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Original Article,

Soybean Phytoestrogen-Rich Extract: A Novel Approach For Lipid Regulation In 4-Vinylcyclohexane Diepoxide-Induced Menopausal Albino Rats

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Abstract

Background/Objective: Atherosclerosis is a key feature in the pathophysiology of arterial forms of cardiovascular disease, and these compounds (isoflavones) may serve as both shields against the disease and disruptors of already-formed plaques. The study aimed to seek for novel approach and alternative way rather than use of statin in lipid regulation in 4-vinylcyclohexane diepoxide-induced menopausal albino rats.
Methods: Thirty (30) female albino Wistar rats were employed in the investigation and each one of the experimaental animal was induced with 80mg/kg of 4-vinylcyclohexene diepoxide before being treated with either normal estradiol therapy (14ug/kg) or varying concentrations of the soybean phytoestrogen-rich extract (200 mg/kg, 400 mg/kg, and 600 mg/kg). The levels of total cholesterol, triglyceride, high density lipoprotein and low density lipoprotein were all measured spectrophotometrically. Statistical software SPSS (IBM) version 23.0 was used to analyze the data.
Results: Results showed dose-dependent reductions in lipid profile levels were seen across all treatment groups ($p<0.05$) with soybean phytoestrogen rich extract. Treatment with phytoestrogen isoflavones significantly decreased high density lipoprotein (HDL) levels in a dose-dependent manner, with no discernible difference between 400mg/kg and 600mg/kg. Except at a concentration of 14ug/kg of estradiol therapy.
Conclusion: Data from this research clearly demonstrate that soybean phytoestrogen-rich extract have increased lipolytic effect on hyperlipidaemia induced with 4-vinylcyclohexene diepoxide in menopause-induced female Wistar rats. Soybean phytoestrogen-rich extract therapy in a high-dose appears to be more effective in lowering lipids compared to hormone replacement therapy as an alternate source of estrogen in treatment of cardiovascular diseases

Keywords: Phytoestrogens, Menopause, Soybean extract, Lipid profile, Atherosclerosis

Introduction:

Atherosclerosis is a key feature in the pathophysiology of arterial forms of cardiovascular disease, and these compounds (isoflavones) may serve as both shields against the disease and disruptors of already-formed plaques. The discovery that the prevalence of cardiovascular disease is lower in Asian communities with high levels of consumption of these phytoestrogens than in those in Western countries lends credence to the idea that phytoestrogens may function in a similar fashion [1] Soybean isoflavone, a C6-C3-C6 phenolic molecule, mimics the structure of the female hormone estrogen. It alleviates several symptoms associated with menopause, including high cholesterol, and an increased risk of cardiovascular disease. When ovaries stop producing eggs, hormone levels shift, causing a variety of unpleasant side effects for women going through menopause. As a result, women experience an increase in the prevalence of conditions like cardiovascular disease in addition to symptoms including hot flashes, night sweats, sleeplessness, genital atrophy, disturbed sleep, and anxiety. Menopause syndrome is a collection of distinct symptoms that occur at the same time[2]. For example, estrogens and phytoestrogens have been recommended as a means of lowering the risk of and death from cardiovascular disease because of the effect they have on ovarian function [3]. Obesity, fat distribution, plasma lipid profile, plasma rheology, and platelet function may all be affected by the drop in estrogen that occurs during menopause[4]. These findings provide supporting evidence for the hypothesis that phytoestrogens can mitigate the risk of cardiovascular disease in women caused by estrogen shortage. The low prevalence of cardiovascular illnesses in Asian cultures with a diet rich in soy is more evidence in favour of this theory[5,6]. However, this protective effect is lost among those who have emigrated to Western nations. Tokede *et al*. (2015) revealed the findings of a meta-analysis showing that isoflavone supplementation did not alter serum lipid profiles[7].

