

Preclinical Protective Activity of Lutein on Diclofenac-induced Hepatotoxicity

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Abstract

Background: Diclofenac (DF), a non-steroidal anti-inflammatory drug, may cause hepatotoxicity. Lutein (LT), a naturally occurring compound, has potential therapeutic activities. The protective activity of LT against DF-induced hepatotoxicity in adult Wistar rats was evaluated in this study. **Methods:** Twenty adult Wistar rats weighing 240-250 g of both sexes were randomized into 4 groups of n=5/group. The rats were given the chemical agents intraperitoneally for 7 days as follows: Group 1 (control) and Group 2 were given sterile water (1 mL/kg/day) and LT (40 mg/kg/day), respectively. Group 3 was given DF (10 mg/kg/day), while Group 4 was supplemented with LT (40 mg/kg/day) prior to the administration of DF (10 mg/kg/day). On day 8, the rats were weighed, euthanized, and blood samples were collected and evaluated for serum biochemical markers. The liver tissues of the rats were weighed and assessed for histological changes and oxidative stress markers. **Results:** DF decreased body weight and increased liver weight significantly at $p < 0.01$ when compared to the control. DF elevated serum lactate dehydrogenase, alkaline phosphatase, gamma glutamyl transferase, conjugated bilirubin, aminotransferases, total bilirubin, and liver malondialdehyde levels significantly at $p < 0.001$ when compared to the control. Liver glutathione, catalase, superoxide dismutase, and glutathione peroxidase levels were significantly ($p < 0.001$) decreased by DF when compared to the control. DF caused hepatocyte necrosis. However, LT supplementation abrogated DF-induced changes in all evaluated parameters significantly at $p < 0.001$. LT supplementation restored liver histology. **Conclusion:** LT shows potential therapeutic benefit against DF-induced hepatotoxicity.

Keywords: Diclofenac, Lutein, Liver toxicity, Protection, Rats

Introduction

The liver regulates various important functions, including the metabolism of drugs and chemicals. The functions of the liver can be impaired through hepatotoxicity as a consequence of its interactions with drugs and chemicals [1]. Drug-induced hepatotoxicity refers to the unexpected, adverse effects of medicines on the liver. It is a major health concern and is responsible for most cases of acute liver failure with up to a 50% fatality rate [2]. Idiosyncratic drug-induced hepatotoxicity is the main type of hepatotoxicity, leading to liver dysfunction, acute liver failure, and death [3]. Unfortunately, aside from drug cessation and liver transplantation, there are limited and effective treatments for drug-induced hepatotoxicity. More importantly, the idiosyncratic nature of drug-induced

hepatotoxicity presents a significant challenge to its management due to the difficulty in predicting its incidence and the underlying mechanism [4,5].

Diclofenac (DF) is a phenylacetic acid non-steroidal anti-inflammatory drug (NSAID) that possesses anti-inflammatory, antinociceptive, analgesic, and antipyretic properties. DF is widely used for the treatment of rheumatoid arthritis and pain [6]. Its mechanism of action involves the inhibition of cyclooxygenase- (COX-) 2 enzymes, with a greater impact on COX-1. Despite its well-tolerated nature and remarkable clinical success, DF has been associated with significant adverse effects, including hepatotoxicity [7]. DF is one of the most common drugs linked to idiosyncratic hepatotoxicity, with a risk of hepatotoxicity in 6 out of 100,000 users.

Furthermore, 8% to 20% of patients may succumb to liver failure associated with the development of jaundice [8]. Metabolic idiosyncrasy and immunologic mechanisms have been linked to DF-associated hepatotoxicity [9], but recent studies have suggested oxidative stress as an additional contributing factor [10,11].

