Cardiocurative Effect of the Seed of *Tetracarpidium Conophorum* (African Walnut) on Wistar Rats with Doxorubicin Induced Myocardial Infarction

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Abstract: **The study investigated the cardiocurative effect of the seed of *Tetracarpidium Conophorum* extract on wistar rats with doxorubicin-induced myocardial infarction. Herbal drugs are used widely even when their biologically active compounds are unknown, probably because of their effectiveness, lesser side effects and affordability. The result of this study will provide harmless and affordable remedy for cardiotoxicity and other oxidative stress induced diseases. Twenty adult wistar rats (140 – 330g) of both sexes were divided into five experimental groups (A, B, C, D, and E). Each group had four rats. Group A, B, C, D, and E represented groups treated with food only, doxorubicin only, (doxorubicin + 6% walnut of feed), (doxorubicin + 12% walnut of feed) and (doxorubicin + Enalapril) respectively. Cardiotoxicity was induced by the cumulative administration of 15mg/kg doxorubicin intraperitoneally during the first alternate seven days (1st, 3rd, 5th, and 7th day). After the treatment period of forty-two days, blood samples and hearts were collected for biochemical and histopathological studies respectively. Serum enzyme and lipid profile were checked. There was significant increase ($p < 0.05$) in aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase, total cholesterol, triglycerides, low-density lipoprotein and very low-density lipoprotein with significant decrease ($p < 0.05$) in high-density lipoprotein in the group induced with doxorubicin without additional treatment when compared with the *Tetracarpidium Conophorum* and Enalapril treated groups. This observation was supported by histopathological report. The repeated administration of doxorubicincaused toxic damage to the myocardium. But treatment with the *Tetracarpidium Conophorum* significantly restored ($p < 0.05$) the myocardium from the toxic damage. Treatment with Enalapril produced the best abatement, followed by the 12% walnut of the feed intake.**

Key Words: **Curative, *Tetracarpidium conophorum*, wistar rats, doxorubicin-induced, cardiotoxicity.**

I. INTRODUCTION

Background of the study

Some of the modern anti-cancer drugs like doxorubicin are associated with myocardial infarction (Asensio-López et al., 2017, Bishop and Liu, (2017, Fernandez-Chas et al., 2018). Consequently, significant myocardial necrosis and heart failure result due to reduced blood supply to the heart during the prolonged use and/or misuse of these anti-cancer drugs (Pecoraro et al., 2016). In view of this, the present study has investigated the cardiocurativeeffect of *Tetracarpidium Conophorum* (African Walnut) on the wistar rats with doxorubicin induced myocardial infarction. Myocardial infarction is the acute condition of necrosis of the myocardium that occurs due to imbalance between coronary blood supply and myocardial demand.Cardiovascular disease is a major cause of disability and premature death in the world, and accounts for a large proportion of global deaths from all causes (Dos Santos et al., 2018). Oxidative stress, which is an imbalance between the generation of oxidants and the antioxidant defence capacity of the body, has been proposed to play important role in the development and progression of myocardial infarction and heart failure (Fernandez-Chas et al., 2018, Guo et al., 2018).Doxorubicin is a member of the Anthracycline drug family, and one of the most frequently used anti-tumor agents, having a variety of therapeutic potency against most of the human tumors, including soft tissue sarcoma, breast cancer, small cell carcinoma of the lung and acute leukemias. This drug is a recombinant IgG mono-clonal antibody that binds to the human epidermal growth factor receptor protein and is used for treatment of breast cancer that over-expresses this growth factor. Its usefulness is limited by the risk of developing myocardial infarction due to its immune suppressive activity (He et al., 2018).

Herbal drugs are used widely even when their biologically active compounds are unknown, probably because of their effectiveness, lesser side effects and affordability. Today the usage of herbal drugs is gaining wider acceptance in medical practice due to their positive contribution and influence on health. The result of this study will provide harmless and affordable remedy for oxidative stress caused diseases, myocardial infarction due to doxorubicin therapy on cancer patient, hypertension, and other cardiovascular diseases.

