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Comparative Biochemical and Pharmacological Assessment of Expired and Unexpired Diclofenac on Egg Albumin-Induced Inflammation in Wistar Rats

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Abstract

A non-steroidal anti-inflammatory medicine (NSAID) called diclofenac is recommended for treatment in rheumatoid arthritis and several non-rheumatoid illnesses that cause pain and inflammation. The relative effectiveness of drugs after their stated expiration dates is up for debate. Furthermore, studies reveal that drugs lose their effectiveness after expiration because the active ingredients may become less potent or more unstable. The purpose of this study was to examine how the medicine diclofenac, both expired and unexpired, affected the inflammation caused by egg albumin in Wistar rats. For the purpose of the efficacy trial, twenty-four wistar rats weighing between 120 and 150 grams were split up into four groups of six rats each. Group C was given outdated diclofenac medication, Group D was given expired diclofenac medication, Group B was given inflammation without treatment, and Group A (control group) was given 0.5 mL/kg distilled water. Rats' paw edema caused by fresh egg albumin was used to measure anti-inflammatory responses. A vernier caliper was positioned at the edge of the phalanges and metatarsals to measure and record the animals' paw thickness. The test medications and controls were administered orally to the rats. Before being administered, the uncoated tablets were broken up and dissolved. A vernier caliper was used to measure and record the anti-inflammatory activity one hour after treatment began. This process was repeated one, two, three, four, and five hours later. Ocular punctures were used to obtain blood, which was then centrifuged to extract serum. Assessments of haematological parameters, biochemical parameters, oxidative stress biomarkers, and histology were conducted. According

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to the findings, the control and unexpired diclofenac groups had considerably higher levels of oxidative stress indicators like SOD, CAT, and GSH than the other groups. Compared to the control and unexpired diclofenac groups, the induced untreated and expired groups had significantly higher MDA levels. HDL concentration was significantly higher in the control and unexpired drug group than in the induced untreated group, but liver function parameters (ALT, AST, ALP) and lipid profile parameters (TAG, CHOL, LDL) were significantly higher in the induced untreated group than in the control, unexpired, and expired groups. All of the groups had different levels of urea, uric acid, and total bilirubin. The control and unexpired groups had considerably higher hematological results (WBC, RBC, neutrophils, hemoglobin, and monocytes) than the diclofenac-expired and induced untreated groups. When compared to the control and group that received unexpired diclofenac, the histopathology results also examined liver damage in both the induced untreated group and the groups that received expired medication. According to the study's findings, the medication diclofenac that has not expired has the strength to perform anti-inflammatory, analgesic, and antipyretic effects. Additionally, there are still some active ingredients in outdated diclofenac that have the same function.

Keywords: Diclofenac; Inflammation; Oxidative Stress Biomarkers; Biochemical Parameters Hematological Parameters

Abbreviations

PPMVL: Patent and Proprietary Medicines Vendors Licenses; NSAIDS: Non-Steroidal Anti-Inflammatory Class of Drugs; COX: Cyclooxygenase; PGs: Prostaglandins; NAFDAC: Nigerian National Agency for Food and Drug Administration and Control; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; GSH: Determination of Reduced Glutathione; DTNB: 5, 5-dithio-bis-2 nitrobenzoic acid; TCA: Trichloroacetic; MDA: Malondialdehyde; TBARS: Thoibarburitic Reactive Substance; Hb: Haemoglobin; CBC: Complete Blood Count; WBC: White Blood Cell; PLT: Platelet; LYMP: Lymphocyte; NEU: Neutrophil, MID: Monocyte, MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase; TP: Total Protein, ALB: Albumin; BIL: Bilirubin; IDB: Indirect Bilirubin; WBC: White Blood Cell; CAT: Estimation of Catalase; SOD: Estimation of Superoxide Dismutase.

Introduction

In Nigeria, the usage of outdated medications has increased significantly. medications that have expired are frequently distributed by holders of Patent and Proprietary Medicines Vendors Licenses (PPMVL), who typically view medications as simply commercial commodities on which they must never lose money [1]. During medical missions to rural places, medical professionals may use imported, expired medications from developed nations. Customers are not aware of their expiration status, despite the fact that the dispensers may be [2]. The relative effectiveness of drugs after their stated expiration dates is up for debate. Since the expiration date simply serves as a guarantee that the medication's stated potency will persist at least until that point, expired drugs do not necessarily lose their potency. Clinical circumstances may occur wherein expired medications may be taken into consideration due to a lack of suitable substitutes [3] or budgetary constraints [4]. Numerous drugs continue to be effective years after their originally stated expiration dates, according to ongoing research [5]. An expedited stability assessment of the medication is used to estimate expiration dates. However, caution is essential because the medication's safety and efficacy must be guaranteed prior to its expiration dates. Such guarantees are withdrawn after this date [6].

Diclofenac is the most well-known NSAID in the world and a member of the Non-Steroidal Anti-Inflammatory Class of Drugs (NSAIDS) [7]. Diclofenac is frequently used to treat mild pain and inflammation, which are typical signs of many illnesses. It has analgesic, antipyretic, and anti-inflammatory properties [8]. Asthma, chronic peptic ulcers, TB, rheumatoid arthritis, periodontitis, ulcerative colitis, Crohn's disease, sinusitis, and active hepatitis are a few illnesses and ailments that involve persistent inflammation. Many illnesses and ailments, such as rheumatoid arthritis, atherosclerosis, periodontitis, hay fever, and some types of cancer, might eventually be brought on by chronic inflammation. Because inflammation can result in stiffness and limited movement, it must be properly controlled [9-11]. People often experience pain when they have inflammation. Depending on how severe the inflammation is, they feel pain, stiffness, discomfort, anguish, and sometimes agony. Pain comes in several forms. It might be characterized as pinching, stabbing, pulsing, throbbing, and steady. Because the swelling presses against the sensitive nerve endings, inflammation is the main source of pain. The brain receives pain signals as a result. Inflammation also involves other metabolic processes [12]. They have an impact on nerve function, which might intensify pain. Cyclooxygenase (COX), an enzyme that transforms arachidonic acid into prostacyclins, thromboxanes, and

prostaglandins (PGs), is inhibited by diclofenac. Diclofenac should be taken for the shortest period of time and at the lowest dose due to its negative effects. In order to boost sensitivity to diclofenac and decrease its dosage, numerous attempts were made to establish a method of mixing it with other substances.

