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Vol. 9(30), pp. 745-755, 15 August, 2015
DOI: 10.5897/AJPP2015. 4389
Article Number: 3905DCB54854
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Effect of aqueous extract of *Annona muricata* seed on atherogenicity in streptozotocin-induced diabetic rats

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Received 21 June, 2015; Accepted 2 August, 2015

Poorly controlled diabetes has been implicated in lipoprotein abnormalities and hypertriglyceridemia in both human and experimental animals, which increase the risk of artherogenicity. The effect of *annona muricata* seed extract was studied in streptozotocin-induced diabetic rats for 30 days in order to evaluate its anti-atherogenic property by investigating lipid profile malondialdehyde and histology of the aorta. The animals were divided into four groups (I-IV). Groups II-IV received a single intraperitoneal injection of streptozotocin (65 mg/kg) dissolved in a citrate buffer (pH 4.5). Group I (control) received citrate buffer only. Groups III and IV received 500 and 750 mg/kg of extract orally, whereas group I and II received rat chow. Result showed that treatment with high dose of extract (750 mg/kg) significantly increased anti-atherogenic percentage (70.7 %), whereas total cholesterol, low density lipoprotein-cholesterol, triglycerides and malondialdehydewere significantly reduced after treatment with 500 and 750 mg/kg respectively. Low-density lipoprotein-cholesterol (LDL-C) was significantly elevated. Histological assessment of the aorta showed widely dilated, moderately thickened wallin extract treated groups. Data showed that biochemical parameters were greatly improved without significant effect on endothelial thickness.

Key words: Annona muricata, streptozotocin, malondialdehyde, lipid profile, anti-atherogenic index.

INTRODUCTION

Hyperlipidemia is one of the risk factors for atherosclerosis. Likewise, diabetes mellitus is associated with an increased risk of atherosclerotic and cardiovascular disease. Study has showed a relationship between diabetes mellitus and hyperlipidemia Gandhi (2001). Data have shown that hyperglycemia contributes heavily to risk of atherosclerosis (Kunjathoor et al., 1996). The most common abnormalities in humans with poorly controlled diabetes are increased lipoprotein abnormalities and hypertriglyceridemia (Ginsberg, 1996).

However, lipoprotein abnormalities associated with diabetes mellitus can increase risk of coronary artery disease (O'Brien et al., 1998), whereas hyperlipidemia enhances oxidative stress (Bhalodia et al., 2010) in which

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increased low-density lipoprotein-cholesterol (LDL-C) may lead to abnormal metabolism and atherosclerotic plaque. In addition, hyperglycemia attenuates antioxidant mechanisms creating oxidative stress state (Gumienczek, 2005) and hyperglycemia shifts antioxidant system toward a pro-oxidant state that can speed up vascular injury (Vasavada and Agarwal, 2005).

High-density lipoprotein-cholesterol (HDL-C) is frequently used as a determinant of the existence of natural antioxidant in the body since it inhibits LDL-C (Van Lenten et al., 2001; Navab et al., 2004) and reactive oxygen species *in vitro* (Lee et al., 2005). Although, lipoproteins in the body are susceptible to oxidation accelerated by advanced glycation end products (Tsai et al., 1994; Rabini et al., 1994) in diabetes, the increased formations of advanced glycation/lipoxidation end products are both implicated in the pathogenesis of diabetes complication, and enhance atherogenic process. In a similar vein, streptozotocin has been shown to increase perivascular and interstitial collagen deposition in the left ventricle and kidney, and surrounding aorta (Miric et al., 2001).

The plant *Annona muricata* (sour sop) belongs to a family of annonaceae. The *A. muricata* is known for its edible fruit which grows to about 5 to 6 m in height (Greenhouse et al., 2008). The fruit has a fleshy endocarp size ranging from 10 to30 cm long and up to 4.5 to 6.8 kg, whitish and acidic to taste especially when ripe and characterized with the presence of abundant seeds. *A. muricata* seed has been used against various cancer cells (Chang, 2001; Liaw, 2002).

Recent studies showed that it improved reproductive functions (Agbai and Nwanegwo, 2013a) and liver enzymes in alloxan induced diabetes (Agbai and Nwanegwo, 2013b), possessed anti-hemolytic (Agbai et al., 2014) and anti-hyperlipidemic properties (Adeyemi et al., 2008), decreased lipid peroxidation and enhanced insulin production (Adewole and Ojewole, 2008).

Therefore, the present study was undertaken to evaluate the effect of *A. muricata* seed extracts on antiatherogenic index and thickness of the aortic wall in streptozotocin induced diabetic rats. Malondialdehyde (MDA) and some lipid profile were investigated based on findings by Yang et al. (2014) that oxidized LDL disrupts the growth and survival human coronary artery endothelial cells through an MDA-dependent pathway.

MATERIALS AND METHODS

Experimental protocol

Twenty male albino wistar rats (Animal house, Madonna University, Nigeria) weighing 200 to 280 g were used in this study. They were randomly divided into four groups (control group I, experimental groups: II, III and IV) and placed in standard wooden cages in a room with a temperature between 32 to 38°C and had access to tap water and normal rat chow (Vital feeds, Nigeria). The animals were acclimatized for two weeks. Experimental procedures involving the animals and care were conducted in conformity with the Madonna University guidelines that are in compliance with National and International laws and guidelines for care and use of laboratory animals.

