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Original Research Article

Cardioprotective Effects of Apocynin and Curcumin against Diclofenac-Induced Cardiotoxicity in Male Wistar Rats via Inhibition of Oxidative Stress

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Abstract

This study explored the protective potential of NADPH-oxidase inhibitors, apocynin and curcumin in diclofenac-induced cardiotoxicity via oxidative stress. A total of 80 male Wistar rats were used for the study. 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. Administration of diclofenac significantly ($p<0.05$) elevated the activities of NAD(P)H oxidases type 2 and malondialdehyde while the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione level were significantly ($p<0.05$) decreased. There was no alteration in the activities of xanthine oxidase. However, pretreatment with 500 and 1000 mg/kg body weight of apocynin or curcumin attenuated all biochemical alterations induced by diclofenac in a dose dependent manner. Pretreatments with apocynin and curcumin inhibitors of NOX 2 was effective in ameliorating diclofenac-induced cardiotoxicity by alleviating the oxidative stress thus, highlighting the therapeutic potentials of apocynin and curcumin in the management of diclofenac-mediated cardiotoxicity.

Keywords: Diclofenac, NADPH-oxidase inhibitors, apocynin, curcumin, oxidative stress, cardiotoxicity.

INTRODUCTION

Cardiovascular diseases have been the prime cause of mortality worldwide for decades and they are projected to remain so (Medsafe, 2019). 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 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 (Mladenka *et al*., 2018).

Non-steroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory, antipyretic and analgesic effects and these effects are explained by the inhibition of prostaglandins via inhibiting cyclooxygenase enzymes (COX). Diclofenac (2-[-2′,6′-(dichlorophenyl) amino] phenyl acetic acid)) is the most prescribed NSAIDs because of its potent anti-inflammatory, antipyretic, analgesic and more recently anticancer effect when compared to other NSAIDs (Balding, 2013; Erdal and Sefa, 2017). It is extensively used for treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and mild to moderate pain. Cardiotoxicity has become the most significant side effect observed with the use of this drug (Erdal and Sefa, 2017; Ojha *et al.*, 2016; Okwakpam *et al.*, 2021).

No mechanism is currently known to be associated with the side effects of the diclofenac directly on the heart. However, recent studies have established that oxidative stress resulting from increased generation of reactive oxygen species may have a major role among these factors (Ghosh *et al*.,

2016). Diclofenac have been shown to induce ROS generation in cardiac tissue (Okwakpam *et al*., 2020).

Oxidative stress is a molecular deregulation in reactive oxygen species (ROS) metabolism involved in the pathogenesis of several diseases. In recent times, oxidative stress is no longer considered as a simple imbalance between the production and scavenging of reactive oxygen species (ROS), but as a dysfunction of enzymes involved in ROS production (Konior *et al*., 2014; Gracia *et al*., 2017).

Although it is well established that oxidative stress plays a major role in development of cardiovascular diseases, hardly any clinical study testing antioxidant supplementation to prevent or treat cardiovascular diseases resulted in improved outcomes (Gori *et al*., 2011; Davies and Holt, 2018). In contrast, mortality was even increased in some trials using vitamin E and vitamin C supplementation (Rodiño-Janeiro *et al*., 2013; Ye *et al*., 2013; Zhang *et al*., 2020). Next to potentially causing reductive stress and thus worsening cardiovascular outcome rather than improving it, the lack of specificity of antioxidants towards a certain ROS at a specific site might have contributed to their clinical failure (Rodiño-Janeiro *et al*., 2013; Ye *et al*., 2013; Davies and Holt, 2018). Because antioxidant supplementation proved to be non-effective or even detrimental, another therapeutic strategy to fight oxidative stress evolved: Targeting the sources of pathophysiological ROS rather than trying to scavenge ROS in a generalized fashion after they have been produced. Therefore, prevention of ROS formation, by targeting specific sources of superoxide anion and other ROS, might prove beneficial. Potential targets include xanthine oxidase, lipoxygenases, uncoupled nitric oxide synthase (NOS), Nicotinamide adenine dinucleotide (NADPH) oxidases (NOX) and the mitochondrial oxidases. The majority of these enzymes only produce ROS after they have been damaged by ROS. In contrast, NADPH oxidases produce ROS as their primary and sole function.

NOX enzymes have been implicated in multiple diseases associated with oxidative stress including cardiovascular disease.

Many natural products are enzyme inhibitors; the finding and development are dynamic areas of pharmacology and biochemistry. Apocynin (4-hydroxy-3-methoxyacetophenone) also known as acetovanillone is a naturally occurring acetophenone, found in the roots of *Apocynum cannabinum and Picrorhiza kurroa* with a molecular weight of 166.17g/mol. Apocynin has been used as an efficient inhibitor of the complex NADPH-oxidase in many experimental models involving phagocytic and nonphagocytic cells (Simonyi *et al*., 2011; Impellizzeri *et al*., 2011; Tain *et al*., 2012; Kim *et al*., 2013 and Mouzaoui *et al*., 2014). Curcumin is a phenolic yellow pigment constituent found in the rhizomatous parts of *Curcuma longa* (turmeric), commonly considered as its most active constituent (Abarikwu *et al*., 2014; Prasad *et al*., 2014). It possesses various pharmacologic and biological properties and is usually used in Nigeria and in most tropical and subtropical regions of the world as a spice and medical agent (Prasad *et al*., 2014).

However, whether preventing oxidative stress via inhibition of NOX 2 with apocynin and curcumin can reduce cardiotoxicity induced by diclofenac and by what possible mechanism is the enzyme NADPH oxidase activated in this setting has not been explored. Hence, the aim of this study, is to provide novel evidence that NADPH oxidase play critical role in diclofenac induced cardiotoxicity and to indicate that directly targeting NOX 2, is a viable potential pharmaceutical owing to the current therapeutic importance of diclofenac. Results obtained from this study are expected to bring relief to various patients and general public.

