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Research Article

Ethyl acetate fraction of *Mucuna pruriens* leaves mitigates diclofenac-induced hepatotoxicity via modulation of biochemical and histological parameters changes in Wistar Rats

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Abstract

Mucuna pruriens contains saponins and flavonoids, which help to decrease cholesterol, treat hypertension, provide protein and vitamins and prevent premature ageing. This research followed the NIH guidelines (NIH publication 85–23, revised in 1996). Rats weighing 200–250 g were assigned into six groups (n = 6), normal saline only (control), normal saline (NS), Ethyl acetate fraction of *Mucuna pruriens* leaves (EAFMP) (100, 200 and 400 mg/kg) and Silymarin (100 mg/kg) treated orally for five days, diclofenac (DFN) was administered on days 3 and 4 via intraperitoneal route, biochemical and histology parameters were determined in serum and liver. This research revealed that treatment with EAFMP reversed the elevation of liver enzymes, total bilirubin, LDL and total cholesterol and lipid peroxidation; liver SOD, GSH, and CAT were elevated in EAFMP and Silymarin groups. The hepatic histological lesions in EAFMP were reduced in a dose-dependent manner. This research shows that EAFMP attenuates the deleterious effect of diclofenac-induced liver toxicity in rats.

Keywords

Diclofenac, Silymarin, *Mucuna pruriens*, hepatotoxicity, rats

Introduction

The liver is a vital organ that performs many bodily functions, including protein synthesis, triglyceride and cholesterol formation, glycogen synthesis, and bile production. The liver metabolizes various poisons, including synthetic and natural toxins (Jameson 2018). Hepatotoxicity is the liver damage brought on by drug exposure. Numerous substances have been linked to mild to severe liver problems (Unzueta and

Vargas 2013). Drug-induced hepatic injury reduces liver function depending on the extent of the harm and the histological site damage (Sriutta et al. 2018). Additionally, conventional medical procedures and herbal treatments could be hepatotoxic (Unzueta and Vargas 2013). Most people take non-steroidal anti-inflammatory drugs (NSAIDs). However, some have been linked to liver damage (Masubuchi et al. 1998; Boelsterli 2013). Diclofenac, sulindac, and acetylsalicylic acid are non-steroidal anti-inflammatory medicines more frequently linked to liver damage (Marija et al. 2012). Diclofenac (DFN) is an example of an NSAID with painkilling, anti-inflammatory, antipyretic, and antinociceptive qualities (Mazumdar et al. 2006). It is widely employed to ease the pain brought on by rheumatoid arthritis. Despite the therapeutic benefits of DFN, it causes adverse side effects, including s gastrointestinal ulceration and damage to the liver, kidneys, lungs, and cardiac tissues (Tomic et al. 2008; Harirforoosh et al. 2016). According to Todd and Sorkin (1998) and Adeyemi et al. (2018), the mechanism of DFN-induced hepatotoxicity has been linked to an increase in ROS generation by DFN metabolites through the creation of redox imbalance and malfunction of the mitochondria (Moreno-Sanchez et al. 1999). Therefore, it is necessary to investigate potential treatment agents to prevent or reduce DFN-induced hepatotoxicity.

In tropical and subtropical areas of the world, the genus *Mucuna pruriens* belong to a member of the Fabaceae family and subfamily Papilionaceae. *Mucuna pruriens* has been studied for its beneficial medicinal properties in various contexts, including its anti-diabetic, aphrodisiac, anti-neoplastic, anti-epileptic, and anti-microbial activities (Sathiyanarayanan et al. 2007). Additionally, prior research suggested that *Mucuna pruriens* promotes the formation of antibodies, protecting against the toxicity of snake venom (Tan et al. 2014). Previous studies have demonstrated the importance of *M. pruriens* extract as a natural antioxidant source, suggesting that it may help halt the progression of various oxidative stressors (Satheesh et al. 2010). However, the aforementioned properties and preliminary studies of *Mucuna pruriens* prompt the investigation of the potential benefit of ethyl acetate fraction of *Mucuna pruriens* (EAFMP) in mitigating DFN-induced hepatic function parameters, lipid, protein, bilirubin, antioxidant/pro-oxidant indices, and histological modification in Wistar rats.