II. MATERIALS AND METHOD

Experimental animals

Adult wistar rats (140 – 330g) of both sexes were gotten from the animal house of the University of Nigeria teaching hospital, and caged in a well-ventilated animal house of the department of Anatomy, University of Nigeria, Enugu Campus at 25 ± 5^{o}C under 12:12 hours light & dark cycle. The animals were divided into five experimental groups (A, B, C, D, and E). Each group had four rats, and was allowed to acclimatize for two weeks before the experiment. The animals had free access to standard rat chow (Grower's mash) and water *ad libitum*. All animal experiments were conducted in compliance with the humane animal care standards outlined in the 'Guide to the care and use of Animals in Research and Teaching' as approved by the Institute of Laboratory Animals Resources National Research Council, DHHS, Pub. No. NIH 86-123 (1985). Body weights were recorded every week until the end of the experiment.

Experimental protocol

The animals were divided into five experimental groups (A, B, C, D, and E), as shown below.

Table 1: Experimental protocol

GROUPS	TREATMENT
A: Food and water (Negative control)	Day 1 to 42: Grower's mash and water *ad libitum* daily, and normal saline.
B: Doxorubicin (Positive control)	Day 1, 3, 5, 7: 3.75mg/kg Doxorubicin injection on alternate first seven days of the experiment.
C: 6% walnut of feed + Doxorubicin	Day 1, 3, 5, 7: Pretreated with 3.75mg/kg Doxorubicin on alternate first seven days of the experiment. Day 8 to 42: 6% walnut of food intake.
D: 12% walnut of feed + Doxorubicin	Day 1, 3, 5, 7: Pretreated with 3.75mg/kg Doxorubicin on alternate first seven days of the experiment. Day 8 to 42: 12% walnut of food intake.
E: Enalapril + Doxorubicin (Positive control group)	Day 1, 3, 5, 7: Pretreated with 3.75mg/kg Doxorubicin on alternate first seven days of the experiment. Day 8 to 42: 0.64mg/kg Enalapril.

Doxorubicin

All drugs and reagents used in this study were of analytical grade. Doxorubicin was obtained from Olive Pharmacy, Trans-Ekulu Enugu, Enugu State of Nigeria.

Reconstitution: 50mg Lyophilized powder was reconstituted with 10ml Sterile Water for Injection to give a final concentration of 5mg/ml.

Dosage: 3.75mg/kg body weight doxorubicin was administered for alternated four days through intraperitoneal route, which constituted the cumulative dose of 15mg/kg body weight that causes myocardial infarction (Wang et al., 2018).

Storage/Stability: Intact vials (lyophilized powder) and reconstituted solution was kept stable for ≤ 15 days under refrigeration (2° to 8°C /or 36° to 46°F) and was also protected from light.

Enalapril

Enalapril was obtained from Olive Pharmacy, Trans-Ekulu Enugu, Enugu State of Nigeria.

Administration: Oral administration.

Dosage: 0.64mg/kgENA was administered to the experimental rats (Ma Hongbao and Yang Yan, 2015).

Walnut (Tetracarpidium conophorum)

Fresh walnut pod was obtained from walnut plantation at Opi, Nsukka Local Government Area, Enugu State of Nigeria. A specimen of the walnut was identified by a botanist from Department of Plant Science and Biotechnology, University Of Nigeria, Nsukka, with herbarium voucher specimen number 377a. The nuts were boiled at 100°C for 2 hours.It was then allowed to cool. The shells were removed and the milky coloured nuts were dried. The dried nuts were made into powder with mechanical grinder. The powder was formulated into feed with grower's mash in 6% and 12% weight of feed intake concentration in accordance to Ghorbani et al., 2014 and Ebrahim et al., 2012, and some other researchers' design on animal experiment with walnut.The mix ratio was calculated thus:

The percentage **:** (100 **-** The percentage)= 6% **:** (100 **–** 6%) = 6 **:** 94 = Walnut **:** Feed.

