

Carvacrol Exerts Anti-Inflammatory, Anti-Oxidative Stress and Hepatoprotective Effects Against Diclofenac-Induced Liver Injury in Male Rats

Abstract

Background: Diclofenac (DIC) is an NSAID that can cause toxic effects in animals and humans and carvacrol (CAR) is a monoterpene compound that displays effective pharmacological and biological actions. The purpose of this work was to assess the influences of CAR on DIC-induced liver injury and oxidative stress in male rats. **Methods:** The male Wistar rats were segregated into four groups. Group 1, the control group; Group 2 received DIC-only (10 mg/kg BW, p.o); Group 3, received CAR-only (10 mg/kg BW, p.o), and group 4 received DIC plus CAR. The serum levels as well as the activity of several liver-associated markers, and oxidative and anti-oxidant compounds were tested. The expression of pro-inflammatory mediators was also studied using the qRT-PCR analysis. **Results:** Our results showed that DIC treatment was associated with the elevation in the serum levels of liver-related markers together with the increase in the serum and the hepatic levels of malondialdehyde (MDA) and protein carbonyl (PC). Moreover, DIC reduced the activity of the antioxidant system in the rats and increased lymphocyte infiltration into the hepatocytes. CAR; however, protected the hepatocytes from the toxic effects of DIC by enhancing the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and Glutathione (GSH). By diminishing the expression of tumor necrosis factor (TNF)- α , CAR was also capable of preventing the inflammatory effects of DIC on liver cells. **Conclusions:** The findings of this study indicated that the administration of CAR could alleviate the noxious effects of DIC on the antioxidant defense system and liver tissue.

Keywords: Carvacrol, diclofenac, DIC-induced liver injury, oxidative stress

Introduction

The liver is vital to tissue in the body. The liver's main job is to filter the blood coming from the digestive tract, before passing it to the rest of the body. The liver also detoxifies chemicals and metabolizes drugs.^[1] As it does so, the liver secretes bile that ends up back in the intestines. The liver also makes proteins important for blood clotting and other functions. All these fundamental actions of the liver tissue are essential for homeostasis. Liver damages often occur due to their contribution to detoxification, metabolism, and excretion of drugs and their metabolites, making the liver an important target organ for drug-caused damage.^[2-4] Liver injury is an important health disorder that is created through the use of some drugs. Some therapeutic drugs, such as nonsteroidal anti-inflammatory

drugs (NSAIDs), are drugs that caused liver poisoning.^[5,6]

NSAIDs are often prescribed for relieving symptoms of headaches, sprains and strains, painful periods, flu and colds, arthritis, and other causes of long-term pain and they are among the most commonly used medications globally on a daily basis.^[7] NSAIDs are the most important toxicity-causing medications which in some cases can be fatal.^[8] Diclofenac (DIC), an NSAID, is universally consumed by many people mostly for the treatment of degenerative joint disease, pain, rheumatoid arthritis, trauma inflammation, and dysmenorrhea.^[9,10] Actually, worldwide DIC used annually has been estimated to be nearly 940 tons.^[11] Although DIC is an effective remedial medication, its adverse effects in both humans and animals are related to prostaglandin biosynthesis

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How to cite this article: Barzan M, Heydari M, Mirshekari-Jahangiri H, Firouzi H, Dastan M, Najafi M, *et al.* Carvacrol exerts anti-inflammatory, anti-oxidative stress and hepatoprotective effects against diclofenac-induced liver injury in male rats. *Int J Prev Med* 2023;14:61.

Access this article online

Website:
www.ijpvmjournal.net/www.ijpvm.ir

DOI:
10.4103/ijpvm.ijpvm_178_21

Quick Response Code:



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suppression. Specifically, DIC-mediated hepatotoxicity, nephrotoxicity, and gastrointestinal injuries are the main side effects of the drug that are mostly induced due to the activation of oxidative damage.^[12-14] Understanding that DIC causes its poisonous effects mostly through mechanisms induced by oxidative stress, clinicians and researchers have emphasized the use of antioxidants as natural agents for the treatment of DIC-induced toxicity.

Carvacrol (CAR), 2-methyl-5-isopropyl phenol, is a monoterpene compound found in essential oils of fragrant plants such as thyme, wild bergamot, oregano, and pepperwort with a distinguishing perfume of oregano.^[15] CAR has been broadly used as a food additive for many years. Many studies have demonstrated that CAR displays effective pharmacological and biological actions such as; anticancer, antibacterial, anti-inflammatory, antifungal, antioxidant, spasmolytic, vasorelaxant, and hepatoprotective, both *in vivo* and *in vitro*.^[15] It has been shown that CAR suppressed lipopolysaccharide (LPS)-induced COX-2 mRNA and protein expression in differentiated macrophage-like U937 cells and in bovine aortic endothelial cells (BAEC) activated PPAR α and γ .^[16] The production of nitric oxide (NO), a mediator of inflammation, by intact murine peritoneal macrophages stimulated by LPS was inhibited by CAR probably due to its ability to activate PPAR leading to the inhibition of NF- κ B transcription and subsequently to a decrease in the iNOS levels.^[17] Some research revealed the ameliorative effects of this agent, including ischemia/reperfusion,^[18] gentamicin,^[19] and methotrexate-induced oxidative stress.^[20] Because the use of CAR may be helpful in preventing liver damage caused by DIC, in this study, we evaluated the possible protective effect of CAR against DIC-induced liver injury and oxidative stress.