Materials and methods

Collection of plant material

The leaves of *Mucuna pruriens* were purchased from Oje market, Ibadan, Oyo/State, Nigeria. A specimen was identified and authenticated at the herbarium of the Forestry Research Institute of Nigeria (FRIN) Ibadan with a voucher number of FHI 112974.

Preparation of crude extract

The fresh leaves of *Mucuna pruriens* were air-dried and ground in a food processor. After that, three hundred grams (300 g) of powdered plant material was weighed and macerated in 1.5 L of 100% methanol for 72h with occasional shaking at about 64 °C using the Soxhlet extractor apparatus. The concentrate was then lyophilized (freeze-dried). Fractionation of crude methanol extract of *Mucuna pruriens* was carried out in a separating funnel, using a liquid-liquid fractionation process, with 1000 mL each of n-hexane, dichloromethane, ethyl acetate, and methanol. The resulting fractions were concentrated on a rotary evaporator with reduced pressure, and the various fractions of n-hexane, dichloromethane, ethyl acetate, and methanol were lyophilized. For subsequent investigation, the lyophilized powdered fractions were kept at -20 °C. However, the ethylacetate fraction was the most effective in the authors' preliminary assays.

Experimental animals

The studies and techniques described in the present study followed National Institutes of Health guidelines (NIH Publication No. 85–23, revised in 1996). Wistar rats (200–250 g) used in this study were purchased from the Animal House of the Institute for Advance Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria. The rats were kept in plastic cages in conventional environments (12-hour light/dark cycle, 23 °C, 50–60% relative humidity). Rats were given unlimited access to food and water.

Treatment protocol

Thirty-six (36) rats were randomly divided into six groups (6) and treated with normal saline, graded doses of EAFMP and silymarin for five days. Group A (Control group) received normal saline (NS) only (10 mL/kg, p.o.). Group B received normal saline (10 mL/kg, p.o.), plus DFN (50 mg/kg, i.p.), on days 3 and 4. Groups C, D, E and F received EAFMP 100, 200 and 400 mg/kg p.o and silymarin {standard drug}, (100 mg/kg, p.o.), respectively, plus DFN (50 mg/kg, i.p.) on day 3 and 4.

Collection of serum samples and liver tissue

Rats from various groups were sacrificed on day 6 of the treatment regimen after being sedated with light ether. This was done twenty-four (24) hours following the last treatment. The blood was drawn from the retro-orbital plexus and placed in a vial without anticoagulants. The separated serum sample was centrifuged at 5,000 rpm for 10 minutes, which was then used for biochemical parameters analysis using standard diagnostic kits. The liver tissue was extracted, washed with ice-cold saline, and stored at -20 °C for further analysis.

Histological study

After the rats were sacrificed, the liver was removed. The liver was used for histological investigations after being blotted without blood or tissue fluids. The liver tissue was fixed in 5% formalin for 48 hours, dehydrated by passing through various ethyl alcohol-water combinations, and immersed first in paraffin, then in xylene Bancroft and Layton (2012). Then, using a microtome, slices measuring 15 m thick were cut and put on a glass slide. After being stained for 3–5 minutes with 10% hematoxylin, the liver slices were rinsed under running water to make the staining more pronounced. The sections were then counterstained for 2 minutes with 10% eosin. The slices were examined using a photomicroscope at a magnification × 400, and the desired spots were photographed.

Biochemical assays

Total protein (TP), Total bilirubin (TBIL) and Albumin-A (ALB-A) in serum were measured according to the principles of biochemical analysis described by Vasudevan and Sreekumari (2007). Serum lipid profiles parameters, including total cholesterol (TCHOL), triglycerides (TRIG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), liver enzymes such as Alanine aminotransaminase (ALT), Aspartate aminotransaminase (AST), and alkaline phosphatase (ALP), were tested using Randox kits, using the procedures in the manual. All the activities of oxidative stress and antioxidant markers were estimated in the hepatic tissue. The Buyukuslu et al. (2006) method measured the hepatic superoxide dismutase (SOD) activity using a spectrophotometer at 480 nm. The Michelson (1991) method was used to test the catalase (CAT) activity, and the sample's absorbance was measured at 240 nm. The concentration of reduced glutathione (GSH) in liver sub-cellular fractions was measured as described by Mori et al. (2004); the absorbance was measured at 412 nm against blank. The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) using the Theophile et al. (2006) method, which measured the absorbance at 532 nm.

