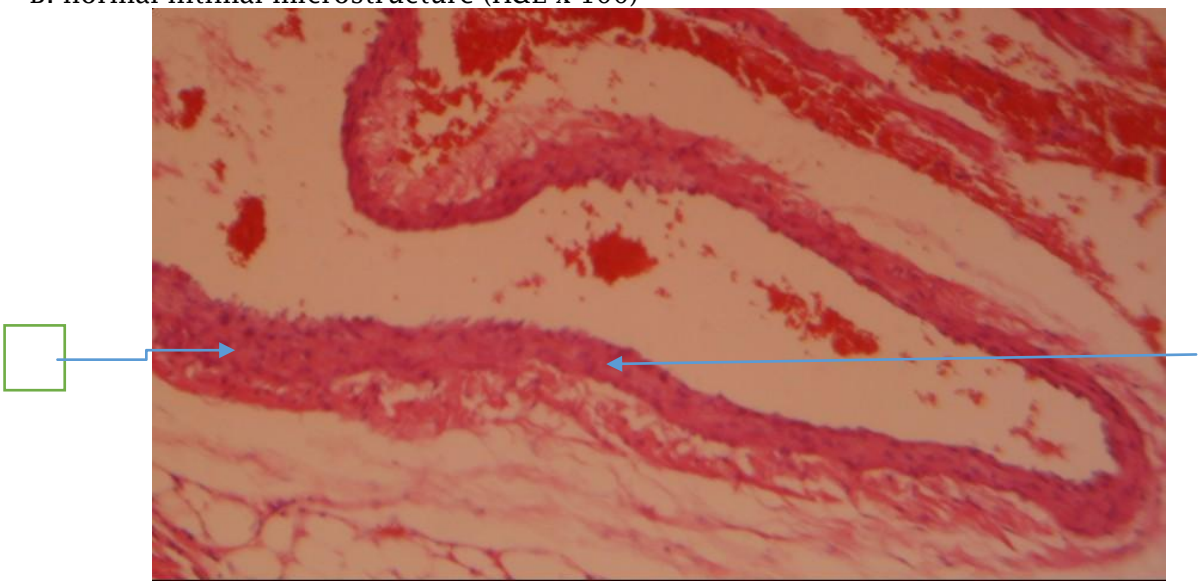

Plate 11. Aorta of rat given combined diet plus 1,100mg/kg Yoyo bitters. A: normal tunica media and B: normal intimal microstructure (H&E x 100)

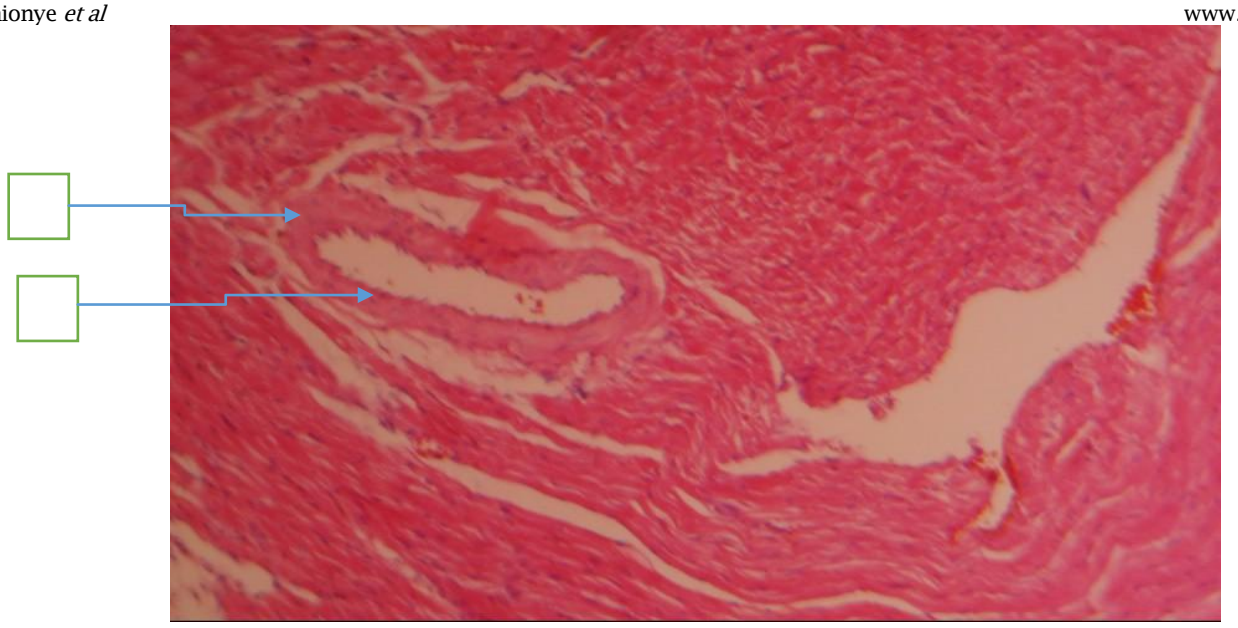

Plate 12. Branch of Aorta of rat given the combined diet plus 2,200mg/kg Yoyo bitters. A: normal tunica media and B: normal intimal microstructure (H&E x 100)

