

Original Research Article

Inhibition of NADPH Oxidase with Apocynin and Curcumin: A Novel Treatment Model for Diclofenac-Induced Cardiotoxicity in Male Wistar Rats

ABSTRACT

This study explored the protective potential of apocynin and curcumin in diclofenac-induced cardiotoxicity utilizing cardiac enzymes, pro-inflammatory markers, and along with histopathological endpoints. A total of 123 male Wistar rats were used for the study. 43 rats were used for the determination of the median lethal dose (LD₅₀) of apocynin and curcumin while 80 rats were randomly divided into 8 groups of 10 rats each. Group 1(control) received distilled water while others received orally, per mg/kg body weight of treatments as follows: group 2(1000, apocynin), group 3(1000, curcumin), group 4(10, diclofenac), group 5(500, apocynin and 10, diclofenac), group 6(1000, apocynin and 10, diclofenac), group 7(500, curcumin and 10, diclofenac) and group 8(1000, curcumin and 10, diclofenac). The treatments were administered daily for 14 and 28 days. LD₅₀ up to 5000mg/kg body weight of apocynin and curcumin did not show any fatality in animals. Administration of diclofenac significantly ($p < 0.05$) elevated the activities of creatinine kinase-MB, lactate dehydrogenase, levels of troponin-T, and tumor necrosis factor. There was no alteration in the activities of Interleukin-1 β . The histological results also showed cardiac insults such as cardiac muscle having severe fibro collagenous stroma, reduced myocytes density, and thick wall blood vessels. However, pretreatment with 500 and 1000 mg/kg body weight of apocynin or curcumin attenuated all biochemical alterations and histological lesions induced by diclofenac in a dose-dependent manner. Pretreatments with apocynin and curcumin inhibitors of NADPH oxidases were effective in ameliorating diclofenac-induced cardiotoxicity suppressing inflammation and restoring normal histological architecture, thus, highlighting the therapeutic potentials of apocynin and curcumin in the management of diclofenac-mediated cardiotoxicity.

Keywords: Apocynin, curcumin, NADPH oxidases inhibitors, cardiac enzymes, pro-inflammatory markers, cardiotoxicity

INTRODUCTION

Diclofenac (2-[-2',6'-(dichlorophenyl) amino] phenylacetic acid)) is the most prescribed Non-steroidal anti-inflammatory drugs (NSAIDs) because of its potent anti-inflammatory, antipyretic, analgesic, and more recently anticancer effect when compared to other NSAIDs [1,2]. These effects are explained by the inhibition of prostaglandins via inhibiting cyclooxygenase enzymes (COX). Despite these benefits, Cardiotoxicity has become the most significant side effect

observed with the use of this drug [2-4]. Drug-induced cardiotoxicity is a major clinical problem, often detected only after the introduction of the drug in clinical practice [5].

Cardiovascular disease is the term used to describe diseases affecting the heart and circulatory system. These include coronary heart disease (heart attacks, cerebrovascular disease(stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease, and heart failure [3].

Cardiovascular diseases are among the most significant determinants of morbidity and mortality in developed countries; therefore, it is important that any negative impact of drugs or toxins on this system is not understated. Globally, cardiovascular diseases are the number one cause of death and they are projected to remain so. An estimated 17.9 million people died from cardiovascular disease in 2016, representing 30% of all global deaths. Of these deaths, 7.2 million were due to heart attacks, and 5.7 million were due to stroke. About 80% of these deaths occurred in low- and middle-income countries. If current trends are allowed to continue, by 2030 an estimated 23.6 million people will die from cardiovascular disease [6].

Since the origin of human civilization, plants, and herbs have been traditionally used in the treatment of various diseases and ailments [7]. Apocynin was originally extracted from the root of the medicinal herb *Picrorhiza kurroa*, from the Himalayan Mountains; however, nowadays apocynin can be obtained commercially from several companies, which facilitates the broad application of this relatively non-toxic phytochemical. Curcumin. Curcumin is a phenolic yellow pigment constituent found in the rhizomatous parts of *Curcuma longa* [7]. Several *in vitro* and *in vivo* studies have demonstrated curcumin's protective actions [8-10]. Apocynin and curcumin have also been reported to be potent NADPH oxidases inhibitors [10,11], The efficacy of apocynin and curcumin in attenuating diclofenac-induced cardiotoxicity has not been fully explored. The aim of this study is to explore the efficacy of NADPH oxidases inhibitors: apocynin and curcumin in diclofenac-induced cardiotoxicity by assessing serum activities of cardiac ad pro-inflammatory biomarkers.

MATERIALS AND METHODS

Drugs and Chemicals

Diclofenac sodium manufactured by Laborate Pharmaceuticals Ltd, India with Batch number EDKF1-001 and NAFDAC Registration number A4-0035HP/DRUGS/MIS/04/87 and apocynin obtained from Sigma-Aldrich (St. Louis, MO, USA) was used for this study. All other reagents and chemicals used in this study were of analytical grade and were commercially available.

Plant Material

The rhizomes of *Curcuma longa* (turmeric) were procured from Choba market in Obio Akpor Local Government Area of Rivers State, Nigeria. The rhizome was identified and authenticated by a plant taxonomist in the Department of Plant Science and Biotechnology, Rivers State University, Nkpolu- Oroworukwo, Rivers State. A sample of the *Cucurma longa* rhizome was deposited at the herbarium of the Department of Plant Science and Biotechnology, Rivers State University, Nkpolu- Oroworukwo, Rivers State with voucher number PSB-085.

Preparation of ethanolic extract of turmeric

The collected rhizomes of *Curcuma longa* (turmeric) were chopped into small pellets, dried at room temperature, and ground to powder in a locally fabricated mill. Two hundred and fifty grams of the powdered sample was macerated in 1.5 liters of ethanol for 48 hours and was filtered twice; first with a sieve and then with a filter paper (Whatman No.1) to obtain a filtrate which is the extract in solution. The filtrate was thereafter concentrated in a hot air oven at 40⁰ C temperature to obtain an oily, reddish extract that weighed 21.67 grams and represented a percentage yield of 8.67. The extract was preserved in a refrigerator until needed.

% Yield was calculated as = $\frac{\text{weight of crude extract obtained (g)}}{\text{weight of starting pulverized dry leaf extracted (g)}} \times 100$

Experimental Animals

One hundred and twenty-three (123) adult male Wistar rats weighing between 180 and 200 g were obtained from a private commercial farm in Etche, Rivers State. Forty-three of the adult male Wistar rats were used for the determination of the median lethal dose of apocynin and curcumin while the remaining eighty Wistar rats were used for the biochemical studies. The rats were acclimatized in aluminum cages and housed in the animal house of the Department of Biochemistry, Rivers State University for a period of seven (7) days. The rats were acclimatized for one week prior to the commencement of the study. They were provided with clean drinking water and fed *ad-libitum* with commercially available poultry feed pellets (Topfeed®), produced by Premier Feed Mill Sapele, Nigeria (FMN). Experimental protocols were in accordance with the principles and procedures of laboratory animal use and care as enshrined by the Natural Research Council [12].

Median Lethal Dose (LD₅₀) Estimation

Median Lethal Dose (LD₅₀) Evaluation of Apocynin

The median lethal dose (LD₅₀) was determined by adopting a modified method of Karber as described by Ijioma *et al.* [13]. Twenty-five adult Wistar rats (180-200 g) assigned to 5 groups of 5 rats were administered graded daily oral doses of apocynin according to the order (Group 1 = 1000 mg/kg, Group 2 = 2000 mg/kg, Group 3 = 3000 mg/kg, Group 4 = 4000 mg/kg and Group 5 = 5000 mg/kg body weight). The rats were thereafter returned to their respective cages and allowed free access to feed and water. Each group received a single dose of apocynin and the general signs and symptoms of toxicity such as ingestion of food and water, alterations in physical appearance, behavioural changes, and mortality were noted within 24 hours and a further 7 days. The number of deaths recorded in each group within 24 hours and a further 7 days were noted and were used to evaluate the LD₅₀ by substitution into Karber's arithmetic formula.

$$LD_{50} = LD_{100} - \frac{\sum(Dd \times Md)}{N}$$

Where;

LD₁₀₀ = Dose that killed all animals in a group

LD_{50} = Dose that killed 50% of animals in a group

$\sum (Dd \times Md)$ = Sum of all products of dose difference and mean death

N = Number of animals in each group.