Materials and Methods

Animal care

To evaluate whether CAR could prevent DIC-induced liver injury, thirty-two 6 to 8 weeks old Wistar rats with an estimated weight of around 200 ± 200 g were purchased from Pasteur Institute (Tehran, Iran). The standard condition was provided for all the rats, including $60 \pm 10\%$ humidity, $22 \pm 2^\circ\text{C}$ environment temperature, and 12 h light/dark cycle. Rats also had access to the appropriate amount of food and water. Of note, animal care, and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH) and Ethics Committee of Iran University of Medical Sciences, Tehran, Iran (Ethical number IR. IUMS. REC. 99. 453), respectively.

Experimental design

Experimental animals were accidentally allocated to four groups (8 rats each), and 10 mg/kg CAR was administered following 10 mg/kg DIC administration by oral gavage for 14 consecutive days. The doses of DIC and CAR in this research were designated with references to formerly published studies.^[4,21,22] DIC and CAR dissolved in distilled water and 5% DMSO (v/v) respectively before administration to experimental animals daily. In the group that received both DIC and CAR, DIC was administered first and then CAR administration was performed at 1-hour intervals. **Group 1** (control group) received distilled water (0.5 mL as the solvent of DIC) and 5% DMSO solution (0.5 mL as the solvent of CAR) by gavage with an interval of 1 h to provide an equal shock in the control group as opposed to other groups. **Group 2** (DIC-alone) received DIC (10 mg/kg BW, p.o.) and 0.5 mL 5% DMSO solution (solvent of CAR) with an interval of 1 h. **Group 3** (CAR-alone) received 0.5 mL distilled water (solvent of DIC) and CAR (10 mg/kg BW, p.o.) with an interval of 1 h. **Group 4** (DIC + CAR) received DIC (10 mg/kg BW, p.o.) and CAR (10 mg/kg, p.o.) with an interval of 1 h. It has been reported that the median lethal dose of CAR in rats is 810 mg/kg of body weight when administered by oral gavage.^[23] After the drug treatment and keeping the animals fast for 12 h, rats were killed, so that their liver tissues could be separated for further biochemical analysis. Their blood samples were also collected by the cardiac puncture method.

Serum markers of liver injury

To investigate the effects of the drugs on liver-associated serum markers, first, the serum was provided from the rat's blood specimen. Then, the serum levels of Total bilirubin, ALP (alkaline phosphatase), alanine aminotransferase (ALT), aspartate aminotransferase (AST) were measured using diagnostic kits (Pars Azmoon Co, Iran), and an auto-analyzer system (BT3000, Italy).

Investigating plasma antioxidant capacity

For evaluating the effects of drugs, either alone or in combination, on the plasma antioxidant capacity, we used the ferric reducing/antioxidant power (FRAP) method according to the procedure that was explained previously.^[24]

Biochemical analyses

A slice of liver specimens was weighed and homogenized in ice-cold phosphate buffer (pH 7.2). The homogenates were centrifuged at 4500 rpm/10 min applying a cooling centrifuge at 4°C , and then the supernatants were applied to

assess the biochemical markers (MDA, GSH, CAT, SOD, GPx, and nitrite). All tests were performed in triplicate.

Liver and serum MDA were evaluated by determining the formation of thiobarbituric acid (TBA) (MERK, Germany) reactive substances.^[25] To do so, we mixed 100 μ L of the liver homogenate or serum with the equivalent amount of sodium dodecyl sulfate (SDS) (MERK, Germany) and 2.5 mL of TBA. The mixture was then heated at 95°C for 1 h and then centrifuged at 4000 rpm for 10 min. The optical density (OD) was then measured at 532 nm.

The content of glutathione (GSH) in liver tissue was quantified according to the Ellman protocol^[26] based on the use of 5,5-dithiobis-2-nitrobenzoic acid (DTNB or Ellman reagent). Briefly, 100 μ L of supernatant was diluted with 4.0 mL of Tris buffer (0.4 M, pH: 8.9), and 0.1 mL of DTNB, and the resulting sample was shaken. GSH level was assessed by a spectrophotometer at 412 nm, and the liver content of GSH was reported as μ mole/g wet tissue.

The method developed by Flohé was used to evaluate the enzymatic activity of SOD in liver tissue.^[27] The inhibition of nitro blue tetrazolium (NBT) (Sigma-Aldrich Company, St. Louis) by SOD in each sample was quantified spectrophotometrically at 560 nm. All total protein samples were measured using the Bradford protocol.^[28] Data were reported as U/mg protein.

The CAT activity was assessed using the method of Aebi.^[29] The activity of CAT was described as the level of the enzyme needed to catalyze 1 nmol of H₂O₂ (MERK, Germany) per unit. The disintegration of H₂O₂ in the presence of CAT was evaluated at 240 nm.

