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The suppressive role of *Ginkgo biloba* against diclofenac induced hepatic injury.

Sally M. E. Ramadan*, Hanaa M. Serag and Gamal M. F. Edrees

1. Zoology Department-Faculty of Science-Mansoura university

2. Professor of animal physiology

Zoology Department-Faculty of Science-Mansoura university

3. Professor of animal physiology

Zoology Department-Faculty of Science-Mansoura university

** sally_mahmoud_2012@yahoo.com*

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Abstract Many physiological problems can injure people who are treated with diclofenac (DF), Ginkgo biloba (G.b) has to exhibit a hepatic prevention effect against this injury. To estimate the dangerous role of DF and the protective role of G.b, rats treated with DF (10 mg/kg), and/or G.b at dose (100, 200, 400 mg/kg/b.wt) by intragastric administration for one and two weeks. Biochemical analysis and immunohistochemical studies were estimated. The results revealed that, DF led to an increase in the concentration of AST, ALT, and γ-GT accompanied by a decline of GSH, SOD, CAT, GPx, prostaglandin, TNF-α, IL-1 β, and IL-6. In addition to alterations in immune regulatory symptoms. Notably, supplementary G.b significantly protects the liver from these abnormalities. In conclusion, it seems that the mechanism of action of DF to induce hepatic injury may be through stimulating ROS generation and declining the antioxidant systems, triggering ionic disturbances. G.b administration can scavenge ROS and free radicals, restore the antioxidant status and exhibit a significant chemoprotective effect against DF-produced hepatotoxicity.

keywords: Diclofenac, *Ginkgo biloba*, Transaminases, Oxidative stress and anti-oxidant, anti-inflammatory markers.

1.Introduction

1.1 General background

The liver detoxifies various metabolites, helps energy provision and regulates the homeostasis of the body (33). Hepatotoxicity by chemicals causes damage to the liver where it becomes unable to perform its normal synthetic and metabolic functions, acute or chronic (26). Non-steroidal anti-inflammatory drugs (NSAIDs) represent the most popular drugs currently prescribed and used worldwide (23). Diclofenac (Voltaren) is 2-[(2,6-dichlorophenyl) amino] benzene acetic acid, is the most common NSAID prescribed for the pain and inflammation suppress in addition to injury-related inflammation post-surgery and physical trauma, (34). Diclofenac has adverse effects that include, heart disease, stroke, kidney problems, ulceration, gastrointestinal disorders, liver injury, hypersensitive reactions and hemato-toxicity, (20). The main mechanisms of action of NSAIDs involve the inhibition of cyclooxygenase activity (COX). Cyclooxygenase is required to convert arachidonic acid into thromboxanes, prostaglandins, and prostacyclins, (13). There are two cyclooxygenase isoenzymes, COX-1 and COX-2, thereby preventing the synthesis of their downstream effector prostaglandins and thromboxane A2, (40).

(24) reported that the extract of *Ginkgo biloba* leaves has an effective antioxidant, possesses cardioprotective, anti-asthmatic, antidiabetic, and potent central nervous system activities. Gb contains 24% flavonol glycosides and 6% terpene lactones. The flavonoid fraction is primarily composed of quercetin, kaempferol, and isorhamnetin. The terpenoid fraction primarily contains ginkgolides A, B, C, J, and M, as well as bilobalide, (35). *Ginkgo*

biloba shows a strong antioxidative property that directly scavenges ROS, (39).

Aim of the work: The purpose of the present study, was to investigate the efficacy of a natural plant *Ginkgo biloba* extract as a hepatoprotective agent against hepatic injury induced by Diclofenac in male rats.

2. Materials and methods

2.1. Experimental animals:

Seventy-two adult male Wistar rats, weighing 150-180 g were used, they were obtained from Helwan animal Farm, Cairo, Egypt. Rats were housed in stainless steel cages in an artificially illuminated and thermally controlled room (22- 25°C and 12 h light / dark cycle).