Rats of experimental groups II, III and IV received a single intra peritoneal injection of 65 mg/kg freshly prepared streptozotocin (Sigma Chemicals, USA), after dissolving 30 mg/ml of the drug in 0.5 mol/L of citrate buffer (pH = 4.5). Four control rats received only citrate buffer. Two weeks later, glucose was measured from the blood collected from the tail vein after a small incision on the tail. They all exhibited blood glucose above 10 mmol/L. Rats did not receive insulin treatment during the present study.

The extract was administered orally using oral gavage to groups III and IV at dose concentration of 500 mg/kg and 750 mg/kg respectively for four weeks after preliminary test using doses of extract(<500 mg/kg). The group I (control) and II (diabetes only) received normalrat chow for four weeks. Rats were sacrificed under chloroform anesthesia after completion of the study. Blood sample was taken from the heart and collected in a labeled EDTA bottles. The heart was harvested and aorta excised.

Collection of plant and preparation of plant extract

The edible fruits of *A. muricata* were bought from a local market in Rivers State. The seeds were removed from the fruits, washed and dried in an electric oven at 45°C (Gallenkamp) for two weeks. The seeds were ground into coarse particles. About 100 g of the coarse form was macerated into 500 ml of water and placed in a mechanical shaker for 48 h to make a mixture. The mixture was filtered with Whatman filter paper (No 1). The filtrate was then concentrated using rotator evaporator (Buchi), and was further concentrated to dryness in an electric oven (40°C). The extract was refrigerated (4°C) until ready for use.

Biochemical measurements

All the biochemical analysis was performed based on Randox diagnostic kits (Randox Laboratories Limited, Antrim, UK). MDA was measured using Thiobarbituric Acid Assay method. Glucose was measured using the glucose oxidase method (Trinder, 1969), triglycerides was measured using the colorimetric method of analysis as described by Tietz (1990) and enzymatic end point method for total cholesterol, and HDL as described by Allain et al. (1974). LDL estimation was calculated from the total cholesterol, triglycerides and HDL as described by Friedewald et al. (1972). Antiatherogenic index was calculated according to method used in a paper by Guido and Joseph (1992). The aortic ring was routinely processed and stained with Haematoxylin and Eosin.

Statistics

Results are expressed as means ± SEM. Statistical significance of differences between control and experimental groups. ANOVA was used to analyze results. Any significant ANOVA were further analyzed by Tukey post hoc test. P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The present study attempts to evaluate the effectiveness of the *A. muricata* seed extract at different dose concentrationin improving anti-atherogenicity on the aorta. Results (Table 1) showed varying degrees of effectiveness of the extract on biochemical assessment

Table 1. General data showing biochemical changes four weeks after treatment of *A. muricata* extract.

Groups	Glucose (mg/dl)	MDA (M/cm)	Total- C (mmol/L)	LDL (mg/dl)	TG (mmol/L)	HDL (mg/dl)	AAI (%)
I	106.33±10.26	0.0038± 2.4×10^{-4}	$3.80\pm9.7\times10^{-4}$	$0.58\pm1.41\times10^{-1}$	$3.47\pm1.6\times10^{-1}$	1.69±0.22	80.1
II	282.02±4.03	$0.014\pm8.1\times10^{-4}$	$9.72\pm1.3\times10^{-2}$	$1.19\pm1.6\times10^{-2}$	$3.08\pm5.8\times10^{-4}$	0.47±0.02	5.08
III	92.40±13.07	$0.0085\pm7.3\times10^{-4}$	$5.87\pm4.0\times10^{-3}$	$0.41\pm3.8\times10^{-2}$	$2.69\pm2.0\times10^{-2}$	1.13±0.09	23.8
IV	80.62±12.10	$0.0023\pm2.4\times10^{-4}$	$2.97\pm1.7\times10^{-3}$	$0.36\pm7.5\times10^{-2}$	$1.47\pm3.2\times10^{-1}$	1.23±0.19	70.7

MDA = malondialdehyde, Total-C = Total cholesterol, LDL-C = low density lipoprotein-cholesterol, TG = Triglycerides, HDL-C = High density lipoprotein-cholesterol, AAI = Anti-atherogenic index.

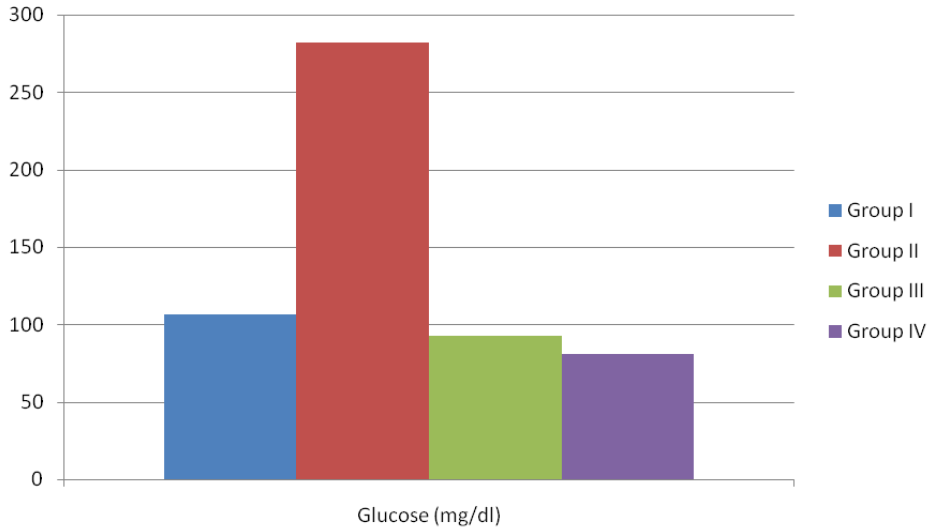

Figure 1. Plasma glucose concentration in control, diabetic and extract-treated rats extract.

during the entire experiment. The higher dose concentration of extract markedly reduced the levels of MDA, total cholesterol, LDL and glucose, with a decrease in triglycerides and HDL levels respectively.