Median Lethal Dose (LD_{50}) Value Determination of Turmeric Extract (curcumin)

The method of Lorke [14] was employed with little modification (use of rats instead of mice). Two test phases were carried out on eighteen adult Wistar rats used. In the first phase, 3 groups of 3 rats each were administered 10, 100, and 1000 mg/kg of the extract as treatments for groups 1, 2, and 3 respectively. With zero mortalities recorded, the acute toxicity evaluation proceeded to the second phase where another set of 9 rats was assigned to 3 groups of 3 rats each were used. The first group was administered 2000 mg/kg of the extract, the second received 3200 mg/kg while the third got 5000 mg/kg. All treatments were done via the oral route. The animals were observed for toxicity signs and mortalities within 24 hours and a further 7 days. Mortalities recorded within the period were used to calculate the acute toxicity value of turmeric extract using Lorke's formula stated below as was employed;

$$LD_{50} = \sqrt{D_0 \times D_{100}}$$

Where;

LD_{50} = Lethal Median Dose

D_0 = Highest dose that gave no mortality

D_{100} = Lowest dose that produced 100% mortality.

Experimental Design

The eighty (80) male Wistar rats were randomly allocated to eight groups consisting of ten rats each. Group 1 was used as control while groups 2 to 8 served as treatment groups receiving a specific dose of diclofenac (DIC), apocynin (APO), or curcumin. Treatments were given daily for 14 and 28 days. The experiment lasted for four weeks (28 days). Group I served as normal control animals; rats received distilled water for 14 and 28 days. Group 2; rats received apocynin

(1000 mg/kg/day). Group 3; animals were given curcumin (1000 mg/kg/day). Group 4; rats received diclofenac (10 mg/kg/day). Group 5 (treated group); animals were pre-treated with apocynin (500 mg/kg/day) 30 minutes before diclofenac (10 mg/kg/day) administration. Group 6 (treated group); animals were pre-treated with apocynin (1000 mg/kg/day) 30 minutes before administration of diclofenac (10mg/kg/day). Group 7 (treated group); rats were pre-treated with curcumin (500mg/kg/day) 30 minutes before diclofenac (10mg/kg/day) administration. Group 8 (treated group); rats were pre-treated with curcumin (1000mg/kg/day) 30 minutes before diclofenac (10mg/kg/day) administration.

Drug Administration

Diclofenac was dissolved in distilled water while apocynin and curcumin were dissolved in water and three drops of tween 20 before daily oral administration by dose to each animal in the group using a stomach cannula for four weeks as follows 10mg/kg diclofenac, this dose of Diclofenac was reported to be cardiotoxic [15], 500mg or 1000mg/kg of apocynin and 500mg or 1000mg/kg of Curcumin (extrapolated from LD₅₀ >5000mg/kg). Apocynin and curcumin were given 30mins before the administration of diclofenac.

Sample Collection

The collection of blood samples and heart tissues was conducted in two phases

Phase 1: samples were collected after two weeks (15th day).

Phase 2: samples were collected after four weeks (29th day).

For each phase three rats from each group were sacrificed by cervical dislocation and blood samples were collected into clean specimen bottles for biochemical analysis while the heart was gently and carefully divided into two halves (each consisting of the atrium and ventricle) using a new surgical blade. The left half of the heart was briskly rinsed in ice-cold 1.15% potassium chloride solution in order to preserve the stress enzyme activities of the heart before being placed in a clean sample bottle which itself was kept in an ice-pack-filled cooler. This is to prevent the breakdown of the stress enzymes in the organ. The other right halve of the heart tissue were kept

in plain bottles containing 10% formalin for preservation prior to the histopathological processing.

Preparation of Sample

The bottle containing the blood sample was allowed to stand for one hour to enable the blood to clot thereafter blood sample collected was centrifuged at 3000g for 10 minutes to separate clear sera from the clotted blood. The serum obtained was used for assays of the following biochemical parameters: serum cardiac troponin T, CK-MB, LDH, IL-1 β and TNF- α .

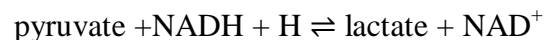
Determination Cardiac Biomarkers

Determination of Serum Creatine kinases -Myocardial Band (CK-MB) Activity

The serum activity of CK-MB was ascertained using highly sensitive CK-MB enzyme-linked immunosorbent assay (ELISA) kit MBS2515061 (MyBiosource.inc. Company, San Diego, CA. USA). Serum level of cardiac troponin (T) was also detected using high sensitivity Rat cTnT enzyme-linked immunosorbent assay (ELISA) kit MBS730382 (MyBiosource.com Company, San Diego, CA. USA). The assays were carried out with the aid of an ELISA kit Microplate Reader according to the manufacturer's procedure and expressed as pg/ml.

Determination of Serum Lactate Dehydrogenase (LDH) Activity

The serum LDH activity was determined Kinetically using Randox test kits. Kinetic determination of lactate dehydrogenase was according to the following reaction:



The amount of NAD⁺ produced is directly proportional to the lactate dehydrogenase activity present in the serum which is determined at an absorbance of 340nm.

0.02ml of sample and 1.0ml of reagent (Buffer/substrate and NADH were added into a test tube and mixed thoroughly at a temperature of 37^oc. The initial absorbance was read after 0.5 minutes

and the start timer was at the same time. The reading of the absorbance was repeated after 1, 2, and 3 minutes at a wavelength of 340nm.

Calculation: Lactate dehydrogenase activity(U/L) = (Δ OD/min) x 16030.

Pro-inflammatory Markers

Determination of Serum Tumor Necrosis Factor- α (TNF- α)

The serum level of TNF- α was measured using the high sensitivity Rat TNF- α enzyme-linked immunosorbent assay (ELISA) kit CSB-E11987r (CUSABIO, Biotechnology Company Sweden) while the Serum level of IL-1 β was assessed using Rat IL-1 β ELISA kit CSB-E08055r (CUSABIO, Biotechnology Company Sweden) according to the manufacturer's instructions.

Histological Evaluation of the Heart Tissue of Experimental Animals

Histopathological examination was done using the method of Ijioma *et al.* [13]. The heart tissues were fixed in 10% formal saline, dehydrated embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The slides were viewed under a light microscope and selected images were captured using Moticam 2.0 digital camera attached to a computer.

Statistical Analysis

Data obtained from the study were subjected to a one-way analysis of variance (ANOVA) followed by post hoc Tukey's test using GraphPad Prism 9.0 software and comparisons were done at 0.05 significance level. Values were presented as mean \pm standard error of the mean (S.E.M). Correlation analysis was carried out using the statistical package for social science (SPSS) version 25.

RESULT

Acute Toxicity of Apocynin

The result of the acute toxicity of apocynin is presented in Table 1. No death was recorded within the 24 hours and a further 7 days of the acute toxicity study, even at the highest dose of 5000 mg/kg body weight. The rats instead had a normal disposition and were emotionally stable. They were calm and clustering together, exhibited normal physical activity and agility and they all survived the period of the study.

Acute Toxicity of Turmeric Extract (*Curcumin longa*)

No death was recorded within the 24 hours and a further 7 days of the acute toxicity study, even at the highest dose of 5000 mg/kg body weight. The rats instead had a normal disposition and were emotionally stable. They were calm and clustering together, exhibited normal physical activity and agility and they all survived the period of the study as shown in Tables 2 and 3.

Table 1: Acute Toxicity Evaluation of Apocynin

Dose (mg/kg)	Number of Deaths	Percentage mortality	Dose Difference (DD)	Mean Death (MD)	DD X MD
1000	0	0	1000	0	0
2000	0	0	1000	0	0
3000	0	0	1000	0	0
4000	0	0	1000	0	0
5000	0	0

$$\sum (Dd \times Md) = 0$$

The extract is relatively safe at doses administered. $LD_{50} > 5000$ mg/kg body weight

Table 2: Phase 1 Result of Acute Toxicity Evaluation of Turmeric Extract (curcumin)

Group	Dose (mg/kg)	No. of death	Observation
1	10	0/3	Clustering and calm
2	100	0/3	Clustering and calm
3	1000	0/3	Clustering and calm

Table 3: Phase 2 Result of Acute Toxicity Evaluation of Turmeric Extract (curcumin)

Group	Dose (mg/kg)	No. of death	Observation
1	2000	0/3	Normal physical activity and agility
2	3200	0/3	Clustering, and calmness
3	5000	0/3	Clustering and calmness

LD₅₀ = >5000 mg/kg body weight.

Extract is relatively safe at doses administered. LD₅₀ > 5000 mg/kg body weight

Effect of Apocynin and Curcumin on Serum Creatine kinase-MB (CK-MB) Activity of Diclofenac-induced Cardiotoxicity in Adult Male Wistar Rats

From Figure 1. rats in groups 2 (1000 mg/kg bwt apocynin) and 3 (1000 mg/kg bwt Curcumin) maintained normal creatine kinase MB activity for 14 days (13.01 ± 0.06 ng/ml and 13.01 ± 0.06 ng/ml) and 28 days (13.02 ± 0.13 ng/ml and 13.24 ± 0.59 ng/ml) with no significant ($p < 0.05$) difference when compared to group 1 (control).