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 *Curcuma 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 40^0 C temperature to obtain an oily, reddish extract which weighed 21.67 grams and represented a percentage

yield of 8.67. The extract was preserved in a refrigerator until needed.

Experimental Animals

Eighty (80) adult male Wistar rats weighing between 180 and 200 g were obtained from a private commercial farm in Etche, Rivers State. 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 Natural Research Council (Natural Research Council of the National Academies, 2011).

Experimental Design

The eighty (80) male Wistar rats were randomly allocated to eight groups consisting of ten rats each. Group1was used as control while groups 2 to 8 served as treatment groups receiving 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) 30minutes before diclofenac (10 mg/kg/day) administration. Group 6 (treated group); animals were pre-treated with apocynin (1000 mg/kg/day) 30minutes before administration of diclofenac (10 mg/kg/day). Group 7 (treated group); rats were pre-treated with curcumin (500 mg/kg/day) 30minutes before diclofenac (10 mg/kg/day) administration. Group 8 (treated group); rats were pre-treated with Curcumin (1000 mg/kg/day) 30minutes before diclofenac (10 mg/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 (Okwakpam *et al*., 2020), 500mg or 1000mg/kg of apocynin and 500mg or 1000mg/kg of Curcumin (extrapolated from LD_{50} >5000mg/kg). Apocynin and curcumin were given 30mins before the administration of diclofenac.

Sample Collection

The collection of heart tissues was conducted in two phases on15th and 29th day. For each phase three rats from each group were sacrificed by cervical dislocation and 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.

Preparation of Heart Tissue Homogenate

Phosphate buffer was prepared by dissolving 3.4 g of sodium mono hydrogen phosphate and 21g of sodium dihydrogen phosphate in one liter of water. The pH of the buffer solution was adjusted to 7.4 via drop by drop addition of either NaOH or HCl. One gram of the heart tissue was crushed in a laboratory mortar and homogenized in 5 ml of the prepared phosphate buffer of pH value 7.4. The resulting homogenized tissue in phosphate buffer was then centrifuged in a bench centrifuge at 5000 rpm for 15 minutes. The supernatant was then transferred into a fresh plain bottle, labeled appropriately and preserved at extremely low temperature until needed**.** The heart homogenate was used for assay of the following biochemical parameter tissue activity of NOX 2, XOD, SOD, CAT, GSH- Px, GSH and MDA.

Determination of Oxidative Stress Markers

Prooxidant Enzyme Biomarkers

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity and xanthine oxidase were assayed in heart homogenates using CSB-E13614r ELISA kit (CUSABIO, Biotechnology Company, Sweden) with the aid of ELISA plate reader as stated in the manufacturer's manual.

Antioxidant Enzymes Biomarkers

The activities of Superoxide dismutase (E.C.1.15.1.1.) and Catalase (CAT, E.C.1.11.1.6.) glutathione were measured by using the sensitive rat SOD Enzyme-linked Immunosorbent Assay (ELISA) kit KT-60703 (Kamiya Biomedical Company) and MBS701713 (MyBiosource inc, Company, Southern California, USA) respectively with the aid of ELISA kit Microplate Reader according to the manufacturer's protocol. Reduced glutathione (GSH) content of cardiac tissues homogenate were determined using a high sensitivity and excellent specificity rat glutathione enzyme-linked immunosorbent assay (ELISA) Kit MBS724319 (MyBiosource.com Company, San Diego, CA. USA) while the Malondialdehyde (MDA) level of cardiac tissues homogenate was measured as index of lipid peroxidation using high specificity ELISA kit MBS9389391 (My BioSource Inc., Company, San

Diego, CA. USA) and presented as activity units per mg of protein (units/mg proteins).

Protein Quantification

Total Protein in the cardiac tissue was quantified using Randox kit Biuret method as described by Tietz (1995) with bovine serum albumin as the standard and expressed as mg protein/ml.

Statistical Analysis

Data obtained from the study were subjected to 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 ± standard error of mean (S.E.M). Correlation analysis was carried out using statistical package for social science (SPSS) version 25.

RESULTS

Effect of Apocynin and Curcumin on Heart Homogenate Nicotinamide Adenine Dinucleotide Phosphate Oxidase Type 2 (NOX2) Activity of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

Figure 1 shows the effect of apocynin and curcumin on cardiac nicotinamide adenine dinucleotide phosphate oxidase type 2(NOX 2) 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 NAD(P)H oxidase type 2(NOX 2) activity for 14 days (47.80 ± 1.49 pg/mg protein and 48.68 ± 0.82 pg/mg protein) and 28days (47.27 ± 0.50 pg/ mg protein and 53.53 ± 1.06 pg/ mg protein ml) with no significant ($p < 0.05$) difference when compared to group 1(control). Diclofenac treated rats (10mg/kg/bwt diclofenac) in group 4 showed a significant ($p<0.05$) increase in NAD(P)H oxidase type 2(NOX 2) activity when compared to group 1(control) for14 days (73.66 ± 1.17 pg/ mg protein) and 28 days (84.33 ± 3.00 pg/ mg protein) 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 ($p<0.05$) NAD(P)H oxidase type 2 (NOX 2) for 14 days (47.97 ± 0.73 pg/ mg protein and 43.24 ± 0.84 pg/ mg protein) and 28 days (49.73 ± 1.41 pg/ mg protein and 47.50 ± 0.35 pg/ mg protein) 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 ($p<0.05$) NAD(P)H oxidase type 2(NOX 2) activity for 14 days (54.63 ± 1.30 pg/ mg protein and 51.62 ± 0.65 pg/ mg protein) and 28 days (53.08 ± 0.12 pg/ mg protein and 50.00 ± 0.26 pg/ mg protein) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