According to research, medications lose their effectiveness after they expire because the active ingredients may become less potent or become relatively unstable. Some, however, either stay the same, deteriorate, or transform into more harmful compounds that are harmful to health [13,14]. The literature on this topic has significant gaps [15]. Few research, for instance, have examined "expected" or "acceptable" levels of medication expiration. If a medicine is stored according to the manufacturer's recommended storage conditions, it should meet the applicable standards of identity, purity, strength, and quality at the time of use, as indicated by the expiration date supplied by the manufacturer [16]. This study aimed to determine the harm that expired Diclofenac can do to organs and tissues, as well as the effectiveness of both expired and unexpired forms of the anti-inflammatory medicine in inflamed wistar rats.

Materials and Methods

Collection of Anti-Inflammatory Drugs

The Nigerian National Agency for Food and Drug Administration and Control (NAFDAC) warehouse and pharmacies in Mushin, Lagos, Nigeria, provided samples of diclofenac that were both expired and unexpired. The hospital-pack diclofenac samples that were recovered were six months after their expiration date and had never been opened.

Experimental Animals

Albino wistar rats of both sexes weighing between 120 and 150g were bought from the College of Medicine, University of Lagos, and Idi-Araba animal house. They were acclimated for two weeks in the animal house under normal conditions, which included a 12-hour dark-light cycle, a temperature range of 22 to 29°C, and unrestricted access to drinking water and a typical pellet diet. As shown below, the rats were split into four (4) groups at random, each consisting of six (6) animals. Before being administered, one tablet of diclofenac potassium (1.43 mg/kg) in 20 milliliters of water was crushed and dissolved. The test medications were administered orally by gavage to the rats in groups C and D.

- Group A: Healthy control received 0.5 ml/kg distilled water;
- Group B: Induced untreated (Inflammation without treatment);
- Group C: Administered expired diclofenac;
- Group D: Administered unexpired diclofenac.

Induction of Inflammation

The fresh egg albumin-induced rat paw edema method of Yu, et al. was used to generate inflammation. The experimental rats' left hind paw received an injection of 0.1 cc fresh egg albumin. A vernier caliper was used to measure the anti-inflammatory activity after one hour of treatment. A vernier caliper was positioned at the edge of the phalanges and metatarsals to measure and record the animals' paw thickness size at 0 hours. Following treatment, the measurements and records were made again one, two, three, four, and five hours later.gavage.

Collection of Blood Samples

After six hours of therapy, 0.1 ml blood samples were obtained via retro-orbital puncture. Biochemical tests were estimated using non-heparinized blood samples. After allowing the blood samples to clot, the sera were separated using centrifugation for ten minutes at 3000 rpm. The Randox kit (Randox Laboratory Limited, Ardmore, UK) was used to measure the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, triacyl glycerol, cholesterol, HDL, LDL, VLDL, creatinine, and urea.

Estimation of Catalase (CAT) Activity

Colorimetric Determination of H_2O_2: Different amounts of H_2O_2 ranging from 10 to 100µmoles were taken in small test tubes and 2 ml of dichromate/acetic acid added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made up to 3ml and the optical density measured with a spectrophotometer at 570nm. The concentrations of the standard were plotted against absorbance.

Determination of Catalase Activity: 0.2ml of the sample was mixed with 0.8ml distilled water to give a 1:5 dilution. The assay mixture containing 2ml H_2O_2 solution (800µmoles) and 2.5ml of phosphate buffer, pH 7.4. 1ml of properly diluted sample was mixed with the reaction mixture by a gentle swirling motion at room temperature. A portion of the reaction mixture (1ml) was withdrawn and blown into 2ml dichromate/acetic acid reagent at 60 seconds intervals for 3 min. The hydrogen peroxide content of the withdrawn sample was determined by the method described by Weydert, et al. [17]. Catalase activity was obtained by plotting the

standard curve and the concentration of the remaining H_2O_2 extrapolated from the curve, taking cognizance of the equations below:

$$H_2O_2 \text{consumed by catalase} = \text{Initial}(200\mu\text{moles}) - H_2O_2\text{remaining}(\mu\text{moles})$$

$$\text{Catalase activity} = H_2O_2\text{consumed}(\mu moles)/mg \text{ protein}$$

Estimation of Superoxide Dismutase (SOD) Activity

0.2ml of the sample was diluted with 0.8ml of distilled water to make a 1:5 dilution. An aliquot (0.2ml) of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette will contain 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation:

Increase in absorbance per minute = $\frac{A_3 - A_0}{2}$

Where A_0 = absorbance after 30 seconds,

A_3 = absorbance after 120 seconds.

$$\%\text{inhibition} = \frac{100 \times \text{increase in absorbance for substrate}}{\text{Increase in absorbance for blank}}$$

One unit of SOD activity is described as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

Determination of Reduced Glutathione (GSH)

0.2ml of the sample was diluted with 0.8ml of distilled water to make a 1:5 dilution. 2ml of trichloroacetic (TCA) was added and centrifuge for 10minute. 1ml of the supernatant was taken and 1.5ml and 0.5ml of phosphate of buffer and 5, 5-dithio-bis-2 nitrobenzoic acid (DTNB) were added respectively. To the Blank, 1.5ml of phosphate buffer was added to 1ml of distilled water with 0.5ml of DTNB. Absorbance was taken at 412nm.

Calculation:

$$\frac{\text{Absorbance of test} \times \text{Total assay volume}}{\text{Extinction coefficient of GSH}\left(1.34 \times 10\text{^}4\text{m}\text{^} - 1\text{cm}\text{^} - 1\right) \times \text{sample volume}}$$

Lipid Peroxidation Assay

Quantitative estimation of levels of malondialdehyde (MDA), a Thoibarburitic Reactive Substance (TBARS), was used to quantify the level of lipid peroxidation in the plasma and liver, following procedures outlined by Weydert, et al. [17]. Part of the assay combination is 0.2 ml of sample (either blood or liver homogenate), together with 0.04 ml of 8.1% SDS, 0.3 ml of 20% glacial acetic acid (pH 3.5 adjusted with NaOH), and 0.3 ml of 0.8% thiobarbituric acid aqueous solution. Before adding 0.2 ml of distilled water and 1.0 ml of n-butanol, the mixture was heated in a water bath at 95°C for 1 hour and cooled under tap water. The upper organic layer was scanned at 532 nm to determine the MDA (μmol) equivalents after centrifuging the mixture at 3000 rpm for 10 minutes. Zooming in at x100 and x400 magnifications, the camera.