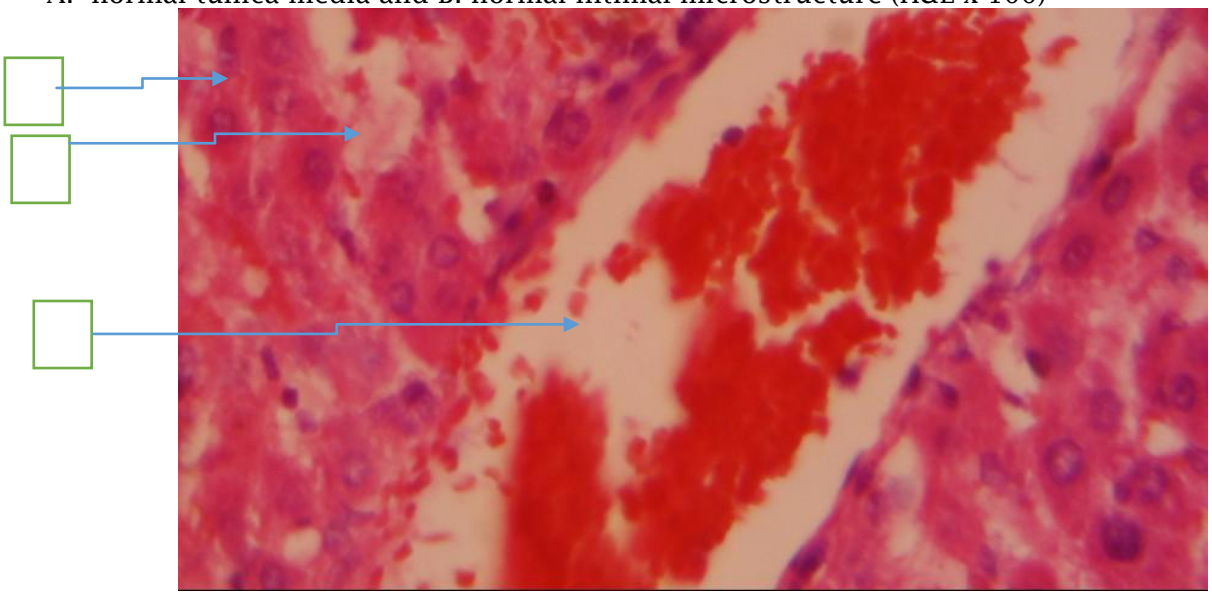

Plate 13. Liver of Control Rat for the combined diet groups. Control. A: Hepatocytes, B: sinusoids, C: Portal vein (H&E x 400).

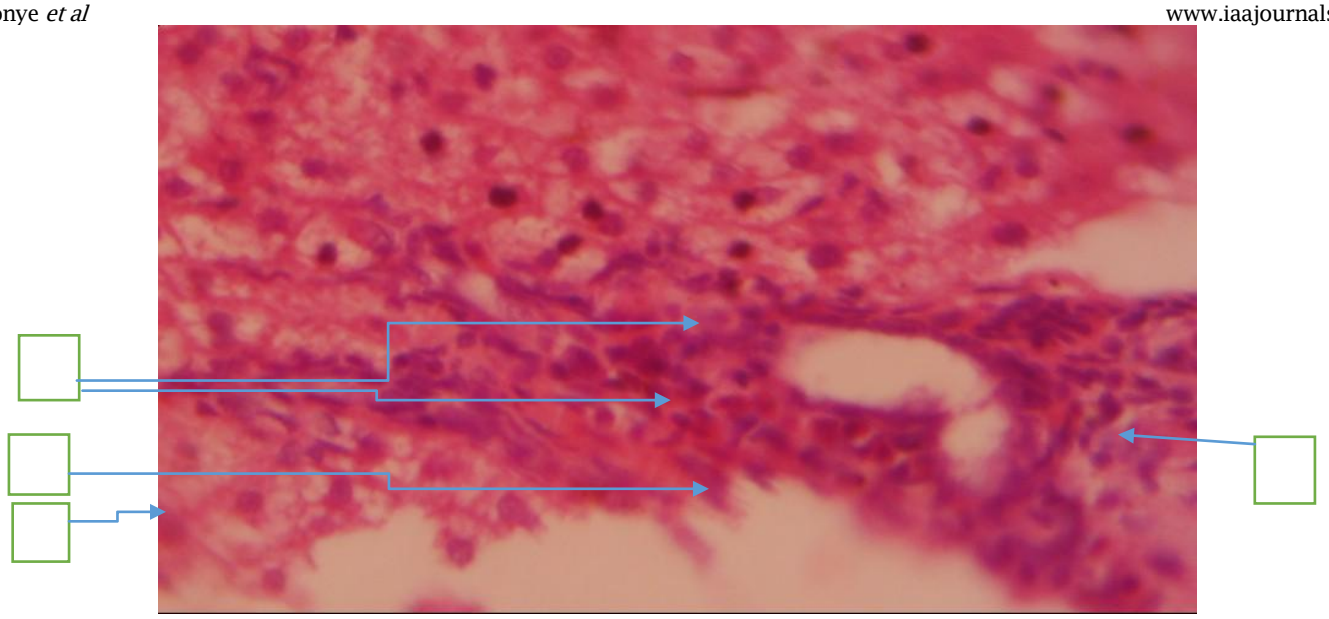

Plate 14. Liver of rat fed the combined diet of high salt and high fat, showing. A: Periportal inflammatory infiltrates. B: vascular ulceration and C: congestion with D: hepatocyte degeneration (H&E x 400)