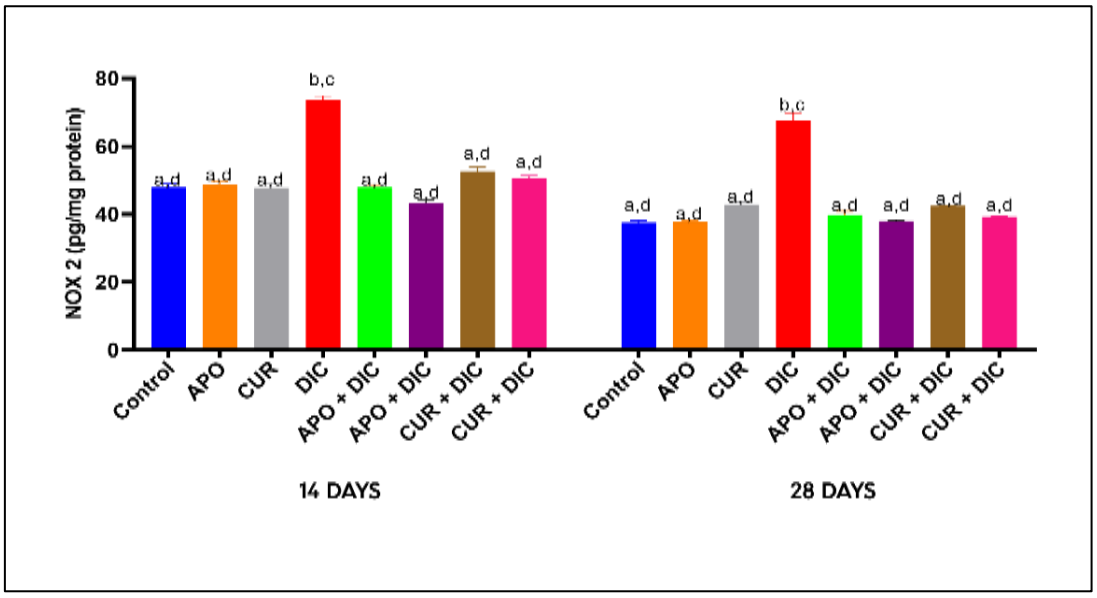

Figure 1: Effect of apocynin (APO) and curcumin (CUR) on cardiac nicotinamide adenine dinucleotide phosphate type 2 (NOX2) activity of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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.

Effect of Apocynin and Curcumin on Heart Homogenate Xanthine Oxidase Activity of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

Figure 2 shows the effect of apocynin and curcumin on cardiac xanthine oxidase activity of diclofenac-induced cardiotoxicity in adult male Wistar rats. Rats in group 2 and 3 receiving 1000mg/kg bwt apocynin and Curcumin respectively maintained normal cardiac xanthine oxidase activity for 14 days (3.71 ± 0.07nmol/mg protein/min and 3.78 ± 0.07 nmol/mg protein/min) and 28day (3.70 ± 0.03 nmol/min/ mg protein and 3.70 ± 0.03 nmol/min/ mg protein) with no significant ($p<0.05$) difference when compared to group 1(control). Group 4 (10mg/kg/bwt diclofenac) rats showed no significant ($p<0.05$) alteration in xanthine oxidase activity when compared to group 1(normal control) for14 days (4.08 ± 0.05 nmol/mg protein/min) and 28 days (4.28 ± 0.01) nmol/mg protein/min 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 did not significantly ($p < 0.05$) alter xanthine oxidase activity for 14 days (3.74 ± 0.19 nmol/min/ mg protein and 3.62 ± 0.31) nmol/mg protein/min and 28 days (3.54 ± 0.11 nmol/mg protein/min and 3.53 ± 0.11nmol/mg protein/min 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 did not significantly ($p<0.05$) alter cardiac xanthine oxidase activity for 14 days (3.92 ± 0.08 nmol/mg protein/min and 3.85 ± 0.11 nmol/mg protein/min) and 28 days (3.66 ± 0.01 nmol/mg protein/min and 3.63 ± 0.03nmol/mg protein/min) when compared to diclofenac treated rats (group 4).