Statistical analysis

The values were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey, multiple comparison tests, was used for all statistical analyses. Statistical significance was defined as $p<0.05$.

Results

Effect of EAFMP on Diclofenac induced hepatotoxicity on serum ALT, AST and ALP

The effect of DFN, EAFMP and SLM on ALT, AST and ALP in serum is presented in Table 1. The group administered with NS+DFN showed a significant ($p<0.05$) elevation in serum ALT, AST and ALP relative to the control. Treatment with graded doses of EAFMP (100, 200 and 400 mg/kg) showed a decrease in serum ALT, AST and ALP, with a significant reduction ($p<0.05$) at EAFMP 400 mg/kg when compared with NS + DFN group. However, the group administered with SLM 100mg/kg + DFN showed a noticeable reduction ($p<0.05$) in serum ALT, AST and ALP in comparison with NS + DFN treated group (Table 1).

Effect of EAFMP on Diclofenac induced Hepatotoxicity on Total Protein, Albumin-A and Total Bilirubin.

As presented in Table 2, the group treated with NS + DFN revealed a decrease in TP, ALB-A and an increase in TBIL relative to the control. Treatment with graded doses of EAFMP (100, 200 and 400 mg/kg) showed an increase in TP and ALB-A, with a decrease in TBIL relative to NS + DFN. Similarly, increased serum TP, ALB-A and a decrease in TBIL were observed in SLM 100mg/kg+ DFN group (Table 2).

Table 1. Showing the effect of EAFMP on Diclofenac induced hepatotoxicity on serum Liver enzymes.

Treatments	Units	Groups					
		Control (NS)	NS + DFN	EAFMP(100 mg/kg) + DFN	EAFMP (200 mg/kg) + DFN	EAFMP (400 mg/kg) + DFN	SLM (100 mg/kg) +DFN
AST	U/L	8.00±1.53	21.00±0.58*	19.67±1.20	15.00±0.58	12.00±0.58**	9.00±0.58**
ALT	U/L	7.03±0.88	14.67±0.88*	11.23±0.88	10.00±0.58	9.50±0.76**	9.33±0.67**
ALP	U/L	12.33±1.67	27.00±0.57*	24.67±0.33	19.00±0.58	15.33±1.45**	13.33±0.68**

Results are expressed as mean ± SEM * $p < 0.05$ = compared with control: ** $p < 0.05$ = compared with NS+DFN.

Table 2. Showing the effect of EAFMP on diclofenac induced hepatotoxicity on TP, ALB-A and TBIL.

Treatments	Units	Groups					
		Control (NS)	NS + DFN	EAFMP(100 mg/kg) + DFN	EAFMP (200 mg/kg) + DFN	EAFMP (400 mg/kg) + DFN	SLM (100 mg/kg) +DFN
TP	g/dL	47.67±2.33	33.23±0.28	39.23±0.56	40.33±0.88	43.67±1.20	45.00±0.58
ALB-A	g/dL	19.00±1.00	12.67±0.88	15.60±0.88	17.13±0.88	18.27±2.60	18.90±1.53
TBIL	mg/dL	0.02±0.01	0.07±0.01*	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.01

Results are expressed as mean ± SEM * $p < 0.05$ = compared with control.

Effect of EAFMP on diclofenac-induced hepatotoxicity on lipid profiles

The group treated with NS+DFN showed a noticeable ($p<0.05$) increase in total cholesterol, triglyceride and LDL with a decrease in HDL relative to control. As presented in Table 3, groups administered with graded doses of EAFMP (100, 200 and 400 mg/kg) plus DFN reduced total cholesterol, triglyceride and LDL, while EAFMP 200 and 400 mg/kg DFN significantly ($p<0.05$) increased HDL when compared with NS+DFN group. Likewise, SLM 100 mg/kg + DFN group showed a reduction in total cholesterol, triglyceride and LDL with a significant increase ($p<0.05$) increase in HDL.