Chemicals: Diclofenac sodium (Voltaren) ampoules were produced by Novartis Pharma Company; each ampoule was diluted in 2 ml saline immediately before intra-muscular (i.m) administration. All other diagnostic kits and chemicals used were highly purified and purchased from specific agents.

Ginkgo biloba L. family (Ginkgoaceae), were purchased from Orchidia Pharmaceutical Company. The powder of *Ginkgo biloba* leaves was confirmed and analyzed by Orchidia Pharmaceutical Company, Cairo, Egypt.

2.2. Preparation of aqueous extract of *Ginkgo biloba*:

Fine quality of *Ginkgo biloba* leaves powder was used for the preparation of an aqueous extract. Fifteen grams of *Ginkgo biloba* leaves powder were added to 500 ml cold water and mixed in an electric mixture for 20 minutes. The mixture was centrifuged, and the clear supernatant was carefully removed and kept in a refrigerator at 2–8°C as a final extract for subsequent experimental treatments, (5).

2.3. Experimental design:

After one week of acclimation before the experimental work, rats were randomly divided into the following groups: Control group (Cont); Animals received basal diet. *Ginkgo biloba* treated group (G.b); Animals received freshly prepared *Ginkgo biloba* extract orally using a gastric tube at a dose of 400 mg/kg b.w/day. Diclofenac treated group (DF); Animals daily injected intramuscularly (i.m) Diclofenac at a dose of 10 mg/kg b.wt. DF+G.b100 mg/kg treated group; Animals were i.m daily injected by Diclofenac and in the same time received *Ginkgo biloba* 100 mg/kg. DF+G.b 200 mg/kg treated group; Animals were i.m daily injected Diclofenac and received orally freshly prepared *Ginkgo biloba* dose of 200 mg/kg. DF+G.b 400 treated group; Animals i.m daily injected Diclofenac and received *Ginkgo biloba* 400 mg/kg.

All the above-treated groups were divided into two subgroups, the first treated for one week and the other treated for two weeks.

2.4. Blood and tissue samples collection:

At the end of each experimental period of each subgroup, overnight fasted animals were sacrificed after slight diethyl ether anesthesia by cervical dislocation. Blood samples were collected in clean centrifuge glass tube. After complete coagulation, tubes were centrifuged for 20 minutes at 860 xg. Sera were separated and immediately frozen at -20°C for further biochemical analysis. Then, animals were dissected, liver was carefully removed and cleaned using saline solution, weighed and known weight of it was homogenized in a 10% w/v in distilled water using homogenizer surrounded by ice jacket. These homogenates were kept at -20°C for further biochemical assay.

2.5. Biochemical parameters:

Liver function serum enzymatic concentration, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated colorimetrically according to the method described by (32). Serum gamma-glutamyl transferase (γ-GT) was determined colorimetrically according to the method of (37).

2.6. Antioxidant status and oxidative stress assays:

The concentration of hepatic malondialdehyde (MDA) was evaluated photometrically according to the procedure of (27). Liver content of reduced glutathione (GSH) was estimated colourimetrically by the method of (31). The activities of hepatic superoxide dismutase (SOD) and catalase

(CAT) were estimated after (25) and (1), respectively. Glutathione peroxidase (GPx) activity was determined as described by (29).

2.7. Inflammatory markers:

For the quantitative determination of serum endogenic rat prostaglandin E2 (PG-E2) concentrations. Using ELISA Kit purchased by cusabio (Catalog Number.CSB-E07967r).

The content of interleukin-1 β (IL-1β) and interleukin-6 (IL-6) in hepatic tissue was determined quantitatively using a Rat ELISA kit provided by Ray Biotech (Norcross, Georgia, USA). ELIZA, which is a solid phase enzyme amplified sensitivity immunoassay, used for the detection of tissue Tumor necrosis factors-α (TNF-α) content.

3. Statistical analysis

All data were statistically analyzed by using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, California, USA). Results were expressed as means ± standard errors (SE). Statistical were one-way analysis of variance (ANOVA) test and post comparisons were carried out with Duncan test followed by Neuman Keulspost hoc test, The values of $p \leq 0.05$ were considered statistically significant, (22).