= 12% **:** (100 **–** 12%) = 12 **:** 88 = Walnut **:** Feed.

$$\therefore \text{Weight of walnut to be mixed with feed} = \frac{\%\ \text{Walnut of feed intake} \ \mathbf{X}\ \text{Weight of feed to be mixed with walnut}}{100\ -\ \%\ \text{Walnut of feed intake}}$$

Phytochemical analysis of the boiled walnut seed

The presence of phytochemical constituents in the aqueous extract ofwalnut (*tetracarpidium conophorum*) was evaluated at Brain-phosphorylationship scientific solution services, Ogui road, Enugu. Extract was tested for the presence of the following bioactive substances:alkaloid, saponins (Harborne, 1996), (Harborne, 2005), flavonoids (Sofowora, 1982), phenolic content(Lin and Tang, 2007), Test for tannins (Trease and Evans, 2002).

Biochemical and histological studies

After 42 days of the experimental period, the animals were anaesthesised under mild chloroform anaesthetic. The blood samples were collected immediately for biochemical assay, and the heart tissues were quickly harvested, washed in ice-cold saline, dried on filter paper, and fixed in 10% formol-saline for histological procedures.

Biochemical studies: Serum collected was separated by centrifuging for 10,000 rvp for 20 min. The activities of serum aspartate transaminase and alanine transaminase were respectively determined colorimetrically and spectrophotometrically by the method of(Tietz, 1995). The creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were also determined by the methods of (Tietz, 1995). The levels of total Cholesterol, triglycerides (TGs), and serum high density lipoprotein (HDL) were estimated by the methods of (Belcher et al., 1991).Serum low density lipoproteins (LDL) and very low density lipoproteins (VLDL) were calculated as LDL = total cholesterol − (HDL cholesterol + VLDL cholesterol) and VLDL=triglycerides/5 respectively.

Histological studies: The hearts were removed, washed immediately with saline and then fixed in 10% formal saline. The hearts fixedin10% formal saline were embedded inparaffin, sections cut at 5 mm and stained with hematoxylin and eosin. These sectionswere then examined under a lightdigital microscope for histoarchitectural changes.

Statistics

SPSS for Windows version 21 was used, and all results were reported as mean values ± standard deviation (SD). Descriptive statistics were done for all the variables in the various groups with a paired samples test, and ($p < 0.05$) was considered statistically significant.

III. RESULTS

Phytochemical result of the boiled seed of T. Conophorum

Table 2: Phytochemical result of the boiled dried seed of *Tetracarpidium conophorum*

Constituents	Bioassay	Mg/g
Alkaloids	+	1.545
Flavonoids	++	5.801
Phenols	+	2.620
Saponins	+++	11.415
Tannins	+	0.689

General observation on the animals

There was no death in the group fed with only grower's mash and groups treated with *Tetracarpidium conophorum*. However, a mortality rate of 25% occured in doxorubicin-induced myocardial toxicity group. Doxorubicin treated group also showed decrease in the feed and water intake during the drug treatment period when compared with other groups.

Results on the biochemical test

Table 3 and fig. 1 show mean serum enzyme level.The level of the (CK-MB, ALT, AST and LDH) in the serum significantly decreased($p < 0.05$) in the group treated with *Tetracarpidium conophorum* when compared with the group treated with doxorubicin. Treatment with *Tetracarpidium conorphorum* after DOX significantly decreased ($p < 0.05$)the serum marker enzyme.Also, the level of the serum enzyme significantly decreased($p < 0.05$) in the group treated with Enalaprilwhen compared with the group treated with doxorubicin.There was no significant increase ($p > 0.05$)in the group treated with 12% walnut (*T.C*) of the feed intake when compared with Enalapril.There was no significant increase ($p > 0.05$)in the group treated with 12% walnut (*T.C*) of the feed intake when compared to the group fed with grower's mash only.

