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Potential protective role of carob ethanolic extract against diclofenac sodium-induced acute hepatorenal toxicity in male rats: Biochemical and histopathological studies.

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ABSTRACT

Phenylacetic acid molecules, non-steroidal anti-inflammatory medications (NSAIDs) including diclofenac sodium (DFS), are used to treat osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and severe muscular rigidity. However, overdosing of DFS can be harmful to the kidneys and liver. This study aimed to assess the protective effect of carob ethanolic extract against DFS-induced hepatorenal damage in rats. Four equal groups of male rats were randomly assigned, with ten members each. Control group was administered with normal saline orally. Carob group was administered carob ethanolic extract orally at a dose of 500 mg/kg for 7 days. DFS group received 5 mg/kg diclofenac sodium orally for 7 days. DFS + Carob group rats received 5 mg /kg from DFS with 500 mg/kg from Carob ethanolic extract orally for 7 days. Blood and tissue samples were collected at the end of the experiment for determination of liver and kidney functions and histological analysis. The obtained results demonstrated a significant increase in serum liver and kidney function biomarkers, and a decrease in albumin concentration in the DFS group when compared to the control group. However, administration of carob ethanolic extract with DFS exhibited an obvious ameliorating effect against severe biochemical and histopathological alterations induced by DFS. In conclusion, our result indicated that carob ethanolic extract improved liver and kidney functions. Therefore, it can be used as a preventive treatment against DFS-induced hepatorenal toxicity in rats.

1. INTRODUCTION

Diclofenac sodium (DFS) is a phenylacetic acid derivative used to treat pain, inflammation, and musculoskeletal disorders in people and animals. Diclofenac sodium, one of the most widely used non-steroidal anti-inflammatory medications (NSAIDs) (Adeyemi and Olayaki, 2018), can have several serious adverse effects e.g., renal damage, hepatotoxicity, gastrointestinal injury, and cardiovascular hazards, despite its wide range of therapeutic benefits (Chatterjee et al., 2015; Owumi and Dim, 2019; Moore, 2020).

There is evidence that diclofenac-induced hepatorenal damage is linked to the generation of reactive oxygen species (ROS), oxidation of proteins and thiols, and mitochondrial dysfunction leading to tissue damage and apoptosis. However, the mechanisms of hepatorenal toxicity caused by the drug are not fully understood (Masubuchi et al., 2002; Santos-Alves et al., 2014). The production of reactive metabolites, 5-hydroxy diclofenac, and N, 5-dihydroxy diclofenac, by diclofenac, was demonstrated to have negative effects. These metabolites may disturb the body's normal balance and promote an excess of reactive oxygen and nitrogen species (RONS) in the tissues, followed by oxidative stress and damage to their hepatorenal tissue (Bort et al., 1999).

Currently, there is a growing interest in supplements derived from natural, conventional, and non-conventional sources as possible sources of biologically active substances with proven health properties for incorporation into the human diet (Baumel et al., 2018).

Ceratonia siliqua, often known as carob, is an evergreen tree that falls under the family Fabaceae. Although it originated in western Asia, it eventually extended to all of the Mediterranean basins, the western coasts of the Americas, South Africa, and the southern sections of Australia after becoming domesticated. (Azab, 2017). Traditional uses of carob include the treatment of diarrhea, coughing, warts, and diuresis (Kivçak et al., 2002). Pods, bean flour, seed gums, carob chocolate, and syrup are the most commercially successful carob products (Youssef et al., 2013).

Carob is rich in proteins, lipids, carbohydrates, polyphenols, and tannins. Its seeds and pods are especially high in ellagitannin, gallotannin, and condensed tannins (proanthocyanidin). These phytochemicals show scavenging properties against a variety of illnesses brought on by assaults by free radicals. (Abulyazid et al., 2017). Additionally, water extracts from carob pods showed a high antioxidative activity in different *in vitro* tests because of the presence of proanthocyanidins, gallic acid, catechin, epicatechin gallate, epigallocatechin gallate and quercetin glycosides (Papagiannopoulos et al., 2004). Furthermore, the presence of carotenoids in the pod extract improves the antioxidant capacity, which may propose a synergistic effect

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between polyphenols and carotenoid compounds (Karim and Azlan, 2012).
Carob is used in multiple industries: food, pharmaceutics, and cosmetics (Durazzo et al., 2014). Numerous studies have demonstrated the multiple biological actions of carob, including antibacterial, anticancer, and antioxidant effects (Custódio et al., 2009; Meziani et al., 2015). In traditional medicine, carob pods and seeds are frequently used for their analgesic, anti-constipation, anti-glucose absorption, gastrointestinal propulsion, and antidiarrheal properties (Rtibi et al., 2017). Therefore, the current study was designed to investigate the effect of carob ethanolic extract probable ameliorating role against DFS-induced hepatorenal toxicity in rats.