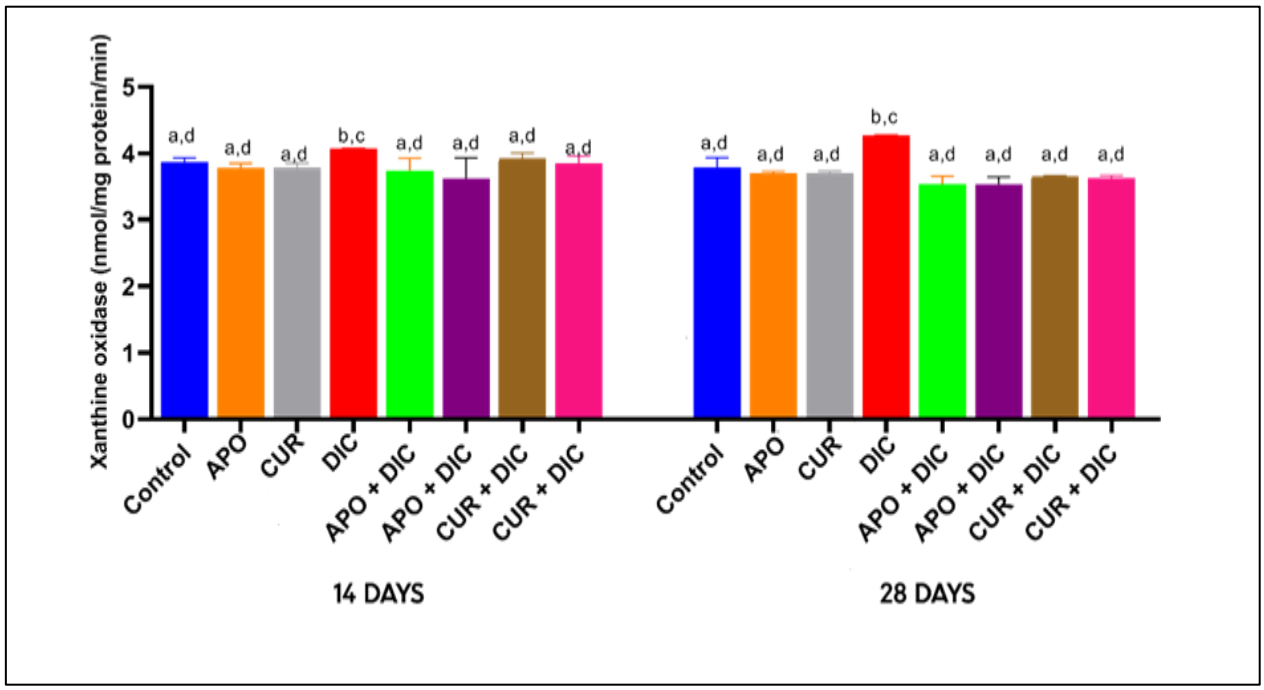

Figure 2: Effect of apocynin (APO) and curcumin (CUR) on heart homogenate xanthine oxidase activity of Diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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**.**

Effect of Apocynin and Curcumin on Heart Homogenate Superoxide Dismutase Activity of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

Figure 3 shows the effect of apocynin and curcumin on cardiac superoxide dismutase 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 cardiac superoxide dismutase activity for 14 days (58.43 ± 0.78 U/mg protein and 58.43 ± 0.78 U/mg protein) and 28day (58.36 ± 0.50 U/mg protein and 57.24 ± 0.24 U/mg protein) with no significant ($p<0.05$) difference when compared to group 1(control).

Group 4 (10mg/kg/bwt diclofenac only) rats in showed a significant ($p < 0.05$) decrease in superoxide dismutase activity when compared to group 1(normal control) for14 days (36.63 ± 0.23 U/mg protein) and 28 days (38.68 ± 0.74 U/mg protein) 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 increased ($p<0.05$) superoxide dismutase activity for 14 days (56.93 ± 1.16 U/mg protein and 57.49 ± 0.35 U/mg protein) and 28 days (57.71 ± 0.30 U/mg protein and 58.14 ± 0.43 U/mg protein) 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 increased ($p<0.05$) cardiac superoxide dismutase activity for 14 days (57.29 ± 0.14 U/mg protein and 57.80 ± 0.41 U/mg protein) and 28 days (58.00 ± 0.84 U/mg protein and 58.53 ± 0.54 U/mg protein) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

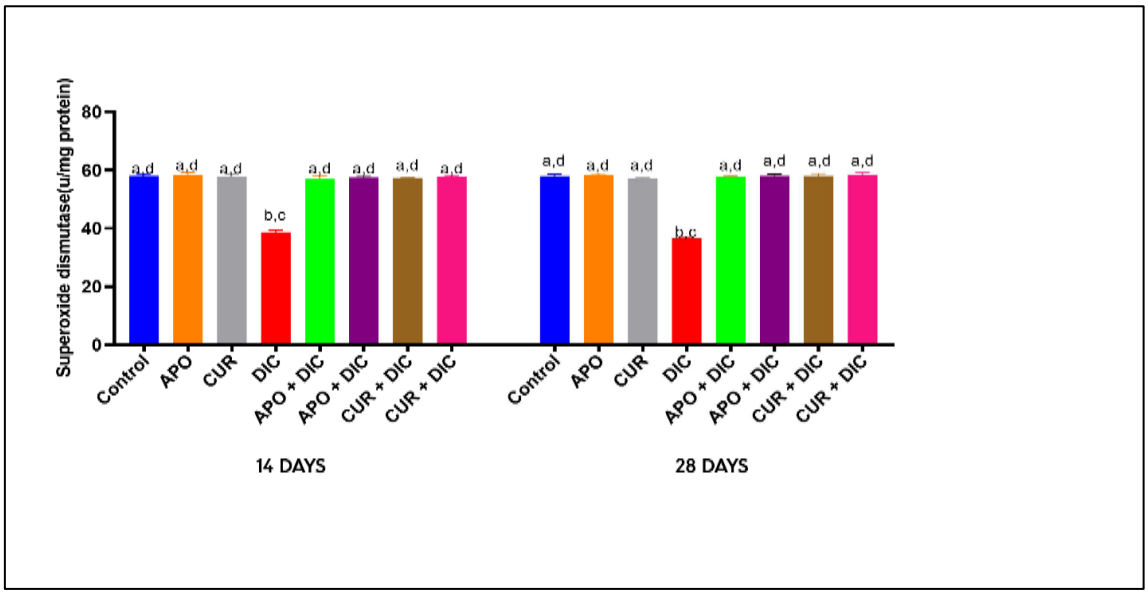

Figure 3: Effect of apocynin (APO) and curcumin (CUR) on heart homogenate superoxide dismutase activity of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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.