Table 3: Mean Rat Serum enzyme

TREATMENT	CK-MB (ıμ/L)	ALT (ıμ/L)	AST (ıμ/L)	LDH (ıμ/L)
A: Positive control : Food only	157.11±5.94$^{b D}$	48.00 ±4.41$^{b D}$	66.19±8.63$^{b D}$	109.58±11.48$^{b D}$
B: Negative control : DOX only	491.21±11.46$^{a d}$	86.18 ± 5.83$^{a d}$	201.73±10.04$^{a d}$	247.41±21.38$^{a d}$
C: DOX + 6% walnut of feed	238.41±7.21$^{a b d}$	53.71±5.83$^{b A D}$	75.51±4.10$^{b A D}$	143.42±16.40$^{a b d}$
D: DOX + 12% walnut of feed	169.40±13.77$^{b d A}$	49.42±7.24$^{b A D}$	69.28±3.11$^{b A D}$	120.90±14.71$^{b d A}$
E: DOX + Enalapril	150.14±8.91$^{b A}$	50.19±8.78$^{b A}$	69.35±2.69$^{b A}$	105.20±11.89$^{b A}$

Values are mean ± SD; *n=4* in each group, ($p < 0.05$) = statistically significant.

a = Significant when compared with the positive control *(A)*, *b* = Significant when compared with the negative control *(B)*, *d* = Significant when compared with the standard drug *(E)*, *A* = Not significant when compared with the positive control *(A)*, *B* = Not significant when compared with the negative control *(B)*, *D* = Not significant when compared with the standard drug *(E)*.

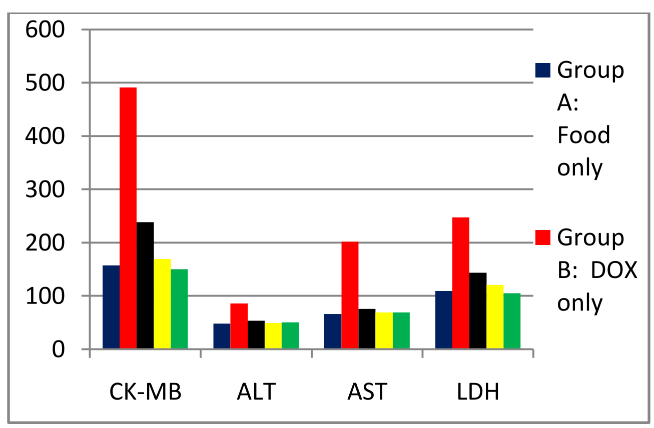

Fig. 1: Component bar graph showing serum marker enzyme level

Table 4 and fig. 2 show mean plasma lipid profile level.There was significant decreasee ($p < 0.05$) in the level of the CHOL, TRI, LDL and VLDL with increased HDL in the group treated with *Tetracarpidium conophorum* when compared with the group treated with doxorubicin. There was no significant increasee ($p > 0.05$)in the level of the CHOL, TRI, LDL and VLDL with decreased HDL in the group treated with *Tetracarpidium conophorum* when compared withEnalapril.But treatment with *Tetracarpidium conorphorum* (Walnut) after DOX-induced toxicity significantly decreased ($p > 0.05$)the level of CHOL, TRI, LDL and VLDL with increased HDL.Also, there was no significant increase ($p > 0.05$)in the level of the CHOL, TRI, LDL and VLDL with decreased HDL in the group treated with *Tetracarpidium conophorum* when compared with the group fed with grower's mash only.

Table 4: Mean Rat Lipid profile

TREAT-MENT	CHOL (mg/dl)	TRI (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
A: Positive control: Food only	105.23± $2.40^{b D}$	49.11± $2.99^{b D}$	41.17± $8.65^{b D}$	29.80± $4.89^{b d}$	19.14± $1.36^{b D}$
B: Negative control: DOX only	137.21± $11.46^{a d}$	81.19± $5.81^{a d}$	29.73± $1.56^{a d}$	79.49± $7.26^{a d}$	31.99± $2.64^{a d}$
C: DOX + 6% walnut of feed	114.41± $7.21^{a b d}$	57.71± $2.99^{b d A}$	35.51± $4.10^{d A B}$	52.67± $9.95^{a b d}$	26.24± $1.36^{a b d}$
D: DOX + 12% walnut of feed	97.40± $5.29^{b A D}$	45.42± $7.24^{b A D}$	42.28± $0.28^{b A D}$	45.46± $18.67^{b A D}$	20.65± $3.29^{b A D}$
E: DOX + Enalapril	102.64± $6.79^{b A}$	41.19± $8.78^{b A}$	43.85± $2.69^{b A}$	40.07± $0.11^{b A}$	18.73± $3.99^{b A}$