Group 4 (10 mg/kg/bwt diclofenac only) rats showed a significant ($p < 0.05$) increase in creatine kinase MB activity when compared to group 1 (control) for 14 days (20.53 ± 0.22 ng/ml) and 28 days (27.51 ± 0.23 ng/ml) duration of the study. However, groups 5 and 6 pretreated with 500 mg/kg bwt and 1000 mg/kg bwt of apocynin respectively before administration of 10 mg/kg diclofenac, significantly attenuated ($p < 0.05$) increases in creatine kinase MB activity for 14 days (13.63 ± 0.80 ng/ml and 13.58 ± 0.88 ng/ml) and 28 days (13.25 ± 0.22 ng/ml and 12.99 ± 0.06 ng/ml) when compared to group 4 (10 mg/kg bwt diclofenac) rats in a dose-dependent manner.

Similarly, groups 7 and 8 pretreated with 500 mg/kg bwt and 1000 mg/kg bwt of curcumin respectively before administration of 10 mg/kg diclofenac, also significantly attenuated ($p < 0.05$) increases in creatinine-kinase MB activity for 14 days (15.79 ± 0.39 ng/ml and 13.81 ± 0.61 ng/ml) and 28 days (13.65 ± 0.10 ng/ml and 13.03 ± 0.00 ng/ml) when compared to diclofenac only-treated rats (group 4) in a dose dependent manner.

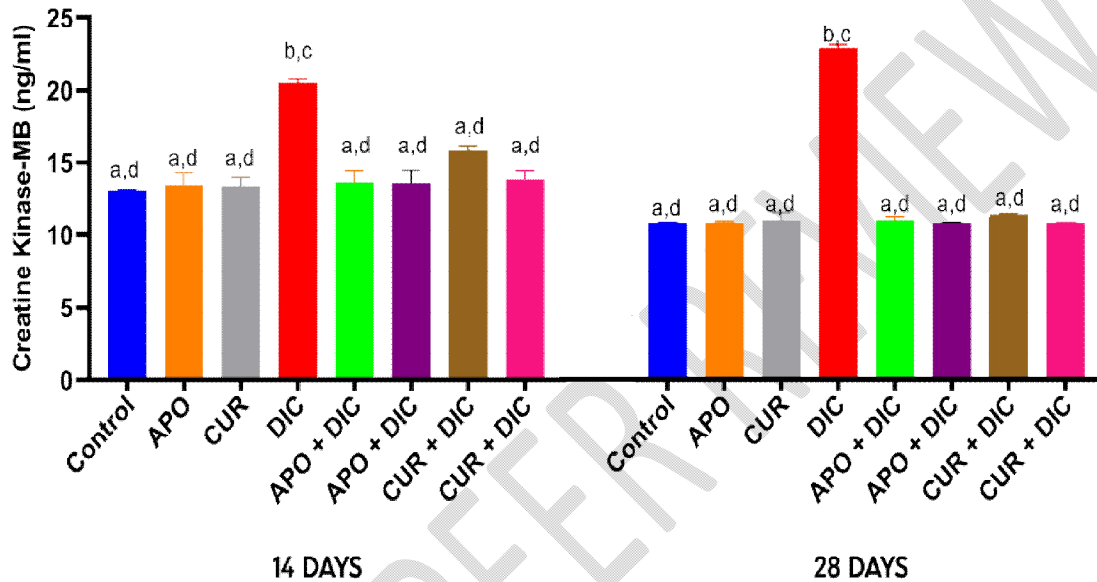


Fig. 1: Effect of apocynin (APO) and curcumin (CUR) on serum creatine kinase-MB (CK-MB) activity of Diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats.

Values are represented as mean \pm standard error mean (SEM) $n=10$. The same superscript as group 1 (control) down the group (2, 3, 4, 5, 6, 7 and 8) shows no significant difference between the group 1 and other groups while same superscript as group 4 (10mg/kg diclofenac only) down the group shows no significant difference between group 4 and treated groups at $p < 0.05$. Group 1- Control, group 2- APO (1000 mg/kg), group 3- CUR (1000 mg/kg), group 4- DIC (10mg/kg), group 5- APO (500 mg/kg) + DIC, group 6 - APO (1000mg/kg) + DIC, group 7- CUR (500mg/kg) + DIC, group 8- CUR (1000mg/kg) + DIC.

Effect of Apocynin and Curcumin on Serum Lactate Dehydrogenase (LDH) Activity of Diclofenac-induced Cardiotoxicity in Adult Male Wistar Rats

Figure 2 shows the effect of Apocynin and Curcumin on serum lactate dehydrogenase (LDH) activity of diclofenac-induced cardiotoxicity in adult male Wistar rats. Animals in groups 2 and 3 receiving 1000mg/kg bwt apocynin and curcumin respectively maintained normal lactate dehydrogenase activity for 14 days (241.07 ± 11.77 U/I and 236.22 ± 1.03 U/I) and 28 days (241.17 ± 3.14 U/I and 232.97 ± 3.75 U/I) with no significant ($p < 0.05$) difference when compared to group 1 (control).

Group 4 (10mg/kg bwt diclofenac only) showed a significant ($p < 0.05$) increase in lactate dehydrogenase activity when compared to group 1 (control) for 14 days (432.62 ± 6.51 U/I) and 28 days (540.62 ± 10.91 U/I) duration of the study. However, groups 5 and 6 pretreated with 500 mg/kg bwt and 1000 mg/kg bwt of apocynin respectively before administration of 10 mg/kg diclofenac significantly decreased ($p < 0.05$) lactate dehydrogenase activity for 14 days (253.01 ± 5.87 U/I and 247.65 ± 5.50 U/I) and 28 days (250.82 ± 2.89 U/I and 234.99 ± 7.39 U/I) when compared to diclofenac treated rats (group 4) in a dose dependent manner. Similarly, group 7 and 8 pretreated with 500 mg/kg bwt and 1000 mg/kg bwt of curcumin respectively before administration of 10 mg/kg diclofenac also significantly decreased ($p < 0.05$) lactate dehydrogenase activity for 14 days (260.58 ± 2.53 U/I and 250.90 ± 2.10 U/I) and 28 days (253.18 ± 6.90 U/I and 252.59 ± 1.28 U/I) when compared to diclofenac- treated rats (group 4) in a dose dependent manner.

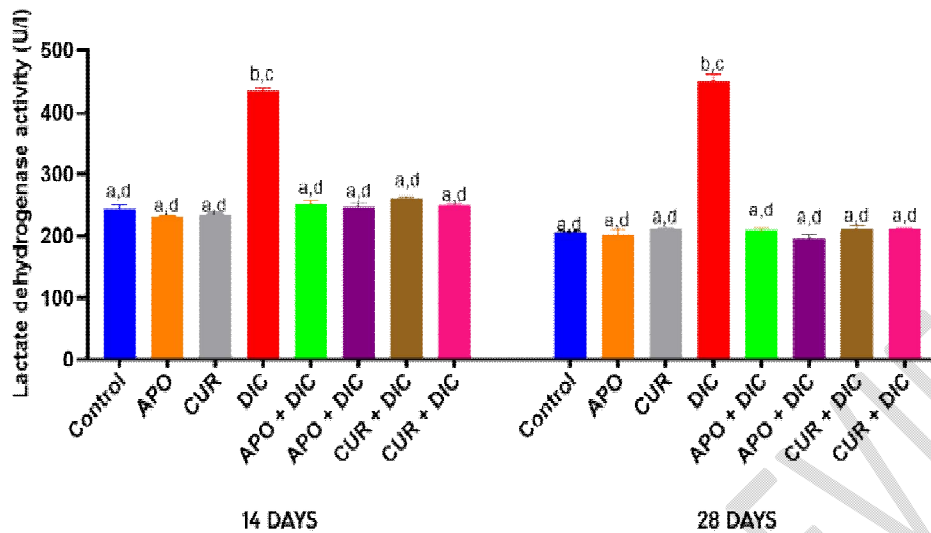


Fig. 2: Effect of apocynin (APO) and curcumin (CUR) on serum Lactate dehydrogenase (LDH) activity of Diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

Values are represented as mean \pm standard error mean (SEM) $n=10$. The same superscript as group 1 (control) down the group (2, 3, 4, 5, 6, 7 and 8) shows no significant difference between the group 1 and other groups while same superscript as group 4 (10mg/kg diclofenac only) down the group shows no significant difference between group 4 and treated groups at $p < 0.05$. Group 1- Control, group 2- APO (1000 mg/kg), group 3- CUR (1000 mg/kg), group 4- DIC (10mg/kg), group 5- APO (500 mg/kg) + DIC, group 6 - APO (1000mg/kg) + DIC, group 7- CUR (500mg/kg) + DIC, group 8- CUR (1000mg/kg) + DIC.