To evaluate the activity of GPx, we incubated the samples with NaN₃ and H₂O₂. The measurement of GSH reduction was done according to the procedure described previously.^[25]

The level of serum protein carbonyl (PC) was measured according to the method of Reznick and Packer at 360 nm using guanidine hydrochloride (6 M) and the result was displayed in nmol dinitrophenyl hydrazine (DNPH)/mg protein.^[30]

The amount of Nitrite was quantified by determining the OD at 548 nm after incubating the samples with Greiss (Sigma-Aldrich Company, St. Louis) reagent as described previously.^[25]

Evaluating the amount of TNF- α in serum

To test the amount of TNF- α in the serum of drug-treated rats, we used an ELISA-based TNF- α kit (BT-Laboratory, China).

Analyzing gene expression using real-time PCR

After extracting RNAs from the liver tissue using RNX-Plus Solution (Sina Clon, Iran), and evaluating the quality of RNAs using Nanodrop2000 (Thermo, USA), the relevant

cDNAs were synthesized using the PrimeScript™ reagent kit (Takara Bio Inc. Japan). The prepared cDNAs were then amplified by SYBR® Green PCR Master Mix (Qiagen Co., Germany) in the presence of the primers, which were designed by Oligo 7.0 software and NCBI BLAST. Table 1 summarized the sequence of used primers in the present study. The thermal cycling condition of qRT-PCR analysis was as follows; denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 25 s. The alteration in gene expression was measured according to the 2^{- $\Delta\Delta$ ct} formula.^[31]

Histopathological study

For histological examinations, sections of liver tissue were fixed in 10% formalin for one week at 4°C. Tissues were removed from formalin after 1 week and dehydration-rehydration processes were performed to fix the samples. After the tissues were placed in a paraffin block (Merck, Germany), tissue sections were prepared using a microtome device (AMR 400, Amos Scientific, Australia) and then the slides were stained with hematoxylin-eosin. Each slide was then examined by a pathologist using a light microscope (Nikon Eclipse E400 microscope with a digital camera, USA) at different magnifications

Statistical Analysis

All the experiments in the present study were performed in triplicate to obtain statistically meaningful results. Data are expressed as Mean \pm SD according to one-way ANOVA and Tukey's post hoc tests. SPSS 18 software was also used for data analysis. A probability level of $P < 0.05$ was considered statistically significant.

Results

Effect of CAR on serum biochemical parameters

We found that DIC-only treatment for 14 days increased the serum levels of total bilirubin, ALP, ALT, and AST levels in DIC-alone exposed animals ($P < 0.05$) compared to the control animals [Table 2]; suggestive of the probable adverse effect of the drug in liver function. However, it seems that CAR could remarkably prevent DIC-induced liver toxicity, as the serum levels of aforesaid parameters was significantly diminished, when rats were exposed to CAR post-DIC treatment.

Effect of CAR on plasma antioxidant capacity, nitrite content, and MDA levels

As presented in Table 3, our data showed that the plasma antioxidant capacity was significantly decreased in rats, which were treated with DIC for 14 days, as compared to the control group ($P < 0.05$). In agreement with this finding, the significant increase in serum levels of nitrite, MDA, and liver MDA also suggested that DIC could remarkably induce liver injury, at least partly, through inducing oxidative stress. On the other hand, we found that when rats were

Table 1: Nucleotide sequences of primers used for real-time RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β -actin	CTTCTACAATGAGCTGCGTGTGGCC	GGAGCAATGATCTTGATCTTCATGG
TNF- α	CTGGCGTGTTCATCCGTTCC	GGCTCTGAGGAGTAGACGATAA

Table 2: Effect of carvacrol on some serum biochemical parameters in the experimental groups

Parameters	Group 1	Group 2	Group 3	Group 4
ALT (U/L)	72.4 \pm 8.3	129.5 \pm 4.6 ^a	72.1 \pm 7.6 ^b	73.5 \pm 6.9 ^b
AST (U/L)	134.7 \pm 8	282.5 \pm 25.3 ^a	136.9 \pm 6.9 ^b	137.9 \pm 4.1 ^b
ALP (U/L)	185.9 \pm 17.5	449.7 \pm 47.9 ^a	191.6 \pm 21.3 ^b	193.4 \pm 13.8 ^b
Total Bilirubin (mg/dl)	0.86 \pm 0.06	2.27 \pm 0.71 ^a	0.84 \pm 0.11 ^b	0.89 \pm 0.08 ^b

Data are expressed as mean \pm SD ($n=8$) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control; group 2: diclofenac only; group 3: carvacrol only and group 4 were treated by diclofenac plus carvacrol. ^a $P<0.05$ versus control group (Group 1). ^b $P<0.05$ versus diclofenac-only administered group (Group 2)

Table 3: Effect of carvacrol on ferric reducing/antioxidant power (FRAP), Nitrite content and malondialdehyde (MDA) levels in the experimental groups

Parameters	Group 1	Group 2	Group 3	Group 4
Serum FRAP (μ M)	609.1 \pm 99.2	389.2 \pm 40.6 ^a	644.2 \pm 122.5 ^b	601 \pm 67.5 ^b
Nitrite content (μ M/mg tissue)	7.01 \pm 0.36	14.09 \pm 1.27 ^a	6.86 \pm 0.54 ^b	6.91 \pm 0.32 ^b
Serum MDA (nmol/L)	1.03 \pm 0.16	5.95 \pm 0.91 ^a	1.05 \pm 0.19 ^b	1.28 \pm 0.27 ^b
Liver MDA (nmol/mg protein)	1.64 \pm 0.24	3.90 \pm 0.33 ^a	1.54 \pm 0.25 ^b	1.66 \pm 0.25 ^b
Protein carbonyl (nmol NADPH/mg protein)	4.94 \pm 0.86	12.22 \pm 0.85 ^a	5.06 \pm 0.91 ^b	5.86 \pm 0.92 ^b