Effect of Apocynin and Curcumin on Heart Homogenate Catalase Activity of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

Figure 4 shows the effect of apocynin and curcumin on cardiac catalase activity of diclofenac-induced cardiotoxicity in adult male Wistar rats. Animals in group 2 and 3 receiving 1000mg/kg bwt apocynin and curcumin respectively maintained normal cardiac catalase activity for 14 days (41.48 ± 0.46 U/mg protein and 41.29 ± 0.60 U/mg protein) and 28day (43.79 ± 0.88 U/mg protein and 42.96 ± 0.39 U/mg protein) with no significant ($p < 0.05$) difference when compared to group 1 (control). Group 4 (10mg/kg/bwt diclofenac) rats showed a significant ($p<0.05$) decrease in catalase activity when compared to group 1(control) for14 days (23.58 ± 0.29 U/mg protein) and 28 days (16.06 ± 0.37 U/mg protein) 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 increased ($p<0.05$) catalase for 14 days (38.60 ± 2.17 U/mg protein and 39.44 ± 3.07 U/mg protein) and 28 days (41.19 ± 0.15 U/mg protein and 42.95 ± 0.95 U/mg protein) 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 ($p<0.05$) increased cardiac catalase activity for 14 days (38.35 ± 2.02 U/mg protein and 39.25 ± 1.24 U/mg protein) and 28 days (41.70 ± 0.83 U/mg protein and 42.49 ± 0.45 U/mg protein) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

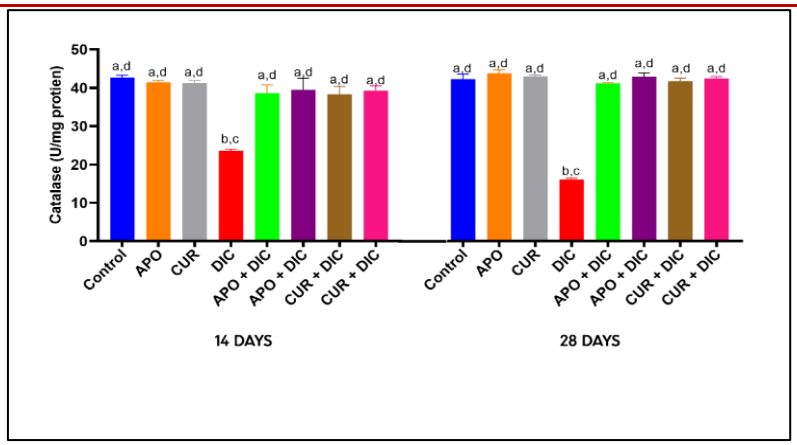

Figure 4: Effect of apocynin (APO) and curcumin (CUR) on heart homogenate catalase activity of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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.

Effect of Apocynin and Curcumin on Heart Homogenate Glutathione Peroxidase Activity of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

Figure 5 shows the effect of apocynin and curcumin on cardiac glutathione peroxidase 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 cardiac xanthine oxidase activity for 14 days (108.00 ± 1.53 U/mg protein and 92.67 ± 0.84 U/mg protein) and 28day (101.57 ± 0.34 U/mg protein and 103.34 ± 1.62 U/mg protein) with no significant ($p<0.05$) difference when compared to group 1(control). Group 4rats (10mg/kg bwt diclofenac) rats showed a significant ($p<0.05$) decrease in glutathione peroxidase activity when compared to group 1(normal control) for14 days (74.35 ± 0.43 U/mg protein) and 28 days (73.35 ± 0.43 U/mg protein) 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 increased ($p<0.05$) glutathione peroxidase for 14 days (98.07 ± 0.70 U/mg protein and 93.12 ± 3.12 U/mg protein) and 28 days (97.55 ± 1.94 U/mg protein and 98.50 ± 1.88 U/mg protein) 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 increased ($p<0.05$) cardiac glutathione peroxidase activity for 14 days (89.75 ± 0.49 U/mg protein and 92.31 ± 2.53 U/mg protein) and 28 days (92.83 ± 0.36 U/mg protein and 93.95 ± 2.50 U/mg protein) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

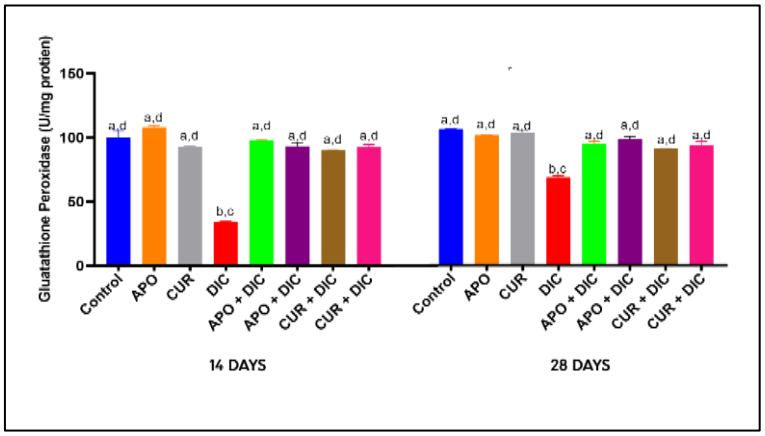

Figure 5: Effect of apocynin (APO) and curcumin (CUR) on heart homogenate glutathione peroxidase activity of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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.

Effect of Apocynin and Curcumin on Heart Homogenate Glutathione Level of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

Figure 6 shows the effect of apocynin and curcumin on cardiac glutathione level of diclofenac-induced cardiotoxicity in adult male Wistar rats. Groups 2 and 3 receiving 1000mg/kg bwt apocynin and curcumin respectively maintained normal cardiac glutathione level for 14 days (26.43 ± 0.86 U/mg protein and 26.35 ± 0.93 U/mg protein) and 28day (26.47 ± 0.27 U/mg protein and 26.43 ± 0.23 U/mg protein) with no significant ($p < 0.05$) difference when compared to group 1(control).