As expected glucose levels was markedly increased (Figure 1) in group II rats (282.02±4.03 mg/dl $P < 0.05$). However, the blood glucose concentration markedly declined in treated groups III (92.40±13.07 mg/dl) and group IV (80.62±12.10 mg/dl) compared with control group I (106.33±10.26 mg/dl, $P > 0.05$) corroborating with recent studies by Rout et al. (2013) and Alhaya et al. (2014). The mechanism of action may be dependent on the ability of the extract to indirectly enhance insulin production and endogenous antioxidants in diabetes mellitus (Adewole and Caxton-Martins, 2006; Adewole and Ojewole, 2008).

MDA which is a lipid peroxidation product frequently used to determine oxidant/antioxidant balance in diabetes (Kalaivanam et al., 2006). In order to ascertain the antioxidant property of *A.* muricata, MDA was estimated. Results in Figure 3 showed MDA was significantly reduced in the extract treated groups III (0.0085±7.3 x 10^{-4} M/cm) and IV (0.0023±2.4 x 10^{-4} M/cm). Phytochemical constituent of *A. muricata* seed extract includes flavonoids which have been implicated in reducing MDA and preventing oxidative damage in hypercholesterolemic condition (Vasquez-Castilla et al., 2013).The reduction in MDA levels in the present study corroborated with works by Florence et al. (2014)

Studies have shown that HDL particles usually attach to cell surface and remove cholesterol, which is readily diminished in diabetes (Brites et al., 2009). This reduction is dependent on the functional activity of lecithin-cholesterol acyltransferase (LCAT) produced in the liver that assist HDL-C to remove free cholesterol from tissues. LCAT activity is diminished in diabetes mellitus (Nakhjavani et al., 2013). LCAT reduction has been reported to be dependent on the degree of diabetes mellitus (Ghanei et al., 2007,).Total cholesterol (Figure 2) was significantly reduced in group IV rats (2.97±1.7 $x10^{-3}$ mmol/L, $P < 0.05$) treated with high dose of extract. Furthermore, it has been shown that LCAT increases in concentration and activity in type I diabetic patients with improved metabolic control (Weight et al., 1993). These

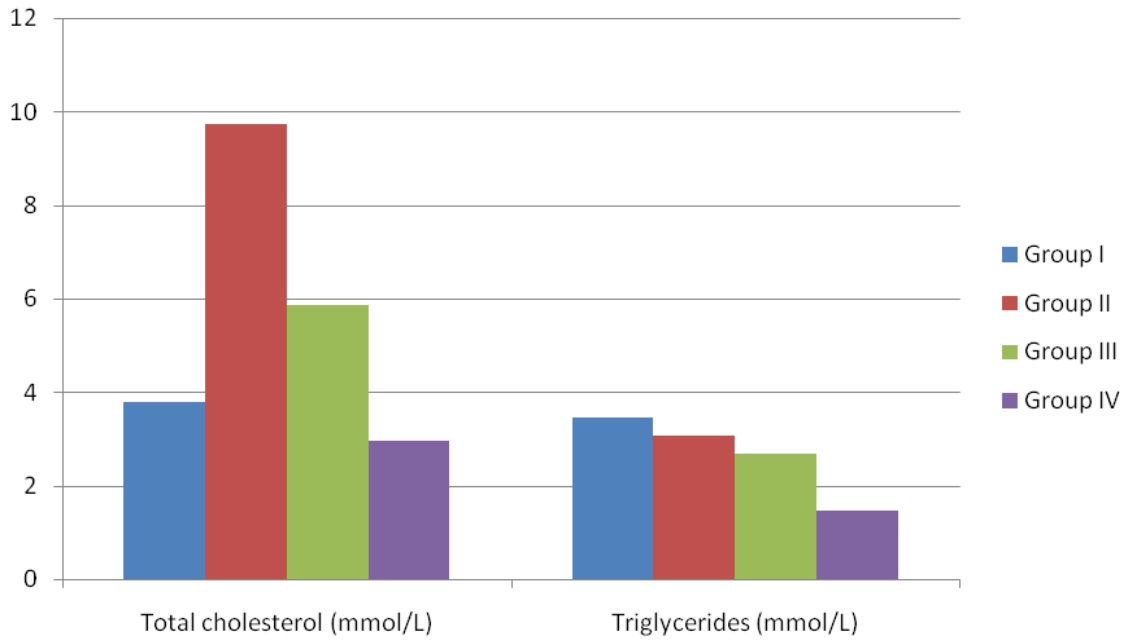

Figure 2. Total cholesterol and triglycerides concentrations in control, diabetic and extract-treated rats.