Lutein (LT) is a fat-soluble carotenoid pigment, containing 40 carbons with a sequence of conjugated double bonds. LT is a tetraterpenoid present in some fruits, vegetables, egg yolk, and corn. LT is an important and effective functional molecule with varieties of biological properties that are beneficial to human health [12]. The biological activities of LT include anti-inflammatory, antiapoptotic, and antioxidative properties [13]. As an antioxidant, LT grabs and neutralizes hydroxyl radicals, and other reactive oxygen species (ROS), which keeps biomolecules from getting damaged [14]. LT's anti-inflammatory activity has been attributed to the inhibition of inflammatory mediators like tumor necrosis factor (TNF- α) and nuclear factor kappa B (NF- κ B) [13]. Studies have shown that LT has notable protective activity against toxicities caused by drugs and chemicals. LT has been shown to protect animals' liver from damage caused by alcohol [15], arsenic [16], and cyclophosphamide [17]. However, there is no scientific literature on the protective effect of LT on DF-induced hepatotoxicity, which the current study evaluated in adult Wistar rats.

Materials and Methods

Drug administration

Twenty adult Wistar rats of both sexes with an average weight of 240–250 g were obtained from the animal handling unit of the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria. The rats were divided into 4 groups of 5 rats each and were housed in clean plastic cages under standard conditions, including a temperature of $24 \pm 2^\circ\text{C}$ and a 12-hour light/dark cycle with unlimited access to food and water. Diclofenac sodium (Sunesta Life Sciences, India) and Lutein (Puritan Pride, Lutigold, US) were used in the study. The rats were administered with the chemical agents intraperitoneally (i.p.) for 7 days as follows: Group 1 (control) and Group 2 were given sterile water (1 mL/kg/day) and LT (40 mg/kg/day) [16] respectively. Group 3 was given DF (10 mg/kg/day) [18], while Group 4 was supplemented with LT (40 mg/kg/day) prior to the administration of DF (10 mg/kg/day).

Sample collection

On day 8, the rats were weighed and lightly anesthetized using diethyl ether. Blood samples (5 mL) were collected from the heart in sterilized centrifuge tubes, which were centrifuged at 3,000 rpm for 10 minutes. The sera were collected for biochemical analysis. Liver samples were collected through dissection, weighed, and used for histological study and oxidative stress marker assay. The liver of the rats for the

oxidative stress marker assay were rinsed in ice-cold saline and homogenized in phosphate buffer using a homogenizer (WiseTis® HG-15D, China). The homogenates were centrifuged for 30 minutes at 4°C at 800 g, decanted, and the supernatants were collected and used.

Evaluations of liver tissue and serum biochemical markers

Alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphate (ALP), total bilirubin (TB), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), and conjugated bilirubin (CB) levels were measured using an automatic chemical analyzer (Konelab™ PRIME 60i, Thermo Scientific, Vantaa, Finland).

Assay of liver oxidative stress markers

Malondialdehyde (MDA) was measured following the protocol by Buege and Aust, 1978 [19]. Glutathione peroxidase (GPx) level was estimated using the protocol by Rotruck *et al.*, 1978 [20]. Glutathione (GSH) level was assessed following the procedure by Sedlak and Lindsay 1968 [21]. Superoxide dismutase (SOD) levels were measured using the procedure by Sun and Zigman, 1978 [22]. Catalase (CAT) level was evaluated following the method by Aebi 1984 [23].

Histology of the liver

The liver tissues of the rats were fixed in 10% neutral buffered formalin for 48 hours. Subsequently, the liver tissues were dehydrated in a gradual ethanol (50–100%) solution, processed, and embedded in paraffin wax. The tissues were sectioned (4–5 mm thick) using a rotary microtome, stained with hematoxylin and eosin (H&E), and examined using a light microscope (Olympus BX51, Tokyo, Japan).

Data analysis

Results were expressed as mean $\pm$ SD (Standard Deviation) for $n=5$. Data was analyzed using a student t-test followed by a Dunnett test using GraphPad Prism® version 8.0 for Windows® 10 (GraphPad Software, San Diego, CA, USA). Probability values <0.05 , 0.01 , and 0.001 were considered significant.