The values of MDA obtained were calculated using the formula:

$$\frac{\text{Absorbance of test} \times \text{Total assay volume}}{\text{Extinction coefficient of MDA}\left(1.56 \times 105\text{M} - 1\text{cm} - 1\right) \times \text{sample volume}}$$

Haematological Assessment

Among the many haematological indices tested for are hemoglobin (Hb), complete blood count (CBC), white blood cell (WBC) count, platelet (PLT) count, lymphocyte (LYMP) count, neutrophil (NEU) count, monocyte (MID) count, mean corpuscular hemoglobin (MCH) count, and mean corpuscular hemoglobin concentration (MCHC), among many others. The Sysmex KX-21N automated machine (Sysmex Corporation, Kobe, Hyogo, Japan) was used to analyze the whole blood samples in accordance with the instructions provided by the manufacturer. For aspiration, the sample was mixed and placed in contact with the sample probe. After two "beep beep" buzzers and the LCD screen showing "ANALYZING" were heard, the sample was removed. After that, the device ran an automated analysis and showed the outcomes on the LCD.

Biochemical Examination

Liver Function Test: The serum samples were also analyzed for various liver function parameters, including Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Total protein (TP), Albumin (ALB) and Bilirubin (BIL) using a UV/visible spectrophotometer.

Assessment of Aspartate Aminotransferase (AST) Activity: Aspartate Aminotransferase activity in the serum was measured using the procedures described by Reitma, et al. [18]. A Randox reagent kit was used for this analysis.

Assessment of Alanine Aminotransferase (ALT) Activity: Alanine Aminotransferase activity in the serum was investigated using the method described by Reitman, et al. [18] using a Randox reagent kit.

Assessment of Alkaline Phosphatase (ALP) Activity: Alkaline Phosphatase (ALP) serum activity was assessed

using the Agappe reagent kit, following the procedure by Schlebusch, et al. [19].

Determination of Total Protein (TP): The Randox reagent kit was utilized to determine the total protein concentration, following the method outlined by Weichselbaum [20].

Determination of Albumin (ABL): The Agappe kit was employed to determine the serumalbumin concentration, following the method described by Doumas, et al. [21].

Determination of total bilirubin (TB): The estimation of Total Bilirubin was performed using the formula provided below:

$$\text{Total Bilirubin}(\text{TB}) = \text{Direct Bilirubin} + \text{Indirect Bilirubin}(\text{IDB})$$

Kidney Function Test: Serum kidney function biomarker such as urea and creatinine were determined as described by Imo, et al. [22].

Lipid Profile Analysis: The serum samples were used for the analysis of several lipid parameters, including Low-Density Lipoprotein Cholesterol (LDL-C), High-Density Lipoprotein-Cholesterol (HDL-C), Triacylglycerol (TAG), and Total Cholesterol (TC). These parameters were determined to assess the rats' lipid profile and evaluate the effects of treatment on their cholesterol and lipid levels.

Determination of Total Cholesterol (TC): Total cholesterol in the serum was determined using the method described by Allain, et al. [23] using an Agappe reagent kit. It contains specific reagents and chemicals necessary for accurately measuring total cholesterol levels.

Determination of Triglycerides (TAG): Triglycerides were analyzed using the enzymatic colorimetric method by Allain, et al. [23] and an Agappe reagent kit.

Determination of HDL-cholesterol (HDL-C): HDL-cholesterol was determined using the method described by Assmann [24], which involves an Agappe reagent kit.

Estimation of LDL-cholesterol: LDL-cholesterol was determined by the Friedewald equation, as described by Friedewald, et al. [25]. The equation is presented as follows:

$$\text{LDL} - \text{C} = \text{TC} - \text{HDL} - \text{C} - \text{TG} / 5$$

Where:
LDL-C: Low-Density Lipoprotein Cholesterol;
TC: Total Cholesterol;
HDL-C: High-Density Lipoprotein-Cholesterol;
TAG: Triacylglycerol.

Histopathological Examination

Using the modified methods established by Mohammed, et al. [1], the histological assessment of hepatic, renal, and cutaneous tissues was completed. Using a desiccator, the animals were anesthetized using chloroform. After the rats were dissected, the organs were removed: the liver and kidneys. The skin was removed beforehand. Their condition was closely monitored for any signs of obvious disease. Immediately after being isolated, the organs were preserved in 10% buffered formalin. The tissues were cut into 5 µm sections using a Leica Model RM1225 automated tissue processor, then immersed in paraffin and stained with H&E. Using photomicrographs captured with a Motic 9.0 Megapixels Microscope Camera at x100 and x400 magnifications, the histological examination of the experimental rats' microscopic architecture on the H&E stained slides was conducted.

Statistical Analysis

The results obtained was statistically computed using GRAPHPAD and differences between the groups was analyzed using analysis of variance (ANOVA); and values was expressed as mean ± standard Deviation (SD). Data were further analyzed by Tukey Honest Significant Difference (Tukey's HSD) test. Statistical Differences was considered statistically significant at $p < 0.05$.

Results

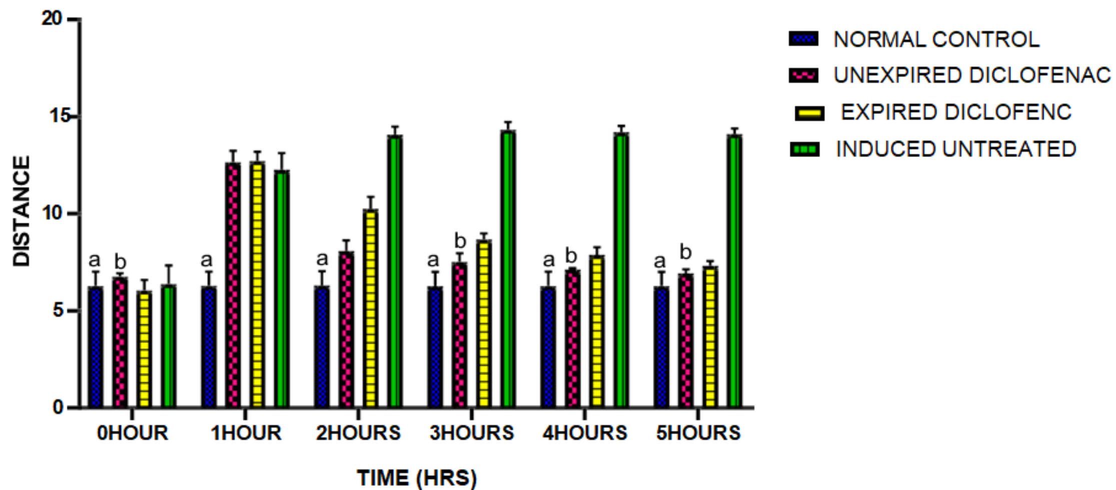
Effect of Expired and Unexpired Diclofenac on Paw Size of experimental rats Following the Administration of Egg Albumin with Time

The result captured in Figure 1 below reveals the there was no significant difference between the control group at different time intervals. However, there was significant increase in group administered with unexpired diclofenac after 1 and 2 hours when compared with 0hr, but after 2hrs above the paw size decreased as compared to 1hr. Administration with expired diclofenac also saw the paw size increase significantly from 1, 2, 3, 4, and 5hrs as compared to 0hr, but after 2hrs above the paw size decreased as compared to 1hr. Paw size of group induced untreated increased significantly as the time increased.