Researchers have found that estrogens have varying effects on atherosclerosis and its associated clinical outcomes[8,9]. The primary evidence on the effects of isoflavones on the cardiovascular system, from both the laboratory and the clinic, has been reviewed by Cano *et al*. (2010)[9] and Gonzalez and Duran (2014)[1]. According to previous studies, two clinical trials have looked at the connection between isoflavone intake and clinical cardiovascular events in a population without cardiovascular illness at baseline[10,11]. One study found that Japanese women, particularly postmenopausal women in particular, had a lower risk of stroke and heart attack when they consumed a large amount of isoflavones[11]. The second study looked at the relationship between low phytestrogen intake and cardiovascular disease risks in a Western population[10]. There is no evidence that any phytoestrogen studied protects against cardiovascular disease in humans[12]. All things considered, it is unclear whether or not early postmenopausal administration of phytoestrogens offers protection against cardiovascular disease. It is plausible that isoflavones do not recapitulate the potential effect of estrogens on the risk for stroke,[9] however the data is less for phytoestrogens than it is for estrogens.

Consuming soy isoflavones in their whole, natural form in soy protein is necessary for their beneficial effects on cholesterol levels. Small but significant alterations were documented in normocholesterolemic and moderately hypercholesterolemic women[13,14], although the effect was largest in hypercholesterolemic women. Even though soy[15] isoflavone supplements may not reduce plasma cholesterol, they may have other beneficial effects on the cardiovascular system, such as enhanced arterial compliance. The prevalence of cardiovascular disease especially heart attack among Nigeria called for the need to sought for alternative way and novel approach to ameliorate and regulate lipid profile, the risk factor of atherosclerosis. The study aimed to seek for novel approach and alternative way rather than use of statin in lipid regulation in 4-vinylcyclohexane diepoxide-induced menopausal albino rats.

Materials and methods:

Experimental Animals

This is an experimental study design conducted at the Faculty of Pharmacy laboratory and animal house, University of Benin, Benin City. It involved thirty (30) females matured (aged 6-8 weeks) Wistar rats (*Rattus norvegicus*) weighing between 120 and 240g, which was menopause induced with 4-vinyl cyclohexane diepoxide. The

animals were kept at the animal house at the University of Benin, Benin City for two weeks to stabilize before the experiments began. The Wistar rats were given a conventional rodent cube diet from Ewu feeds and flour mills limited Ewu, Edo state, Nigeria, and have free access to water *ad libitum.* Before each experiment, all animals were fasted overnight. Because of their ability to mimic the symptoms of perimenopause and postmenopause in humans—such as estrous acyclicity and fluctuating, then undetectable, estrogen levels—Wistar rats were chosen for this study to enable the separation of the impact of hormone levels from that of ageing. The study aimed to seek for novel approach and alternative way rather than use of statin in lipid regulation in 4-vinylcyclohexane diepoxide-induced menopausal albino rats.

Ethical Consideration

The approval for the study was sought from the Animal Studies Ethic Review Committee of the Faculty of Pharmacy, Department of Pharmacology and Toxicology, University of Benin, Benin City. The approval was given after experimental protocols (where animals were housed and cared for under natural lighting conditions). Because some of the Laboratory investigations were done at the Federal University of Technology, Akura, a second Ethical approval and clearance was obtained from the ethics and research committee of Federal University of Technology Akure, Ondo (protocol number FUTA/ETH/21/14).

Soyabean Purchase, Authentication and Preparation of Soybeans Flour

The soybeans were purchased from the Oba market in Benin-City, and were authenticated by a plant taxonomist at the Department of Plant Biology and Biotechnology (PBB) laboratory, University of Benin, Benin City, and was given a voucher number (UBH-G628).

In preparing the flour, the soybean seeds were carefully picked, separated from debris and rinsed in water. After washing, the grains were transferred to a large, clean bowl, and left to soak overnight. The soybean chaff was washed off, drained to eliminate as much water as possible, and dried in the sun until they were completely dried. The grains were heated in a frying pan over medium heat, and stir until they turned brown, but being careful not to allow them to burn. Once the beans have browned, the grains were removed immediately. The roasted soybeans were immediately ground into a fine powder in a Kitchen blender. A quick transfer from a hot frying pan into the blender ensures the seeds grind smoothly to powder. The powder was stored in an air tight container. The method of Cvejic *et al.* (2009)[16] was used with modification. Here, a known quantity of the Soybean flour was loaded into a thimble and placed in the Soxhlet extractor chamber until it was defatted using hexane, in the Soxhlet extractor. After defatting, the powder was dried and then re-extracted with methanol, using the Soxhlet extractor to obtain the methanol extract (phytoestrogen - rich extract). The extract was concentrated using a rotary evaporator and then dried completely using a thermostatically controlled hot air oven. **Experimental design: (4-vinylcyclohexene diepoxide (VCD) induced menopausal wistar rats)**

Animals were divided into six (6) groups of five (5) animals in each group and induced intraperitoneally with 80mg/kg of VCD, obtained during the preliminary study.