Results

Effects of lutein on the body and liver weights of diclofenac-administered rats

The body and liver weights were normal ($p>0.05$) following the administration of LT when compared to the control (**Table 1**). In contrast, liver weight increased while body weight decreased significantly ($p<0.05$) following the administration of DF (**Table 1**). However, the liver and body weights were significantly ($p<0.05$) restored by LT supplementation when compared to DF (**Table 1**).

Table 1. Effects of lutein on the body and liver weights of diclofenac-administered rats.				
Group	Dose (mg/kg)	Body Weight (g)	Absolute Liver Weight (g)	Relative Liver Weight (%)
1	Control	258.67 ± 5.89	5.69 ± 0.25	2.20 ± 0.07
2	LT 40	265.60 ± 3.44	5.78 ± 0.10	2.18 ± 0.06
3	DF 10	210.20 ± 7.09*	9.03 ± 0.87*	4.10 ± 0.40*
4	LT 40 + DF 10	256.80 ± 2.95**	6.34 ± 0.15**	2.46 ± 0.05**
Data as mean ± SD; n=5; SD: Standard Deviation, LT: Lutein, DF: Diclofenac. *p<0.01 and **p<0.05 Significant difference when compared to the control and DF, respectively (Student T-test and Dunnett test).				

Effects of lutein on the biochemical markers of diclofenac-administered rats

The administration of LT had no significant (p>0.05) effects on serum AST, LDH, ALT, GGT, TB, ALP, and CB levels when compared to the control (Table 2). On the other hand, the administration of DF significantly (p<0.001) elevated serum AST, LDH, ALT, GGT, TB, ALP, and CB levels when compared to the control values (Table 2). However, supplementation with LT significantly (p<0.001) restored serum AST, LDH, ALT, GGT, TB, ALP, and CB levels when compared to DF (Table 2).

Effect of lutein on the liver tissue biochemical markers of diclofenac-administered rats

Liver tissue AST, LDH, ALT, GGT, and ALP levels were normal (p>0.05) following the administration of LT when compared to the control (Table 3). On the other hand, the aforementioned

liver tissue biochemical markers were significantly elevated (p<0.001) following the administration of LT when compared to the control (Table 3). However, the liver tissue's biochemical markers were significantly restored (p<0.001) by LT supplementation when compared to DF (Table 3).

Effect of lutein on the liver oxidative stress markers of diclofenac-administered rats

Liver CAT, GPx, SOD, GSH, and MDA levels were normal (p>0.05) after the administration of LT when compared to the control (Table 4). In contrast, liver CAT, GPx, SOD, and GSH levels were significantly decreased (p<0.001), whereas liver MDA levels were significantly elevated (p<0.001) following the administration of DF when compared to the control values (Table 4). However, LT supplementation significantly (p<0.001) restored liver CAT, GPx, SOD, GSH, and MDA levels when compared to DF (Table 4).

Table 2. Effect of lutein on the serum biochemical markers of diclofenac-administered rats.								
Group	Treatment (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	LDH (U/L)	TB (g/dL)	CB (g/dL)
1	Control	33.40 ± 4.28	28.60 ± 3.30	38.80 ± 4.03	21.90 ± 4.09	42.60 ± 4.58	8.32 ± 0.60	4.68 ± 0.39
2	LT 40	32.60 ± 4.39	28.00 ± 3.49	38.50 ± 4.74	21.56 ± 3.76	42.10 ± 5.89	8.00 ± 0.59	4.46 ± 0.44
3	DF10	59.80 ± 6.46*	54.60 ± 4.77*	61.40 ± 7.22*	46.86 ± 6.85*	93.40 ± 8.34*	13.12 ± 1.54*	10.70 ± 0.97*
4	LT 40+DF 10	35.00 ± 6.24**	31.60 ± 4.51**	40.20 ± 2.95**	24.26 ± 3.02**	44.60 ± 6.56**	8.58 ± 0.31**	5.02 ± 0.37**
Data as mean ± SD; n=5; SD: Standard Deviation; DF: Diclofenac; LT: Lutein; ALT: Alanine Aminotransferase; LDH: Lactate Dehydrogenase; ALP: Alkaline Phosphate; AST: Aspartate Aminotransferase; GGT: Gamma Glutamyl Transferase; TB: Total Bilirubin; CB: Conjugated Bilirubin; *p<0.001 and **p<0.001, Significant difference when compared to the control and DF, respectively (Student T-test and Dunnett test).								