Effect of Expired and Unexpired Diclofenac on Reduced Glutathione Levels in Egg Albumin-Induced Inflammation in Wistar Rats

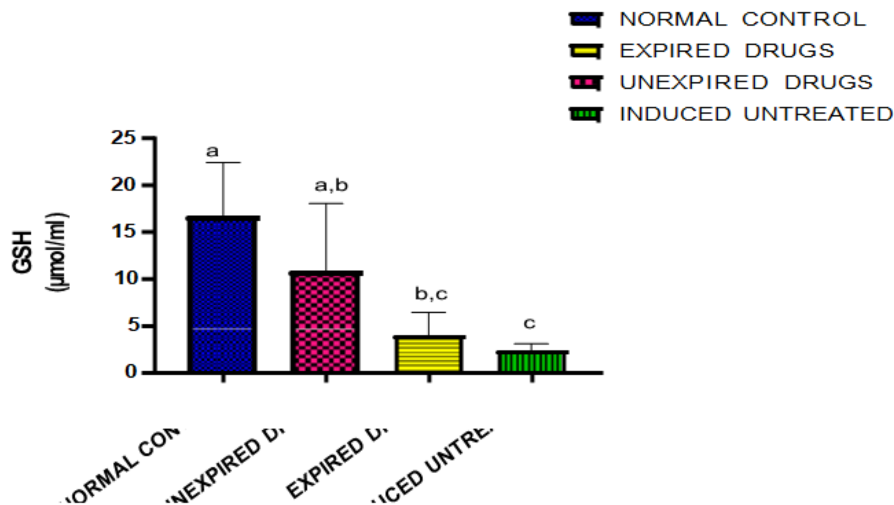
The Figure 2 presented below shows the effect of expired and unexpired diclofenac on reduced glutathione levels in in egg albumin-induced inflammation in wistar rats There was significant increase in the level of GSH in normal control

group as compared to the groups administered with expired diclofenac and induced untreated. In addition, the level of GSH in groups administered with unexpired diclofenac was significantly higher in comparison to the group untreated following induction.



*Bars tagged with the same alphabet(s) are not significantly different at 95% confidence interval (p>0.05).

Figure 1: Effect of expired and unexpired diclofenac on paw size of experimental rats following the administration of egg albumin with time.

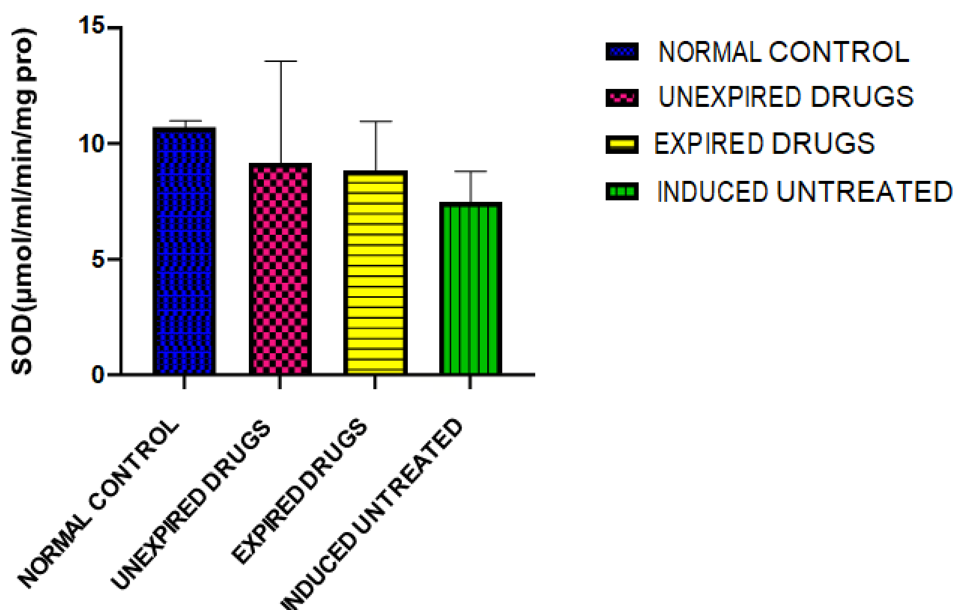


*Bar tagged with same alphabet(s) are not significantly different at 95% confidence interval (p>0.05)

Figure 2: Effect of expired and unexpired diclofenac on reduced glutathione levels in egg albumin-induced inflammation in wistar rats.

Effect of Expired and Unexpired Diclofenac on Superoxide Dismutase Levels in Egg Albumin-Induced Inflammation in Wistar Rats

Figure 3 below shows the result of the effect of expired and unexpired diclofenac on superoxide dismutase levels in egg albumin-induced inflammation in wistar rats It was observed that there was an increase in the level of Superoxide dismutase in the control group compared to the groups administered with expired drug, unexpired and induced untreated. However, the change was not significant.

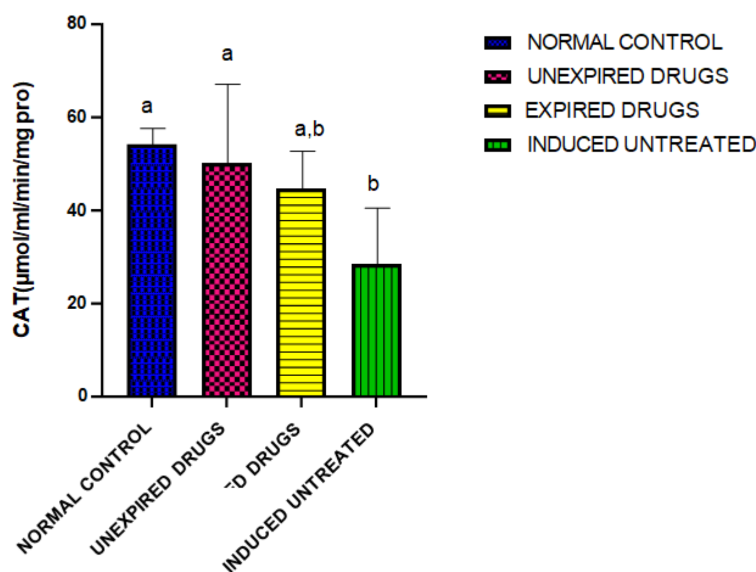


*Bar tagged with no alphabet(s) are not significant different at 95% confidence interval (p>0.05)

Figure 3: Effect of expired and unexpired diclofenac on superoxide dismutase levels in egg albumin-induced inflammation in wistar rats.