Group 1: 80 mg/kg of 4-vinylcyclohexene diepoxide + 200mg/kg phytoestrogen - rich extract.

Group 2: 80 mg/kg of 4-vinylcyclohexene diepoxide + 400 mg/kg of phytoestrogen - rich extract.

Group 3:80 mg/kg of 4-vinylcyclohexene diepoxide + 600 mg/kg of phytoestrogen - rich extract.

Group 4: 80 mg/kg of 4-vinylcyclohexene diepoxide + 14µg/100g estrogen of body weight

Group 5: 80 mg/kg of 4-vinylcyclohexene diepoxide (positive control)

Group 6: Normal rats (negative control)

The soybean was administered daily at single dose for 28 days using oral gavage. The animals were observed closely for sign of toxic manifestation and toxicity, and none of the wistar rats died after 28 days of treatment. The study was carried out between 1st March- August 30th, 2023.

Inclusion/Exclusion criteria

Sexually matured female Wistar rats were used in the study while those less than 6 weeks' old and male Wistar rats were excluded.

Collection of samples

At the end of the 28-day treatment period, the animals were sacrificed under chloroform anesthesia, blood sample was collected directly

from the abdominal aorta and the heart chamber with a needle mounted on a 10 mL syringe (Agary pharmaceutical LTD, Nigeria) into lithium heparin anticoagulant and plane sample bottles. Biochemical analysis was performed on the serum sample obtained after centrifugation of whole blood at 2500 rpm for 10 min. The serum they were kept frozen at -20^0 degrees Celsius until biochemical analysis was performed. Methods

Total cholesterol, triglyceride, high density lipoprotein were determined by enzymatic assay using spectrophotometric technique (NEUUV-08, Palo Alto, CA) described by Allain *et al.*, (1974)[17], Fossati and Precipe (1982)[18], Cheesbrough (2010)[19] respectively while low density lipoprotein (LDL) was calculated using Friedewald formula[20] to determine the plasma low density lipoprotein.

LDL-C = (TC)- (HDL-C)- (TG)/2.2 where all values are expressed in mg/dl.

Statistical Analysis

The data were statistically analysed using SPSS Software (IBM) version 23.0. The various results obtained from this study were expressed as Mean ± Standard deviation (SD). The differences between the groups were determined by one-way ANOVA. The Tukey-Kramer Multiple Comparisons Test was used as the post hoc test for determination of significant difference between Means. A P-value (<0.05) was considered to be statistically significant and P-value (>0.05) was considered not statistically significant.

Result:

Table 1 displays the effects of soyabean phytoestrogen-rich extract (200mg/kg, 400mg/kg, and 600mg/kg) and estradiol (14ug/100g) on the lipid profile levels in female rats treated with 80mg/kg VCD. Total cholesterol (F=9.95, p<0.001), triglyceride (F=7.49, p<0.001), high density lipoprotein (F=7.18, p<0.001), and low density lipoprotein (F=3.89, p<0.001) were all found to differ significantly between groups using one-way ANOVA and Tukey's post hoc test. Total cholesterol, triglyceride, high-density lipoprotein and low-density lipoprotein levels decrease significantly (p<0.05) between group 1 to group 3 but the control group (4ug/100g estradiol) shown significant increase in total cholesterol, triglyceride, high-density lipoprotein and low-density lipoprotein. Results showed dose-dependent reductions in lipid profile levels were seen across all treatment groups (p<0.05) with soybean phytoestrogen rich extract. Treatment with phytoestrogen isoflavones significantly decreased high density lipoprotein (HDL) levels in a dose-dependent manner, with no discernible difference between 400mg/kg and 600mg/kg. Except at a concentration of 14ug/kg of estradiol therapy.

Figure 1: displays the error bar chart of lipid profile with treatment with soybean phytoestrogen rich extract at varying concentration (200mg/kg, 400mg/kg and 600mg/kg).It show that at high dose-dependent concentration of soyabean phytoestrogen rich extract, total cholesterol, triglyceride, high density lipoprotein were significantly decrease compared to standard estradiol therapy that increases the levels of lipid profile.

