European Review for Medical and Pharmacological Sciences 2022; 26: 8551-8566

β-caryophyllene ameliorates hepatic ischemia reperfusion-induced injury: the involvement of Keap1/Nrf2/HO 1/NQO 1 and TLR4/NF-κB/ NLRP3 signaling pathways

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Abstract. – OBJECTIVE: Hepatic ischemia-reperfusion (H I/R) injury is a frequent clinical event during which the leading contributing players are inflammation and oxidative stress responses. β-caryophyllene (BCP), a natural bicyclic sesquiterpene, is an essential oil component of different plant species and edibles. This study aims to identify whether BCP pretreatment could avert H I/R injury with inspections of the underlying mechanisms.

MATERIALS AND METHODS: Animals were devised into five groups; Sham and BCP + Sham; the animals were administered saline or BCP (200 mg/kg, orally) respectively; H I/R group, the animals were administered saline orally for 14 days before induction of H I/R; BCP100 and BCP200, the animals were administered BCP (100 and 200 mg/kg, respectively) for 14 days, followed by induction of H I/R.

RESULTS: H I/R showed markedly increased ALT, AST, MDA, and lowered antioxidant enzyme activities, while the Nrf2/HO1/NQO1 pathway components were significantly augmented. The TLR4/NF-κB/NLRP3 elements were deterred, and subsequently, escalations in the inflammatory mediators (IL-1β, IL-6, and TNF-α), adhesion molecule ICAM-1, neutrophils infiltration (MPO), and apoptotic markers were observed. Pretreatment with BCP amplified the antioxidant enzyme activities and Keap1/Nrf2/HO1/NQO1 pathway components. BCP pretreatment lowered TLR4/ NF-κB/NLRP3 pathway elements, which mitigated inflammatory mediators, ICAM-1, MPO, and apoptotic markers.

CONCLUSIONS: The protective effect of BCP against hepatic I/R induced injury might be accomplished via mitigation of oxidative stress by regulating the Keap1/Nrf2/HO1/NQO1 pathway and inhibition of the inflammatory process via manipulating the TLR4/ NF-κB/ NLRP3, reflected by inflammatory markers, neutrophils recruitment, and adhesion molecules reduction. BCP might be a potential therapeutic agent for alleviating hepatic I/R-induced injury.

Key Words:
Hepatic ischemia-reperfusion, β-caryophyllene, Inflammatory mediators, Reactive oxygen species, In-silico study.

Introduction

Hepatic ischemia-reperfusion (H I/R) injury occurs in different clinical scenarios, for instance, during hepatectomy, liver trauma surgery, and hepatic transplantation, which results in postoperative hepatic dysfunction and failure[1-3]. During the H I/R condition, reactive oxygen species (ROS) are generated, causing hepatocytes to undergo inflammation and apoptosis cascades bringing about liver cell damage[4]. As a response to hepatocytes' exposure to ROS, the liver undergoes complicated oxidative stress response status, which comprises the upregulation of defensive genes to limit hepatocytes' injury. Nuclear factor E2related factor 2 (Nrf2)/antioxidant response element (ARE) is a principle endogenous antioxidant stress pathway, which regulates more than 200 coding endogenous protective genes[1]. These defensive genes encompass antioxidant and phase II detoxification enzymes, e.g., catalase, heme oxygenase1 (HO1), superoxide dismutase (SOD), and NAD(P)H dehydrogenase quinone (NQO1 and 2)[5]. Numerous

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earlier studies reported that Nrf2/ARE pathway plays an imperative role in the protection against H I/R injury in animals[2,5,6]. Nrf2 is a cytosolic protein; it binds to its adaptor Kelch-like ECH-associated[7] protein 1 (Keap1) to produce the inactive complex; Nrf2-Keap1. Specific amino acid motifs (ETGE) from the Nrf2 protein interact with the Kelch domain of Keap1to create the complex. The Nrf2 becomes ubiquitylated and degraded by proteolysis if the complex is maintained. However, when the confirmation of Keap1 in the complex is changed due to ROS, the Nrf2 is stabilized and then detaches from the complex to enter the cell nucleus and activates the transcription of several ROS- driven genes[8].

Hepatocytes release damage-associated molecular patterns (DAMP) in their death processes, triggering inflammasomes, which further activate inflammatory responses leading to massive cell death[9]. Toll-like receptor (TLR)4/Nuclear factor (NF)-κB/NOD-like receptor protein (NLRP)3 signaling pathway is a detrimental cascade, implicated in inflammation and apoptosis concomitant with H I/R induced injury. TLR4 stimulation *via* DAMP triggers the NF-κB, which mediate the release of numerous cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, and cellular adhesion molecules[10]. Additionally, NF-κB activation is critical for the NLR family, and the NLRP3 inflammasome expression augmentation implicated in liver diseases[11], including H I/R induced injury[12]. Drugs and ischemic pretreatment approaches are frequently used in clinical practice to prevent or limit H I/R induced injury; however, research on drug pretreatment to protect against H I/Rhas not been addressed with significant breakthroughs.