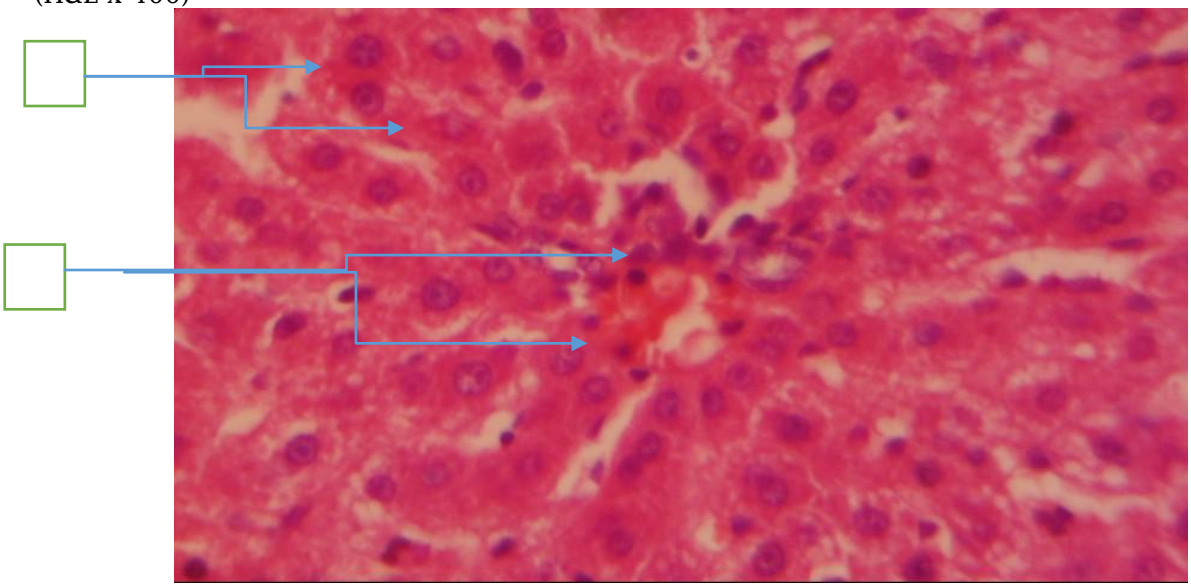

Plate 15. Liver of rat given combined diet plus Lisinopril-Atorvastatin. A: normal hepatocytes and B: normal portal microstructure (H&E x 400)

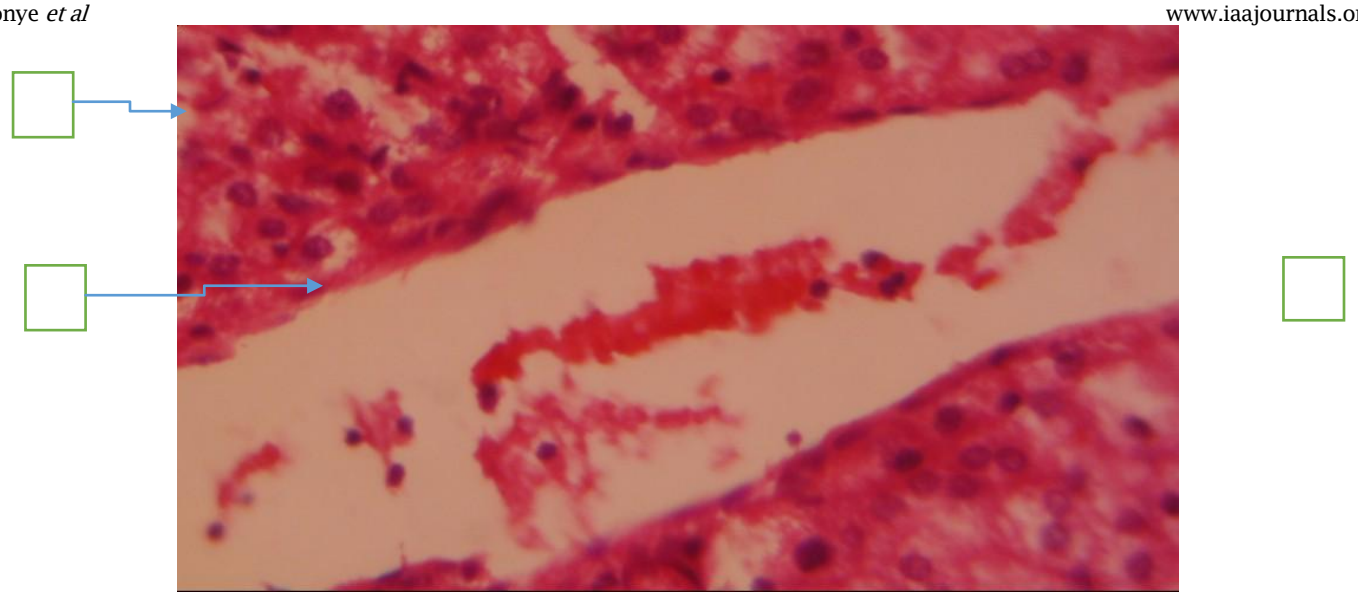

Plate 16. Liver of rat given the combined diet plus 600mg/kg Yoyo bitters. A: normal hepatocytes and B: normal portal microstructure (H&E x 400)