Values are mean ± SD; n=4 in each group, ($p < 0.05$) = Statistically significant.

a = Significant when compared with the positive control (A), b = Significant when compared with the negative control (B), d = Significant when compared with the standard drug (E), A = Not significant when compared with the positive control (A), B = Not significant when compared with the negative control (B), D = Not significant when compared with the standard drug (E).

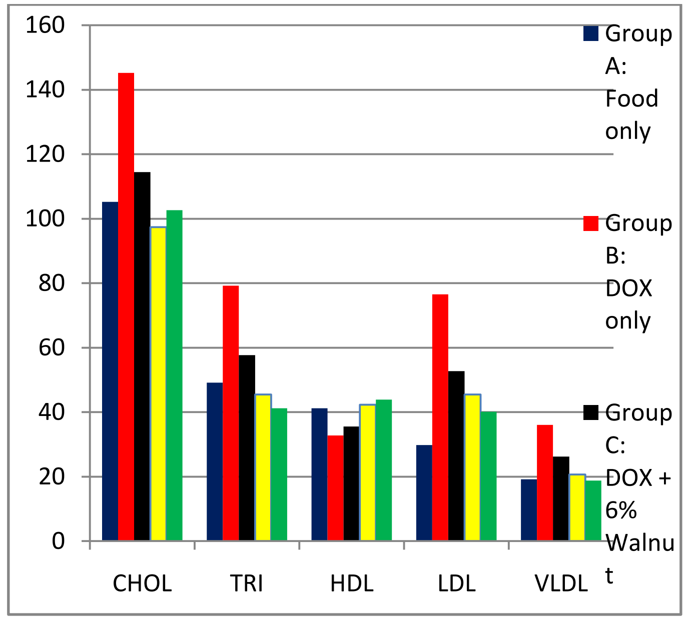

Fig. 2: Component bar graph showing lipid profile level

Histographs

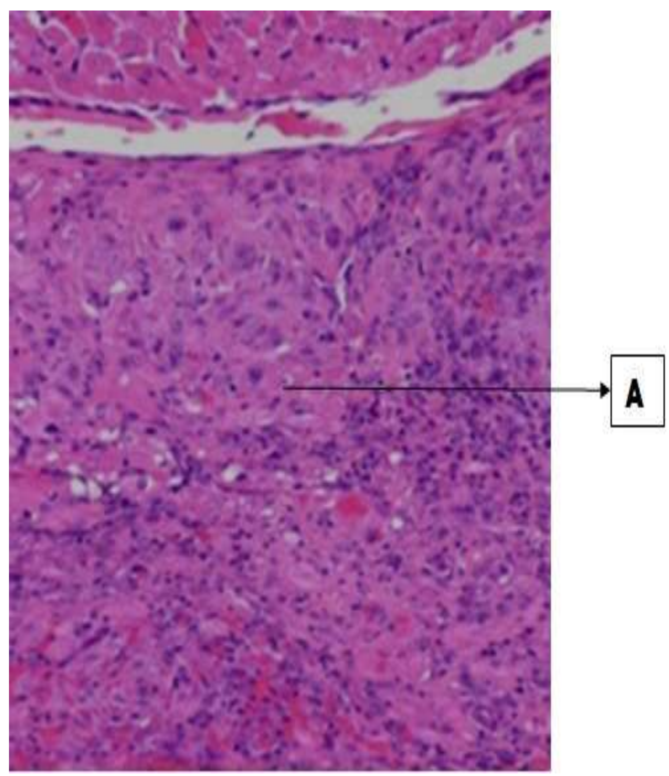

PLATE 1: Showing the heart of the rat treated with only growers mash. **A**, normal myocardial architecture (H&E x 100)