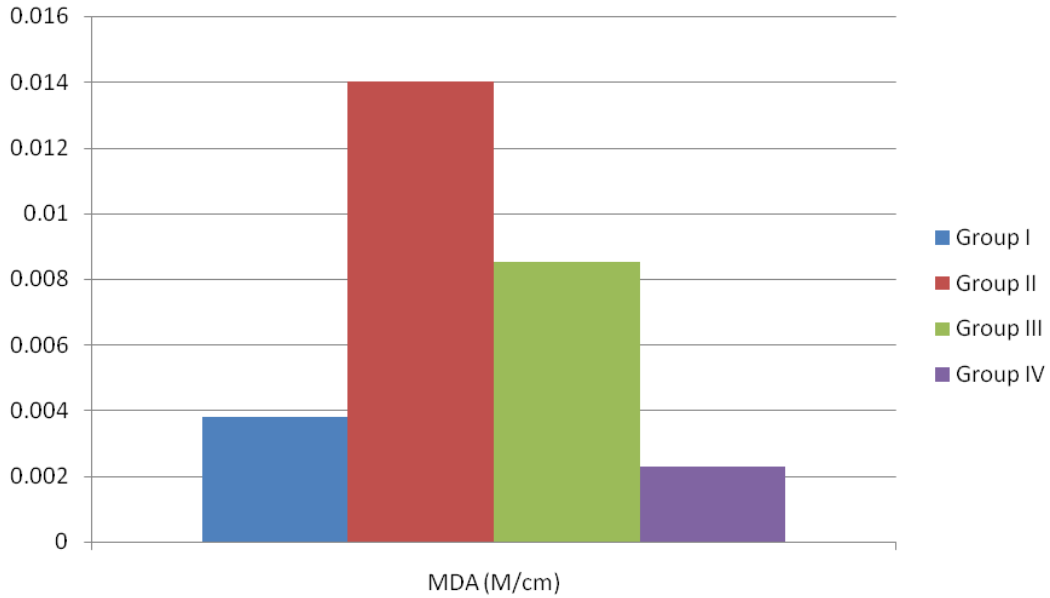

Figure 3. MDA concentration in control, diabetic and extract-treated rats.

suggest that mechanism of action of extract could be via LCAT activity. Plant sterols also a phytochemical constituent of *A. muricata* seed extract have been shown to prevent cardiovascular disease by decreasing cholesterol level in the plasma (Parraga et al., 2011). The HDL level (Figure 4) was significantly elevated in group III (1.13 ± 0.09 mg/dl) and group IV (1.23±0.19 mg/dl) respectively, possibly due to improved metabolic control of the seed extract of *A. muricata*.

Lipoprotein lipase is low in both type I and II diabetes, resulting in altered lipoprotein levels and elevated triglycerides (Taskinen, 1987). Studies also showed that over-expression of lipoprotein lipase inhibited diabetic hypertriglyceridemia and hypercholesterolemia but

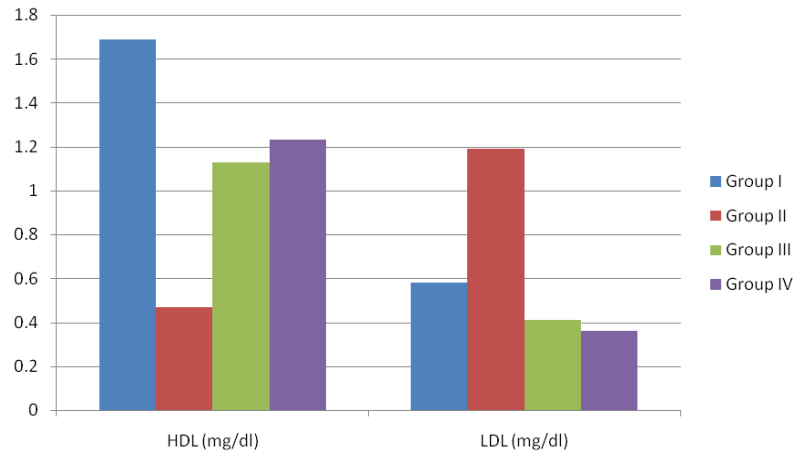

Figure 4. HDL and LDL levels in control, diabetic and extract-treated rats.

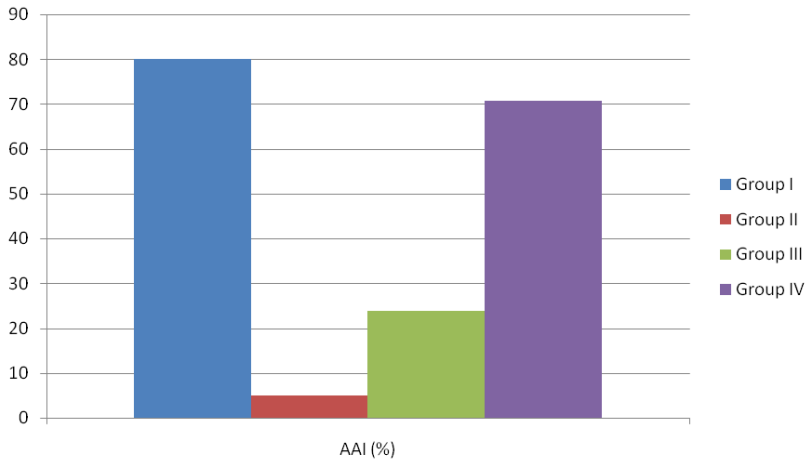

Figure 5. Antiatherogenic index of control, diabetic and extract-treated rats.

enhanced hydrolysis of triglycerides (Shimada et al., 1995). Triglycerides levels (Figure 2) were significantly reduced ($P < 0.05$) in group III ($2.69\pm2.0 \times 10^{-2}$ mmol/L) and group IV ($1.47\pm3.2 \times 10^{-1}$ mg/dl) possibly as a result of the influence of the seed extract. Phytochemical constituent of the extract alkaloid could lead to be hypolipidemia, although the alkaloid fraction of *Hunteria umbellate* seed significantly reduced serum LDL-C, TG and TC in triton-induced hyperlipidemic rats (Adeneye and Crooks, 2015).