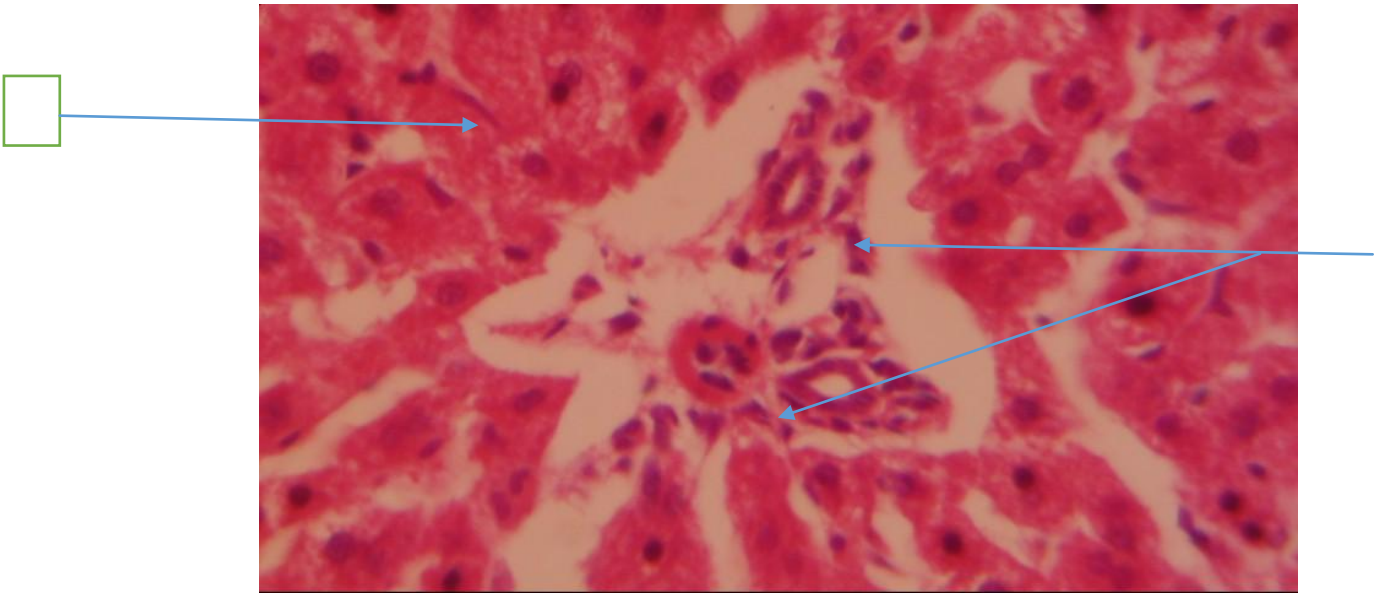

Plate 17. Liver of rat given the combined diet plus 1,100mg/kg Yoyo bitters. A: normal hepatocytes and B: normal portal microstructure with mild periportal inflammatory infiltrates (H&E x 400)

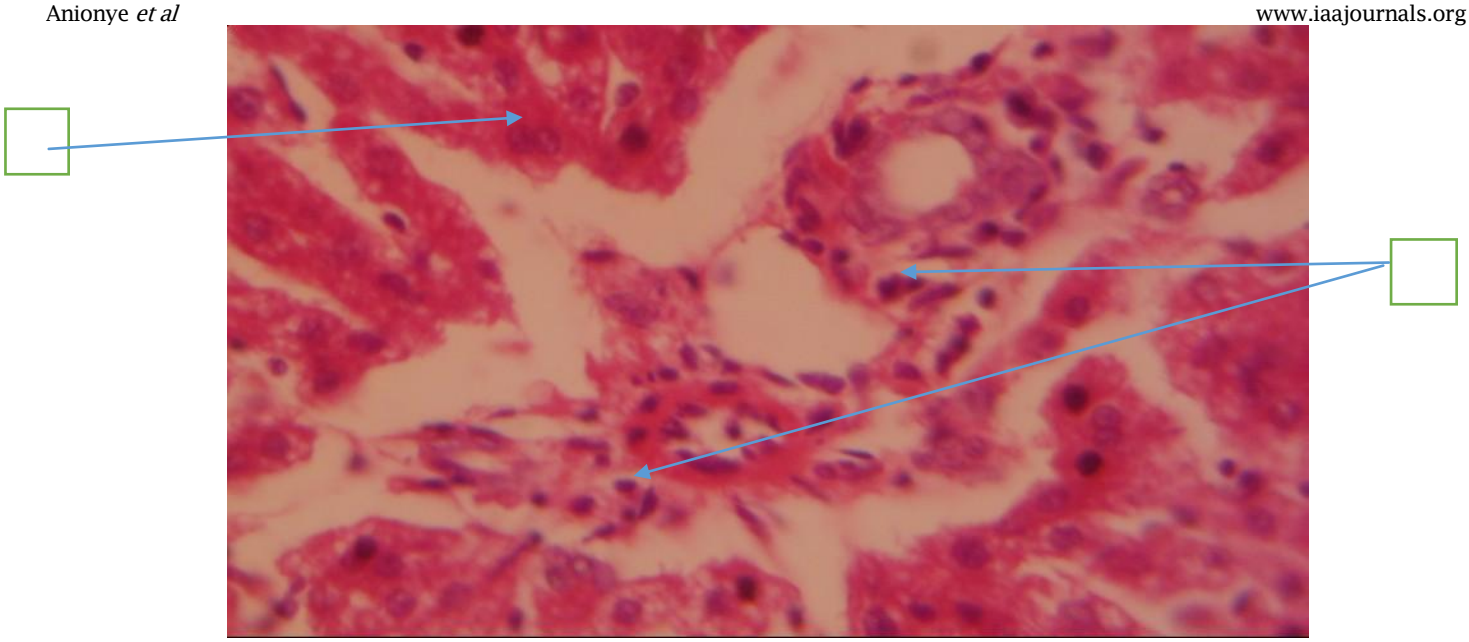

Plate 18. Liver of rat given the combined diet plus 2,200mg/kg Yoyo bitters. A: normal hepatocytes and B: normal portal microstructure with mild periportal inflammatory infiltrates (H&E x 400)