Effect of Expired and Unexpired Diclofenac on Catalase Levels in Egg Albumin-Induced Inflammation in Wistar Rats

Figure 4 reveals results on the effect of expired and unexpired diclofenac on catalase levels in egg albumin-induced inflammation in wistar rats. It was observed that there was significant increase in the level of catalase in the control group compared to the group induced and untreated. In addition, the level of catalase in the group administered with unexpired diclofenac was significantly higher in comparison to the group induced and untreated.

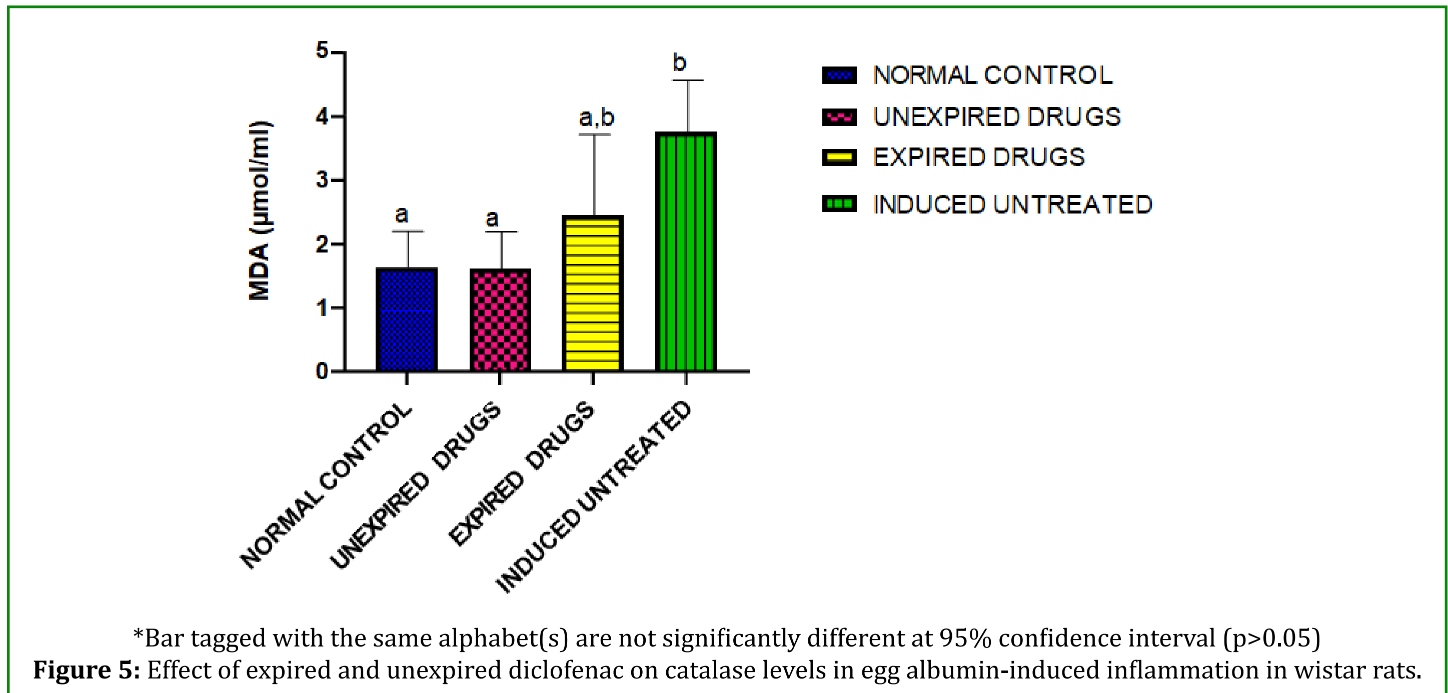


*Bar tagged with no alphabet(s) are not significantly different at 95% confidence interval (p>0.05)

Figure 4: Effect of expired and unexpired diclofenac on catalase levels in egg albumin-induced inflammation in wistar rats.

Effect of Expired and Unexpired Diclofenac on Catalase Levels in Egg Albumin-Induced Inflammation in Wistar Rats

Figure 5 reveals results showing the effect of expired and unexpired diclofenac on malondialdehyde levels in egg albumin-induced inflammation in wistar rats. It was seen that there was significant decrease in the level of malondialdehyde in the controlgroup as compared to the group induced and untreated. In addition, the level of MDA in group administered with unexpired ddiclofenac was significantly lower in comparison to the group induced and untreated.



*Bar tagged with the same alphabet(s) are not significantly different at 95% confidence interval (p>0.05)

Figure 5: Effect of expired and unexpired diclofenac on catalase levels in egg albumin-induced inflammation in wistar rats.

Hematological Assessment of Egg Albumin-Induced Inflammation in Wistar Rats Treated with Expired and Unexpired Diclofenac

From Table 1 presented below, Red Blood Count (RBC) of control group significantly increased when compared with the group treated with unexpired diclofenac and the group induced untreated. Also, the group treated with expired diclofenacwassignificantlyhigherincomparisonwithinduced untreated group. White Blood Cell (WBC) was significantly higher in in the control group than the group treated with expired diclofenac and induced not treated group, however, the group treated with unexpired diclofenac increased significantly when compared with expired and induced untreated group. HCT level was significantly increased in the control group compared with induced untreated group, but induced untreated group was significantly decreased when compared with groups treated with expired and unexpired diclofenac respectively. MCV, MCH, MCHC, NEU and HBG levels were higher in the control group than the other groups but they are not significant. Finally, lymph % of the control group was significantly increased in comparison to induced untreated group, although groups treated with expired and unexpired diclofenac were significantly higher than the induced untreated group.

Biochemical Status of Egg Albumin-Induced Inflammation in Wistar Rats Treated with Expired and Unexpired Diclofenac

Table 2 presented below shows the result of liver and kidney function parameters of egg albumin-induced inflammation of wistar rats treated with expired and unexpired diclofenac. The urea level of the control group was significantly higher than the group treated with expired diclofenac. The group treated with expired diclofenac however, was significantly increased in comparison with the group treated with unexpired diclofenac, although lower significantly when compared with the induced untreated group. Creatinine level of the group administered with unexpired diclofenac was significantly lower than the group administered with expired diclofenac and induced untreated group. Amazingly, Alanine amino transferase level of the group administered with unexpired diclofenac was higher than other groups but not significant. AST level of the control group was significantly lower than the other groups. ALP level of the control was significantly lower than the group induced and not treated but the group administered with unexpired drug significantly lower than the groups administered with expired and induced untreated respectively. The ALB level of

the normal control group was significantly higher than the groups administered with unexpired diclofenac and induced untreated group. The group administered with expired diclofenac saw their albumin level significantly increased than the group induced not treated. TP level of group treated with expired diclofenac significantly increased in comparison to the group administered with unexpired diclofenac, control and induced untreated group. The group treated with expired diclofenac had the highest bilirubin level when compared to other group but not significant.