Studies have shown a strong association between the risk of coronary artery diseases, high levels of LDL-C and low levels of HDL-C (Castelli, 1988; Igweh et al., 2005). Because plasma atherogenic index has been employed as a significant predictor of atherosclerosis (Dobiasova and Frohlich, 2001; Tan et al., 2004), the anti-atherogenic properties of *A. muricata* seed extract was evaluated. Results in Figure 5 showed that the anti-atherogenic index was readily elevated in group IV treated with high dose concentration of extract (70.7 %) compared with groups II and III (5.08 and 23.8%) which showed significant decreases respectively. This increased percentage in anti-atherogenic index at high dose concentration corroborated with the present findings showing that total cholesterol was significantly reduced only at high dose concentration of the extract.

Although, the extract significantly increased HDL-C and reduced LDL-C in the treated groups, it is plausible to state that the increased antiatherogenic index could be a function of LCAT activity. It is well-known diabetes accelerates atherosclerosis and the degree of atherosclerosis is commonly evaluated by the degree of thickness of the arteries, blood flow. Figures 6-7 showed the photomicrograph of normal aorta (control) with well arranged smooth muscle without inflammation. Figures 8 to 9 showed the aorta of the diabetic rats with thickened blood

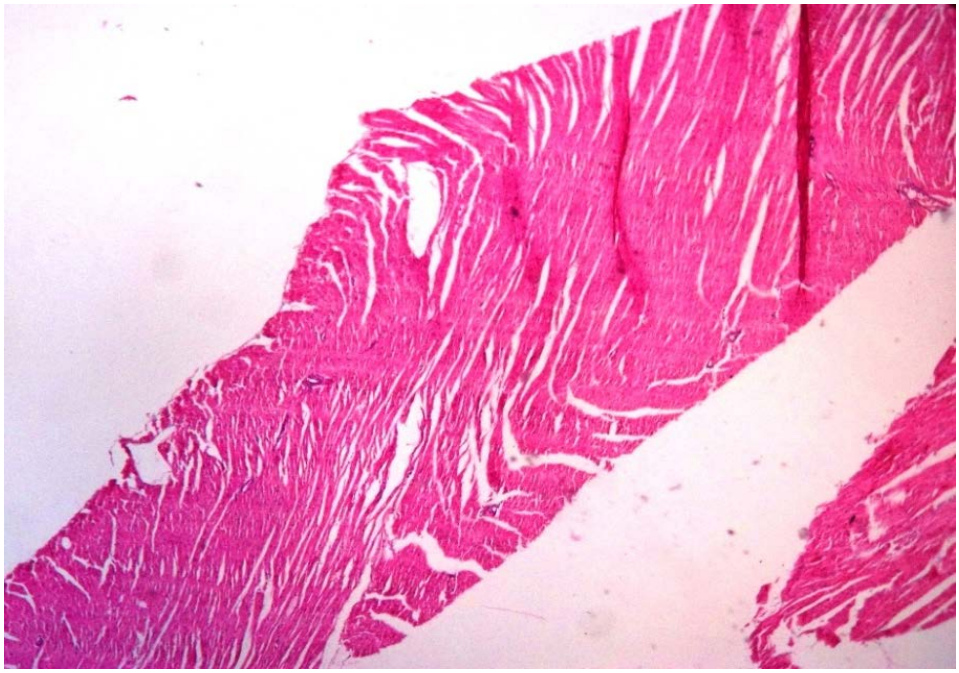

Figure 6.Photomicrograph showing the aorta of control group I rats (H & E x 40) with well arranged smooth muscle fibers punctuated by few small sized and thin walled blood vessels. No inflammation.