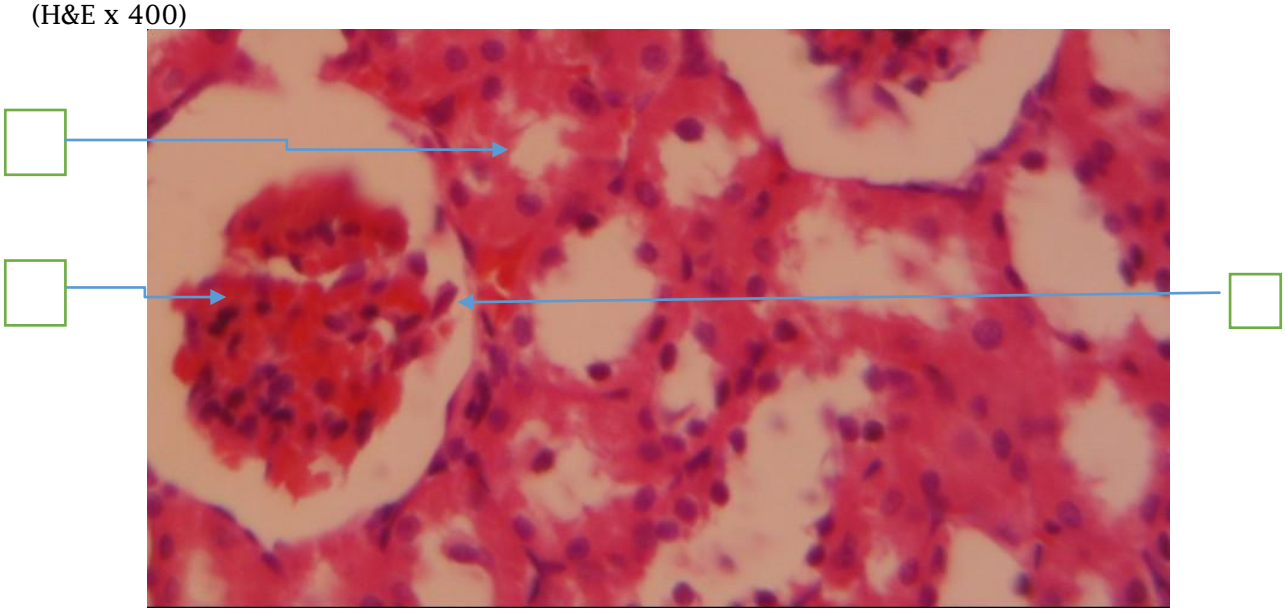

Plate 19. Kidney of Control Rat for the combined diet groups. A: Tubules, B: glomerulus and C: interstitial space (H&E x 400)

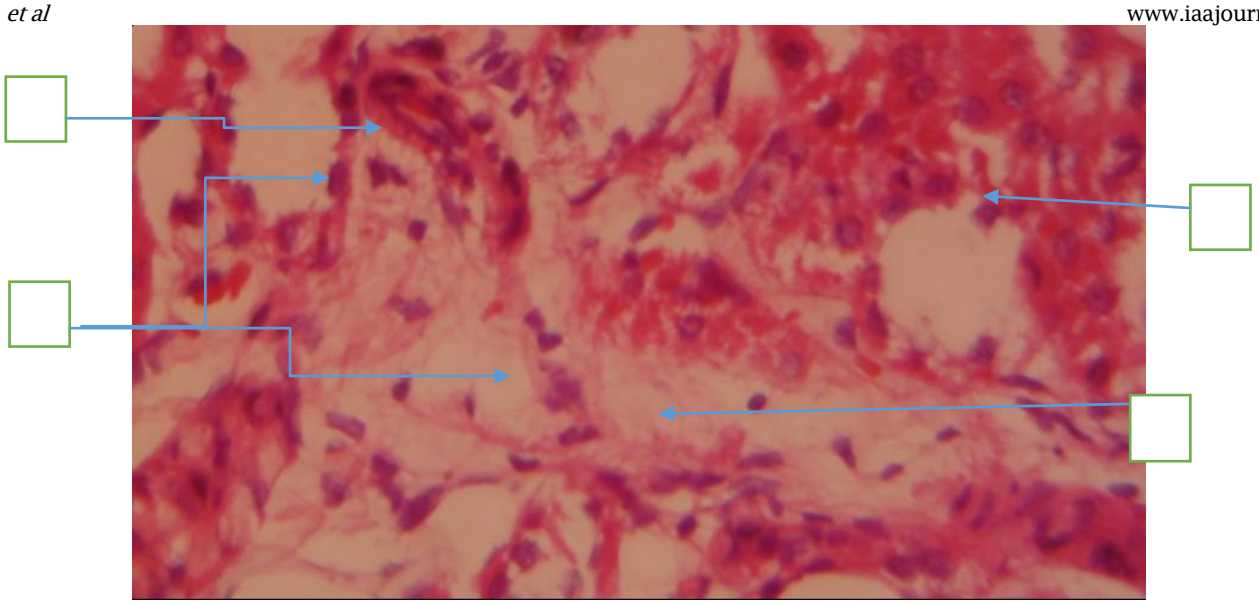

Plate 20. Kidney of rat fed the combined diet. A: distorted vascular architecture/ vasoconstriction. B: perivascular inflammatory infiltrates and C: interstitial congestion D: tubular necrosis and oedema (H&E x 400)

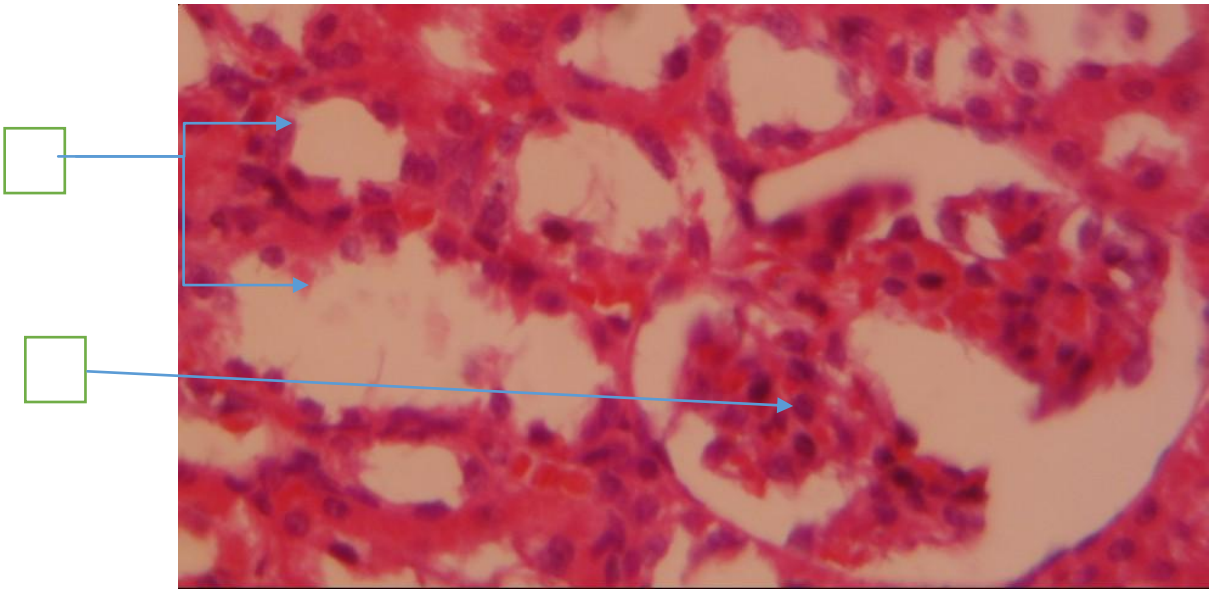

Plate 21. Kidney of rat given the combined diet plus combined drug of lisinopril and atorvastatin. A: normal tubular and B: normal glomerular architecture (H&E x 400)