Table 3. Effect of lutein on the liver tissue biochemical markers of diclofenac-administered rats.						
Group	Treatment (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	LDH (U/L)
1	Control	225.42 ± 35.21	210.71 ± 27.43	265.42 ± 23.22	211.55 ± 24.11	220.52 ± 21.32
2	LT 40	219.37 ± 34.41	209.62 ± 25.42	257.61 ± 25.14	207.75 ± 20.43	217.21 ± 32.20
3	DF10	475.22 ± 22.33*	421.67 ± 30.32*	491.72 ± 27.33*	399.81 ± 22.51*	389.45 ± 24.31*
4	LT 40+DF 10	245.41 ± 26.63**	238.22 ± 22.67**	280.81 ± 20.21**	234.44 ± 27.24**	241.33 ± 20.42**
Data as mean ± SD; n=5; SD: Standard Deviation; DF: Diclofenac; LT: Lutein; ALT: Alanine Aminotransferase; LDH: Lactate Dehydrogenase; ALP: Alkaline Phosphate; AST: Aspartate Aminotransferase; GGT: Gamma Glutamyl Transferase; *p<0.001 and **p<0.001, Significant difference when compared to the control and DF, respectively (Student T-test and Dunnett test).						

Table 4. Effect of lutein on the liver oxidative markers of diclofenac-administered rats.						
Groups	Dose (mg/kg)	SOD (u/mg protein)	GPx (u/mg protein)	CAT (µg/mg protein)	GSH (u/mg protein)	MDA (nmol/mg protein)
1	Control	34.11 ± 4.22	17.76 ± 1.71	22.71 ± 3.49	22.17 ± 3.46	0.35 ± 0.03
2	LT 40	34.73 ± 3.63	17.23 ± 1.80	23.73 ± 2.07	22.22 ± 2.43	0.34 ± 0.04
3	DF10	12.18 ± 1.25*	8.92 ± 0.80*	11.85 ± 1.11*	9.78 ± 0.55*	0.79 ± 0.04*
4	LT40 +DF10	31.96 ± 4.74**	16.58 ± 2.00**	20.64 ± 0.44**	20.66 ± 1.17**	0.38 ± 0.03**

Data as mean ± SD; n=5; SD: Standard Deviation; LT: Lutein; DF: Diclofenac; SOD: Superoxide Dismutase; GPx: Glutathione Peroxidase; GSH: Glutathione; CAT: Catalase; MDA: Malondialdehyde; *p<0.001 and **p<0.001 Significant difference when compared to the control and DF, respectively (Student T-test and Dunnett test).