2. MATERIAL AND METHODS

2.1. Experimental animals:
In the current investigation, forty male rats weighing 180±40 g and 7–8 weeks old were used. Rats were acquired from the Laboratory Animal Research Center, Faculty of Veterinary Medicine, Benha University. For a week prior to the start of the experiment, the animals in cages had a normal meal, access to fresh water, and bedding made of hardwood shavings under appropriate living conditions (21 to 24 °C, 12 h light/dark cycle and humidity not exceeding 60%. The Animal Care and Use Committee, Faculty of Veterinary Medicine, Benha University, Egypt, approved the experimental protocols (BUFVTM 06-07-23).

2.2. Diclofenac:
Diclofenac sodium (DFS) oral tablet was acquired from PHARCO Company. Diclofenac sodium, a drug with a 50 mg concentration, is marketed under the name Diclofen. The tablets were crushed, suspended in distilled water, and given to male rats at a dose level of 5 mg/kg body weight daily for 7 days (Al-Hayder et al., 2022).

2.3. Preparation of Carob ethanolic extract
Carobs (*Ceratonia siliqua*) were purchased from the local herbal market in Cairo, Egypt. Then, they were washed, all the seeds removed, and ground to a fine powder. The plant extract was prepared by addition of ethanol 70% to the fine powder in a firmly closed jar and kept for 3-5 days at room temperature with vigorous shaking twice a day. The mixture was filtered using filter paper and a rotatory evaporator was used to evaporate the alcohol and obtain the pure extract. The residues were re-extracted by the same method to get the whole constituents of the plant. It is suspended in distilled water and given to male rats at a dose level of 500 mg/kg body weight daily for 7 days (Atta et al., 2023).

2.3. Experimental Design:
Forty male rats were randomly divided into four equal groups (10 rats /group).
Control group: rats were received 1 ml normal saline orally;
Carob group: rats were given orally 500 mg/kg b.wt from carob ethanolic extract for 7 days (Atta et al., 2023);
Diclofenac group: rats were received orally 5 mg/kg b.wt DFS dissolved in distilled water for 7 days (Al-Hayder et al., 2022);
Diclofenac + Carob group: received 5 mg/kg DFS dissolved in distilled water with 500 mg/kg b.wt from carob ethanolic extract for 7 days.

2.4. Sampling:
2.4.1. Blood samples
Blood samples were taken from the medial canthus of the eye using a capillary tube. Serum separation was achieved by centrifuging the collected samples for 15 minutes at 3000 rpm. The serum was kept at -20 °C for subsequent biochemical evaluation.

2.4.2. Tissue specimens
After blood samples rats were euthanized, the liver and kidney tissue specimens were removed for histopathological examination according to Bancroft and Layton (2013).

2.5. Biochemical analysis:
Liver enzyme activities were measured using commercial kits. Serum ALT and AST measurements were estimated using the protocol described by Huang et al. (2006). Additionally, the albumin concentration was measured using spectrophotometry following the guidelines provided by Doumas et al. (1971). The techniques of Kaplan (1984) and Schirmeister et al. (1984) were used to measure the concentrations of serum urea and creatinine, respectively. Furthermore, uric acid concentrations were measured using spectrophotometry in accordance with the guidelines provided by (Schultz, 1984).

2.6. Histopathological analysis
Small tissue specimens were collected from the liver and kidneys and immediately fixed in preserved in 10% neutral buffered formalin, dehydrated in ascending strengths of ethanol concentrations, cleared in xylene, and embedded in paraffin wax. To analyze general tissue structure, the paraffin blocks were sectioned using a rotatory microtome at a thickness of 4-6 µm and then stained with hematoxylin and eosin. A Leica microscope (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland) was used to examine these sections.

2.7. Statistical analysis:
The one-way ANOVA analysis of variance was utilized to analyse the data which is shown as Mean ± S.E. Duncan's test was used as post hoc for multiple comparisons between groups using SPSS for windows ver. 25. Mean values with various superscripts are significantly different at P<0.05.

3. RESULTS

3.1. Biochemical results:
Oral administration of DFS resulted in a significant increase in ALT and AST enzyme activities and urea, creatinine and uric acid levels along with a significant decrease in albumin levels compared to control group. However, oral carob ethanolic extract administration alongside with diclofenac ameliorated the elevation in liver enzyme activities and kidney parameters compared to the diclofenac group. Additionally, the carob ethanolic extract induced a significant increase in albumin level (Figure 1).