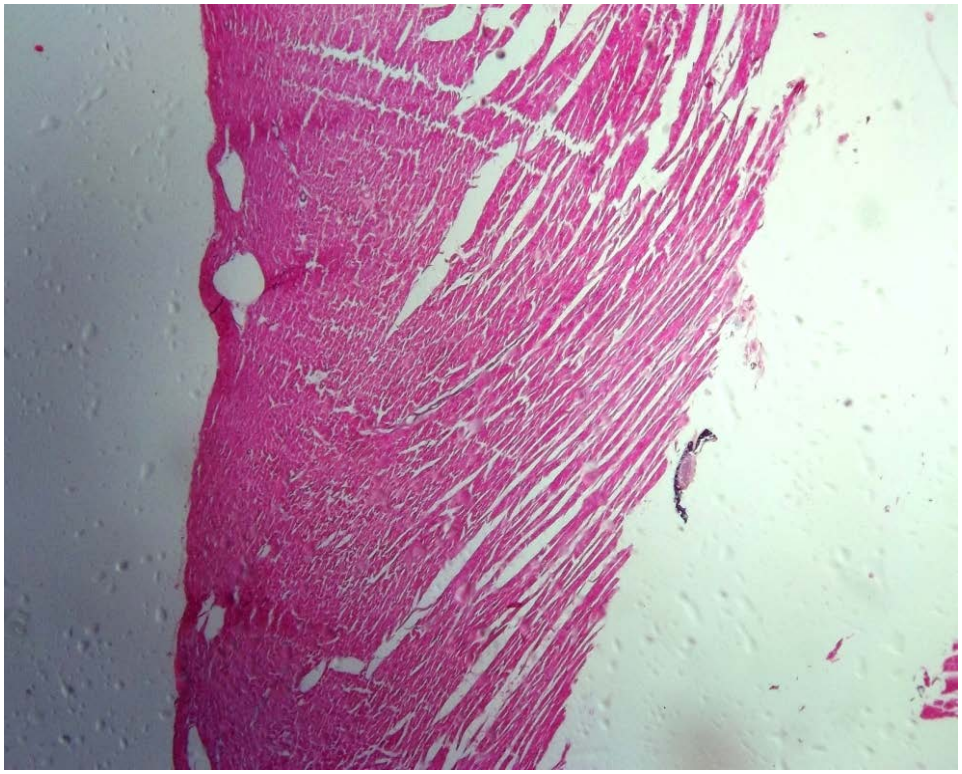

Figure 7. Photomicrograph showing the aorta of control group I rats (H & E x 40) with regular fiber arrangement. Dilated, empty and thin-walled vessels without inflammation.

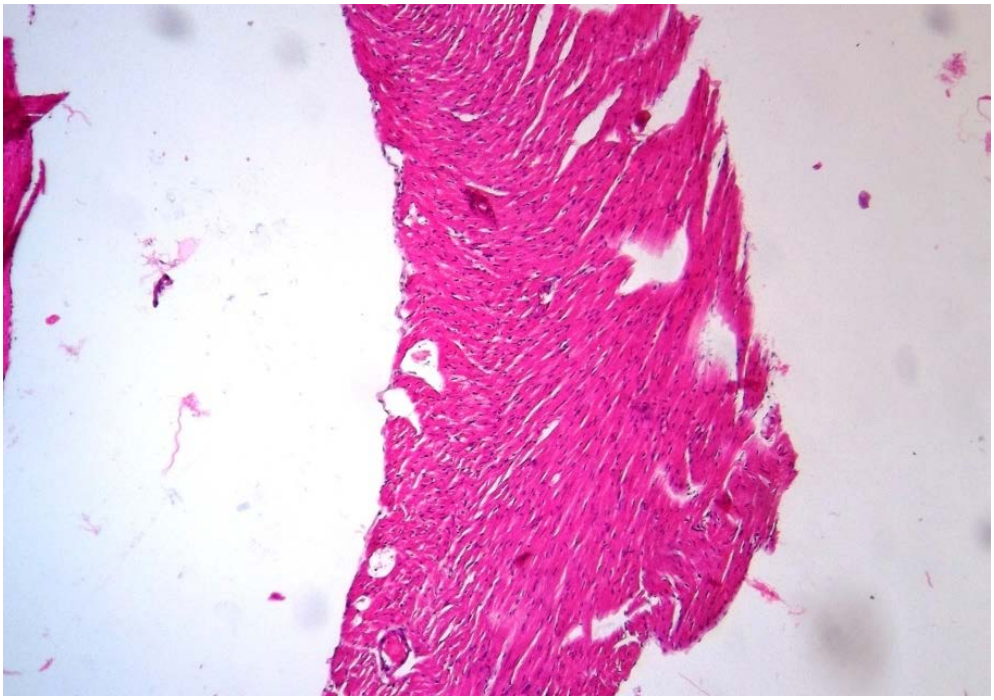

Figure 8. Photomicrograph showing the aorta of group II diabetic rats (H & E x 100) with well arranged smooth muscle fibers having some mildly dilated and thin walled blood vessels some which are congested. No inflammation.