Data are expressed as mean \pm SD ($n=8$) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control; group 2: diclofenac only; group 3: carvacrol only and group 4 were treated by diclofenac plus carvacrol. ^a $P<0.05$ versus control group (Group 1). ^b $P<0.05$ versus diclofenac-only administered group (Group 2)

exposed to CAR after DIC, not only there was a significant elevation in plasma antioxidant capacity ($P < 0.05$) but also the serum levels of nitrite content, MDA, and liver MDA robustly declined as compared to the DIC-alone treated group ($P < 0.05$) [Table 3].

Effect of carvacrol on serum protein carbonyl

The amount of serum PC elevated substantially ($P < 0.05$) in the animals exposed to DIC-only when compared with the control group [Table 3]. However, the amount of serum PC in animals treated with CAR after exposure to DIC considerably diminished ($P < 0.05$) relative to the animals exposed to DIC alone. There was no considerable change between animals administrated with CAR after exposure to DIC and control animals.

Effect of carvacrol on CAT, SOD, GPx activities, and GSH level

Rats that were treated with a single agent of DIC showed a significant decrease in liver CAT and SOD activities, as compared to the control group ($P < 0.05$) [Table 4]. However, the results obtained in the DIC-plus-CAR group were completely different, as CAR could remarkably elevate liver SOD and CAT activities in rats, as compared to the DIC-alone group ($P < 0.05$) [Table 4]. To confirm the results, we also evaluated the effects of CAR and DIC, either alone or in combination, on liver GPx activity. We found that while DIC reduced the intracellular amount of GSH

in the liver tissue ($P < 0.05$), the administration of CAR at the concentration of 10 mg/kg after DIC exposure could remarkably ameliorate the anti-oxidant condition of liver tissue by elevating the level of GSH ($P < 0.05$) [Table 4].

Effect of carvacrol on serum level and gene expression of TNF- α

Having established the effects of CAR and DIC on the anti-oxidant activity of liver tissue in rats, it was of particular interest to evaluate the impacts of both drugs on the serum level as well as the expression of TNF- α . DIC not only remarkably increased the expression level of TNF- α but also led to an increase in the serum level of this inflammatory cytokine in rats [Figure 1] ($P < 0.01$). Interestingly, when we treated rats with CAR after DIC exposure we found that both the expression level and the serum level of TNF- α significantly reduced as compared to DIC-treated rats [Figure 1] ($P < 0.01$). It should be noted that CAR was able to diminish the serum level of TNF- α in rats after DIC exposure to the same level that was observed in the control group.

Histopathological findings

We also evaluated whether DIC and CAR, either as a single agent or in combination, could change the morphology of the liver in rats. As presented in Figure 2, while both the control group and CAR-only treated groups showed to have a normal morphology of the liver, the liver morphology of DIC-only

Table 4: Effect of carvacrol on catalase (CAT) activity, superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity and Intracellular glutathione (GSH) level in the experimental groups

Parameters	Group 1	Group 2	Group 3	Group 4
CAT (U/mg protein)	178.4±12.1	53.6±6.9 ^a	179.7±14.5 ^b	179.7±11.2 ^b
SOD (U/mg protein)	33.45±3.3	14.3±1.2 ^a	33.9±3.4 ^b	34.9±2.4 ^b
GPx (U/mg protein)	25.8±2.6	14.8±1.1 ^a	26.4±2.2 ^b	26.4±1.1 ^b
GSH (μmol/g tissue)	13.4±1.7	5.8±0.5 ^a	13.3±1.4 ^b	14.1±1.3 ^b

Data are expressed as mean±SD ($n=8$) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control; group 2: diclofenac-only; group 3: carvacrol-only and group 4 were treated by diclofenac plus carvacrol. ^a $P<0.05$ versus control group (Group 1). ^b $P<0.05$ versus diclofenac-only administered group (Group 2)

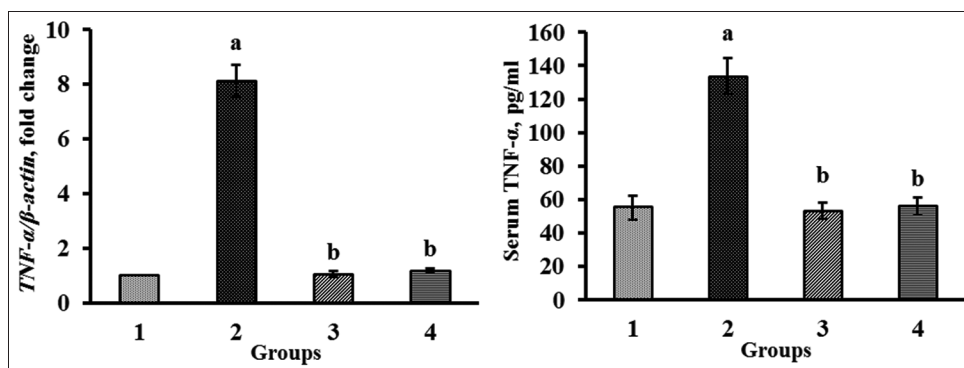


Figure 1: Effect of carvacrol on serum tumor necrosis factor- α (TNF- α) and expression of TNF- α . Each value represents the mean \pm SD of eight rats. Group 1: control; group 2: diclofenac-only; group 3: carvacrol-only and group 4 were treated by diclofenac plus carvacrol. ^a $p < 0.05$ versus control group (Group 1). ^b $p < 0.05$ versus diclofenac-only administered group (Group 2)