Effect of Apocynin and Curcumin on Serum Cardiac Troponin- T level of Diclofenac-induced Cardiotoxicity in Adult Male Wistar Rats

Figure 3 shows the effect of apocynin and curcumin serum cardiac troponin- T level of diclofenac-induced cardiotoxicity in adult male Wistar rats. Groups 2 and 3 receiving 1000mg/kg/ bwt of apocynin and curcumin respectively maintained normal cardiac troponin level for 14 days (52.54 ± 0.48 pg/ml and 56.12 ± 2.40 pg/ml) and 28 days (52.11 ± 1.33 pg/ml and 55.90 ± 0.74 pg/ml) with no significant ($p < 0.05$) difference when compared to group 1 (control).

Group 4 (10mg/kg/bwt diclofenac) rats showed a significant ($p < 0.05$) increase in cardiac troponin level when compared to group 1 (control) for 14 days (78.58 ± 1.21 pg/ml) and 28 days (82.24 ± 0.54 pg/ml) duration of the study. However, group 5 and 6 pretreated with 500 mg/kg/bwt and 1000 mg/kg/bwt of apocynin respectively before administration of 10 mg/kg diclofenac significantly decreased ($p < 0.05$) cardiac troponin levels for 14 days (54.40 ± 4.84 pg/ml and 53.88 ± 2.61 pg/ml) and 28 days (53.98 ± 0.44 pg/ml and 53.46 ± 0.09 pg/ml) when compared to diclofenac treated rats (group 4) in a dose dependent manner. Similarly, group 7 and 8 pretreated with 500 mg/kg/bwt and 1000 mg/kg bwt of curcumin respectively before administration of 10 mg/kg diclofenac also significantly attenuated ($p < 0.05$) increases in cardiac troponin level for 14 days (59.24 ± 2.84 pg/ml and 57.30 ± 1.65 pg/ml) and 28 days (57.30 ± 0.27 pg/ml and 56.88 ± 0.2 pg/ml) when compared to diclofenac treated rats (group 4) in a dose dependent manner

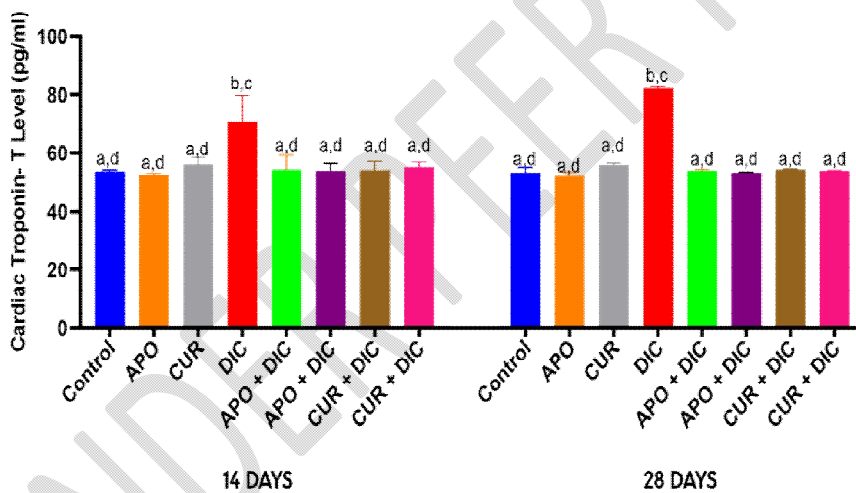


Fig. 3: Effect of apocynin (APO) and curcumin (CUR) on serum cardiac troponin- T level of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats.

Values are in triplicates and represented as mean \pm standard error mean (SEM) $n=10$. The same superscript as group 1 (control) down the group (2, 3, 4, 5, 6, 7 and 8) shows no significant difference between the group 1 and other groups while same superscript as group 4 (10mg/kg diclofenac only) down the group shows no significant difference between group 4 and treated groups at $p < 0.05$. Group 1-Control, group 2-APO (1000 mg/kg), group 3- CUR (1000 mg/kg),

group 4-DIC (10mg/kg), group 5-APO (500 mg/kg) + DIC, group 6 - APO (1000mg/kg) + DIC, group 7- CUR (500mg/kg) + DIC, group 8- CUR (1000mg/kg) + DIC.

Effect of Apocynin and Curcumin on Serum Interleukin-1beta (IL-1 β) Level of Diclofenac-induced Cardiotoxicity in Adult male Wistar Rats

Figure 4; shows the effect of apocynin and curcumin on serum interleukin-1beta (IL-1 β) level of diclofenac-induced cardiotoxicity in adult male Wistar rats. No variations were revealed in the serum interleukin-1beta level of various groups. The result showed that groups 2 and 3 receiving 1000mg/kg bwt of apocynin and curcumin respectively showed normal Interleukin-1Beta (IL-1 β) level for 14 days (87.76 ± 1.28 pg/ml and 83.30 ± 0.80 pg/ml) and 28 days (88.04 ± 0.89 pg/ml and 83.20 ± 0.74 pg/ml) with no significant ($p < 0.05$) difference when compared to group 1(control).

Group 4 (10mg/kg/bwt diclofenac only) rats showed no significant ($p < 0.05$) change in Interleukin-1Beta (IL-1 β) level when compared to group 1(control) for 14 days (87.97 ± 2.23 pg/ml) and 28 days (90.86 ± 0.09 pg/ml) duration of the study. Similarly, no significant change in interleukin-1Beta (IL-1 β) level was observed in group 5 and 6 rats pretreated with 500 mg/kg bwt and 1000 mg/kg bwt of apocynin respectively before administration of 10 mg/kg diclofenac for 14 days (81.43 ± 1.05 pg/ml and 85.87 ± 1.72 pg/ml) and 28 days (82.20 ± 0.44 pg/ml and 86.25 ± 0.32 pg/ml) when compared to diclofenac treated rats (group 4). Similarly, group 7 and 8 pretreated with 500mg/kg/bwt and 1000mg/kg/bwt of curcumin respectively before administration of 10 mg/kg diclofenac showed no significant ($p < 0.05$) change in interleukin-1Beta (IL-1 β) for 14 days (82.63 ± 2.66 pg/ml and 86.90 ± 2.53 pg/ml) and 28 days (79.92 ± 0.89 pg/ml and 79.92 ± 0.89 pg/ml) when compared to diclofenac intoxicated rats (group 4).

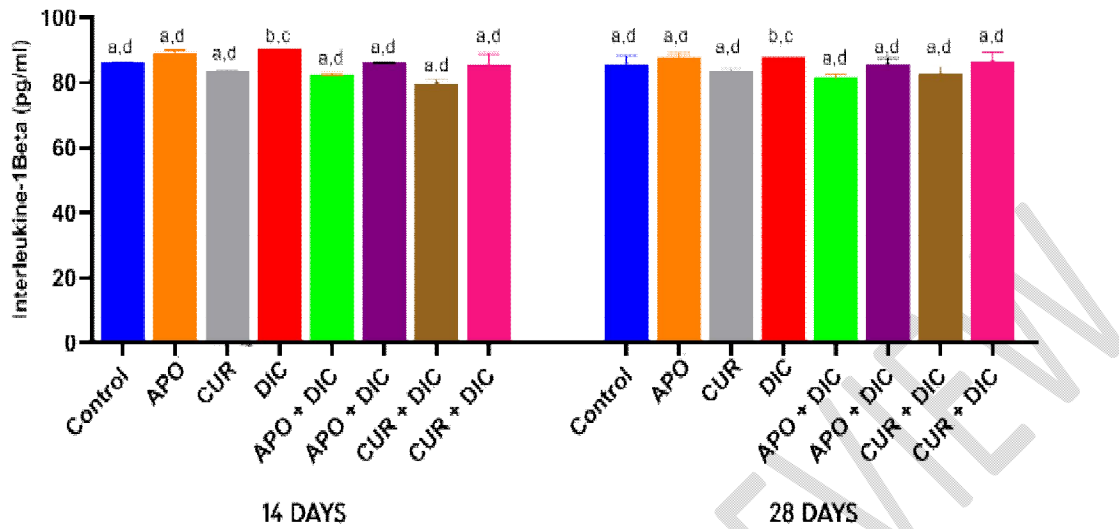


Fig. 4: Effect of apocynin (APO) and curcumin (CUR) on serum Interleukin-1Beta (IL-1 β) level of Diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats.