Group 4 (10mg/kg/bwt diclofenac) rats showed a significant ($p < 0.05$) decrease in glutathione level when compared to group 1(normal control) for14 days (16.66 ± 0.55) and 28 days (14.15 ± 0.32) 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 increased ($p < 0.05$) glutathione level for 14 days (25.66 ± 0.55 U/mg protein and 26.01 ± 0.95 U/mg protein) and 28 days (25.72 ± 0.36 U/mg protein and 26.87 ± 0.73 U/mg protein) 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 increased ($p<0.05$) cardiac glutathione level for 14 days (24.07 ± 0.53 U/mg protein and 25.43 ± 1.29 U/mg protein) and 28 days (25.92 ± 0.96 U/mg protein and 25.67 ± 1.87 U/mg protein) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

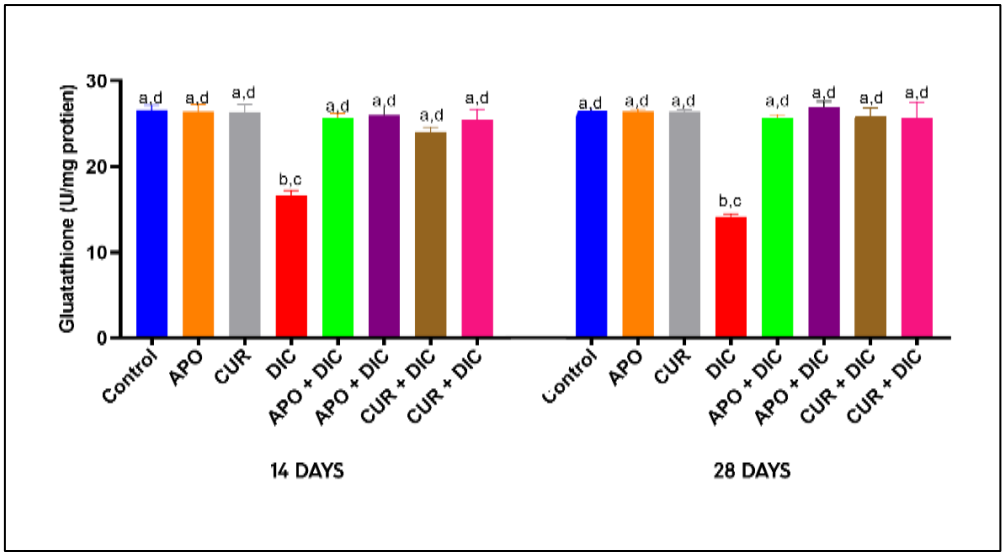

Figure 6: Effect of apocynin (APO) and curcumin (CUR) on heart homogenate glutathione level of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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.

Effect of Apocynin and Curcumin on Heart Homogenate Malondialdehyde Level of Diclofenac-Induced Cardiotoxicity in Adult Male Wistar Rats

The results as showed in figure 7 demonstrates that groups 2 and 3 receiving 1000mg/kg/ bwt of apocynin and curcumin respectively showed normal cardiac malondialdehyde level for 14 days (0.38 ± 0.02 U/mg protein and 0.37 ± 0.01 U/mg protein) and 28 days (0.38 ± 0.02 U/mg protein and 0.37 ± 0.01 U/mg protein) with no significant ($p < 0.05$) difference when compared to group 1(control). Group 4 (10mg/kg/bwt diclofenac) rats significantly ($p<0.05$) increased cardiac malondialdehyde level as a marker for oxidative

damage to lipid and consequently for lipid peroxidation to be 1.50 ± 0.08 for 14 days and1.60 ± 0.04 for 28 days duration of the study when compared to group 1 (control). 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 ($p<0.05$) reduced cardiac lipid peroxides for 14 days (0.61 ± 0.0 U/mg protein 3 and 0.59 ± 0.02 U/mg protein) and 28 days (0.45 ± 0.02 U/mg protein and 0.41 ± 0.01 U/mg protein) 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 ($p<0.05$) cardiac lipid peroxides for 14 days (0.61 ± 0.03 U/mg protein and 0.58 ± 0.01 U/mg protein) and 28 days (0.44 ± 0.03 U/mg protein and 0.42 ± 0.02 U/mg protein) when compared to diclofenac treated rats (group 4) in a dose dependent manner.

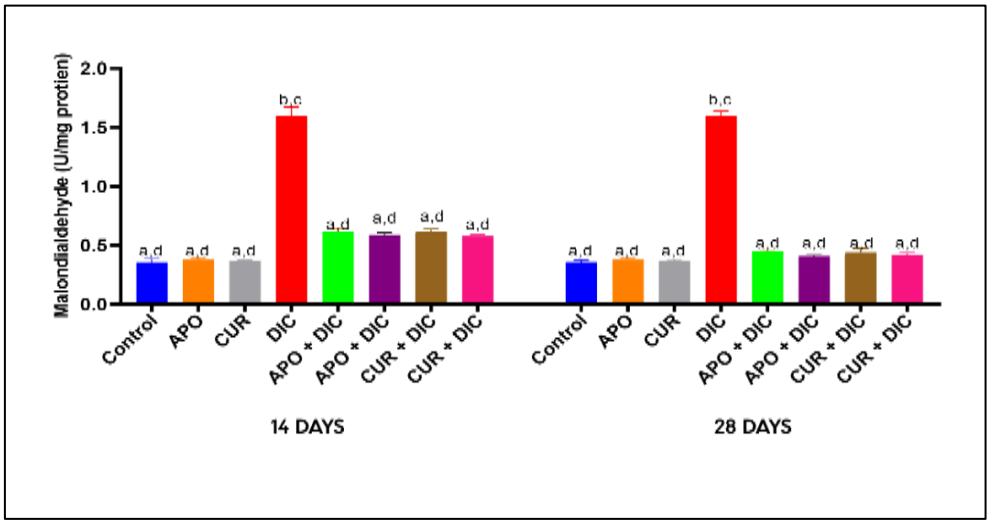

Figure 7: Effect of apocynin (APO) and curcumin (CUR) on heart homogenate malondialdehyde level of diclofenac (DIC)-induced cardiotoxicity in adult male Wistar rats

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.