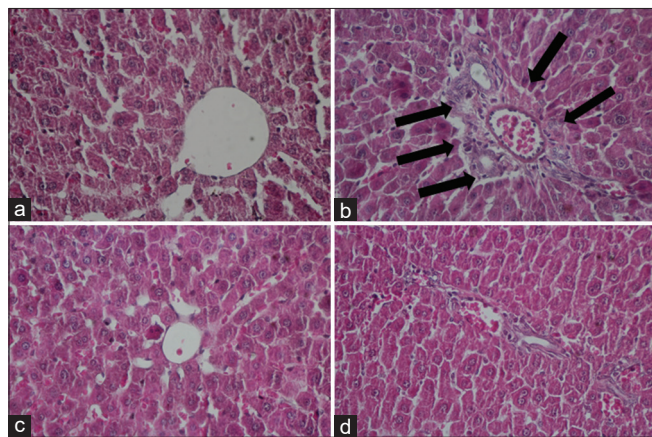


Figure 2: Effects of carvacrol on the liver histology of experimental groups. (a) Control group with normal structure; (b) diclofenac-only administered rats show lymphocyte infiltration (the black arrows); (c) carvacrol-only administered rats; (d) diclofenac-administered rats supplemented with carvacrol

treated rats showed the infiltration of lymphocyte cells in the tissue [Figure 2a, b, and c]. Of note, the administration of CAR to rats after DIC exposure significantly eliminated the population of lymphocytes and degenerated cells in the liver tissue [Figure 2d]; indicative of the ameliorative impacts of CAR on the devastating effects of DIC in the rats.

Discussion

The current research discloses novel evidence regarding the protective effects of CAR on DIC-induced hepatotoxicity.

The significant increase observed in the levels of ALT, AST, ALP, and total bilirubin content in DIC-only treated animals in comparison with control animals confirmed the hepatotoxic potential of DIC. This is in accordance with prior studies showing an elevation in ALT, AST, ALP, and total bilirubin levels in DIC-exposed humans and experimental animals.^[32,33] The diminution levels of total bilirubin, AST, ALT, and ALP in the CAR treated group suggested that this component has protective effects against DIC-induced hepatotoxicity. These findings are sustained by the prior findings, which have indicated that CAR attenuates selected drugs and chemical-induced hepatotoxicity.^[34-36]

The amount of malondialdehyde (MDA) is an indicator of lipid peroxidation (LPO), which shows the activation of oxidative stress within the cells.^[37] In this study, the amount of MDA was considerably augmented in the DIC-treated rats, which was in accordance with the results of the earlier research.^[38,39] Furthermore, treatment with CAR after exposure to DIC not only caused a rise in FRAP level but also caused a decline in MDA content in the liver tissues and sera. The protective impact of CAR on the liver tissue could be attributed to the ability of the agent in neutralizing free radicals. The same results were obtained when we evaluated the effects of both CAR and DIC on the nitrite level of rats and we found that DIC could elevate the level of nitrite in rats. This finding was in total agreement with the previous results that disclosed that NO plays a vital role in DIC-induced damage.^[14,40] Substantial diminution of

nitrite level in CAR-administrated rats revealed that CAR presented hepatoprotection in rats administrated with DIC by lowering NO content and accordingly nitrosative stress.

The enzymes of SOD and CAT are substantial factors in the system of antioxidant defense. Two superoxide radicals ($O_2^{\bullet-}$) combined with SOD produce Hydrogen peroxide (H_2O_2). Finally, hydrogen peroxide in peroxisomes is converted by CAT to H_2O and oxygen molecules.^[41] Several surveys have disclosed that DIC diminishes the activity of antioxidant enzymes in the liver tissue.^[42,43] On the other hand, there is a compelling number of studies indicating that CAR has the ability to reinforce the activity of enzymes that are responsible for regulating anti-oxidant responses.^[21,44] The results of the present study also showed that while DIC decreased the activity of SOD and CAT in rats and thereby produced oxidative stress, this was CAR that could evolve an anti-oxidative defense against DIC-induced hepatotoxicity through elevating the activity of SOD and CAT.