Values are represented as mean \pm standard error mean (SEM) n=10. The same superscript as group 1 (control) down the group (2, 3, 4, 5, 6, 7 and 8) shows no significant difference between the group 1 and other groups while same superscript as group 4 (10mg/kg diclofenac only) down the group shows no significant difference between group 4 and treated groups at $p < 0.05$. Group 1- Control, group 2- APO (1000 mg/kg), group 3- CUR (1000 mg/kg), group 4- DIC (10mg/kg), group 5- APO (500 mg/kg) + DIC, group 6 - APO (1000mg/kg) + DIC, group 7- CUR (500mg/kg) + DIC, group 8- CUR (1000mg/kg) + DIC.

Effect of Apocynin and Curcumin on Serum Tumor Necrotic Factor-alpha (TNF- α) Levels of Diclofenac-induced Cardiotoxicity in Adult Male Wistar Rats.

Figure 5 shows the effect of apocynin and curcumin on serum tumor necrotic factor-alpha (TNF- α) level of diclofenac-induced cardiotoxicity in adult male Wistar rats. Group 2 and 3 receiving 1000mg/kg/ bwt of apocynin and curcumin respectively showed normal cardiac tumor necrotic factor-alpha (TNF- α) level for 14 days (120.22 ± 0.84 pg/ml and 117.90 ± 0.95 pg/ml) and 28 days (119.80 ± 0.37 pg/ml and 117.93 ± 0.89 pg/ml) with no significant ($p < 0.05$) difference when compared to group 1 (control).

Group 4 (10mg/kg/bwt diclofenac only) rats showed a significant ($p < 0.05$) increase in cardiac serum Tumor necrotic factor-alpha level when compared to group 1 (normal control) for 14 days

(167.10±1.09pg/ml) and 28 days (211.33±1.15pg/ml) duration of the study. However, group 5 and 6 pretreated with 500mg/kg/bwt and 1000mg/kg/bwt of apocynin respectively before administration of 10 mg/kg diclofenac significantly decreased ($p<0.05$) cardiac troponin level for 14 days (115.10±2.74pg/ml and 115.35±0.61pg/ml) and 28 days (120.40±0.34pg/ml and 119.91±0.88pg/ml) when compared to diclofenac treated rats (group 4) in a dose dependent manner. Similarly, group 7 and 8 pretreated with 500 mg/kg/bwt and 1000 mg/kg/bwt of curcumin respectively before administration of 10mg/kg diclofenac also significantly decreased ($p<0.05$) cardiac troponin level for 14 days (113.43±1.88pg/ml and 113.40±1.94pg/ml) and 28 days (121.60±0.41pg/ml and 120.13±0.21pg/ml) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

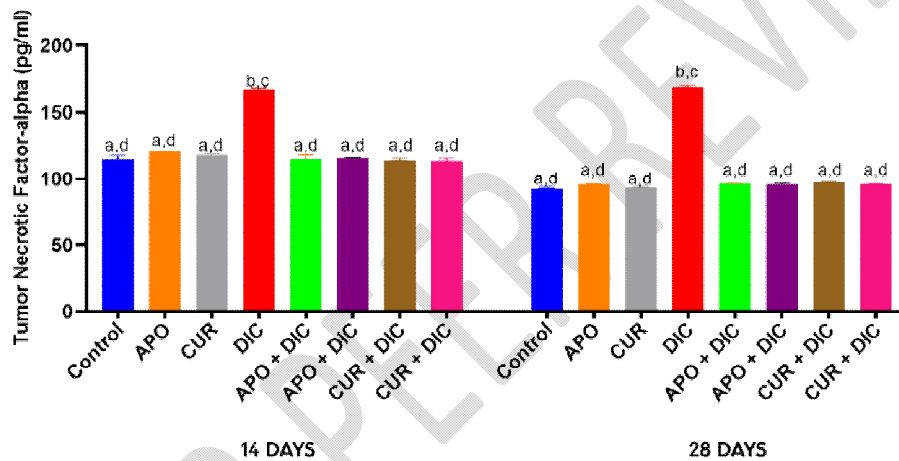


Fig. 5: Effect of apocynin (APO) and curcumin (CUR) on serum tumor necrotic factor-alpha (TNF-α) level of Diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

Values are represented as mean ± standard error mean (SEM) n=10. The same superscript as group 1 (control) down the group (2, 3, 4, 5, 6, 7 and 8) shows no significant difference between the group 1 and other groups while same superscript as group 4 (10mg/kg diclofenac only) down the group shows no significant difference between group 4 and treated groups at $p < 0.05$. Group 1- Control, group 2-APO (1000 mg/kg), group 3- CUR (1000 mg/kg), group 4- DIC (10mg/kg), group 5-APO (500 mg/kg) + DIC, group 6 - APO (1000mg/kg) + DIC, group 7- CUR (500mg/kg) + DIC, group 8- CUR (1000mg/kg) + DIC.

Histological evaluation of heart tissue of Wistar rats after 14-days treatment

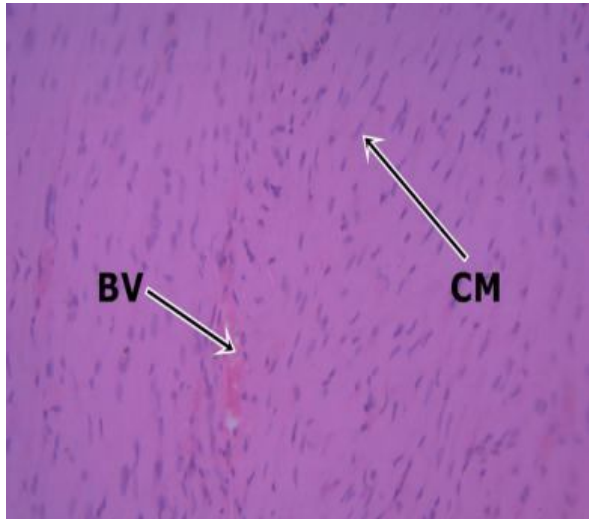


Plate 1: Histopathological section of heart (x400) of control rats. Result shows normal histology having compact myocardial muscle (CM) bundles with inconspicuous fibro collagenous stroma. The myocyte density is high that are moderately plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

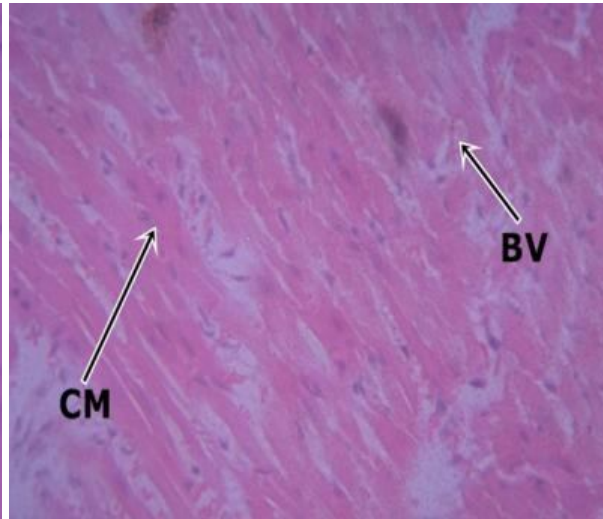


Plate 2: Histopathological section (x400) of heart of Wistar rats that received 1000 mg/kg/day of apocynin (group 2). Result shows normal histology having compact myocardial muscle (CM) bundles with inconspicuous fibrocollagenous stroma. The myocyte density is high that are moderately plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

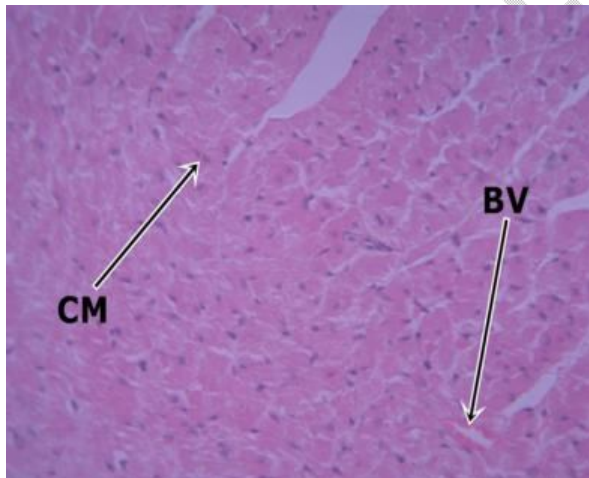


Plate 3: Histopathological section (x400) of heart of Wistar rats that received 1000 mg/kg/day of curcumin (group 3). Result shows normal histology having compact myocardial muscle (CM) bundles with inconspicuous fibrocollagenous stroma. The myocyte density is high that are moderately plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

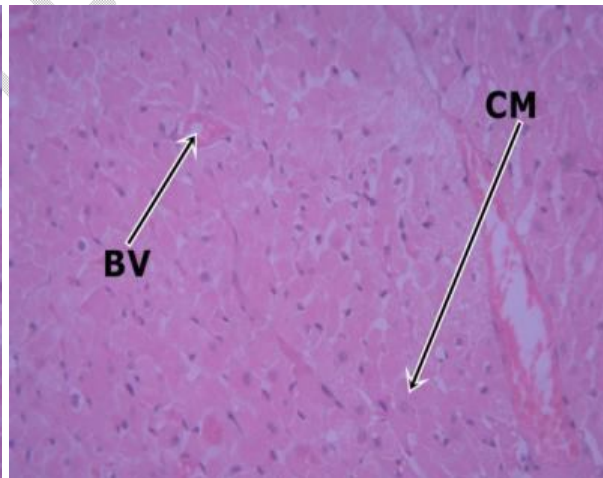


Plate 4: Histopathological section (x400) of heart of Wistar rats that were treated with 10 mg/kg/day of diclofenac (group 4). Result shows severely impaired cardiac muscle (CM) bundles with conspicuous fibrocollagenous stroma. There is reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thick-walled and lined by endothelial cells.