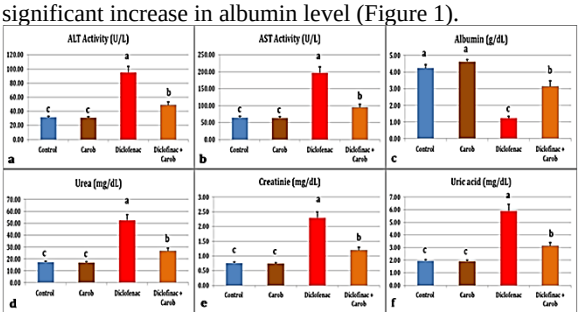

Figure 1 Effect of DFS and /or Carob on liver and kidney functions in male rats. a: serum ALT activity (U/L), b: serum AST activity (U/L), c: serum albumin (g/dl), d: serum urea level (mg/dL); e: serum creatinine level (mg/dL); f: serum uric acid level (mg/dL). Mean values with letters are significantly different at P<0.05.

3.2. Histopathological results:
3.2.1. Liver tissue
Almost all examined liver sections in the control and carob

groups revealed normal histoarchitecture of the liver where well-organized hepatic cords with polygonal hepatocytes around the central veins were observed (Fig. 1A). These hepatic cells had vesicular nuclei in the center and eosinophilic cytoplasm (Figure 2 a, b).
Conversely, the examined liver section of the DFS-administered rats revealed severe degeneration along hepatic tissue with loss of hepatic cord organization. Most hepatocytes appear with deep-stained pyknotic nuclei and eosinophilic cytoplasm. Obvious dilatation of the central veins and sinusoids was observed. Severe hepatocellular degeneration was also recorded (Figure 2c). On the other hand, after the administration of carob with DFS, there was an improvement in hepatic histo-architecture that was evidenced by an intact central vein and its endothelial lining. All hepatic cord organizations were also restored with intact hepatocytes, except a few hepatic cells exhibited detected karyorrhexis of the nuclei. Hepatic sinusoids posed mild congestion. Mild micro-vesicular steatosis was also seen in Figure (2d).

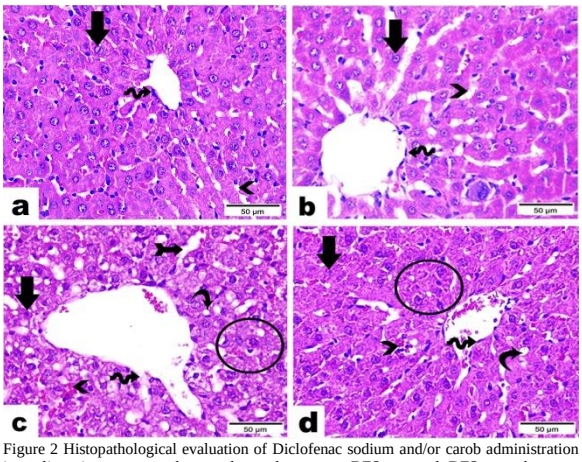

Figure 2 Histopathological evaluation of Diclofenac sodium and/or carob administration in rat liver tissue a: control group, b: carob group, c: DFS group, d: DFS + carob group. Wave arrows: hepatic tissue, arrows: hepatic cords, arrowheads: between hepatic cords and hepatic sinusoid, arrow with tail: hepatic sinusoids, curvy arrow: micro-vesicular steatosis

3.2.2. Kidney tissue
The histological examination of the control and carob group exhibited the regular structure of renal cortex tissue with intact renal corpuscles, proximal convoluted tubules, and distal convoluted tubules (Figure 3 a, b). Conversely, DFS-administrated rats showed severe degeneration along renal cortex tissue assembling as hypertrophy of renal corpuscle, cellular vacuolation along glomerulus, and deterioration along bowmen's capsule, serious interstitial congested blood capillary, as well as congestion of blood vessels. Renal tubules lost their regular structure that presented through severe tubular dilatation with flat nuclei, epithelial desquamation, and deep pyknotic nuclei with cytoplasmic vacuolation in figure (3c). In contrast, the administration of carob with DFS revealed obvious improvement in renal cortex structure with intact renal capsule but with hypertrophy. Normal and intact bowmen's capsule is observed, in addition to moderate interstitial hemorrhage. Most renal tubules lining epithelium appeared intact, except some appeared with few epithelial desquamations, and others were still seen dilated with flattening epithelial lining in figure (3d).