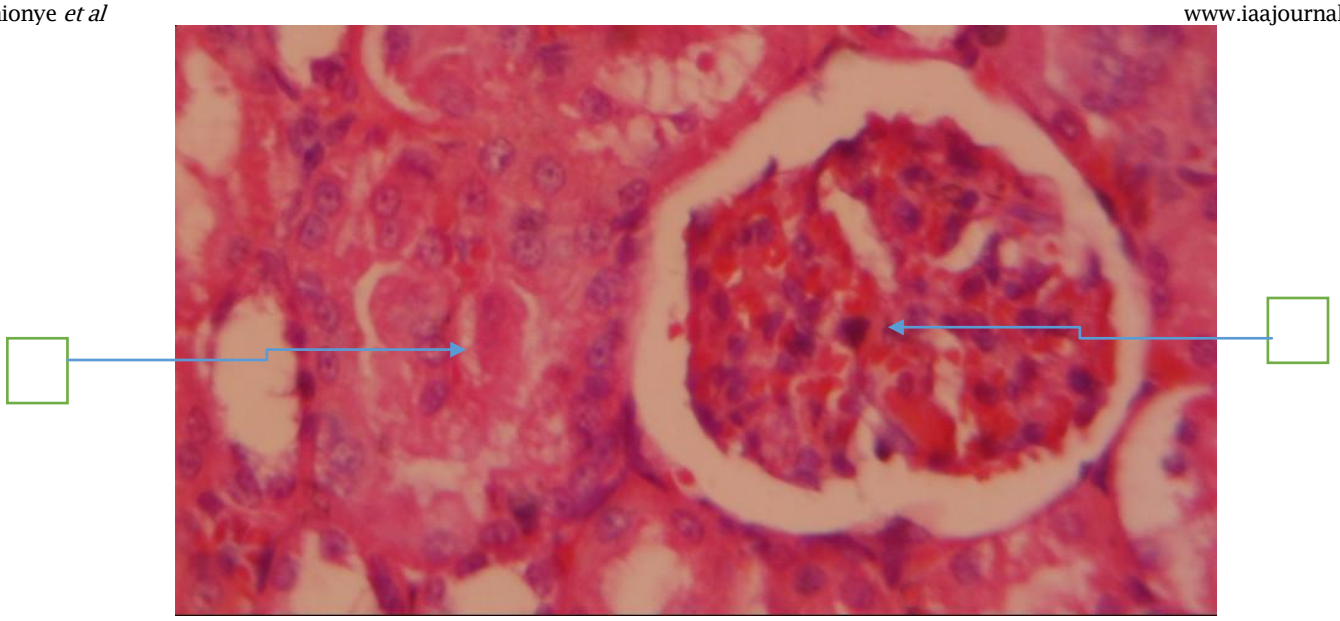

Plate 22. Kidney of rat given the combined diet plus 600mg/kg Yoyo bitters. A: resolving focal tubular necrosis and B: normal glomerulus (H&E x 400)

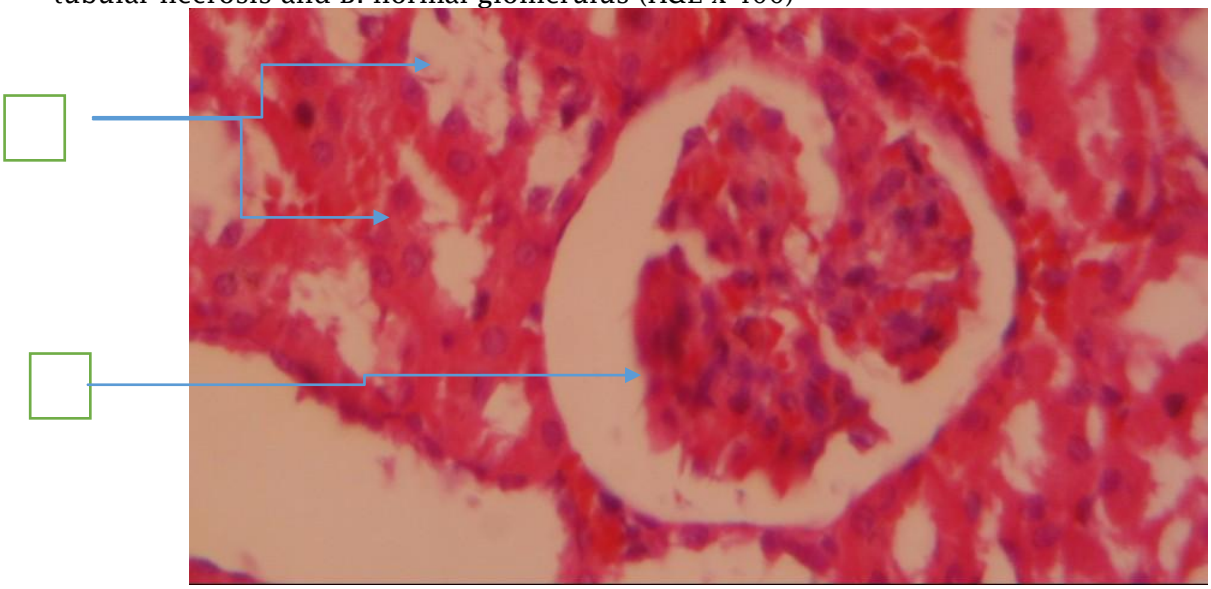

Plate 23. Kidney of rat given the combined diet plus 1,100mg/kg Yoyo bitters. A: normal tubular and B: normal glomerular microstructure (H&E x 400)