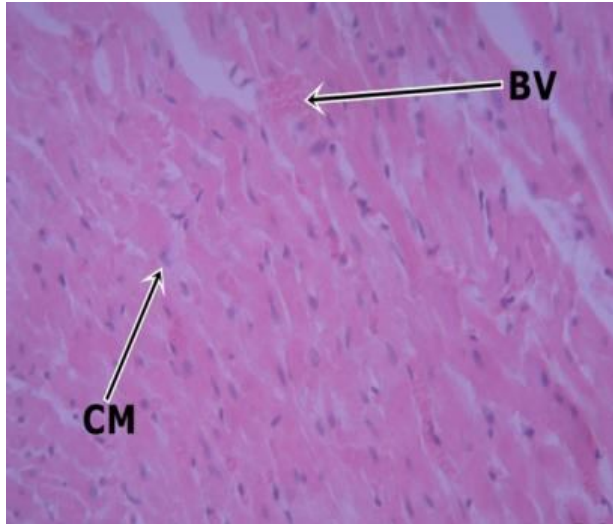


Plate 5: Histopathological section (x400) of heart of Wistar rats that were pretreated with 500 mg/kg/day of apocynin before receiving 10 mg/kg/day of diclofenac (group 5). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is mildly reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

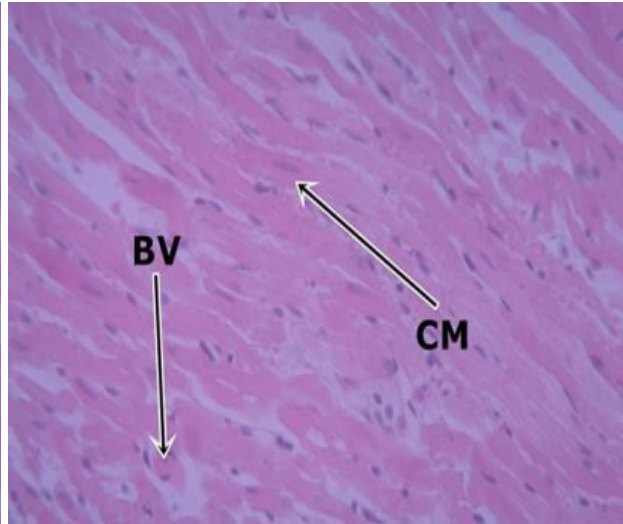


Plate 6: Histopathological section (x400) of heart of Wistar rats that were pretreated with 1000 mg/kg/day of apocynin before the administration of 10 mg/kg/day of diclofenac (group 6). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is moderately reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

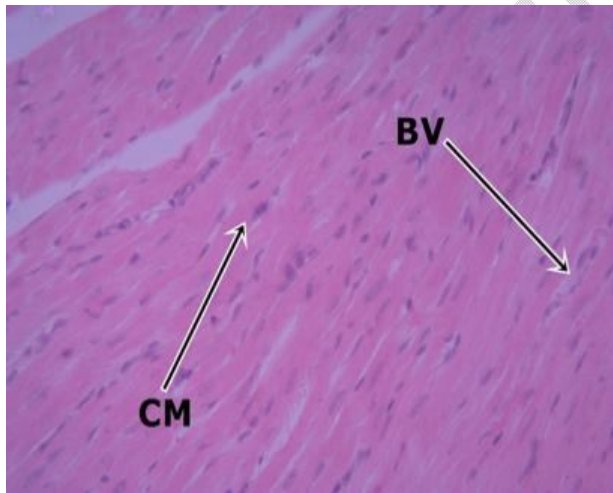


Plate 7: Histopathological section (x400) of heart of Wistar that were pretreated with 500 mg/kg/day of curcumin before the administration of 10 mg/kg/day of diclofenac (group 7). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is mildly reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

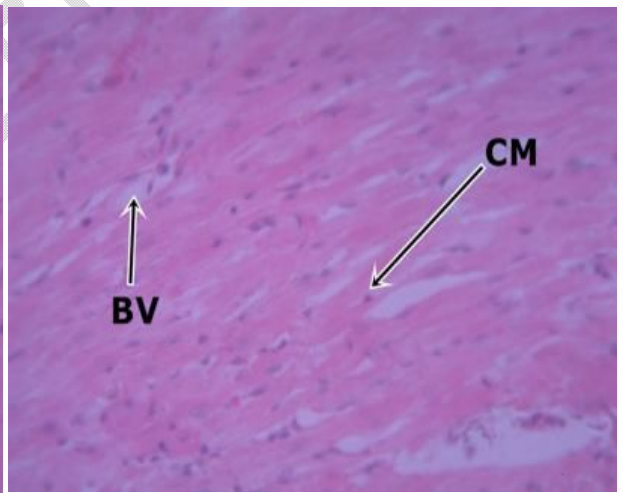


Plate 8: Histopathological section (x400) of heart of Wistar that were pretreated with 1000 mg/kg/day of curcumin before the administration of 10 mg/kg/day of diclofenac (group 8). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is moderately reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

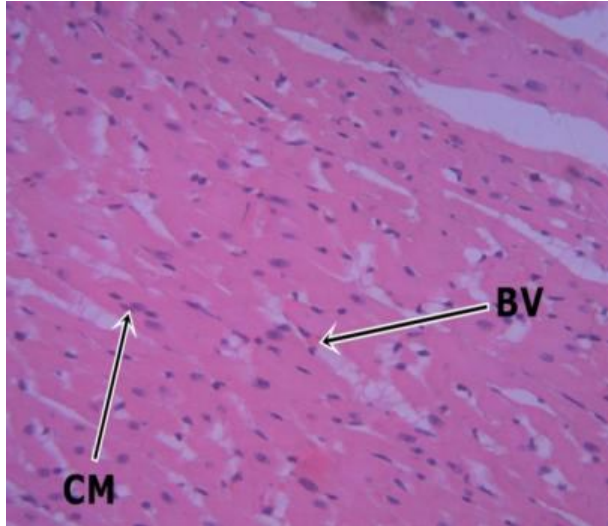


Plate 9: Histopathological section (x400) of heart of Wistar that received distilled water vehicle and served as control (group 1). Result shows normal histology having compact myocardial muscle (CM) bundles with inconspicuous fibrocollagenous stroma. The myocyte density is high and moderately plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

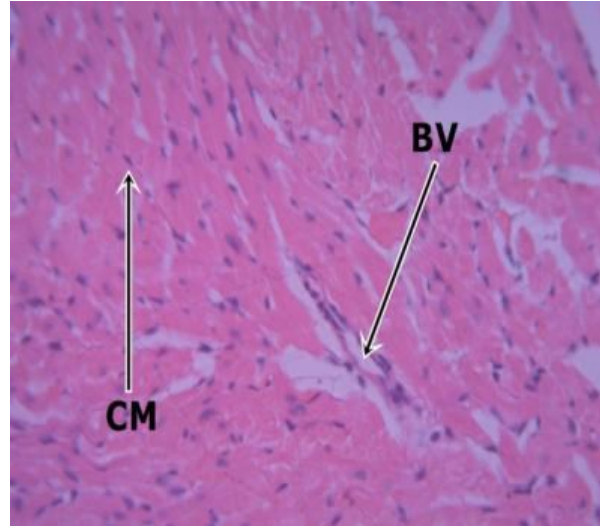


Plate 10: Histopathological section (x400) of heart of Wistar that received 1000 mg/kg/day of apocynin (group 2). Result shows normal histology having compact myocardial muscle (CM) bundles with inconspicuous fibrocollagenous stroma. The myocyte density is high and moderately plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