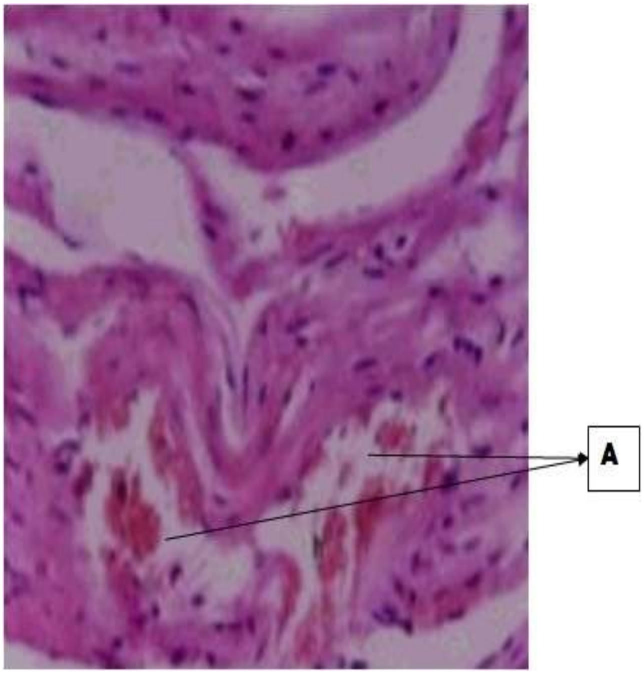

PLATE 2: Showing the heart of the rat treated with only doxorubicin. A, myocardiac necrosis around the blood vessels (H&E x 400)

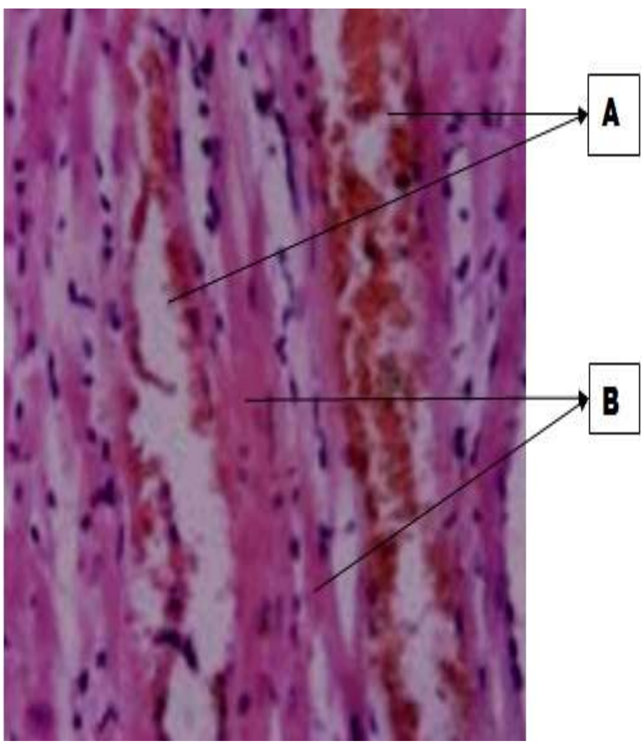

PLATE 3: Showing the heart of the rat treated with doxorubicin, and later with 6% walnut of the feed intake. A, mild dilation of the coronary artery, B, mild recovery of the myocardiac fibre (H&E x 400)