Additionally, the inordinate creation of free radicals as a result of DIC administration was associated with the elevated protein-oxidation reaction, which in turn led to the augmentation of PC contents, indicating that protein oxidation could be one of the mechanisms that participated in DIC-induced liver injury. These observations are in accordance with earlier research.^[14,45] In this study, the administration of CAR decreased DIC-mediated oxidative stress in the liver tissue by reducing the amount of PC. It should be noted that previous studies declared that CAR serves as a ROS scavenger and is capable to stabilize membrane structures. The main mechanism(s) for the reduction of DIC-induced liver damage can be attributed to the antioxidant properties of CAR, which is responsible for scavenging reactive

GSH, an endogenous antioxidant, plays a chief function in protecting cells from oxidative stress-induced tissue injury. Thus far, several models have determined that DIC could induce oxidative stress by altering the intracellular level of GSH within the cells.^[46,47] In agreement, we also found that DIC-induced liver damage results in a considerable augmentation in the amount of GSH in liver tissues relative to the control animals. In contrast, a considerable restoration of GSH content was discovered in CAR administrated animals. The restoration of GSH content caused by CAR could be due to either the ability of the drug to augmentation of GSH level or due to the protective effect of the drug on oxidative stress.

To investigate the role of CAR on GSH metabolism, we tested the effect of the drug on GPx, an enzyme that deactivates peroxides by converting GSH to oxidized glutathione (GSSG). DIC-caused differences in the activity of GPx have been explained formerly in the liver and renal tissues.^[42,46] In the current research, DIC-caused diminution of GPx activity probably can be because of the defense

mechanism against diminution LPO and considerable elevation in GPx activity in the treated animals with CAR can be due to the diminution level of LPO or oxidative stress.

Several surveys have also revealed that DIC motivates the movement of monocytes and macrophages, which results in the production of diverse pro-inflammatory cytokines, including $IL-1\beta$, $NF-\kappa\beta$, and $TNF-\alpha$.^[14,42] Some reports also disclosed that $TNF-\alpha$ plays an important role in the formation of inflammatory responses, especially in liver tissue.^[48,49] The data of the present investigation confirmed that exposure to DIC considerably elevated the $TNF-\alpha$ gene expression in the liver tissue. Conversely, administration of CAR after exposure to DIC considerably diminished $TNF-\alpha$ gene expression. The findings of the present research indicated that CAR could improve the liver damage induced by DIC by suppressing the inflammatory response. When tissue injury takes place, leucocytes rapidly vamoose to locations of injury and start an inflammatory response. Consequently, leucocyte infiltration was considered an indication of an inflammatory response.^[50,51] As previously shown in the histological study, leucocyte infiltration was considerably raised in the liver tissue of DIC-administrated animals. Nevertheless, in this research administration of CAR noticeably diminished leucocyte infiltration in the liver of the animals receiving DIC. Accordingly, histological examinations revealed that CAR could diminish the DIC-caused inflammatory response in the liver tissues. The main mechanism(s) for the reduction of DIC-induced liver inflammation can be attributed to the antioxidant and anti-inflammatory properties of CAR, which is responsible for scavenging ROS.

Conclusions

Our data revealed that administration of CAR to rats that were previously exposed to DIC could remarkably ameliorate the devastating impact of DIC on liver function, as this agent significantly increased the levels of CAT, GSH, GPx, and SOD, while it reduced the serum levels of total bilirubin, ALT, AST, ALP, PC, and MDA. Apart from the restoration of anti-oxidant compounds within the serum of rats, CAR was also shown to have the ability to reduce DIC-induced elevated levels of $TNF-\alpha$. This finding adds another dimension to the hepatoprotective property of CAR, this time from the perspective of an anti-inflammatory agent. Overall, our findings suggested that CAR could be a good candidate to be administrated alongside DIC to prevent its unfavorable hepatotoxicity side effects.

Acknowledgments

The authors would like to express their gratitude to those who have helped in the Biochemistry Department of Iran University of Medical Sciences.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Received: 03 May 21 **Accepted:** 27 Oct 22