Effect of EAFMP on Diclofenac induced hepatotoxicity on GSH, MDA, CAT and SOD

Table 4 showed a decrease ($p<0.05$) in GSH, CAT, and SOD levels in NS +DFN treated groups relative to control; on the contrary, MDA level was elevated ($p<0.05$) in NS +DFN. As presented in Table 4, EAFMP (100, 200 and 400 mg/kg) showed a dose-dependent increase in GSH, CAT and SOD levels relative to NS +DFN. On the contrary, EAFMP 400 mg/kg showed a significant reduction in MDA level. Silymarin 100mg/kg +DFN treated group showed a significant ($p<0.05$) increase in GSH, CAT and SOD and decreased MDA levels.

Effect of EAFMP on Diclofenac induced hepatotoxicity on Liver Histology

The histological results are established in Fig. 1. The microscopic examination established no observable lesion of hepatic tissue from the normal saline-treated group (Fig. 1A). On the other hand, NS+DFN-treated group showed atrophy of cords, random hepatocellular coagulative necrosis and inflammation relative to the normal saline-treated group, which showed hepatotoxicity (Fig. 1B). EAFMP 100 mg/kg + DFN treatment demonstrated moderate peri-acinar hepatocellular coagulation necrosis (Fig. 1C). The EAFMP 200 mg/kg + DFN-treated group showed an improvement in liver histological changes when compared with the NS+ DFN-administered group but still exhibited moderate centrilobular cord atrophy and Kupffer cell hyperplasia (Fig. 1D). Similarly, the group treated with EAFMP 400 mg/kg + DFN improved the liver histological changes compared with NS+ DFN by demonstrating mild cord atrophy (Fig. 1E). However, the liver of the group treated with SLM 100 mg/kg + DFN showed no observable lesion (Fig. 1F).

(A) Control animals (Normal Saline) show no visible lesions ; (B) Normal Saline+ DFN treated animals show atrophy of cords (blue arrow), random hepatocellular coagulative necrosis (black arrow) and inflammation (red arrow); (C) EAFMP 100 mg/kg + DFN) show moderate peri-acinar hepatocellular coagulation necrosis (black arrow); (D) EAFMP 200 mg/kg + DFN) show moderate centrilobular cord atrophy (blue arrow) and Kupffer cell hyperplasia (yellow arrow); (E) EAFMP 400 mg/kg + DFN) show mild cord atrophy (blue arrow); (F) SLM 100 mg/kg + DFN) show no observable lesion.

Discussion

The liver is an organ involved in many metabolic processes and susceptible to xenobiotic injury because of its critical role in xenobiotic metabolism. Hepatotoxic medications like acetaminophen can damage the liver (Buraimoh et al. 2011). Diclofenac is a well-known NSAID frequently used to treat pain and inflammation, and there has been much concern regarding the hepatotoxicity effect of DFN. The deposition of DFN, as well as its bioactivation, produces intermediate reactive species, which cause the production of oxidative stress, inflammation, tissue necrosis and deterioration that are likely responsible for liver damage (Adeyemi et al. 2018).

Table 3. Showing the effect of EAFMP on Diclofenac induced hepatotoxicity on TCHOL, TRIG, HDL and LDL.

Treatments	Units	Groups					
		Control (NS)	NS + DFN	EAFMP(100 mg/kg) + DFN	EAFMP (200 mg/kg) + DFN	EAFMP (400 mg/kg) + DFN	SLM (100 mg/kg) +DFN
TCHOL	mg/dL	83.33±1.15	154.33±1.00*	124.00±2.52	117.19±3.46	102±1.15	93.00±1.73
TRIG	mg/dL	51.33±1.53	127.73±2.65*	81.67±2.08	86.67±2.08	90.67±2.30	96.34±1.53
HDL	mg/dL	52.33±2.52	18.98±1.53*	32.67±1.53	41.67±0.58**	43.33±2.08**	44.00±0.00**
LDL	mg/dL	43.00±2.65	107.96±3.00*	85.00±2.00	80.67±1.53	71.33±0.58	63.67±0.57

Results are expressed as mean ±SEM * $p < 0.05$ = compared with control; ** $p < 0.05$ = compared with NS+DFN.