4. Results and Discussion

4.1. Serum ALT, AST and γ-GT concentration in different animal groups.

After one week or two weeks**,** the administration with *Ginkgo biloba* extract (G.b) alone exerted no significant change in the concentration of serum AST, ALT and γ-GT, but the diclofenac injected group (DF) showed a significant increase in the concentration of these enzymes as compared with the control group. Co-administration of G.b with their different doses (100, 200, and 400 mg/kg) with DF suppress to some extent the effect of DF on these enzymes relative to that of the DF group. We notice that *Ginkgo biloba* extract administered 400 mg/kg after two weeks has the best protective effect against the injured liver damage.

4.2. Hepatic MDA, GSH content and SOD, CAT, GPx activities in different animal groups.

The results shown in table 4.2. indicate that, in both examined times, one or two weeks, rats treated with G.b alone showed normal content of liver MDA, GSH, and activities of SOD, CAT, GPx while those administered with DF showed a significant increase in MDA content, and a decrease in the GSH content in addition to SOD, CAT and GPx activities when compared to the control group. The administration of *Ginkgo biloba* extracts with different doses (100, 200, 400 mg/kg) to the rats treated with DF reduce the hepatic MDA content and improve in hepatic content of GSH, SOD, CAT, and GPx when compared to that treated with DF only. The highest dose 400 mg/kg for a long time 2 weeks showed more effective protection than the other doses.

4.3. Serum prostaglandin and hepatic interleukin-1β, interleukin-6 and tumor necrosis factors- α (TNF- α) concentration in different experimental groups.

Rats treated with G.b alone at the 1st week or 2nd week showed no significant changes in serum prostaglandin and hepatic interleukin-1β, interleukin-6, and TNF- α concentration, while that administered with DF alone produced a highly significant decrease in prostaglandin concentration when compared to the control group but a significant increase in the other parameters. The administration of G.b extract with different doses (100, 200, and 400 mg/kg) to the rats treated with DF showed a protection role in serum prostaglandin and hepatic IL-1β, IL-6, and TNF- α concentrations when compared to that treated with DF group.

Rats injected with DF group at the 2nd week a recorded significant decrease in serum prostaglandin and hepatic IL-1β, IL-6 and TNF- α concentration when compared to the

control group. On the other hand, the G.b administration pre-DF resulted in an increase in these parameters when compared to that injected with DF only.

Table 4.1. Serum ALT, AST and γ-GT concentration in different animal groups

		Cont.	G.b	DF	DF+G.b100mg/kg	DF+G.b 200mg/kg	DF+G.b400mg/kg
AST (U/l)	1week	71.50±6.00	70.02±6.80	140.00±12.01[a]	134.00±9.45[a,b]	127.20±12.71[a,b]	105.60±9.57[a,b]
	2weeks	72.05±7.40	69.09±11.01	162.00±13.64[a]	128.80±11.72[a,b]	110.80±10.21[a,b]	98.80±8.31[a,b]
ALT (U/l)	1week	31.00±6.45	29.28±5.37	71.00±6.26[**a**]	64.00±7.41[**a**,b]	50.80±5.49[**a,b**]	45.60±6.06[**a,b**]
	2weeks	30.00±4.51	28.50±4.73	88.33±8.76[**a**]	60.60±9.93[**a,b**]	44.20±6.86[**a,b**]	40.45±7.50[**a,b**]
γ-GT (U/l)	1week	3.60±0.19	3.53±0.20	6.20±0.40[**a**]	5.90±0.30[**a,b**]	5.10±0.35[**a,b**]	4.80±0.40[**a,b**]
	2weeks	3.62±0.40	3.49±0.32	7.30±0.37[**a**]	5.40±0.40[**a,b**]	4.90±0.46[**a,b**]	4.30±0.32[**a,b**]

a and **b** significant changes at p ≤0.05 compared to control and diclofenac groups respectively.

Cont: Control, **G.b**: *Ginkgo biloba*, **DF**: Diclofenac

Table 4.2. Hepatic MDA, GSH content and SOD, CAT, GPx activities in different animal groups.