β-caryophyllene (BCP) is one of the main components of the essential oils extracted from several edible plant spices, such as black pepper, rosemary, and cinnamon. Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) approved this bicyclic sesquiterpene to be utilized as a flavor enhancer and in the cosmetics[13,14]. BCP is a safe, non-toxic natural compound, with a lethal dose (LD50) greater than 5 g/kg in rodent[13]. BCP was verified to have numerous pharmacological actions, including neuroprotective[15], antioxidant[16], anti-inflammatory[17], cholesterol-lowering effect[18], cytotoxic activity against various cancer cell lines, and antimicrobial[19] activities. Furthermore, BCP attenuated doxorubicin-induced chronic cardiotoxicity[20], MSU-induced gouty arthritis and inflammation[17], MPTP induced Parkinson's disease[15,21], and hyperoxaluria-induced kidney dysfunction[22], and cerebral ischemia/ reperfusion injury[23,24]. BCP protected rat liver from carbon tetrachloride-induced fibrosis[16], D-galactosamine and lipopolysaccharide (LSP)[25], aflatoxin B1[26], ketoprofen[27] induced hepatic injuries.

The current study explored the defense effect provided by BCP pretreatment against H I/R-induced injury. Moreover, the study has proposed two mechanistic pathways, Keap1/Nrf2/HO1/NQO1 and TLR4/NF-κB/NLRP3, through which BCP might exert this protective effect against such an insult.

Materials and Methods

Animals

Wistar male rats (age: 6-8 weeks; weight: 210-230 g) were obtained from Experimental Animal Research Centre, King Saud University, Riyadh, KSA. The animals were maintained with standard laboratory food and water *ad libitum* in ventilated cages system (12 h light/dark cycles, 20.3-23.1°C) throughout the whole experiment.

Ethical Statement

The experimental protocol (KFU-REC-2022-FEB-EA000418) was permitted by the Institutional Animal Care and Use Committee of King Faisal University. All the experiments were executed in harmony with the relevant procedures and regulations of the Ethical Conduct for the Use of Animals in Research at King Faisal University.

H I/R Injury Surgery

H I/R induction was executed as described earlier[2,28], where the animals were anesthetized using isoflurane with oxygen (2%, 0.5 L/h) and placed in the supine position. A midline opening of the upper abdomen was operated to expose the hepatic portal. The branches of the left and middle portal veins and hepatic arteries were clipped using a noninvasive vascular clamp, resulting in 70% warm ischemia. The hepatic lobe color in the blocked area altered from bright red to pale white, demonstrating efficacious hepatic ischemia induction. After 60 min, the clamp was detached, allowing the blood flow to be restored and permitted reperfusion for 6 h.

Experimental Design

Animals (n=6/group) were arbitrarily allocated into five groups. In the sham group, the animals

were administered saline orally daily for 14 days before the operation. In the BCP + Sham group, the animals were given BCP (200 mg/kg) orally for 14 days before the operation. The operation included liver exposure in the sham and sham + BCP (200 mg/kg) groups without blocking blood flow. In the H I/R group, the animals were administered saline orally daily for 14 days before induction of H I/R. The fourth and the fifth groups (BCP 100 + H I/R and BCP 200 + H I/R) were administered BCP orally (100 and 200 mg/kg, respectively) for 14 days, followed by induction of H I/R. BCP dose selection was according to previously mentioned studies[16].

Samples Preparation

At the end of the reperfusion period, blood samples were harvested from the abdominal aorta of the anesthetized animals, and the sera were separated for alanine aminotransferase (ALT) aspartate aminotransferase (AST) measurement using the diagnostic kits. Additionally, animals' livers were collected and divided into four fractions. The first part was homogenized in ice-cold saline and deposited directly at -80°C to be utilized in the biochemical and ELISA assay. The second fraction was homogenized with lysis buffer to be used in the western blot analysis. In contrast, the third part was preserved in 10% formalin for 24 h for the histopathological and immunohistochemical (IHC) analysis. The last portion was mixed in RNA later solution and kept at -80°C to be used for gene expression assay via quantitative real-time polymerase chain reaction (qRT-PCR).