Parameters	Normal Control	Unexpired Drug	Expired Drug	Induced Untreated
RBC (M/µ)	7.93 ± 0.21[a]	6.10 ± 0.99[b,c]	7.02 ± 1.5[a,b]	4.6 ± 0.48[c]
WBC (M/µ)	7.83 ± 0.35[a]	7.80 ± 0.91[a]	5.80 ± 0.93	3.74 ± 0.50
HCT (%)	45.47 ± 5.97[a]	42.68 ± 8.07[a,b]	42.48 ± 2.61[a,b]	33.38 ± 0.91
MCV (fl)	66.33 ± 12.62[a]	64.98 ± 19.50[a]	62.30 ± 11.02[a]	53.78 ± 6.88[a]
MCH(pg)	21.1 ± 2.03[a]	20.55 ± 6.62[a]	20.55 ± 3.47[a]	16.55 ± 1.48[a]
MCHC (g/dl)	33.425 ± 2.50[a]	31.78 ± 3.10[a]	33.10 ± 0.93[a]	30.95 ± 0.98[a]
LYMPH (%)	33.65 ± 8.76[a]	25.15 ± 8.11[a,b]	25.00 ± 3.73[a,b]	22.03 ± 1.57[b]
NEU (%)	50.03 ± 3.04[a]	49.70 ± 9.72[a]	42.35 ± 8.53[a]	41.68 ± 4.97[a]
HGB (g/dl)	12.50 ± 0.84[a]	13.43 ± 1.81[a]	12.6925 ± 0.95[a]	11.7 ± 1.05[a]

*Bar tagged with the same alphabet(s) are not significantly different at 95% confidence interval ($p>0.05$)

Table 1: Hematological assessment of egg albumin-induced inflammation in wistar rats treated with expired and unexpired diclofenac.

Parameters	Normal Control	Unexpired Drug	Expired Drug	Induced Untreated
UREA (mmol/dL)	6.90 ± 1.23[a]	4.23 ± 0.98[a,b]	6.00 ± 1.34	6.88 ± 1.85[a,b]
CRE (mg/dL)	26.90 ± 5.50[a]	18.63 ± 2.38	27.15 ± 4.18[a,c]	26.7 ± 6.33[a,c]
ALT (U/L)	24.83 ± 13.61[a]	40.58 ± 50.78[a]	17.15 ± 8.52[a]	21.53 ± 5.94[a]
AST (U/L)	144.53 ± 3.83	164.60 ± 226.27	196.48 ± 10.63[C]	197.93 ± 40.66[C]
ALP (U/L)	95.23 ± 38.31[a]	88.08 ± 32.19[a]	121.83 ± 40.0[a,b]	170.83 ± 88.84[b]
ALB (g/dL)	30.7 ± 1.33[a]	26.35 ± 2.91[b]	30.85 ± 1.06[a]	27.7 ± 0.90[b]
TP (g/dL)	54.15 ± 4.46[a]	48.93 ± 5.28[a]	60.30 ± 2.62[b]	56.85 ± 2.77[a,b]
BIL (mg/dL)	0.50 ± 0.26[a]	0.55 ± 0.26[a]	0.65 ± 0.31[a]	0.53 ± 0.33[a]

*Bar tagged with the same alphabet(s) are not significantly different at 95% confidence interval

Table 2: Biochemical status of egg albumin-induced inflammation in wistar rats treated with expired and unexpired diclofenac.

Parameters	Normal control	Unexpired drug	Expired drug	Induced Untreated
CHOL (mg/dL)	64.19 ± 0.26	58.77 ± 0.10	72.70± 0.02	66.13 ± 0.18
TRIG (mg/dL)	133.35 ± 0.35	75.30 ± 0.06	128.43 ± 0.16	140.83 ± 0.34
HDL (mg/dL)	43.2 ± 0.13	29.4 ± 0.17	19.2 ± 0.07	15.6 ± 0.10
LDL (mg/dL)	55.55± 0.16	52.89± 0.20	68.85± 0.12	70.01± 0.16

*Bar tagged with the same alphabet(s) are not significantly different at 95% confidence interval ($p>0.05$)

Table 3: Effect of expired and unexpired diclofenac on the lipid profile of egg albumin-induced inflammation in wistar rats.

Effect of Expired and Unexpired Diclofenac on the Lipid Profile of Egg Albumin-Induced Inflammation in Wistar Rats

Table 3 shows the result of expired and unexpired diclofenac on the lipid profile of egg albumin-induced inflammation in wistar rats It was observed that cholesterol level of group treated with expired diclofenac was significantly higher than the other groups, but the triacylglycerol level of the induced untreated group was significantly elevated in comparison with the other groups, whereas high density lipoprotein of the control group increased significantly when compared to the other groups. It was observed that the group induced and not treated had the highest level of low density lipoprotein in comparison to the other groups.

Histopathological Status of Egg Albumin-Induced Inflammation in Wistar Rats Treated with Expired and Unexpired Diclofenac

Figure 6 shows photomicrographs of extracted livers from the experimental animals of different groups. No abnormalities were seen in group A and D while the histological sections of the liver tissue of experimental rats in group B and C showed parallel radially arranged plates of hepatocyte with vascular congestion.