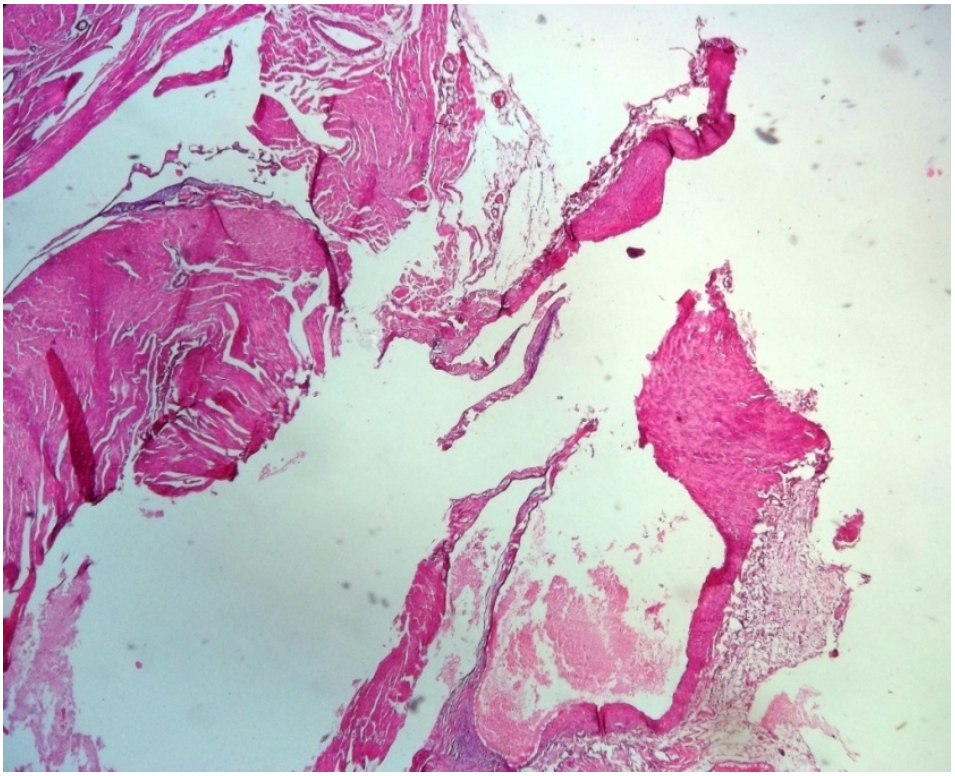

Figure 9. Photomicrograph of the aorta of group II diabetic rats (H & E x 40) with regular fiber arrangement. There are dilated and congested, thick-walled blood vessels with focal perivascular fibrosis and moderate inflammation.

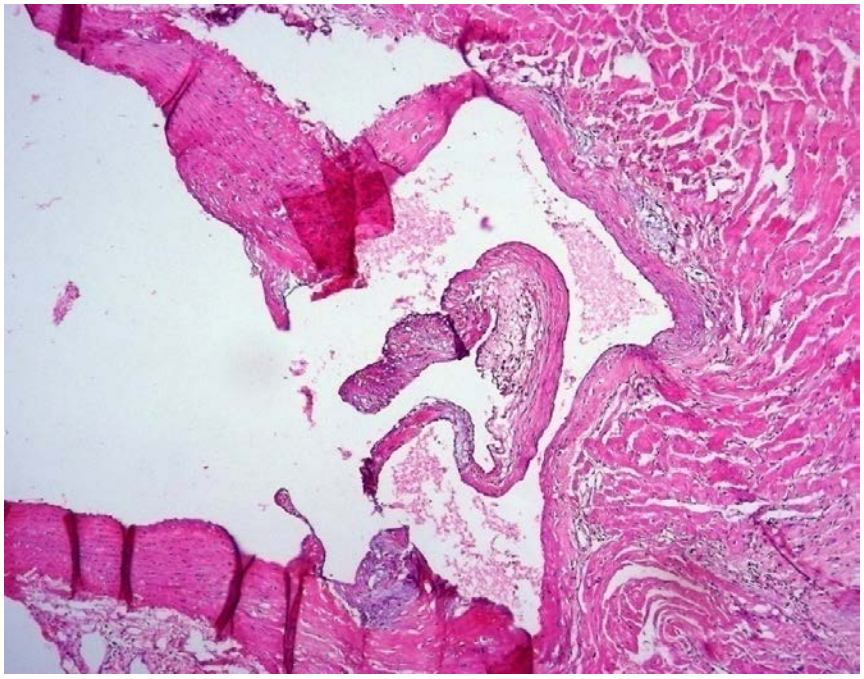

Figure10. Photomicrograph of the aorta of group III extract-treated rats (H & E x 100) with arranged smooth muscle fibers with focal wide separation of the interstitium containing oedema fluid. The blood vessels are widely dilated, containing blood and have moderately thickened wall. There is mild focal infiltration by lymphocytes.

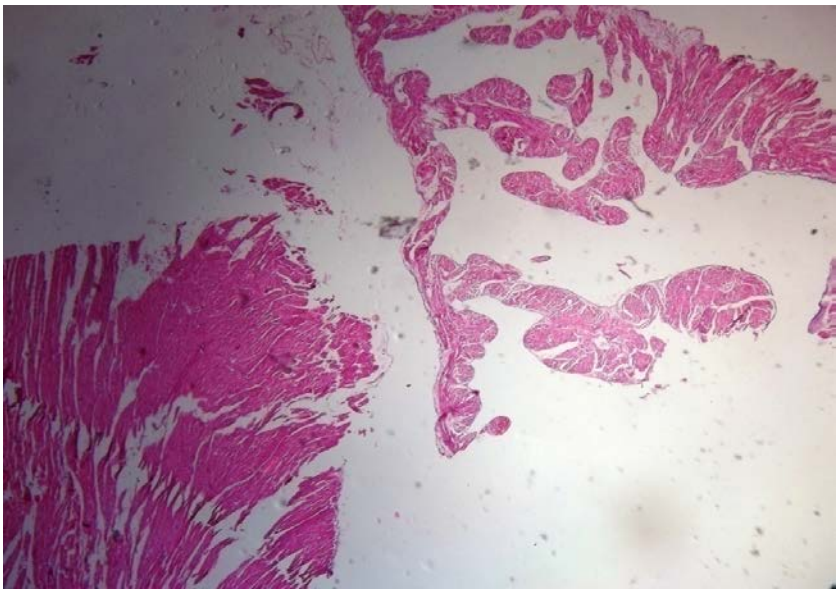

Figure 11. Photomicrograph of the aorta of group III extract-treated rats (H & E x 40) with regular fiber arrangement. There is a focal widely dilated but thin-walled blood vessel. No inflammation.

vessel, perivascular fibrosis and moderate inflammation. Figures 10-13 showed moderately thickened wall with perivascular fibrosis, suggesting that the extract has no significant effect on the thickness of the arteries. The effect of the extract on aorta was not largely evident, although mild effect was observed, suggesting that the effect of the extract to reducing the thickness of the aorta may be dependent on time.