		Cont.	G.b	DF	DF+G.b 100mg/kg	DF+G.b 200mg/kg	DF+G.b 400mg/kg
MDA (nmol/g)	1week	876±9.31	870±17.87	1192±19.10[a]	996±12.01[a,b]	929±13.66[a,b]	899±11.95[b]
	2weeks	875.8±11.03	867.20±15.74	1347±16.82[a]	990±15.81[a,b]	914.40±12.08[a,b]	887±10.98[b]
SOD(U/g)	1week	193.80±9.93	199.20±10.37	122.60±11.39[**a**]	154.60±8.05[**a**,b]	165.20±8.52[**a,b**]	170.40±7.08[**a,b**]
	2weeks	198.40±9.01	211.80±11.83	97.60±10.49[**a**]	157.40±11.59[**a,b**]	171.00±9.11[**a,b**]	177.40±8.33[**a,b**]
CAT (mM/g)	1week	192.40±8.01	196.40±9.94	137.00±10.31[a]	152.50±11.97[a,b]	162.40±9.18[a,b]	167.60±8.49[a,b]
	2weeks	196±9.87	201.20±7.40	108±10.64[a]	157±8.94[a,b]	166±11.68[a,b]	174.50±8.48[a,b]
GSH (mg/g)	1week	5.06±0.40	5.16±0.17	2.80±0.23[**a**]	3.12±0.41[**a,b**]	3.48±0.16[**a,b**]	4.01±0.29[**a,b**]
	2weeks	5.28±0.21	5.62±0.26	2.11±0.06[**a**]	3.34±0.22[**a,b**]	3.90±0.29[**a,b**]	4.25±0.25[**a,b**]
GPx(nmol/min/ml)	1week	784±8.50	791±9.43	565.80±10.89[a]	577±11.17[a,b]	621±9.32[a,b]	640±8.88[a,b]
	2weeks	786±8.41	797.7±9.83	443.40±11.09[a]	603±10.59[a,b]	632±6.11[a,b]	680±9.33[a,b]

a and **b** significant changes at p ≤0.05 compared to control and diclofenac groups respectively.

Cont: Control, **G.b**: *Ginkgo biloba*, **DF**: Diclofenac

Table 4.3. Serum prostaglandin and hepatic interleukin-1β, interleukin-6 and tumor necrosis factors- α (TNF- α) concentration in different experimental groups.

		Cont.	G.b	DF	DF+G.b 100mg/kg	DF+G.b 200mg/kg	DF+G.b 400mg/kg
prostaglandin (pg/ml)	1week	16.00±0.16	16.50±0.19	3.60±0.04[a]	5.32±0.08[a,b]	6.71±0.35[a,b]	7.30±0.50[a,b]
	2weeks	16.40±0.16	16.90±0.12	2.40±0.07[**a**]	6.82±0.24[**a,b**]	7.90±0.32[**a,b**]	9.00±0.25[**a,b**]
HepaticIL-1β (Pg/mg)	1week	88±6.30	84.80±3.90	241±10.50[a]	214±8.5[**a,b**]	181.80±3.63[**a,b**]	120.40±5.60[**a,b**]
	2weeks	87±4.58	80±3.27	264±8.85[**a**]	209.20±5.21[**a,b**]	175.40±3.18[**a,b**]	115±4.99[**a,b**]
Hepatic IL-6(Pg/mg)	1week	2.63±0.04	2.52±0.03	8.07±0.18[a]	6.32±0.12[a,b]	5.84±0.10[a,b]	4.91±0.14[a,b]
	2weeks	2.90±0.04	2.81±0.05	9.64±0.10[a]	6.00±0.09[a,b]	5.11±0.12[a,b]	4.15±0.14[b]
HepaticTNFα(Pg/mg)	1week	10.00±1.13	9.80±1.17	25.00±2.95[a]	24.04±2.05[a,b]	20.80±2.11[a,b]	16.60±1.71[a,b]
	2weeks	9.90±1.52	9.65±1.11	28.00±2.33[a]	22.55±2.18[a,b]	18.20±2.64[a,b]	14.00±1.03[a,b]

a and **b** significant changes at p ≤0.05 compared to control and diclofenac groups respectively.