Table 1: levels of lipid profile in female wistar rats induced with 4-vinylcyclohexane diepoxide (vcd)

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	F-value	P-value
TC (mmol/l)	2.22±0.32^b	1.83±0.14^a	1.42±0.15^a	2.67±0.16^c	2.86±0.06^c	2.57±0.06^b	9.95	0.001
TG (mmol/l)	1.23±0.32^a	0.87±0.14^a	0.42±0.16^a	1.42±0.16^b	1.73±0.16^c	1.74±0.11^c	7.49	0.001
HDL (mmol/l)	1.45±0.15^b	1.21±0.07^a	1.04±0.09^a	1.50±0.09^b	1.73±0.07^c	1.50±0.04^b	7.18	0.001
LDL (mmol/l)	0.21±0.05^a	0.21±0.14^a	0.19±0.01^a	0.49±0.13^b	0.38±0.03^a	0.28±0.03^a	3.89	0.010

Values are expressed in mean ± SD. The value with different superscript showed significant difference from each other (p<0.05) while value with same superscript are not statistically difference from each other (p>0.05). KEY: Group 1 - 80mg/kg

VCD+200mg/kg Soyabean, Group 2 - 80mg/kg VCD+400mg/kg Soyabean, Group 3 – 80mg/kg VCD+600mg/kg Soyabean, Group 4 – 14ug/100g Estradol, Group 5 - 80mg/kg VCD, Group 6 – Control , TC-Total cholesterol, TG- Triglyceride, HDL-High density lipoprotein, LDL- Low density lipoprotein, F-value- Fisher test value(ANOVA)

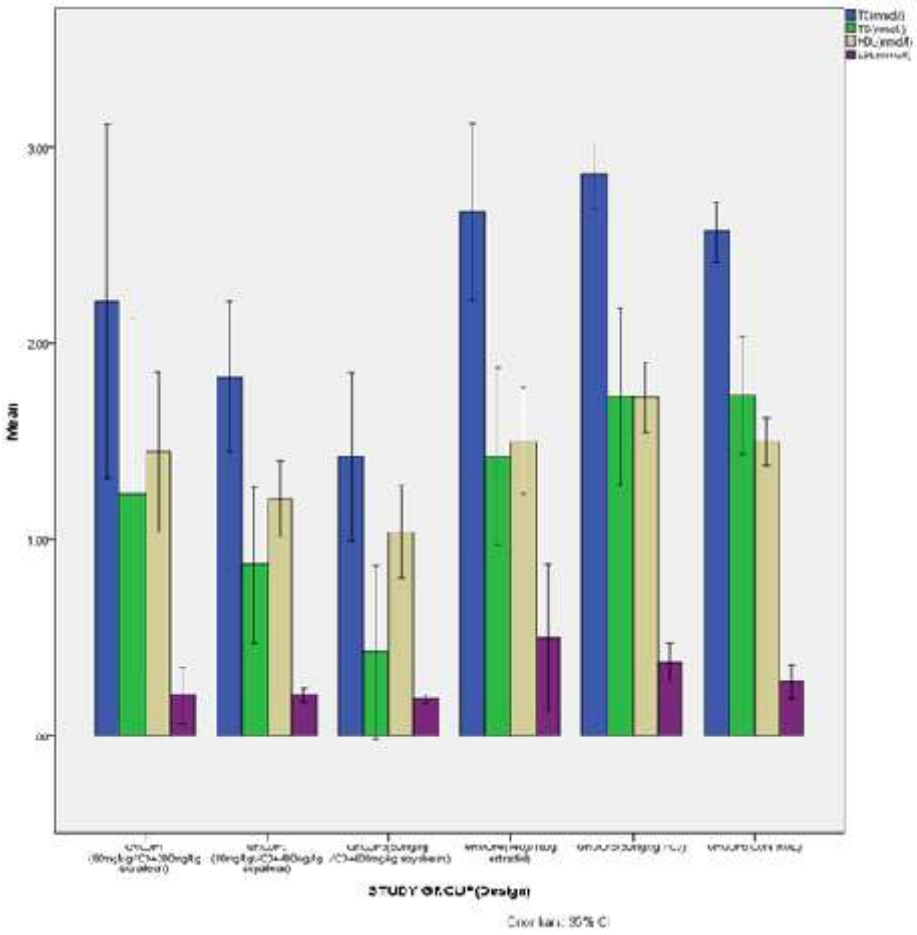

Figure 1: Showing bar chart of lipid profile with treatment with soybean phytoestrogen rich extract at varying concentration (200mg/kg, 400mg/kg and 600mg/kg).