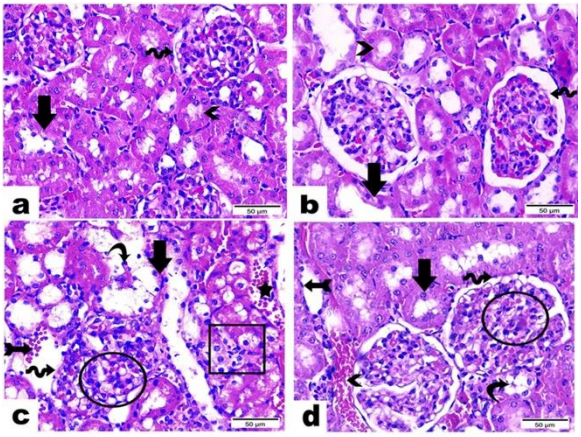

Figure 3 Histopathological evaluation of Diclofenac sodium and/or carob administration in rat kidney tissue (a: control group, b: carob group, c: DFS group, d: DFS + carob group). wave arrows: renal cortex, arrowhead: proximal convolute tubule, arrows: distal convoluted tubule, star: congestion of blood vessel, rectangle: pyknotic nuclei with cytoplasmic vacuolation.

4. DISCUSSION

Non-steroidal anti-inflammatory drugs, like diclofenac, have undesirable consequences on liver and kidney functions. Diclofenac sodium (DFS) is one of the therapeutic drugs that might cause toxic overdose damage to hepato-renal tissues in animals. Due to its prolonged ingestion and regular usage in the treatment of musculoskeletal problems, it may accumulate in human tissues (Owumi and Dim, 2019). The biochemical results of this study have shown that there is an obvious deterioration of hepatic function as the elevation of ALT and AST enzyme activities along with a significant decrease in albumin concentration in rats administrated DFS for one week. These results came in agreement with those of Ogbe et al. (2022), who showed that five days of diclofenac sodium I.M injection (10 mg/kg) resulted in a reduction of albumin and total protein levels, and elevation of total and direct bilirubin concentrations, and liver enzyme activities. Additionally, Mousa et al. (2020) observed in their study the hepatotoxicity induced after oral administration of diclofenac (2.5 mg/kg) to normal rats. One possible mechanism for DFS-induced hepatotoxicity is the production of reactive oxygen species (ROS), which can lead to oxidative stress (Adeyemi and Olayaki, 2018), the alteration of protein integrity, immune-mediated processes, and mitochondrial damage (Masubuchi et al., 2002). Another explanation provided by Kishida et al. (2012) and Gomaa (2018) supports the same theory of cellular injury caused by the reactive metabolites of the drug. Consequently, the decrease in serum albumin could be attributed to the failure of its synthesis by the liver, which suffered from oxidative stress injury.
Regarding renal functions, both biochemical and histopathological examinations revealed a clear state of nephrotoxicity. These results came in line with the previous studies (Ahmed et al., 2017; Izak-Shirian et al., 2022; Kheadr et al., 2021), which recorded renal injury after diclofenac administration to rats. Since diclofenac is metabolized in the liver and its metabolite excreted in the kidney. The disturbed renal function could be explained by the lipid peroxidation and cellular injury induced by the active hydroxy metabolite of diclofenac. This explanation is supported by that of Lonappan et al. (2016). Moreover, the alterations in renal function after diclofenac administration may be attributed to the suppression of renal prostaglandin production by diclofenac. This suppression leads to a decrease in glomerular filtration rate and subsequent deterioration of fluid and electrolyte balance, pathological

lesions in renal tissue as necrosis and inflammation (Nouri and Heidarian, 2019). This was supported by the histopathological findings of the current investigation. Additionally, since the metabolism of diclofenac produces hydroxy radicals and uric acid may act as an electron donor. The elevation of serum uric acid may be explained on the basis that uric acid is required for scavenging free radicals produced by diclofenac (Komhoff et al., 1997).

On the other hand, the biochemical and histopathological findings of the current investigation revealed a remarkable improvement in liver and kidney functions after oral administration of carob ethanolic extract to rats intoxicated with diclofenac sodium. These results came in the same line with those of Abajy et al. (2022) and Martić et al. (2022), who stated that carob extract showed an effective hepatoprotective effect against cyclophosphamide and paracetamol hepatotoxicity, respectively. The recorded nephroprotective effect of carob ethanolic extract in this study came in accordance with the results of the previous study of Martić et al. (2022), who used carob extract (100 mg/kg and 200 mg/kg) against paracetamol toxicity in mice. Moreover, Abajy et al. (2022 and Atta et al. (2023) reported a nephroprotective effect of carob methanolic extract against cyclophosphamide and doxorubicin nephrotoxicity in rats, respectively.

The previously recorded hepatoprotective and nephroprotective effects of carob could be explained by the phytochemical constituents of carob extract, such as caffeic acid and chlorogenic acid, as reported by Fihri et al. (2016). Their antioxidant property boosts the internal antioxidant system and minimizes the production of reactive metabolites of diclofenac.

5. CONCLUSIONS

From the biochemical and histopathological investigations, the current study provided substantial evidence that natural antioxidants as carob may constitute promising hepatorenal protective effects against drug-induced hepatonephro-toxicity. Further studies are recommended to elucidate the possible mechanism of actions by which carob exerts its action.

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