Cont: Control, **G.b**: *Ginkgo biloba*, **DF**: Diclofenac

5. Discussion

Nonsteroidal anti-inflammatory drugs are members of a drug class that reduces pain, prevents blood clots, decreases fever and decreases inflammation. Side effects depend on the specific drug but largely include an increased risk of gastrointestinal bleeds and ulcers, heart attack, liver and kidney disease, reported by (19).

The present findings showed that administration of DF to male rats cause significant impairment in liver functions. Serum AST, ALT, and γ-GT concentrations were significantly increased compared to their corresponding values in the control group. Diclofenac sodium is extremely metabolized in the liver, their metabolites are toxic to the liver (10). The increase in these concentrations in rats treated with diclofenac may be attributed to the membrane damage, hence release these enzymes into circulation, due to leakage and the loss of the functional integrity of membrane architecture that significantly impaired in liver functions, as excess these enzymes concentration the serum, an explanation agrees with the previously reported by Mostafa *et al.*, (2020). This study was intended to examine the protective effect of *Ginkgo biloba* extract with different doses and different times against diclofenac-induced oxidative damage in the rat liver tissues. The DF-induced hepatotoxicity

was suppressed after the administration of Gb extract as indicated from the significant decreases in the serum concentration of AST, ALT, and γ-GT. There were significant restorations of these enzymes' concentration in *the Ginkgo biloba* extract-treated group. The restoration of serum enzyme concentration in DF-induced liver damage by G.b extract may be due to the membrane stabilizing activity and reserves the integrity of the plasma membranes which prevent the leakage of intracellular enzymes and hence restores these enzymes, an explanation which in accordance with (41).

Administration of diclofenac induced a significant increase in lipid peroxidation product malondialdehyde (MDA) in the liver, and a remarkable reduction in its GSH content when compared to that of the control, these results run parallel with (11) these results may be of downregulation of the antioxidant system. The present study demonstrated that rats administered with diclofenac sodium increased production of lipid peroxidation product (MDA) especially in the second week more than that of the first one. This result agrees with, (4) which may be due to a decline in the body antioxidant system and that of body defense mechanism to scavenging the free radicals. The MDA, being a product of lipid peroxidation, is produced in response to the oxidative deterioration of polyunsaturated fatty acids (PUFAs) in liver membranes, its content is enhanced during conditions of the intracellular build-up of reactive oxygen species (ROS) concentration, (18). The administration of G.b extract with different doses may be involved in the elimination of ROS or other reactive by-products generated by DF toxic metabolites in the liver tissues, these explanations coincide with the study of, (17).

The endogenous antioxidant defense system plays an important role in the protection of cells from ROS-mediated oxidative injury (41). Decreases in endogenous antioxidant enzymes such as SOD, CAT and GPx activities were observed in the rat liver of the DF-treated group. Reduction in these antioxidants may be due to the accumulation of cytotoxic metabolites in the rat's hepatic tissues after DF treatment as confirm the result of (28). Diclofenac treatment causes the generation of ROS due to the accumulation of cytotoxic metabolites in the hepatic tissues of rats which increase oxidative stress hence reducing the antioxidant system activities, as reported by, (4).

Our results indicated the decrease in endogenous antioxidant enzymes such as SOD, CAT and GPx content were observed in the liver damage induced by DF at dose 10 mg/kg after one and two weeks, a result that agrees with (30). Conversely, our results indicated that the hepatic GSH content and SOD, CAT and GPx activities were significantly increased in the G.b administered group this effect may be related to the presence of flavonoids in the G.b which can discharge the tissue damage caused by oxygen-derived free radicals, an explanation which in parallel with **(**6).