Discussion:

Atherosclerosis is a key feature in the pathophysiology of arterial forms of cardiovascular disease, and these compounds (isoflavones) may serve as both shields against the disease and disruptors of already-formed plaques. The prevalence of cardiovascular disease especially heart attack among Nigeria called for the need to sought for alternative way and novel approach to ameliorate and regulate lipid profile, the risk factor of atherosclerosis. The study aimed to seek for novel approach and alternative way rather than use of statin in lipid regulation in 4-vinylcyclohexane diepoxide-induced menopausal albino rats.

Results showed that dose-dependent reductions in lipid profile levels were seen across all groups ($p<0.05$). Total cholesterol levels were substantially different ($p<0.05$) between the 200mg/kg phytoestrogen isoflavones group and the other groups, with the exception of the negative control group. The total cholesterol levels of those in groups 2 (400mg/kg) and 3 (600mg/kg) of the phytoestrogen isoflavone were not statistically different. No statistically significant difference in triglyceride reduction was found between doses of phytoestrogen isoflavones ($p>0.05$). Treatment with phytoestrogen isoflavones significantly decreased high density lipoprotein (HDL) levels in a dose-dependent manner, with no discernible difference between 400mg/kg and 600mg/kg. Except at a concentration of 14ug/kg of estradiol therapy. However, Tokede *et al*. (2015)[7] and Sathyapalan *et al*. (2018)[21] found that administering

phytoestrogen-rich extract did not change lipid profiles. While Hsu *et al*. (2007)[22] found a rise in HDL-C levels, our findings contradict that finding. Hormone therapy in postmenopausal women aged 45 to 56 was studied by Hsu *et al*. (2007)[22], who looked at the effects of soya germ extracts on blood lipoproteins. In total, sixty (60) samples were analyzed. Six grammes of soya germ extract were given to the subjects every day for four weeks. After four weeks of soya germ extract intervention, the results show that isoflavone significantly lowers the lipid status that could result in atherosclerosis. This is inferred from a marked increase in plasma HDL-C level and a significant decrease in plasma LDL -C/ HDL-C ratio and LDL (-2.03 mmol 95%, -3.20 to -0.85 mmol). Therefore, it may be stated that soybean phytoestrogen-rich extract successfully increased levels of isoflavones. Yamagata (2019) suggests that isoflavones may help protect the endothelium lining blood arteries by lowering cholesterol levels[23]. A meta-analysis of 38 studies found that those who consumed an average of 50 g of soy protein daily saw reductions in total cholesterol of 9.3%, low-density lipoprotein of 12.9%, and triglycerides of 10.5%. Other studies also found reductions in cholesterol, LDL, and triglycerides,[24,25,26,27] and these results were consistent with one another. Accordingly, rather than a direct modulation of LDL concentrations in the blood, the positive effects of isoflavones in the cardiovascular system may be attributed to their ability to protect against LDL oxidation[28,29]. According to research done by van der Velpen *et al*., (2014)[30], it was revealed that isoflavone supplementation (one based on daidzein and the other on genistein) in postmenopausal women on gene over the course of eight weeks downregulate the expression of genes involved in fat synthesis in mice via activating PPAR. Similar results were seen when C57BL/6J mice with diet-induced obesity were given daidzein[31] in previous study. Besides suppressing lipid production, phytoestrogens have been shown to promote fat breakdown by activating a variety of molecular pathways. The lipolytic compounds genistein and daidzein in soybean phytoestrogen-rich extract were reported to reduce triglyceride (TG) accumulation and adipocyte hypertrophy in rats by inhibiting cyclic adenosine monophosphate phosphodiesterase (cAMP-specific PDE)[32]. By increasing the expression of genes involved in fatty acid oxidation, such as hormone-sensitive lipase, carnitine palmitoyltransferase 1, and uncoupling protein 2, coumestrol was reported to decrease lipid accumulation in 3T3-L1 preadipocytes in previous study. Researchers from previous study looked at data from 9632 men and women who had participated in the First National Health and Nutrition assessment Survey Epidemiologic Follow-up Study (NHEFS) and had not shown any signs of CVD at their baseline assessment. Medical records and death certificates were used to determine the prevalence of CHD and CVD, while a food frequency questionnaire was used to quantify legume consumption over a three-month period. Both coronary heart disease (P=0.002 for trend) and cardiovascular disease (P=0.02 for trend) risk was significantly and inversely linked with legume consumption. There was a 22% reduction in CHD risk (relative risk, 0.78; 95% confidence interval, 0.68-0.90) and an 11% reduction in CVD risk (relative risk, 0.89; 95% confidence interval, 0.80-0.98) among those who ate legumes four or more times per week compared with less than once per week, after adjusting for established CVD risk factors.