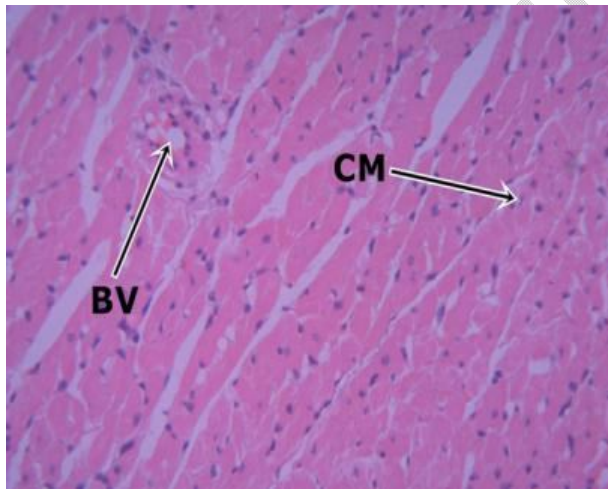


Plate 11: Histopathological section (x400) of heart of Wistar that received 1000 mg/kg/day of curcumin (group 3). Result shows normal histology having compact myocardial muscle (CM) bundles with inconspicuous fibrocollagenous stroma. The myocyte density is high and moderately plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

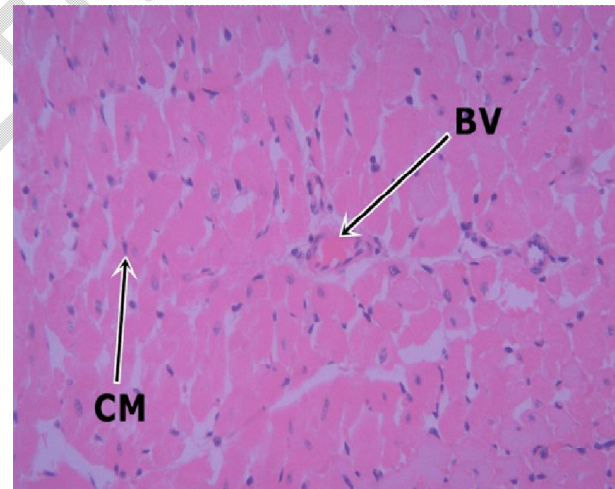


Plate 12: Histopathological section (x400) of heart of Wistar that were treated with 10 mg/kg/day of diclofenac (group 4). Result shows severely impaired cardiac muscle (CM) bundles with conspicuous fibrocollagenous stroma. There is reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thick-walled and lined by endothelial cells.

Histological evaluation of heart tissue of Wistar rats after 28-days treatment

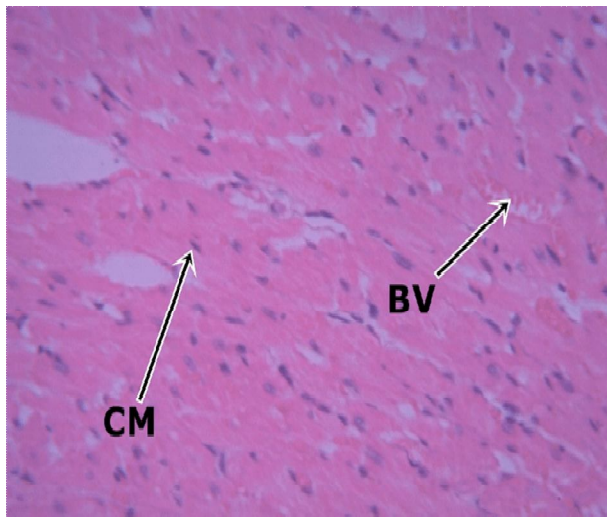


Plate 13: Histopathological section (x400) of heart of Wistar that were pretreated with 500 mg/kg/day of apocynin before receiving 10 mg/kg/day of diclofenac (group 5). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is mildly reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

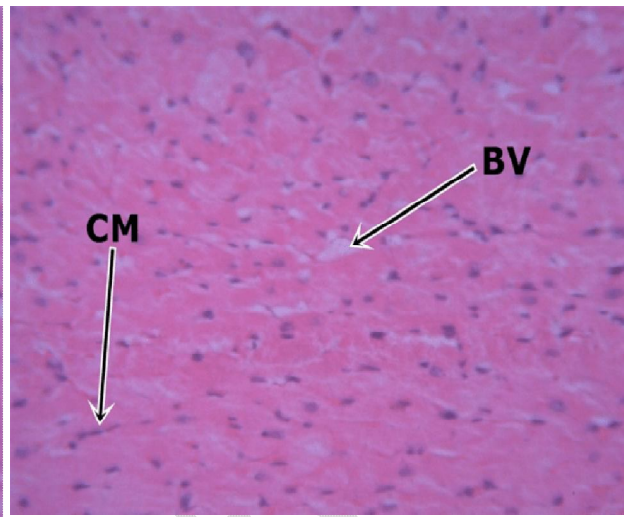


Plate 14: Photomicrograph of histology of heart of rats that were pretreated with 1000 mg/kg/day of apocynin before receiving 10 mg/kg/day of diclofenac (group 6). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is moderately reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

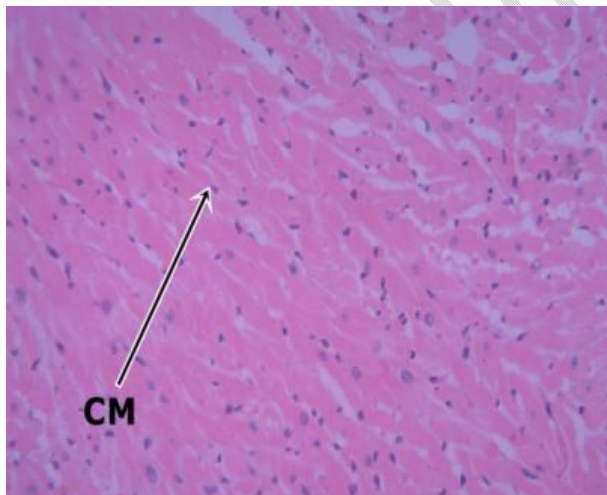


Plate 15: Histopathological section (x400) of heart of Wistar that were pretreated with 500 mg/kg/day of curcumin before receiving 10 mg/kg/day of diclofenac (group 7). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is mildly reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

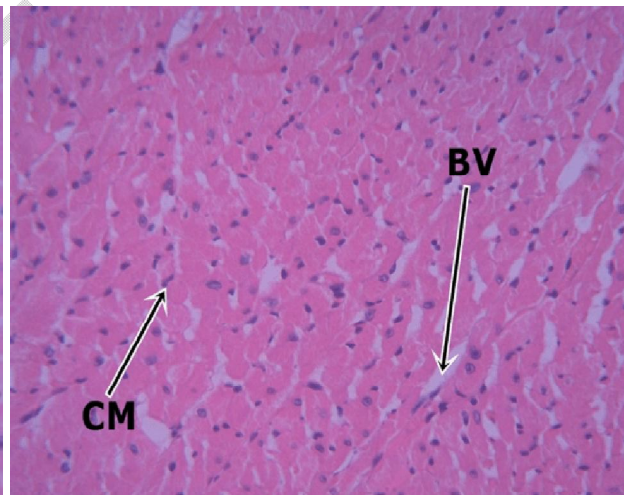


Plate 16: Histopathological section (x400) of heart of Wistar that were pretreated with 1000 mg/kg/day of curcumin before receiving 10 mg/kg/day of diclofenac (group 8). Compared with group 4 rats treated with 10 mg/kg/day of Diclofenac only, result shows improved cardiac muscle (CM) bundles with moderate fibrocollagenous stroma. There is moderately reduced myocyte density that are mildly plump. The blood vessels (BV) have narrow lumina, thin-walled and lined by endothelial cells.

DISCUSSION

Results of this work showed that short and long term non- selective inhibition of cyclooxygenase (COX) enzyme by diclofenac, which is the mechanism by which it exerts its therapeutic effect, had a similar cardiotoxic effect. This finding supports the earlier report of the Medicines Adverse Reactions Committee, which reviewed that all NSAIDs especially the nonselective NSAIDs increase the risk of cardiovascular diseases, and the risk is increased with both short-term and long-term use [3]. Also repeated pretreatment with apocynin or curcumin potent inhibitors of NADPH oxidase before the administration of diclofenac showed a similar significant degree of amelioration on cardiac and pro-inflammatory biomarkers.