The decreases in the specific activities of SOD, CAT and GPx in the liver of DF treated rats were previously recorded by (4). However, the treatments of the DF-intoxicated rats with G.b extract (100, 200 and 400) mg/kg decrease the oxidative stress marker where, the hepatic activities of SOD and GPx have significantly increased these findings are in agreement with (8). The protective role of G.b extract on liver functions may be attributed to its capability to scavenge reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), superoxide anion radical ($O^{\cdot-}$), nitric oxide radical ($NO^{\cdot}$), hydrogen peroxide (H_2O_2), and ferryl ion species, and/or indirectly inhibiting formation of free radicals (9). The increases in hepatic CAT activities concomitant with a decrease in GPx activities may be explained by the major role of CAT in detoxifying hepatic cells and depletion of H_2O_2 and the minor role of GPx, this explanation coincided with (41).

Serum prostaglandin concentration was increased in rats treated with DF, this data may be as a result of inflammatory effects of DF on the rat's induced hepatotoxicity and the inhibitory effect on cyclooxygenase-2 (COX-2) which trigger prostaglandins released, (4). However, the damaged hepatic cell by DF may be protected by G.b extract with its active component flavonoid and terpenoid that have an antioxidant property (7). In addition to the role of G.b in stimulating synthesis GSH hence facilitates prostaglandin formation.

The pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions such as IL-1, IL-6, and TNF- α, (43). The increase of ROS and the impairment of antioxidant defense mechanisms in DF treated group may be perhaps causative factors in inflammatory diseases, (14) where (15) shows the correlation between ROS and inflammation. Interleukin IL-1β and IL-6 are other important cytokines that are released by a variety of cells at the site of injury, (42). Interleukins, including IL-1β and IL-6, have strong influences on inflammatory responses, (21). The obtained rise in IL-1β and IL-6 is associated with liver injury in the DF treated group only are consistent with (36). The increase of proinflammatory cytokines including IL-1β and IL-6 may be due to the role of DF which induce circulation of macrophages and monocytes, (30) after liver tissues inflammation and the formation of oxidative stress that could lead to the activation of NF-kB, an explanation that coincides with (4). The strong suppressor *Ginkgo biloba* extract of IL-1β, IL-6 may be due to its universal inhibitory effects on the production of other pro-inflammatory mediators to different extents in macrophages, (12).

Tumor necrosis factor-α (TNF-α) is considered as one of the keys released cytokines in different inflammatory processes, which activates signaling pathways associated with the inflammatory response. In response to inflammation and infection, TNF-α is produced mainly by the immune system (2). The obtained result shows a significant increase in the concentration of TNF-α in DF-treated rat's livers. This result may contribute to hepatic damage by inducing inflammatory and nitrosative stress responses, (28). But Gb co-treatment with with DF resulted in a significant decrease in the hepatic TNF- α concentration as compared with control group, this result is in accordance with (38). These beneficial effects of Gb can be partially explained by its antioxidant properties by the regulation of the proinflammatory and profibrotic cytokines tumor necrosis factor alpha (TNF-a) as showed by (7).

The present study shows that co-administration of *Ginkgo biloba* with DF inhibited increased pro-inflammatory cytokines concentration (IL-1 β, IL-6 and TNF- α) caused by DF in rats. The anti-inflammatory property of G.b extract could be related to its ability to down-regulate the production of these cytokines concentrations as registered by (16). The decrease in tissues (IL-1, IL-6 and TNF- α) and expression of the TNF-α gene of *Ginkgo biloba* extract may be due to its antioxidant properties, as shown by (3).

6. Conclusion

The present results recorded that, the abuse of diclofenac causes alterations in biochemical and anti-inflammatory parameters in liver tissue. These adverse effects may be due to the oxidative stress-induced liver damage caused by the drug. On the other hand, *Ginkgo biloba* extract seems to be a potent natural antioxidant that demonstrates effectiveness in the prevention of diclofenac-induced hepato-toxicity. It could preserve the liver functions parameters especially with higher doses and a long time. The possible protective mechanism of aqueous extract of *Ginkgo biloba* may be due to many chemical constituents which could scavenge oxidative free radicals, inhibit lipid peroxidation, possess antioxidant activity, anti-inflammatory activity, and then alleviate liver toxicity. Therefore, G.b could be potential to help as a medicament or food supplement for alleviation of liver toxicity.

7. References

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