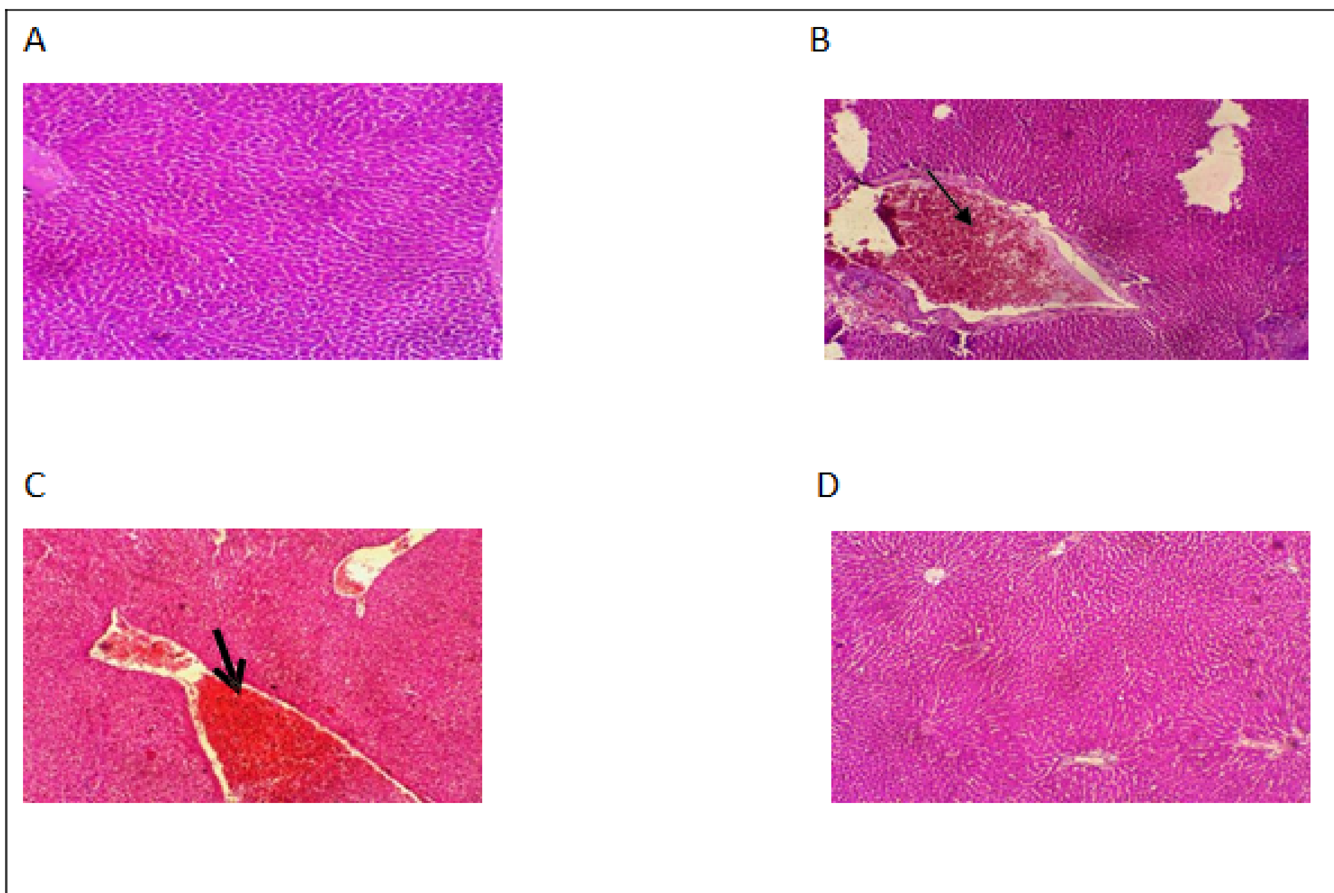


Figure 6: Photomicrographs of extracted liver from the experimental designed animals in groups where (A) Control, (B) Induced untreated, (C) Induced treated with expired diclofenac (D) Induced treated with unexpired diclofenac H & E STAIN X100.

Figure 7 Shows photomicrographs of extracted kidney from the experimental animals of different groups. No abnormalities were seen in group A, C and D while the histological sections of the renal tissue of rats in group B showed parallel radially arranged plates of renal with congested blood vessels.

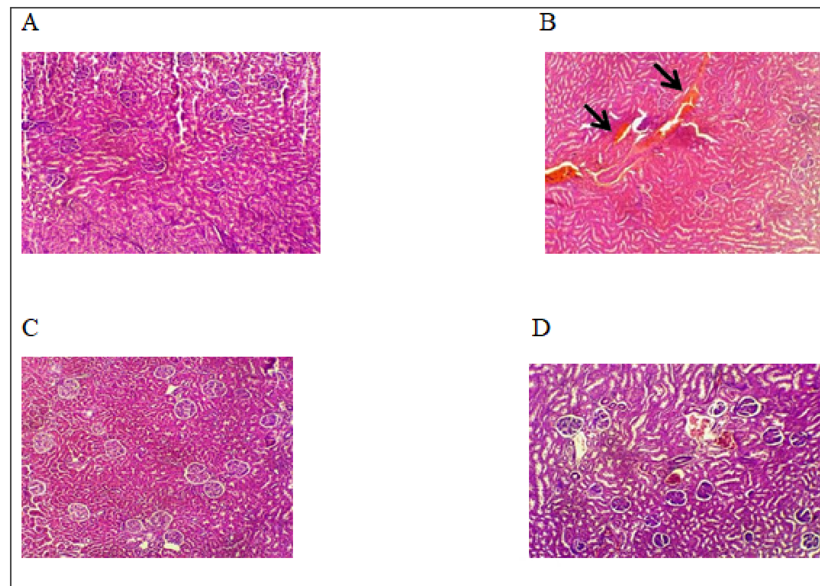


Figure 7: Photomicrographs of extracted kidney from the experimental designed animals in groups where (A) Control, (B) Induced untreated, (C) Induced treated with expired diclofenac, (D) Induced treated with unexpired diclofenac, H & E STAIN X100.

Figure 8 Shows photomicrographs of extracted skin from the experimental animals of different groups. No abnormalities wereseen in group A, C and D while the histological sections of the skin tissue B showed parallel radially arranged plates of skin with congested blood vessels and inflammation.