Table 4. Showing the effect of EAFMP on Diclofenac induced hepatotoxicity on GSH, MDA, CAT and SOD.

Treatments	Units	Groups					
		Control (NS)	NS + DFN	EAFMP(100 mg/kg) + DFN	EAFMP (200 mg/kg) + DFN	EAFMP (400 mg/kg) + DFN	SLM (100 mg/kg) +DFN
GSH	nmols/mg Protein	26.93±1.14	9.04±1.27*	10.41±0.92	14.74±1.21	18.44±0.98**	19.8±0.47**
MDA	nmols/mg Protein	3.27±1.36	10.18±1.00*	6.6±1.50	6.07±0.55	4.8±0.11**	4.2±0.19**
CAT	U/mg Protein	22.29±1.69	9.25±0.4*	19.46±0.17	12.13±0.34	19.54±0.23**	20.62±0.15**
SOD	U/mg Protein	0.33±0.06	0.12±0.05*	0.16±0.04	0.23±0.10**	0.24±0.13**	0.28±0.09**

Results are expressed as mean ± SEM * $p < 0.05$ = compared with control; ** $p < 0.05$ = compared with NS+DFN.

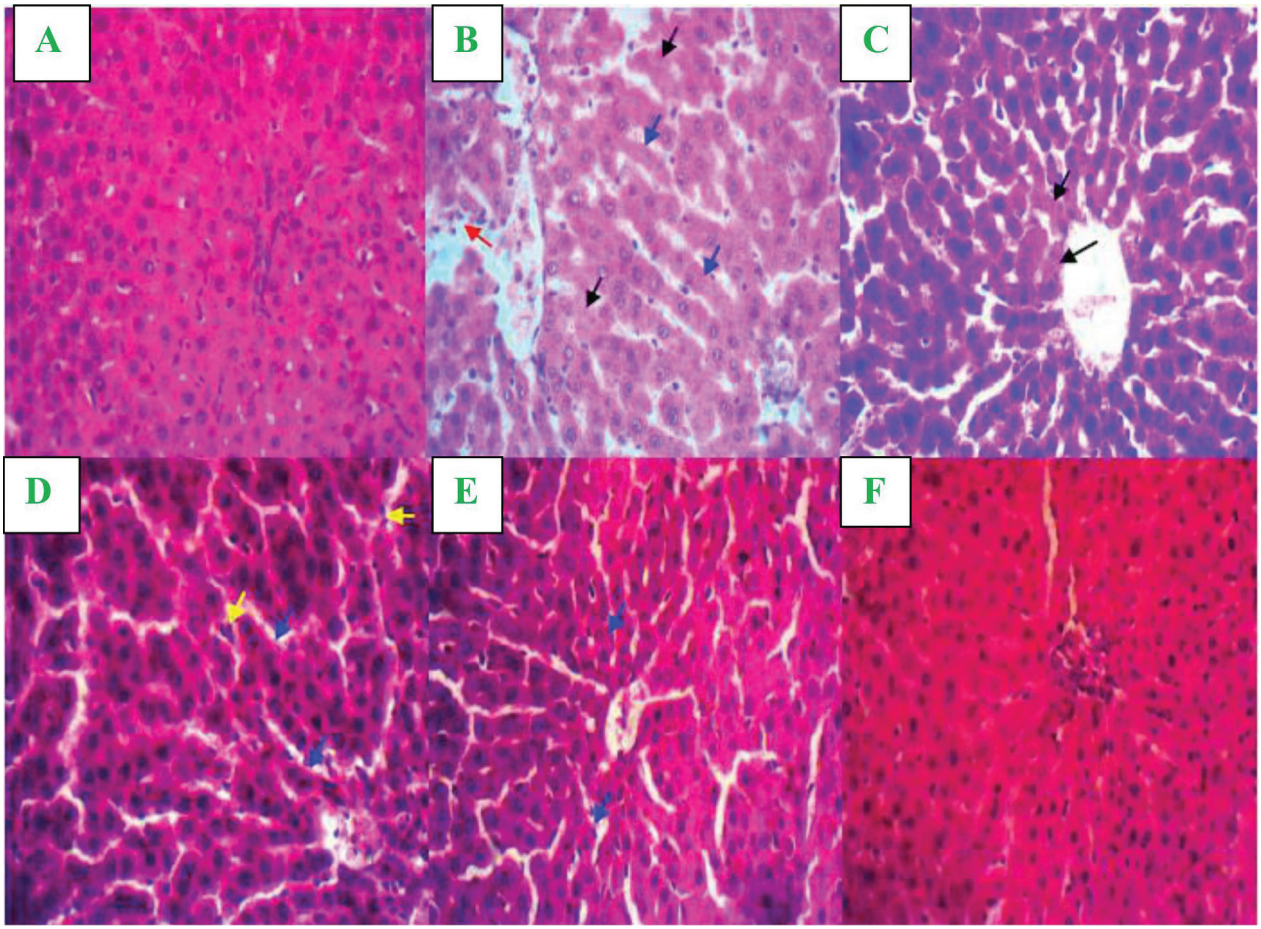

Figure 1. Effects of Diclofenac on the liver of control rats and rats subjected to graded doses of EAFMP and Siltmarin, HE ×400.

Our study investigated the attenuating effects of the EAFMP against diclofenac-induced liver injury and its modulating effects on free radicals and biochemical and histological parameters alterations in Wistar rats. The result of our study revealed that the administration of diclofenac can cause hepatic tissue damage and inflammation. Our findings from the hepato-protective potentials of the ethylacetate fraction of *Mucuna pruriens* against DFN-induced liver toxicity in rats align with earlier research that supported DFN's hepatotoxic effects (Peter et al. 2017). Also, treatment with graded doses of ethylacetate fraction of *Mucuna pruriens* (100, 200 and 400 mg/kg) and Silymarin 100 mg/kg plus diclofenac reversed the physiological abnormalities caused by diclofenac. This suggests that the EAFMP maintained the liver cell membranes' structural integrity, which prevented the release of ALT, AST, and ALP into the bloodstream. However, Ogunmoyole et al. (2021) reported similar findings with ethanol extract of *Mucuna pruriens* leaves using carbon tetrachloride and rifampicin-induced hepatoxicity.