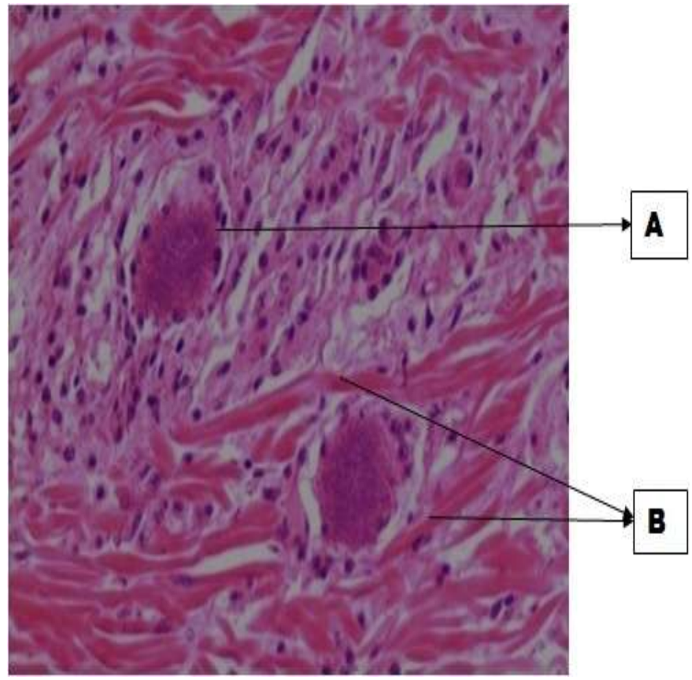

PLATE 4: Showing the heart of the rat treated with doxorubicin and, later with 12% walnut of the feed intake. A, normal dilation of the coronary artery, B, regenerating myocardiac fibre around the coronary artery (H&E x 400)

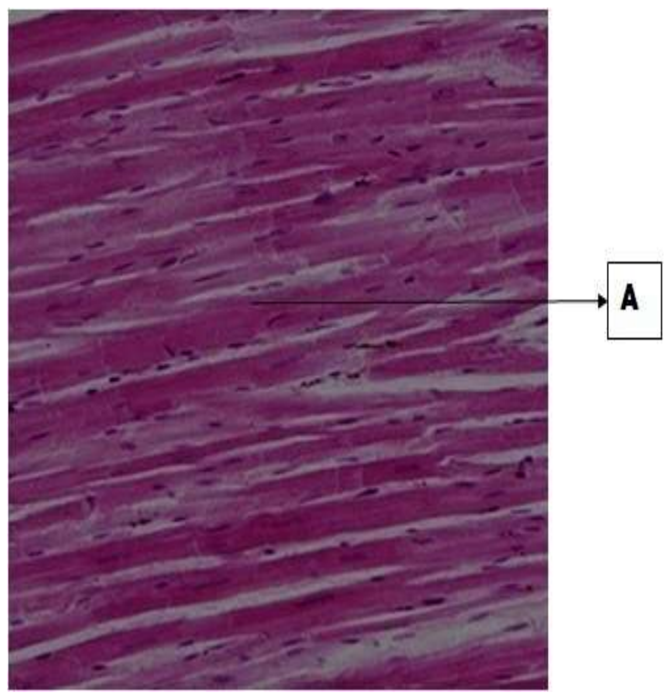

PLATE 5: Showing the heart of the rat treated with doxorubicin and, later with Enalapril. A, normal myocardiac architecture with interstitial connective tissue (H&E x 100)

IV. DISCUSSION

Oxygen-free radicals generated during doxorubicin redox cycling are responsible for the damage that doxorubicin causes to the heart (Asensio-López et al., 2017). Fernandez-

Chas et al., 2018 and many other researchers reported that there is leakage of diagnostic marker enzyme in the serum when there is myocardial infarction, which served as a diagnostic marker.The decreased level of the serum marker enzyme in the walnut treated group with no death of the animal when compared with the doxorubicin indicates that the walnut extract has the ability that may have restored the heart from the infarction.This is in accordance with Clarisse *et al.*, (2017) and Ogbonna *et al.*, (2015) report on the anti-peroxidative activity of walnut extract. Hosseini et al., 2017 reported that decreased level of serum marker enzyme has a negative correlation with myocardial infarction and increased level has a positive correlation. The close level of the serum marker enzyme in the walnut treated group when compared with Enalapril indicates that the walnut extract has potential that may have restored the heart from the infarction. Also when compared to the positive control group fed with only grower's mash.In addition, the administration of *tetracarpidium conorphorum* (walnut) significantly may have restored myocardial infarction as evidenced by the decreased level of CHOL, TRI, LDL and VLDL with increased level of HDL when compared with the doxorubicin group. The increased concentration of cholesterol could be due to a decrease in HDL, since HDL is involved in the transport of cholesterol from tissues to the liver for its catabolism. The observed increase in CHOL, TRI, LDL, VLDL might be due to a decrease in the activity of lipoprotein lipase, which resulted to decreased uptake of triglyceride from the circulation. Shakya Manish et al., 2011 reported that myocardial infarction is associated with altered lipid metabolism. Analike et al., 2017; Shakya Manish et al., 2011 and Akomolafe *et al.*, 2015 showed in their Studies that high level of CHOL, TRG, LDL, and VLDL cholesterol has a positive correlation with myocardal infarction, whereas high level of HDL cholesterol has a negative. In this context, we have observed decreased levels of HDL in doxorubicin-treated group and increased level in walnut treated group, which shows that the walnut has the potential that may have restored the heart from the myocardial infarction.