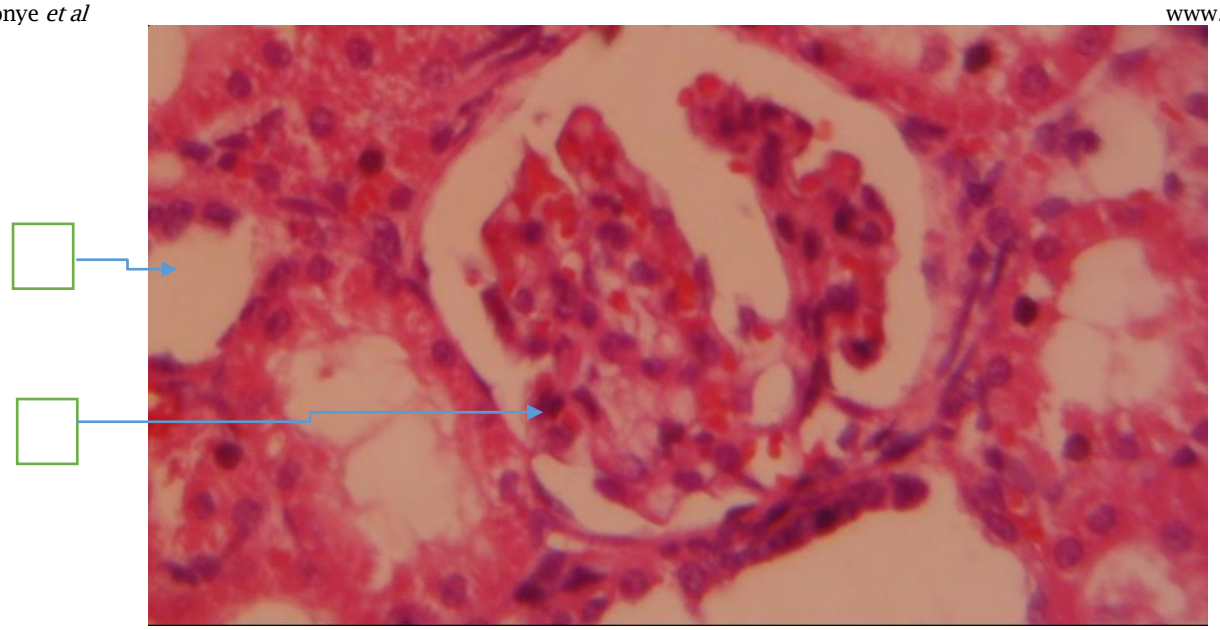

Plate 24. Kidney of rat given the combined diet plus 2,200mg/kg Yoyo bitters. A: normal tubular microstructure and B: normal glomerular microstructure (H&E x 400).

DISCUSSION

Cardiovascular diseases have become an unending scourge to humanity with the developing world where Nigeria belongs being the worst hit [12]. Considering the fact that the World Health Organisation (WHO) has been routing for local preventive measures to these debilitating cardiovascular diseases in sub-Saharan Africa, this study was designed to evaluate the therapeutic potential of Yoyo cleanser bitters in preventing cardiovascular diseases in male albino *Wistar* rats fed a combination of a high-salt and a 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 the herbal bitters will be beneficial in the fight against the scourge of these cardiovascular diseases by ameliorating the inflammation, endothelial dysfunction, oxidative stress, insulin resistance/changes in blood glucose and 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, This study also was designed to compare the effect and relative efficacy of Yoyo cleanser bitters to the anti-hypertensive drug (lisinopril, an ACE-inhibitor) and the anti-hypercholesterolaemic drug (atorvastatin, one of the Statins) given in combination to rats fed a combined high-salt and high-fat diet.

As reflected in Tables 3, the combined diet caused a significant ($p<0.05$) decrease in the food efficiency in the rats despite no significant difference ($p>0.05$) in the mean feed consumed per day between the normal control rats and the rats fed the combined diets respectively. This decreased food efficiency is expressed in a significant ($p<0.05$) weight loss per day for all the groups of rats fed the combined diet. The combined drug and both bitters seem not to have an effect on the food efficiency and daily weight loss, as there was no significant change ($p>0.05$) when the values for food efficiency and the feed consumed per day in their respective groups was compared with the values in the combined diet fed rat groups. The bitters were generally well tolerated as they did not lead to a drastic reduction in food consumption, thus giving credence to the claim that some bitters increase rather than decrease appetite [11]. Since the daily feed consumed by the animals remained the same despite the likely unpalatability of their meals caused by their increased salt and fat content, there may be some innate property in Paxherbal and Yoyo cleanser bitters that contributes to the appetite of the experimental animals that encourages them to continue consuming the meal despite its possible unpalatability. The observed weight loss and the

cause of the decrease in food efficiency was likely caused by the high-salt in the diet concerned. Zhao and his research team [14], explained the weight loss seen in diets high in salt as seen in the combined diet of this study. According to them, the high sodium intake promotes urinary sodium excretion and glycosuria when it stimulates one of the receptors in the family of peroxisome proliferator activated receptors (PPARs), called peroxisome proliferator activated receptor-δ (PPARδ) in adipose tissue. This inhibits sodium-glucose co-transporter 2 (SGLT2) responsible for facilitating glucose reabsorption in the renal tubules leading to loss in glucose and sodium. It also promotes increased plasma adiponectin levels with consequential fatty acid mobilization, breakdown and β-oxidation. All these set the stage for making the observed weight loss, a fait accompli.