One of the markers of liver impairments is serum bilirubin, a test for hepatic excretory function. Bilirubin is a by-product produced by the catabolism of heme, which is usually conjugated by the liver to produce bilirubin's diglucuronide and move from the body through the bile. As a result, increased serum bilirubin is observed when there is liver injury (Dhanotiya 2004). Serum bilirubin is also increased when there is too much breakdown of erythrocytes in case of haemolytic anaemia, in which diclofenac may be the cause. The heme produced from the breakdown of haemoglobin resulting from red blood cells is catabolized by the reticuloendothelial system (RES) to generate bilirubin (Murray 2006).

Our study showed high bilirubin levels in the NS + DFN treated group compared to the control group, a convenient indicator of liver diseases (Achliya et al. 2004). Our results showed a dose-dependent decrease in total bilirubin in groups administered with graded doses of EAFMP (100, 200 and 400 mg/kg) and Silymarin plus diclofenac, which indicates that the liver functions were restored to a level comparable to the control group, the results are consistent with earlier findings (Orinya et al. 2016). The marked changes observed in serum total bilirubin levels due to diclofenac toxicity were reversed by EAFMP and Silymarin-administered groups.

The decrease in serum levels of albumin and, accordingly, total proteins result from the destruction of the synthesizing role of the liver (Vasudevan and Sreekumari 2007). Total protein and albumin levels decrease due to protein-losing nephropathy, haemorrhage, lack of protein in the diet and malabsorption of food nutrients (Kahn and Scott 2010). Furthermore, the decrease in serum total protein and albumin may also be due to blood in the gastrointestinal tract due to the drug's toxic effect and

inadequate food intake. In the present investigation, NS + DFN treated group showed a decrease in serum TP and ALB-A relative to the control. This finding is in agreement with El-Maddawy and El-Ashmawy (2013).