Histopathological report also suggested that*Tetracarpidium conorphorum* (Walnut) may have restored the heart from myocardial infarction, as the heart showed normal appearance, with no inflammatory cell infiltration, and no myocardial fibre degeneration, patchy intimal ulceration, and luminal stenosis and obstruction in the group treated with *T. Conorphorum*. Cardiomyopathy occurred in the doxorubicin treated group, as seen in the micrograph.Infarction occurred in the doxorubicin treated rats, as illustrated by the appearance of myocardial cell degeneration, asymmetrical medial hypertrophy, patchy intimal ulceration, and luminal stenosis and obstruction in the micrograph. Doxorubicin produced massive pathological changes in the myocardium, showing a varying degree of vacuolar damages in the cardiac muscle fibers mainly in the form of degeneration/necrosis of myocardial tissue or myofibrillar loss, vacuolization of the cardiomyocytes, and infiltration of inflammatory cells.But pretreatment with Enalapril produced the most remarkable (best) abatement,followed by12% walnut extract of the feed intake.

V. CONCLUSION

The experimental studies revealed biochemical changes in the serum as well as histological changes after doxorubicin-induced myocardial infarction in the wistar rats. But post-treatment with *Tetracarpidium conorphorum* showed restoration from this doxorubicin-induced myocardial infarction. The administration of *Tetracarpidium conorphorum* (Walnut) after doxorubicin-induced myocardial toxicity showed restoration from doxorubicin-induced elevated serum marker enzymes. This confirms that *Tetracarpidium conorphorum* (Walnut) is responsible for the maintenance of normal structural and/or architectural integrity of cardiac tissue/myocytes through restoring the heart from the myocardial infarction. This ultimately restricted the leakage of diagnostic marker enzymes in the serum, which can be accounted for the membrane stabilizing property of *Tetracarpidium conorphorum* (Walnut).

V. RECOMMENDATION

Herbal drugs are widely used even when their biologically active compounds are unknown, probably because of their effectiveness, lesser side effects and affordability. *Tetracarpidium Conophorum* is cheap and readily available in Africa, with no side effect. It contains omega-3-essential (polyunsaturated) fatty acids, which has been implicated in the normal cardiovascular function (Analike et al., 2017). High content of ascorbic acid in the seed (walnut)also indicates that it can prevent, or minimize the formation of carcinogenic substances from dietary material (Analike et al., 2017). This solves the problem (myocardial infarction) created by the doxorubicin during cancer treatment, and equally helps doxorubicin to perform its task of cancer management. Our study suggests that *Tetracarpidium conorphorum* (walnut) should be considered as a potential safe, useful and affordable substance to limit free radical mediated organ injury like myocardial infarction, especially in cancer patients undergoing treatment with anthracyclines like doxorubicin, and other pharmacologically related therapy. Meanwhile, it is worthwhile to consider this aspect for clinical application in cancer patients at risk of cardiac injury due to doxorubicin therapy, people that are hypertensive, and other people at the risk of cardiovascular diseases.The search for new interventional targets will continue to depend on the knowledge of basic pathophysiological mechanisms based on relevant preclinical models. Further molecular level of investigation should be done using different animal models and different biochemical parameters, so as to assess the possible mode of action of the seed of *Tetracarpidium conorphorum* (Walnut) as cardiocurative agent, which will help in the modeling of a new drug for various diseases.

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