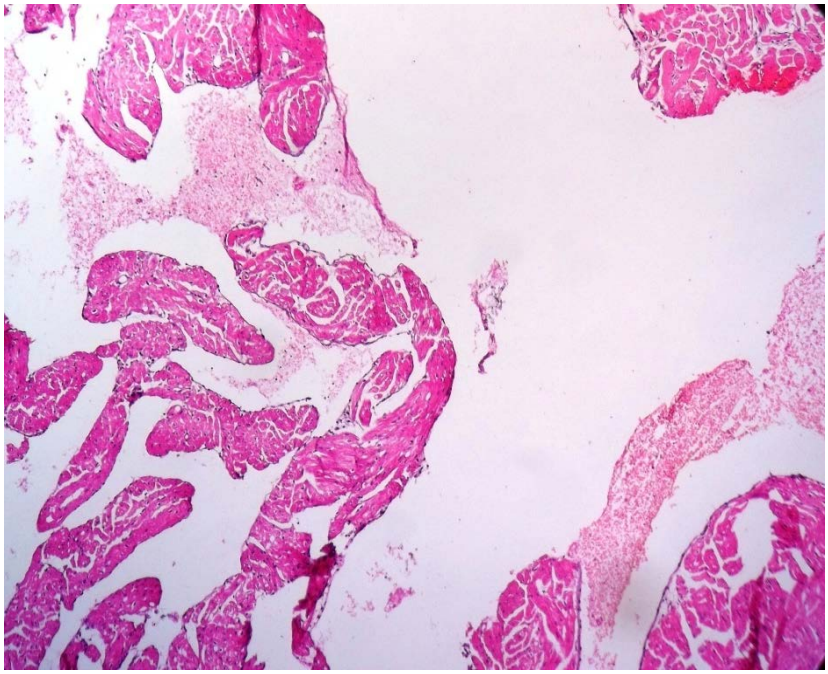

Figure 12. Photomicrograph of the aorta of group IV extract-treated rats (H & E x 40) with regular fiber arrangement with oedema. There is no inflammatory cells infiltration. There are few mildly dilated blood vessels without thickened walls.

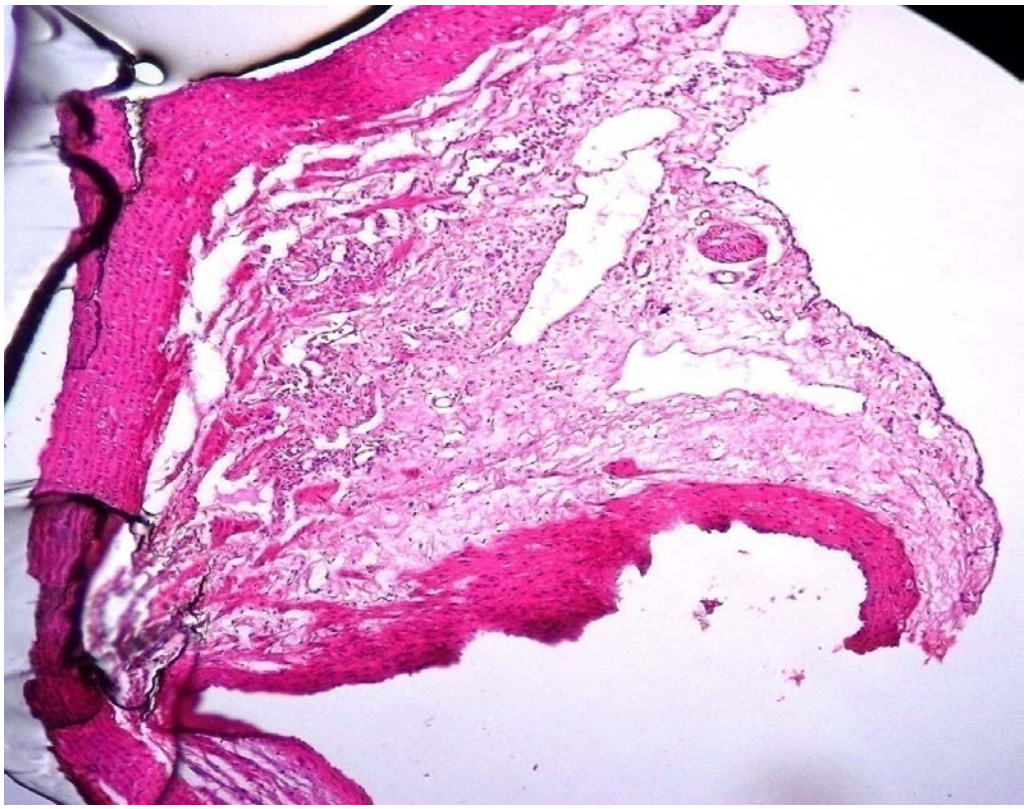

Figure 13. Photomicrograph showing the aorta of group IV extract-treated rats (H & E x 100) with regular fiber arrangement. There is a markedly focal dilated empty and thick-wall blood vessel with perivascular fibrosis and mild inflammation.

Conclusion

The anti-atherogenic index and lipid profile were significantly improved at high dose concentration of extract without a significant improvement on the thickened aortic walls. It is worth stating that reduced lipid profile may not be a single clinical tool in determining the degree of anti-atherogenicity in *A. muricata* seed extract treated rats. Histological assessment could be considered as the first line in determining the extent of regression of atherosclerosis in diabetes mellitus biochemical markers.

Conflict of Interest

The authors have not declared any conflict of interest.

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