The results of this study show that eating legumes like soyabean can significantly lower the risk of developing coronary heart disease in both men and women.

Result from this study showed to validate the prove soybean phytoestrogen rich extract has as cardioprotective effects in menopausal induced rats.

Estrogen affects the risk of obesity-related problems by affecting both the quantity and metabolism of adipose tissue. Clinicians have noticed a steady loss in insulin sensitivity alongside an increase in blood inflammatory markers and an unfavourable lipid profile in postmenopausal women. Ovariectomy has been linked to insulin resistance and increased sensitivity to the harmful effects of a high-fat diet in experimental animals. The physiological concentration of estrogen would prevent this from happening. Clinical trials showed that transdermal administration of estradiol reduced plasma triglyceride (TG) levels by lowering the expression of genes encoding critical lipogenic enzymes (stearoyl-CoA desaturase, fatty acid synthase, acetyl-coenzyme A carboxylase alpha, and fatty acid desaturase 1) which is consistent with result from this study compared with positive control group.

Phytoestrogens' actions resemble the action of estrogen in adipose tissue include the regulation of adipocyte metabolism and secretory activity, in addition to the control of adipogenesis, due to the wide variety of pathways that phytestrogens influence.

Adipocytes derived from ovariectomized rats and 3T3-L1 preadipocytes were also reported to have reduced baseline and insulin-induced lipid production when treated with genistein and coumestrol, respectively[32].

Blood lipid profiles have been shown to improve with increased ingestion of and metabolism of phytoestrogens, according to epidemiological evidence which is in agreement with my finding. From previous study, it was revealed that high isoflavone and lignan intake was associated with significantly lower plasma TG levels in the Framingham Offspring Study, and a high daidzein intake was positively associated with HDL cholesterol in postmenopausal women in a cross-sectional study assessing the influence of isoflavone intake on cardiovascular disease risk factors which was observed in reduction of lipid profile. Furthermore, promising outcomes have been seen in animal investigations of dietary treatments with phytoestrogens to enhance blood lipid profile. Albino wistar rats induced with 4-vinyl cyclohexane diepoxide had considerably lower plasma TG after receiving daidzein, genistein, and glyctin supplements.

Conclusion

This study shows that when compared to the treatment group with phytoestrogen rich extract, menopause-induced female Wistar rats had significantly low levels of total cholesterol, triglyceride, high density lipoprotein and low density lipoprotein In animal models, administration of phytoestrogen -rich extract at varying concentrations exhibited impressive lipolytic effect on lipids thereby helpful in management of cardiovascular disease. Soybean phytoestrogen-rich extract therapy in a high-dose appears to be more effective in lowering lipids compared to hormone replacement therapy as an alternate source of estrogen in treatment of cardiovascular diseases.

Conflict Of Interests

There are no stated conflicts of interest by the authors.

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Authors Contributions

Olaniyan, Edusola Juliana designed and initiated the study, Emokpae, Mathias Abiodun reviewed the manuscript for important intellectual content, Oyakhire, Fidelis Ohiremen assisted in data analysis, Ahmed Liasu Adeagbo assisted in analysis and interpretation of data, Esezobor, Iria Kelly assisted in collection of blood sample from animals, Olaniyan Stephen Olawale and Christian Onosetale Ugege assisted in draft of the manuscript, Akesinro Ayodeji Adebayo assisted in proofreading the manuscript.

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