Published: 27 May 23

References

- Shitara Y, Sato H, Sugiyama Y. Evaluation of drug-drug interaction in the hepatobiliary and renal transport of drugs. *Annu Rev Pharmacol Toxicol* 2005;45:689-723.
- Abdel-Daim MM, Abdeen A. Protective effects of rosuvastatin and vitamin E against fipronil-mediated oxidative damage and apoptosis in rat liver and kidney. *Food Chem Toxicol* 2018;114:69-77.
- Nair SS, Manalil JJ, Ramavarma SK, Suseela IM, Thekkepatt A, Raghavamenon AC. Virgin coconut oil supplementation ameliorates cyclophosphamide-induced systemic toxicity in mice. *Hum Exp Toxicol* 2016;35:205-12.
- Alabi QK, Akomolafe RO. Kolaviron diminishes diclofenac-induced liver and kidney toxicity in wistar rats via suppressing inflammatory events, upregulating antioxidant defenses, and improving hematological indices. *Dose Response* 2020;18:1559325819899256.
- Harirforoosh S, West KO, Murrell DE, Denham JW, Panus PC, Hanley GA. Examination of the pharmacodynamics and pharmacokinetics of a diclofenac poly (lactic-co-glycolic) acid nanoparticle formulation in the rat. *Eur Rev Med Pharmacol Sci* 2016;20:5021-31.
- Tomic Z, Milijasevic B, Sabo A, Dusan L, Jakovljevic V, Mikov M, *et al.* Diclofenac and ketoprofen liver toxicity in rat. *Eur J Drug Metab Pharmacokinet* 2008;33:253-60.
- Maity P, Bindu S, Choubey V, Alam A, Mitra K, Goyal M, *et al.* Lansoprazole protects and heals gastric mucosa from non-steroidal anti-inflammatory drug (NSAID)-induced gastropathy by inhibiting mitochondrial as well as Fas-mediated death pathways with concurrent induction of mucosal cell renewal. *J Biol Chem* 2008;283:14391-401.
- Dhikav V, Singh S, Pande S, Chawla A, Anand KS. Non-steroidal drug-induced gastrointestinal toxicity: Mechanisms and management. *J Indian Acad Clin Med* 2003;4:315-22.
- Ahmed T, Archie SR, Faruk A, Chowdhury FA, Al Shoyaib A, Ahsan CR. Evaluation of the anti-inflammatory activities of diclofenac sodium, prednisolone and atorvastatin in combination with ascorbic acid. *AntiInflamm AntiAllergy Agents Med Chem* 2020;19:291-301.
- Aygün D, Kaplan S, Odaci E, Onger ME, Altunkaynak ME. Toxicity of non-steroidal anti-inflammatory drugs: A review of melatonin and diclofenac sodium association. *Histol Histopathol* 2012;27:417-36.
- Zhang Y, Geißen SU, Gal C. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* 2008;73:1151-61.
- Ahmed AY, Gad AM, El-Raouf OMA. Curcumin ameliorates diclofenac sodium-induced nephrotoxicity in male albino rats. *J Biochem Mol Toxicol* 2017;31:e21951. doi: 10.1002/jbt.21951.
- Simon JP, Evan Prince S. Aqueous leaves extract of *Madhuca longifolia* attenuate diclofenac-induced hepatotoxicity: Impact on oxidative stress, inflammation, and cytokines. *J Cell Biochem* 2018;119:6125-35.
- Esmailzadeh M, Heidarian E, Shaghghi M, Roshanmehr H, Najafi M, Moradi A, *et al.* Gallic acid mitigates diclofenac-induced liver toxicity by modulating oxidative stress and suppressing IL-1 β gene expression in male rats. *Pharm Biol* 2020;58:590-6.
- Suntres ZE, Coccimiglio J, Alipour M. The bioactivity and toxicological actions of carvacrol. *Crit Rev Food Sci Nutr* 2015;55:304-18.
- Hotta M, Nakata R, Katsukawa M, Hori K, Takahashi S, Inoue H. Carvacrol, a component of thyme oil, activates PPAR α and γ and suppresses COX-2 expression [S]. *J Lipid Res* 2010;51:132-9.
- Guimarães AG, Xavier MA, de Santana MT, Camargo EA, Santos CA, Brito FA, *et al.* Carvacrol attenuates mechanical hypernociception and inflammatory response. *Naunyn Schmiedebergs Arch Pharmacol* 2012;385:253-63.
- Uyanoglu M, Canbek M, Ceyhan E, Senturk H, Bayramoglu G, Gunduz O, *et al.* Preventing organ injury with carvacrol after renal ischemia/reperfusion. *J Med Plants Res* 2011;5:72-80.
- Ahmadvand H, Tavafi M, Asadollahi V, Jafaripour L, Hadipour-Moradi F, Mohammadrezaei-Khoramabadi R, *et al.* Protective effect of carvacrol on renal functional and histopathological changes in gentamicin-induced-nephrotoxicity in rats. *Zahedan J Res Med Sci* 2016;18:e6446.
- Bozkurt M, Em S, Oktayoglu P, Turku G, Yuksel H, Saryıldız MA, *et al.* Carvacrol prevents methotrexate-induced renal oxidative injury and renal damage in rats. *Clin Invest Med* 2014;37:E19-25.
- Gunes S, Ayhanci A, Sahinturk V, Altay DU, Uyar R. Carvacrol attenuates cyclophosphamide-induced oxidative stress in rat kidney. *Can J Physiol Pharmacol* 2017;95:844-9.
- Motawi TK, Ahmed SA, El-Boghdady NA, Metwally NS, Nasr NN. Impact of betanin against paracetamol and diclofenac induced hepato-renal damage in rats. *Biomarkers* 2020;25:86-93.
- Hagan EC, Hansen WH, Fitzhugh OG, Jenner PM, Jones WI, Taylor JM, *et al.* Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Food Cosmet Toxicol* 1967;5:141-57.
- Nouri A, Hashemzadeh F, Soltani A, Saghaei E, Amini-Khoei H. Progesterone exerts antidepressant-like effect in a mouse model of maternal separation stress through mitigation of neuroinflammatory response and oxidative stress. *Pharm Biol* 2020;58:64-71.
- Nouri A, Heidarian E, Amini-Khoei H, Abbaszadeh S, Basati G. Quercetin through mitigation of inflammatory response and oxidative stress exerts protective effects in rat model of diclofenac-induced liver toxicity. *J Pharm Pharmacogn Res* 2019;7:200-12.
- Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82:70-7.
- Flohe L, Otting F. Superoxide dismutase assays. *Methods Enzymol* 1984;105:93-104.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
- Aebi H. Catalase. *Methods of Enzymatic Analysis*. Elsevier; 1974. p. 673-84.
- Reznick AZ, Packer L. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods Enzymol* 1994;233:357-63.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 $^{-\Delta\Delta CT}$ method. *Methods* 2001;25:402-8.
- Adeyemi WJ, Olayaki LA. Diclofenac-induced hepatotoxicity:

- Low dose of omega-3 fatty acids have more protective effects. *Toxicol Rep* 2017;5:90-5.
33. Alabi QK, Akomolafe RO, Olukiran OS, Adeyemi WJ, Nafiu AO, Adefisayo MA, *et al.* The Garcinia kola biflavonoid kolaviron attenuates experimental hepatotoxicity induced by diclofenac. *Pathophysiology* 2017;24:281-90.
 34. Mohseni R, Karimi J, Tavilani H, Khodadadi I, Hashemnia M. Carvacrol ameliorates the progression of liver fibrosis through targeting of Hippo and TGF- β signaling pathways in carbon tetrachloride (CCl₄)-induced liver fibrosis in rats. *Immunopharmacol immunotoxicol* 2019;41:163-71.
 35. Aristatile B, Al-Numair KS, Veeramani C, Pugalendi KV. Effect of carvacrol on hepatic marker enzymes and antioxidant status in d-galactosamine-induced hepatotoxicity in rats. *Fundam Clin Pharmacol* 2009;23:757-65.
 36. Bozkurt M, Bodakci MN, Turkcü G, Kuyumcu M, Akkurt M, Sula B, *et al.* Protective effects of carvacrol against methotrexate-induced liver toxicity in rats. *Acta Chir Belg* 2014;114:404-9.
 37. Gawel S, Wardas M, Niedworok E, Wardas P. Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiad Lek* 2004;57:453-5.
 38. Heidarian E, Nouri A. Hepatoprotective effects of silymarin against diclofenac-induced liver toxicity in male rats based on biochemical parameters and histological study. *Arch Physiol Biochem* 2019;127:1-7.
 39. Ogbe RJ, Luka CD, Adoga GI. Effect of aqueous ethanol extract of *Dialium guineense* leaf on diclofenac-induced oxidative stress and hepatorenal injuries in Wistar rats *Comp Clin Path* 2019;28:241-8.
 40. Safari T, Miri S, Gharaei FK, NazriPanjaki A, Saeidienik F, Bagheri H, *et al.* Nitric oxide metabolite changes in gentamicin-induced nephrotoxicity; The effects of antioxidant vitamins. *J Renal inj Prev* 2018;7:201-5.
 41. Wei T, Tian W, Liu F, Xie G. Protective effects of exogenous β -hydroxybutyrate on paraquat toxicity in rat kidney. *Biochem Biophys Res Commun* 2014;447:666-71.
 42. Giridharan R, Lavinya U, Sabina EP. Suppressive effect of *Spirulina fusiformis* on diclofenac-induced hepato-renal injury and gastrointestinal ulcer in Wistar albino rats: A biochemical and histological approach. *Biomed Pharmacother* 2017;88:11-8.
 43. Ramezannezhad P, Nouri A, Heidarian E. Silymarin mitigates diclofenac-induced liver toxicity through inhibition of inflammation and oxidative stress in male rats. *J Herbmec Pharmacol* 2019;8:231-7.
 44. Samarghandian S, Farkhondeh T, Samini F, Borji A. Protective effects of carvacrol against oxidative stress induced by chronic stress in rat's brain, liver, and kidney. *Biochem Res Int* 2016;2016:2645237.
 45. Nouri A, Heidarian E, Nikoukar M. Effects of N-acetyl cysteine on oxidative stress and TNF- α gene expression in diclofenac-induced hepatotoxicity in rats. *Toxicol Mech Methods* 2017;27:561-7.
 46. Ahmad I, Shukla S, Kumar A, Singh BK, Kumar V, Chauhan AK, *et al.* Biochemical and molecular mechanisms of N-acetyl cysteine and silymarin-mediated protection against maneb-and paraquat-induced hepatotoxicity in rats. *Chem-Biol Interact* 2013;201:9-18.
 47. Niu X, de Graaf IA, van de Vegte D, Langelaar-Makkinje M, Sekine S, Groothuis GM. Consequences of Mrp2 deficiency for diclofenac toxicity in the rat intestine ex vivo. *Toxicol In Vitro* 2015;29:168-75.
 48. Nouri A, Heidarian E. Ameliorative effects of N-acetyl cysteine on diclofenac-induced renal injury in male rats based on serum biochemical parameters, oxidative biomarkers, and histopathological study. *J Food Biochem* 2019;43:e12950.
 49. Sharifi-Rigi A, Heidarian E, Amini SA. Protective and anti-inflammatory effects of hydroalcoholic leaf extract of *Origanum vulgare* on oxidative stress, TNF- α gene expression and liver histological changes in paraquat-induced hepatotoxicity in rats. *Arch Physiol Biochem* 2019;125:56-63.
 50. Laskin DL, Laskin JD. Role of macrophages and inflammatory mediators in chemically induced toxicity. *Toxicology* 2001;160:111-8.
 51. Pang L, Ye W, Che XM, Roessler BJ, Betz AL, Yang GY. Reduction of inflammatory response in the mouse brain with adenoviral-mediated transforming growth factor- β 1 expression. *Stroke* 2001;32:544-52.