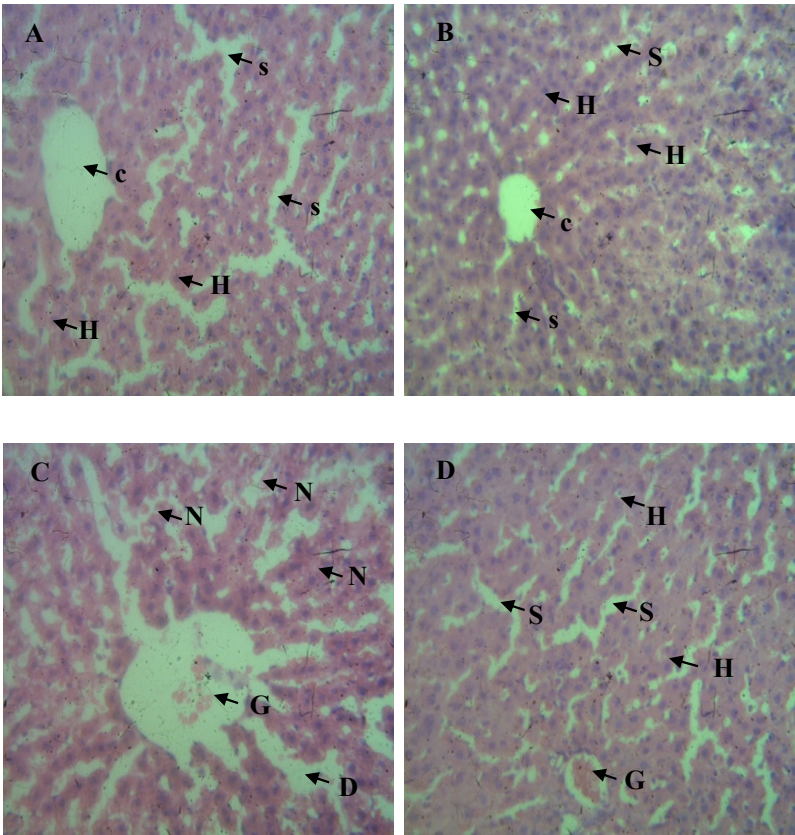


Figure 1. Showed the liver of the experimental rats. **(A)** Control, **(B)** Lutein (40 mg/kg)-administered rats, **(C)** Diclofenac (10 mg/kg)-administered rats, and **(D)** Lutein (40 mg/kg) and Diclofenac (10 mg/kg)- administered rats. H: Normal Hepatocytes; N: Hepatocyte Necrosis; C: Central Vein; G: Congested Central Vein; S: Normal Sinusoids; D: Dilated Sinusoids (Hand E) X40.

Discussion

The liver is a primary target for toxicity due to its essential function in the metabolism and detoxification of drugs and chemicals. The rising trend of drug-induced hepatotoxicity over recent decades has become a serious health concern [24]. DF, an efficacious and affordable drug, has been associated with hepatotoxicity [25]. LT, a naturally occurring compound with potential therapeutic activities, may protect biomolecules from chemical-associated damage [26]. The current study evaluated whether LT supplementation could

protect against DF-induced hepatotoxicity in Wistar rats. In this study, the administration of LT had no deleterious effects on all the evaluated parameters. DF notably decreased body weight and increased the liver weight of the treated rats. A similar decrease in body weight was reported in rats given DF at a dose of 10 mg/kg for 7 days [6]. The observed increase in liver weight in the current study is consistent with the reported observations in rats given DF at a dose of 10 mg/kg for 7 days [27]. This observed increased liver weight may be due to the ability of DF to induce liver inflammation, while decreased body weight may be a consequence of decreased body mass

[27]. Nonetheless, LT supplementation restored body and liver weights. The restored liver weight can be attributed to the anti-inflammatory activity of LT. Several studies have linked LT with remarkable anti-inflammatory activity [13]. The restored body weight may be due to increased appetite for food.

This study observed liver function distortion marked by elevated serum levels of AST, LDH, ALT, GGT, TB, ALP, and CB in rats given DF. These findings are supported by Esmailzadeh *et al.* (2010), who reported similar results in rats given DF at a dose of 50 mg/kg/day for 5 days [28]. Additionally, Abed Al-Kareem *et al.* (2022), found elevated levels of these biochemical markers in rats given 10 mg/kg/day of DF for 14 days [29]. The release of ALP, ALT, and AST into the bloodstream due to DF is a clear indicator of liver damage, likely caused by hepatocyte cell membrane destruction [30]. Bilirubin, a key liver elimination product, is commonly used as a diagnostic marker for liver function. The increased bilirubin levels in DF-administered rats indicate liver dysfunction possibly due to enzyme or transporter inhibition involved in bilirubin processing [31]. However, the current study observed that LT supplementation visibly restored serum levels of AST, LDH, ALT, GGT, TB, ALP, and CB. This could be attributed to the ability of LT to maintain hepatocyte membrane integrity, preventing the release of these biochemical markers into the bloodstream. LT may have also countered the inhibitory effect of DF on enzymes or transporters involved in bilirubin processing.