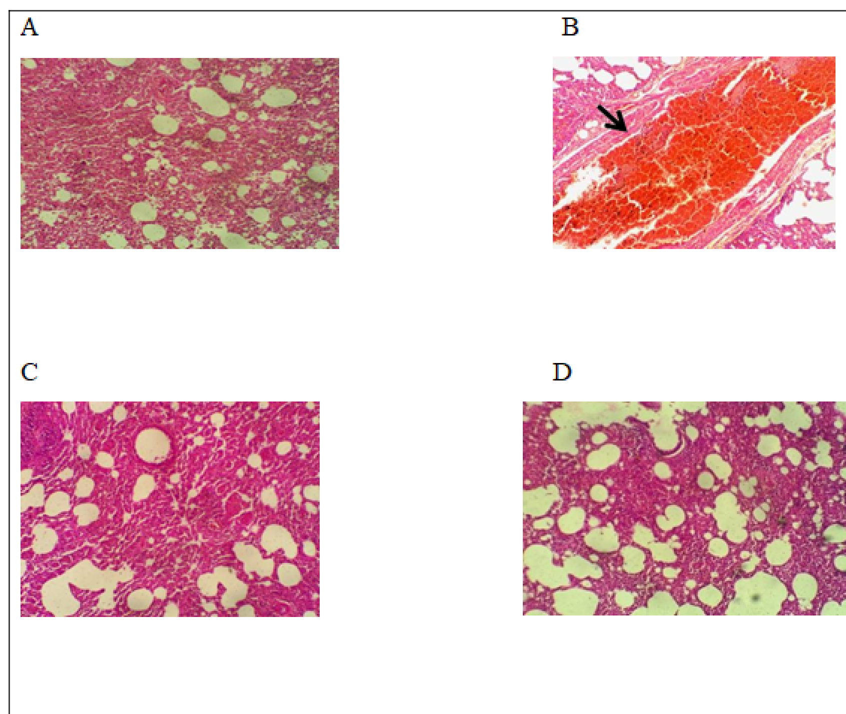


Figure 8: Photomicrographs of extracted skin from the experimental designed animals in groups where (A) Control, (B) Induced untreated, (C) Induced treated with expired diclofenac, (D) Induced treated with unexpired diclofenac, H & E STAIN X100.

Discussion

Several animal studies have shown that diclofenac reduces inflammation-related discomfort and heat, both acute and chronic. According to Czerkies, et al. [26] the medicine has demonstrated a higher weight-for-weight effectiveness compared to aspirin, ibuprofen, naproxen, and phenylbutazone, but it is less effective than piroxicam and comparable to indomethacin. While diclofenac has a typically good therapeutic index (the ratio of gastrointestinal irritant to therapeutic dosages) in animals, it differs significantly to other non-steroidal anti-inflammatory medications (NSAIDs) depending on the model utilized. On the other hand, compared to aspirin, feprazone, indomethacin, and naproxen, diclofenac causes more gastrointestinal harm than fenclofenac, according to controlled trials conducted in healthy people [27].

Compared to the Control group, most of the other groups had noticeably reduced GSH levels, according to the study's findings (Figure 2). When contrasted with groups provoked with inflammation, those given unexpired diclofenac had the highest GSH concentration. This provides more evidence that the drug's action on inflammation resulted in the endogenous generation of reactive oxygen species. The dangers of free radicals and oxidative stress to human health are well-known. A number of lifestyle-related disorders, including as atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and cancers, are known to be influenced by oxidative stress [28-30]. Because oxygen free radicals damage biological components like lipids, proteins, and DNA, oxidative stress is considered hazardous. According to Turner [30], oxidative stress has the potential to impact numerous biological processes, including cell death, virus replication, and inflammatory responses.

All of the groups in this study had lower SOD levels than the Control group. Having said that, the decline was insignificant. The study's results also demonstrated that the control group's catalase level was much higher than those of the stimulated and untreated groups. The group that received unexpired diclofenac also had a considerably higher catalase level than the triggered and untreated groups. According to Van et al. [31] and Ayo, et al. [32], SOD is recognized for its ability to shield tissues from oxygen free radicals by transforming superoxide radicals into molecular oxygen and hydrogen peroxide. Catalase, on the other hand, catalyzes the detoxification of hydrogen peroxide, protecting cell membranes and other biological structures from damage.

In this investigation, the levels of MDA were found to be considerably greater in the induced untreated rats compared to the control group and the group given unexpired diclofenac. Concentrations of TBARS and reduced cellular antioxidant molecule activity are typical causes of elevated lipid peroxide. The outcome that was observed can be explained by this hypothesis [33,34]. Complications including nephropathy and neuropathy can emerge as a result of tissue damage caused by elevated ROS, which damages membrane lipids, proteins, and DNA [34].

The results showed that the negative control group had lower levels of several haematological indicators, including red blood cell (RBC), neutrophil (NK), and monocyte (M) counts. Consistent with previous research, this finding supports the hypothesis put forth by Sechi, et al. [35] that medicinal substance or drug use can affect the normal range of hemotopoiesis parameters, possibly as a result of increased RBC destruction or inhibited haematopoiesis. Treatment of inflamed rats with equal doses of expired and unexpired diclofenac brought parameters to near normal compared to the normal control group, though changes in the other parameters (Hb, HCT, MCV, MCH, MCHC, and lymphocytes) were subtle. This finding is in agreement with the results of Calhoun, et al. [36] and He, et al. [37].

We found that inflamed, untreated rats had higher levels of lipid profile parameters (TC, triglycerides, LDL-C and VDL-C), liver function parameters (AST, ALT, ALP and bilirubin), Urea and creatinine. This could be because the liver sections showed hepatocytic degeneration and fatty changes (cytoplasmic vacuolation). This could be because the liver produces prostagladins from arachidonic acid, which leads to catalytic membrane phospholipid peroxidation and the breakdown of the endoplasmic reticulum. This, in turn, reduces lipid export from the liver cells and causes hepatocyte lipid accumulation [38]. Consistent with previous findings, fatty alterations were observed in liver sections of STZ-induced diabetic rats, as demonstrated by cytoplasmic vacuolizations [39-41]. Consistent with the liver tissue damage observed in our negative control group, prior research has also documented additional liver diseases like hepatocellular necrosis and infiltration of nonspecific inflammatory cells [42].

In this study, the histology results showed that the skin, kidneys, and liver of the induced untreated group were injured. However, when comparing the control and groups given unexpired diclofenac, there was damage in the liver of the ones treated with expired diclofenac. These results is in line with what Tribeskorn, et al. [43] found: that diclofenac causes liver tissue damage in fish. Metabolic processes take place in the liver and play a critical role in the body's overall detoxification process. Toxicology studies employ the anticipated change in hepatocytes of diclofenac-exposed fish. According to Altinok, et al. [44], fish hepatocytes can also serve as a biomarker for water pollution. varying cases of hepatocellular damage result from varying exposure

concentrations and durations [45]. Chronic exposure to egg albumin is also associated with impaired renal function in rats. When compared to other groups, the histopathology results showed that the induced untreated group had damaged skin and kidneys. Diclofenac is believed to primarily cause nephrotoxity by blocking the production of prostaglandins. It does this by blocking either the COX-1 or COX-2 isoforms of the cyclooxygenase enzyme, which are important in prostaglandin synthesis [46]. According to Happi, et al. [47], diclofenac is the most toxic of these compounds, and it is known to harm gastrointestinal and renal tissues in various vertebrate species [48,49].

Conclusion

In conclusion, this study revealed that unexpired diclofenac is potent to exhibit anti-inflammatory, analgesic, and antipyretic functions. Also, the expired diclofenac drug still possesses some active components that serve the same purpose.

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