The Pearson Correlation Coefficient between Elevated NOX 2 and Oxidative Stress in Diclofenac-Induced Cardiotoxicity

The correlations are shown in Table 2. The Pearson correlation analysis indicated a very good positive correlation between elevated cardiac tissue NOX 2 activity, decreased CAT activity ($r = 1.000$, $P = 0.019$) and GSH level ($r = 0.999$, $P = 0.031$). In addition, a significant positive correlations were noted between cardiac tissue NOX 2 activity and SOD activity ($r = 0.998$, $P = 0.041$), NOX-2 activity and MDA activity ($r = 0.997$, $P = 0.052$) and an insignificant negative correlation between cardiac tissue NOX-2 activity and GSH-Px activity(($r = -0.994$, $P = 0.071$)).

Table 1: Correlation coefficient and *P* value between NOX activity and biochemical oxidative parameters

Parameter	r	P
GSH-Px	-0.998	0.741
MDA	0.997	0.052
CAT	1.000	0.019
GSH	0.999	0.031
SOD	0.998	0.041

r value is the Pearson Correlation value while the p value is the significant values. Values are comparison of GSH-Px, MDA, CAT, GSH and SOD to NOX 2. Correlation is significant at $p \leq 0.05$.

DISCUSSION

Given that NOX function, mainly generates ROS and that ROS influences the expression of XOD, the activity of NOX 2 and XOD in the heart of all test group was thus determined. The result obtained is shown in Figure 1 and 2. The result indicates a significant increase in the activity of NOX 2 in the myocardium of rats treated with diclofenac only when compared with the control groups with no significant alteration in the activity of XOD. The result from this study agrees with the works of Huige *et al*., (2008), Li *et al*., (2008) and Okwakpam *et al*., (2020) who in their separate work, confirmed that administration of diclofenac induced increase in the activity of NOX 2

thereby causing excessive ROS production given that NOX function mainly generates ROS in the heart. The result obtained from this study also agrees with the report of Urschel and Cicha (2015) who showed that increased level of TNF-α in cardiac cells enhances the activity of NOX 2 nearly threefold.

The result obtained from this study suggest that diclofenac potentiates cardiac toxicity through mechanism, which includes increase in NADPH oxidase activity, with the ability to further increase reactive oxygen species production, ultimately resulting in cardiac injury.

However, repeated oral pretreatments with apocynin (500 mg/kg or 1000 mg/kg) and curcumin (500 mg/kg or 1000 mg/kg) significantly inhibited the increased activity of NOX 2 in the cardiac tissue induced by diclofenac thereby preventing the hyperactivity of NOX 2. The result obtained from this study supports work of Wang *et al*., (2013), Zhao *et al*., (2014) and Fan *et al*., (2015) whose result showed that apocynin and curcumin, have the ability to inhibit not only the activity of NOX 2 but also the expression of this enzyme. Previous studies have shown that apocynin inhibits NOX 2 through covalent modification of the thiol groups of p47phox essential for NOX 2 activation, there by blocking translocation of the subunit to the membrane (Urschel, and Cicha, 2015) but the route of inhibition of NOX 2 by curcumin is not yet clear.

Small-molecule inhibitors such as apocynin and curcumin are valuable probes for defining the role of a particular enzyme in a given biological process. Application of these compounds to relevant pathophysiological cellular and animal models enables validation of the catalytic activity of that enzyme as a target for pharmacotherapy as demonstrated in this study.

Enzymatic antioxidants which include SOD, CAT and GSH-Px, are crucial in protecting the tissues from oxidative stress damage. SOD, CAT and GSH-Px play association role in the elimination of superoxide radicals, in which SOD transforms them to H_2O_2 while GSH-Px and CAT convert H_2O_2 to water (Ding, 2012; Ho *et al.*, 2013). Therefore, prevent the formation of hydroxyl radicals, which is considered highly toxic molecule.

The assessment of antioxidant enzyme activity is shown in Figure 3,4 and 5. The result obtained from this study further showed that repeated daily oral administration of diclofenac significantly suppressed SOD, CAT and GSH-Px activities in the cardiac tissues of treated rats. These results are similar to the reports of Zhao *et al.*, (2010), Owumi and Dim (2019) and Okwakpam *et al.*, (2020) whose work demonstrated a decrease in the activities of antioxidant enzymes following the administration diclofenac in a dose dependent manner. The declined in the activity of SOD, GSH-Px and CAT activities in diclofenac group was related to inactivation of the enzyme because of the enhanced state of oxidative stress. These results may explain some diclofenac mechanisms to produce toxic effects in the heart. It has been documented that heart tissue is highly susceptible to oxidative stress than other tissues, as the activity of antioxidant enzyme is lower in the heart tissues (Newman, 2018; Tanriverdi *et al*., 2017). Ultimately, cellular damage from persistent oxidative stress leads to impaired tissue function and the onset and progression of a disease state, as has been implicated for a number of diseases, including cardiac diseases (Zhang *et al*., 2020).