The evaluation of serum activity of CK-MB, LDH, and cTnT is an important tool in the clinical analysis as it helps to determine the extent of cardiac damage. CK-MB and cTnT are specific and sensitive cardiac damage markers while LDH is regarded as secondary because of a lack of specificity for the heart [16]. These enzymes together are considered reliable markers of cardiotoxicity and are as such used in monitoring drug induced-cardiotoxicities including diclofenac and their increase in the serum is a marker of cellular outflow and lack of the functional integrity of cell membranes of cardiomyocytes [17,18]. Results from this study which revealed that the serum activities of CK-MB, LDH and cTnT were significantly elevated in treated rats following repeated daily administration of 10mg/kg diclofenac only for 14 days and 28days, is a strong indication that diclofenac-induced cardiac damage was reliably established. The result obtained from this study agrees with the works of Ayse *et al.* [19], Oda *et al.* [20], and Abdel-Daim *et al.* [21] who in their separate works reported a significant increase in the serum activity of CK-MB, LDH, and cTnT in rats and rams following the administration of diclofenac. This effect could be a secondary event to increased lipid peroxidation of cardiac membranes as indicated in the results obtained from this study. This could result in irreversible modification and damage of membrane structures with consequent leakage of cardiac proteins [22].

However, repeated oral pretreatments with apocynin (500mg/kg or 1000mg/kg) and curcumin (500mg/kg or 1000mg/kg) significantly attenuated elevations in serum levels of these cardiac markers, thus, indicating the potential therapeutic role of these agents in mitigating the deleterious effects of acute and sub-chronic administration of diclofenac on the integrity of cardiac myocytes. This result supports the works of Swamy *et al.* [8], El-Sawalhi and Ahmed

[23] whose results showed a significant decrease in the activity of cardiac cTn-T, CK-MB, and LDH in the administration of apocynin and curcumin. The cardioprotective effect demonstrated by these agents could possibly be a result of inhibition of irreversible modification and damage of membrane structures with consequent leakage of cardiac protein.

The assessment of the effects of diclofenac on the expression of inflammatory cytokines such as IL-1 β and TNF- α is shown in Figures 4 and 5. The result obtained shows that repeated daily administration of 10mg/kg diclofenac for 14 days and 28 days did not significantly alter the level of IL-1 β . However, TNF- α level was significantly elevated in rats that received diclofenac only for 14 days and 28 days when compared to the control. The ability of prostaglandin E₂(PGE₂) to reverse the NSAID- induced increase in TNF- α in a study conducted by [24], suggests that it is the NSAID-induced reduction in PGE₂ expression that is responsible for the alterations in cytokine production seen. Originally, TNF- α was thought to be produced mainly by immune cells like activated macrophages and lymphocytes, studies have reported its expression also in other cells such as the cardiac myocytes. The result obtained from this study supports the work of Page *et al.* [24] whose work showed that diclofenac exerts an excellent therapeutic effect in the presence of increased levels of TNF- α . The pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-1 β have been extensively implicated in the pathogenesis of heart failure. It is known that TNF- α is elevated in the myocardium under volume overload and its expression is activated in response to initial myocardial insult (e.g. myocardial infarction) via direct action. Inflammatory cytokines modulate the phenotype and function of all myocardial cells, suppressing contractile function in cardiomyocytes, inducing inflammatory activation in macrophages, stimulating microvascular inflammation and dysfunction, and promoting a matrix-degrading phenotype in fibroblasts. Apart from reducing contractility in cardiac myocytes, TNF- α induces cell hypertrophy manifested by cell enlargement, increased global protein synthesis, and induction of sarcomere organization [25]. The result obtained from this study agrees with the works of Dick and Epelman [26] and Hanna and Frangogiannis [27]. Dick and Epelman [26] in their animal studies also demonstrated that administration or overexpression of TNF- α leads to worsening heart failure, and blockade of TNF- α improves cardiac function in models of heart failure. Second, TNF deletion attenuated dysfunction in a model of left ventricular pressure overload, and TNF- α antagonism reduced adverse remodeling and improved hemodynamics in models of volume overload and post-infarction heart failure. Hanna and Frangogiannis [27]

reported that elevated levels of TNF- α may play a causative role in heart failure. In their study, mice with cardiac-specific overexpression of TNF- α develop dilated cardiomyopathy and infusion of TNF- α impairs cardiac dysfunction in dogs

However, repeated oral pretreatments with apocynin (500mg/kg or 1000mg/kg) and curcumin (500 mg/kg or 1000 mg/kg) significantly inhibited elevation in the levels of TNF- α induced by treatment with diclofenac only thereby preventing the TNF- α challenge. Apocynin and curcumin may also prevent tissue damage in diclofenac-treated rats by inhibiting the elevation of levels of TNF- α in the cardiac cells. This finding is in agreement with the work of Zhao *et al.* (2014) whose result showed that administration of apocynin or curcumin decreased the production of pro-inflammatory cytokine TNF- α thereby protecting the heart [28]. This could be because of the ability of apocynin and curcumin to attenuate myocardial insult imposed by repeated administration of diclofenac. Rats pretreated with apocynin before the administration of diclofenac, showed more reduction in serum level of TNF- α than the group pretreated with curcumin. apocynin and curcumin may also prevent tissue damage in diclofenac-treated rats by inhibiting the elevation of levels of TNF- α in the cardiac cells.

Histopathology of tissue sections of 14 days revealed well intact cardiac tissue with normal cardiac muscle and blood vessels in plate 2 (1000mg/kg apocynin) and plate 3 (1000mg/kg curcumin) treated rats when compared to the control group (plate 1). Repeated daily oral administration of 10 mg/kg of diclofenac for 14 days (Plate 4) resulted in severe cardiac insult with cardiac muscle (CM) having severe fibro collagenous stroma, severely reduced myocyte density that is mildly plump, the blood vessels (BV) have narrow lumina and are thicken-walled and lined by endothelial cells when compared to the control group (Plate 1). The cardiac damage score revealed in the histopathological examination is in accordance with the biochemical changes earlier observed in the diclofenac treated group.

However, the cardioprotective effect of apocynin and curcumin on the diclofenac-induced cardiac injury was further confirmed by the histological findings. Repeated oral pretreatments with apocynin (500 mg/kg or 1000 mg/kg) for 14 days (Plate 5 and Plate 6) significantly improved the diclofenac-induced histopathological alterations (Plate 4) with the cardiac muscle and blood vessel showing nearly normal structure. Similarly, oral pretreatments with curcumin

(500mg/kg or 1000 mg/kg) for 14 days (Plates 7 and 8) significantly improved diclofenac-induced histopathological alterations (Plate 4).

At 28 days, the histological examination showed well intact cardiac tissue with normal cardiac muscle and blood vessels in Plate 10 (1000 mg/kg apocynin) and Plate 11 (1000mg/kg curcumin) treated rats when compared to the control group (Plate 9). Repeated daily oral administration of 10 mg/kg of diclofenac for 28 days (Plate 12) resulted in severe cardiac muscle alteration showing severe fibro collagenous stroma, severely reduced myocyte density that are mildly plump and thicken-walled blood vessels (BV) when compared to the control group (Plate 9). The cardiac damage score revealed in the histopathological examination is in accordance with the biochemical changes earlier observed in the diclofenac-treated group.

However, the cardioprotective effect of apocynin and curcumin on the diclofenac-induced cardiac injury was further confirmed by the histological findings. Repeated oral pretreatments with apocynin (500mg/kg or 1000mg/kg) for 28 days (Plate 13 and Plate 14) significantly improved the diclofenac-induced histopathological alterations (Plate 12) with the cardiac muscle and blood vessels showing nearly normal structure. Similarly, oral pretreatments with curcumin (500mg/kg or 1000mg/kg) for 28 days (Plate 15 and 16) significantly improved diclofenac-induced histopathological alterations (Plate 12).

The protective results observed by apocynin and curcumin pretreatment agrees with the report of Zhao *et al.* [10], Fan *et al.* [11], and Rosa *et al.* [29]. This could be explained most likely by the time element of 30 minutes of apocynin and curcumin administration before induction of myocardial damage by diclofenac, which allowed for the formation of the active dimer required for better protection of heart muscle.

CONCLUSION

These findings show that Acute (14 days) and sub chronic (28 days) pretreatment with apocynin and curcumin in diclofenac induced cardiotoxicity Significantly attenuated diclofenac induced elevation of cardiac biomarkers (cTn-T, CK-MB and LDH probably due to the ability of this specific NOX 2 inhibitor, apocynin and curcumin to prevent membrane injury and subsequent leakage of this cardiac proteins. Ameliorated influx of proinflammatory cytokines TNF- α but not

IL-1 β into the cardiac tissues thereby inhibiting the activation of NOX 2 by TNF- α . TNF- α induces of NOX 2 activation.

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