The combined diet caused a significant ($p<0.05$) increase in the relative organ weights of the liver, the kidneys, the heart and aorta of the rats, as reflected in Tables 4. Generally the combination of lisinopril and atorvastatin and the Yoyo cleanser bitters prevented this increase in these organs, but only that of the heart and aorta was significant ($p<0.05$). This increase in organ weights is a deleterious effect of high-salt and high-fat diets [3, 4]. The result of this study is consistent with earlier studies, where oxidative stress and inflammatory factors from salt and fat have been shown to cause organ fibrosis, hypertrophy and increase in organ weight [13, 18]). It is also consistent with the assertion of [23] that substances with anti-inflammatory and anti-oxidant properties can prevent salt and fat induced damage of cells of the liver and heart, as well as treat them. This thus partly explains why polyherbals with antioxidant and anti-inflammatory properties like is known of Yoyo cleanser bitters can counter the ill effects of a deleterious increase in organ weight caused by a combined high-salt and high-fat diet. The fact that the bitters of this study can prevent or retard the high-salt and high-fat diet induced increase in relative weight of organs, is therefore a positive finding of this present study.

Table 5 shows that the combined diet caused a significant increase ($p<0.05$) in the fasting blood glucose levels in the rats. This is in keeping with earlier studies carried out by [15], [19], [17] and [13]. Their studies indicate that high-salt and high-fat diets induces a kind of insulin resistance usually expressed as an increase in blood sugar level. Generally, the combined drug and the bitters significantly prevented the increase in the blood glucose level ($p<0.05$). This study showed that Yoyo cleanser bitters is as effective as atorvastatin and lisinopril drug combination in inhibiting the high-salt and high-fat diet induced insulin resistance usually expressed as a rise in the level of blood glucose. The ability of the bitters to prevent this insulin resistance and thus resist the elevation of blood sugar is essential because lowering blood sugar levels reduces the risk of coronary heart disease [20]. The ability of the bitters to achieve this is not far-fetched considering the fact they contain plants/herbs with phytochemical and mineral constituents which are known to have anti-diabetic properties [10]. Examples of plants with known blood glucose level lowering properties, contained in the bitters of this study include *Aloe vera, Vernonia amygdalina* and *Gangronema latifolium,* just to mention but a few, [6, 21]. They act synergistically to bring down or prevent a rise in the blood glucose level [15].

The histopathological evaluation of the aorta tissue as shown in Plates 1-6, reveal that the high-salt and high-fat diets cause severe intimal ulceration and mural atrophy at one end as well as asymmetrical vascular constriction and asymmetric media hypertrophy on the other end. The bitters in a dose dependent manner prevented the observed changes to a large extent. They tried to maintain a normal mural architecture and protective vascular dilatation with active congestion. The drugs and Yoyo cleanser bitters exhibited similar efficacy. These findings confirm earlier postulations by [15] that the bitters of this study have “vasculo-protective properties”.

The histopathological evaluation of the heart fed the combined diet as shown in Plates 7-12, reveal that the drugs and bitters in a dose-dependent manner tried to limit (even though not completely) the distortion in the interstitial tissue and vascular architecture, prevent the accumulation of perivascular inflammatory infiltrates and maintain normal myocardial fibres by encouraging active congestion with red blood cells and vascular dilatation. Doing this, they reduced the toxic effect of a high-salt diet and high-fat diet on the heart tissue. This aligns with the possible non-toxic and cardio-protective property postulated earlier about the bitters of this study by [17].

The histopathological evaluation of the liver of rats fed the combined high-salt and high-fat diet

as shown in Plates 13-18, reveal that the diet induces inflammatory features in the liver of the rats characteristic of a developing non-alcoholic steatotic hepatitis (NASH) otherwise known as non-alcoholic fatty liver disease (NAFLD). They cause features of developing damage to the hepatocytes (causing degeneration or devitalisation of the hepatocytes and giving them a ground glass appearance), but also cause severe vascular ulcerations and sinusoidal distortions/congestion. Significant also is the invasion or infiltration of the periportal areas with inflammatory cells in keeping with their activation by the injury promoted by the hypercholesterolaemia/dyslipidaemia and fatty liver that result from the constant consumption of the high-salt and high-fat diet. The bitters in a dose-dependent manner prevented these changes to a large extent. They tried to maintain normal hepatocytes, sinusoids, bile ducts, normal vascular microstructure and reduced the level of periportal infiltration by inflammatory cells. The lisinopril-atorvastatin drugs and the Yoyo cleanser bitters have similar efficacy in preventing the combined diet induced changes. These observed features are again in keeping with earlier postulations of possible hepato-protectivity ascribed to the bitters of this study by [16].

The histopathological evaluation of the kidney of rats fed the combined high-salt and high-fat diet as shown in Plates 19-24, reveal that the diet cause mild to severe vascular alterations and distortions in the interstitial space evidenced by vascular obstruction/constriction and mild interstitial inflammatory infiltrates. They also cause interstitial congestion, tubular necrosis and oedema. The lisinopril-atorvastatin drug combination, and the Yoyo cleanser bitters have similar potency. The bitters in a dose-dependent manner prevented the observed changes to a large extent. They achieved this by resisting the toxic effects of the salt and fat and in so doing kept the tubules, glomeruli and vasculature within their normal architecture, and were in the process of resolving the observed focal tubular necrosis. These reno-protective actions are again in keeping with earlier postulation of possible reno-protective properties of the bitters of this study by [15].

CONCLUSION

This study set out to evaluate the therapeutic potential of Yoyo cleanser 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. Following the idea that our diet lacking in bitters has resulted in a 'bitter deficiency syndrome" which makes us prone to diseases especially metabolic and cardiovascular diseases, it was therefore also a desire of this study to ascertain the effect of daily supplementation of bitters like Yoyo cleanser bitters in our diet. This study revealed that Yoyo cleanser bitters have similar efficacy as the lisinopril and atorvastatin drug combination in preventing the negative effects of hypertension and hypercholesterolaemia. The therapeutic action of Yoyo cleanser bitters, showed that it was able to prevent the development of diet induced insulin resistance, as well as possessing vasculo-protective, cardio-protective, reno-protective and hepato-protective properties.

Considering all these findings, coupled with the fact that they are all factors associated with protection against a wide range of cardiovascular diseases, it can therefore be concluded that the bitters of this study if extrapolated to man, may be a good therapeutic agent, as a dietary supplement in the prevention and management of cardiovascular diseases.

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