Oxidative stress, which involves the imbalance of oxidants and antioxidants in favor of oxidants, has been linked to drug-induced hepatotoxicity. It is often characterized by significant increases in oxidant activities such as reactive oxygen species (ROS) along with depletion or reduced antioxidant activities [30,32]. The present study observed decreases in liver antioxidants (SOD, GPx, CAT, and GSH) in rats given DF. These findings are consistent with previous research on rats given 50 mg/kg/day of DF [33]. Additionally, similar results were reported by Esmailzadeh *et al.* (2020), who showed decreased liver antioxidants in rats administered with DF [28]. However, it is worth noting that LT supplementation was able to restore liver antioxidants.

MDA is a widely used marker of lipid peroxidation. Studies have associated lipid peroxidation with DF-induced hepatotoxicity. The current study discovered increased lipid peroxidation in DF-administered rats marked by elevated levels of liver MDA. In resonance with the observation in this study, Haasan *et al.* (2021) reported increased expression of MDA concentration in DF (3 mg/kg/day) administered rats [7]. Ramezannezhad and others in 2019 also reported increased liver MDA levels in DF-administered rats, which support the findings in the current study [34]. Interestingly, LT supplementation restored liver MDA levels. The observed decrease in liver antioxidants and increase in MDA levels caused by DF are indications of oxidative stress and lipid peroxidation, respectively. This is the result of the functional ability of DF to generate an excessive number of free radicals, which could lead to the depletion

of antioxidants and the breakdown of polyunsaturated fatty acid in the liver [10,11]. LT supplementation restored liver antioxidants and MDA levels, possibly by preventing the production of free radicals by DF, thus inhibiting oxidative stress. Studies have linked LT to its ability to inhibit, scavenge, or quench the activities of free radicals, such as hydroxyl (HO•), peroxy (ROO•), and superoxide anion (O₂•-) [12]. Additional research has shown that LT can enhance the expression of antioxidants and cytoprotective proteins, which can help protect against oxidative stress and cell damage [35].

Furthermore, the current study observed that DF caused hepatocyte necrosis, central vein congestion, and dilated sinusoids in the liver. Similar liver histological alterations were reported by El-Maddawy and El-Ashmawy in 2013 in rats administered with DF (6.3-13.5 mg/kg/day) for 2-8 weeks [36]. Additionally, some scholars reported altered liver histology characterized by hepatocyte necrosis and degeneration in DF-administered rats, which supports the findings of the current study [37,38]. The observed altered liver histology caused by DF may be attributed to the induction of oxidative stress through the excess production of ROS. The exacerbated production of ROS might have activated apoptosis pathways, aggravated cell necrosis through peroxidation of membrane lipids, and damaged other liver biomolecules (DNA, lipids, and proteins), causing structural alterations in the liver [39,40]. However, LT supplementation prevented DF-induced alterations in liver histology. This may be a consequence of the ability of LT to inhibit liver oxidative stress, prevent lipid peroxidation, and safeguard liver biomolecules.

Studies have linked DF-induced hepatotoxicity to metabolic idiosyncrasy and immunologic mechanisms [9] caused by its metabolites. DF is primarily metabolized in the liver to diclofenacacyl-b-D-glucuronide [DF-AG], 4-hydroxydiclofenac (4 OH-DF), and 5-dihydroxy diclofenac (5 OH-DF). 4 OH-DF can be oxidized to p-benzoquinone imines, which can lead to oxidative stress. DF-AG can covalently bind to proteins and trigger an immune response [41], while 5-dihydroxydiclofenac can directly cause liver toxicity [42]. In conclusion, the current study demonstrated that LT supplementation can protect against DF-induced hepatotoxicity and may be clinically effective for DF-associated hepatotoxicity.

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Competing Interests

The authors have no competing interests.

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