In our investigation, the level of low-density lipoprotein in the NS + DFN treated group was considerably more significant than that in the control group, indicating a diclofenac-induced hypercholesterolemia condition. The activity of low-density lipoprotein was strongly correlated with membrane lipid peroxidation. This demonstrates that one effect of oxidative stress might increase low-density lipoprotein activity (Najeeb and Aziz 2015). These results support earlier research on the rise of lipid levels in rats caused by diclofenac toxicity (Das and Roy 2011). Diclofenac's effects may be responsible for the considerable changes in blood triglycerides, total cholesterol and high-density lipoprotein observed in all treated groups. In the current study, rats treated with diclofenac had much higher triglyceride and cholesterol levels than control rats, and their serum high-density lipoprotein levels significantly decreased. This could be explained by the diclofenac's toxic effects, which result in hepatobiliary diseases and decreased cholesterol metabolism. These findings agree with Maity et al. (2012), who reported increased serum cholesterol and triglyceride levels in diclofenac-treated rats. However, our findings showed that the group administered with EAFMP and Silymarin plus diclofenac had a better blood lipid profile when compared with NS + DFN treated group, consistent with an earlier study by Maity et al. (2012).

Administering graded doses of EAFMP (100, 200, and 400 mg/kg) and Silymarin plus diclofenac lowered malondialdehyde to a level comparable to the control group. This shows that EAFMP and Silymarin have anti-lipid peroxidative effects, protecting liver cell integrity (Ramezannezhad et al. 2019). According to our study, the diclofenac-treated group had considerably reduced glutathione, catalase, and superoxide dismutase activity than the control group. These changes are due to the depletion of the antioxidant defence system due to increased oxidative stress markers, which agrees with earlier reports by Haddad et al. (2002); El Sayed et al. (2014). In addition, groups receiving graded doses of EAFMP and Silymarin had increased superoxide dismutase activity compared to the NS+DFN group. This elevation may become significantly more effective in eliminating superoxide radical anions produced due to oxidative stress, as Sangeetha (2016) reported.

Catalase is a crucial enzyme of the antioxidant defence system, and numerous studies have suggested that diclofenac may inhibit the liver's antioxidant enzymes from functioning (Niu et al. 2015). Catalase activity was found to increase in EAFMP and Silymarin-treated groups when compared with the NS+DFN. These results agree with previous findings on the elevation of catalase in rats due to the antioxidant effect of Silymarin (Sangeetha 2016). Glutathione is the primary endogenous antioxidant within cells, crucial in protecting cells from oxidative stress-induced cell damage. In our study, a considerable increase in glutathione activity was seen in the groups treated with graded doses of EAFMP and Silymarin compared to the NS+DFN group, showing that the enzyme is actively involved in scavenging the hydro-peroxides produced as a result of drug-induced liver injury. Our result supports the earlier findings by Niu et al. (2015); Alabi et al. (2017).

In the present study, the histology and the biochemical findings were comparable. Inflammation and hepatocellular coagulative necrosis were observed in the group administered with normal saline and diclofenac, which is consistent with earlier findings by Sangeetha (2016). The group treated with graded doses of EAFMP plus diclofenac demonstrated a protective effect by minimizing hepatocellular necrosis and degeneration and reducing the hepatic histological lesions brought on by diclofenac administration. The same protective effect was seen in the liver of the animals treated with Silymarin.

Conclusions

This study provides information that the Ethylacetate fraction of *Mucuna pruriens* has protective potential against DFN-induced liver damage by decreasing cellular reactive oxygen species release and enhancing liver function enzymes. Furthermore, abnormal biochemical markers and histological modifications of DFN-induced liver toxicity were also ameliorated by the Ethylacetate fraction of *Mucuna pruriens*. Further investigations are necessary to provide additional clinical confirmation of the hepatoprotective effects of EAFMP against diclofenac-induced liver toxicity.

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