However, repeated oral pretreatments with apocynin (500 mg/kg or 1000 mg/kg) and curcumin (500 mg/kg or 1000 mg/kg) significantly increased the activities of SOD, CAT and GSH-Px in the cardiac tissue of rats thereby enhancing the antioxidant capacity of the heart tissue as well as preventing oxidative stress. The result obtained in study agrees with the work of El-Sawalhi and Ahmed (2014), Ojha *et al*., (2016) and Cruz-Alvarez *et al*., (2017), who in their separate studies apocynin demonstrated that apocynin is able to enhance the activity of antioxidant factors and protect the cellular membranes against lipid peroxidation implicated in the pathogenesis of cardiotoxicity.

This probably occurs due to the inhibitory influence of apocynin or curcumin on NOX (Liu *et al.*, 2013; El- Sawalhi and Ahmed, 2014) as well as its ability to trigger mRNA expression of antioxidant enzymes (Ojha *et al*., 2016). Swamy *et al.*, (2012) also reported that curcumin attenuated oxidative stress by increasing the activities SOD, CAT and GSH-Px.

To confirm whether there is a direct relationship between increased oxidative damage and NOX hyperactivity, the level of MDA and GSH content of the heart was determined. The result indicates a significant increase in myocardial MDA content due to ROS generation with subsequent decrease in cardiac GSH content of rats treated with diclofenac when compared to the control group. These data suggested that increase in NOX 2 activity was correlated with oxidative stress in diclofenac-associated cardiac injury.

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes over production of MDA (Singh *et al*., 2014). The role of GSH, a non-enzymatic antioxidant, is very important in fighting the free radicals resulted from toxic chemicals and to conjugate them to fewer toxic products. Depletion of GSH in cardiac tissue indicates GSH utilization, which subsequently leads to oxidative stress. These findings pointed out an obvious change in

prooxidant-antioxidant balance in the heart of rats following acute and sub chronic administration Diclofenac. The result obtained in this study agrees with the findings of Oda *et al.,* (2018) whose result showed that administration of diclofenac significantly increased lipid peroxidation and decreased glutathione level in the heart tissue. Also, the result obtained from this study agrees with the earlier report of Zhao *et al.,* (2014) who suggested that ROS specifically derived from increased activity NOX 2 make a substantial contribution to several key processes underlying the development of cardiac contractile dysfunction and remodeling.

Apocynin and curcumin pretreatments, however, significantly reduced and normalized tissue MDA content when compared to the diclofenac treated group in a dose-dependent manner. Similarly, apocynin and curcumin pretreated groups revealed a significant increase in myocardial GSH content. Therefore, in this present study, apocynin and curcumin were found to protect the heart from the damaging effect of diclofenac and exert significant antioxidant activity via elevation of GSH and inhibition of MDA contents in pretreated groups compared to diclofenac treated group.

This finding is in support with the work of Zhao *et al.,* 2014; Swamy *et al*., 2012; Fan *et al*., 2015 and Cruz-Alvarez *et al.,* (2017) whose result demonstrated considerable decrease in MDA and GSH levels in the administration of apocynin or curcumin. Activated lipid peroxidation is considered a crucial pathogenic event in cardiac disease (Wen *et al*., 2019) as well as it may clarify the association between increased production of MDA and the damaging effect noticed on myocardial cells as evidenced by the increased cardiac enzymes leakage. Moreover, the reduction of the antioxidant GSH content could be justified by their excessive utilization throughout the burst of ROS production.

The protective augmentation shown by apocynin and curcumin against diclofenac-induced oxidative stress could be via inhibition of NOX 2 activity. In apocynin and curcumin pretreated groups, the activities of SOD, CAT, GSH-Px increased as well as levels of GSH via inhibition of NOX 2, thus, the ability of cardiac tissue to overcome the oxidative stress damage caused by administration of diclofenac. All of these biochemical changes were corroborated by remarkable histological lesions.

To investigate the relationship and degree of association between increased oxidative stress and elevated NOX 2 in diclofenac induced cardiotoxicity and comparing the same with control rats, correlation analysis was investigated. Results obtained from the correlation coefficient analysis study established a statistically significant positive relationship between elevated NOX 2 and oxidative stress parameters, except GSH-Px, which showed an insignificant negative correlation. This clearly suggests that NOX 2 is a major contributor to oxidative stress induced by diclofenac in the cardiac tissue and that NOX 2 activity and oxidative stress are two dependent risk factors in the pathological mechanism of diclofenac induced cardiotoxicity. This result agrees with the report of Zhao *et al.,* (2014) and Wen *et al.,* (2019).

The marginally significant positive correlations between elevated NOX 2 and of MDA, CAT, GSH and SOD suggests that NOX 2 is a major contributor to oxidative stress induced by diclofenac in the cardiac tissue This also clearly suggests that elevated NOX 2 activity and oxidative stress are two dependent risk factors in the pathological mechanism of diclofenac induced cardiotoxicity.

Taken together, the results obtained from this study suggest that exposure to diclofenac elicits cardiac injury in rats via mechanism involving NOX 2 induced oxidative stress. Pretreatments with NOX 2 inhibitors, apocynin and curcumin before the administration of diclofenac on the other hand, relieved diclofenac induced cardiac toxicity and attenuated biochemical abnormalities. Apocynin and curcumin, suppressed the expression of pro oxidant enzymes and enhanced overall antioxidant status in rats exposed to diclofenac.

CONCLUSION

While NOX activation alone cannot completely account for oxidative stress-related dysfunction, it may provide a novel potential therapeutic target for diclofenac-induced cardiotoxicity. Thus, apocynin and curcumin, naturally occurring NOX 2 inhibitors, could be considered as promising agents for therapeutic and prophylactic interventions in different cardiovascular disorders, including ischemia, myocardial tissue damage and heart failure.

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