Histopathological and Immunohistochemistry Evaluations

After fixing the ischemic liver fractions with 10% formalin, the fractions were embedded in paraffin, cut into 5 µm thick paraffin slices, dewaxed with xylene twice, fully hydrated with ethanol, and rinsed with tap water. Ischemic hepatic sections were stained with hematoxylin & eosin (H&E). Hepatic injury was evaluated under a light microscope (magnification, ×400) by two independent pathologists blinded to the experimental groups following Suzuki grading[29]. Suzuki grading standard contains five scores (04) related to hepatocyte cytoplasm vacuolization, necrosis, and tissue congestion

For the immunohistochemistry (IHC) procedure, the expression of TLR4 and NF-κB were inspected, as revealed previously by Mohamed et al[30]. Hepatic sections were blocked using 3% hydrogen peroxide (H_2O_2) in methanol (21-25°C, 30 min) and washed with PBS three times. Then, sections were incubated with TLR4 and NF-κB antibodies (1:100, Thermo Fisher Scientific, Cambridge, U.K.) overnight at 4°C, followed by goat anti-rabbit-horseradish peroxidase (HRP) conjugated IgG antibody (1:1000; Cat. no. ab6721; Abcam, Eugene, OR, USA) for 1 hour at 37°C). Finally, the tissue sections were developed with 1% diaminobenzidine (5 min), counterstained with 1% hematoxylin for (2 min, 21-25°C), and mounted with neutral gum. A microscope built-in with a digital camera (Nikon Instruments Inc.) was used to apprehend the hepatic sections. NIS-Elements software was used for quantitative analysis. Firstly, the area of IHC reaction in the picture was selected. Secondly, the average optical density in the designated area of each photograph was assessed. Positive cells were counted under x400 magnification observing ten consecutive non-overlapping fields per animal in a blinded manner.

Assessment of Hepatic Oxidative Stress Status

Malondialdehyde (MDA; ab238537), glutathione peroxidase (GSH-Px; ab102530), and glutathione reductase (GSH-R; ab83461) ELISA kits were acquired from Abcam Inc. (Cambridge, UK). Superoxide dismutase (SOD; MBS036924) and catalase (MBS726781) ELISA kits were obtained from My BioSource (San Diego, CA, USA). All the procedures were executed in agreement with the manufacturer's directions.

In Silico Docking Study

The molecular docking of BCP on Keap1was performed on MOE software, and visualization was done by Discovery Studio visualizer (Accelrys, Inc, San Diego, CA, USA). The procedures started by downloading the 3D crystal structure of Keap1 from the protein data bank (PDB: 5FNU, https://www.rcsb.org/structure/5FNU), followed by preparing the protein structure, adding protons, removing unnecessary water molecules, and fixing missing chains. The binding site of Nrf2 was defined from the co-crystalized ligand position. A validation step was carried out to ensure the docking procedures. The 2D structure of β-Caryophyllene was drawn on ChemDraw Professional 16.0, followed by ligand preparation and energy minimization in MOE to be docked inside the Nrf2 binding site. The best ten docking poses were selected and studied to get the best orientation with the best score compared to the reference ligand.

Protein Expression Experiments (Western Blot)

The whole cytoplasmic and nuclear protein fractions were prepared from hepatic tissues, and western blot analysis was carried out as described previously[31]. Nrf2, NF-κB nucleoprotein was assessed in nuclear lysate fraction by a nuclear protein extraction kit (cat. no. ab113474; Abcam Inc., Cambridge, UK). The total protein of Keap1, HO1, NQO1, TLR4, and NLRP3 was extracted using RIPA lysis buffer. 20 µg protein samples were separated using 10% SDSPAGE and transferred to PVDF membranes (cat. no. IPVH00010; 2022 Merck KGaA, Darmstadt, Germany). Membranes were blocked with 5% skimmed milk (at room temperature for one h), then incubated with primary antibodies at four °C overnight. Membranes were washed with Tris-buffered saline containing 0.1% (v/v) Tween20 (TBST) three times and incubated with horseradishconjugated goatanti-rabbit secondary antibody (1:10,000; cat. no. SA000012; ProteinTech Group, Inc.) at room temperature for one h, then washed again. An enhanced chemiluminescence (ECL) reagent (cat. no. P0018FM, Thermo Fisher Scientific Inc. Waltham, MA USA) was utilized to visualize the bands. Bands were detected with a GeneSys System (Bio Rad Laboratories, Inc.). Band intensities were measured using Image J v1.8.0 software (National Institutes of Health NIH).

Gene Expression Experiments (Real-Time PCR)

Real-time PCR was performed according to the technique described elsewhere[25]. Briefly, the Trizol reagent kit (Invitrogen, Waltham, MA, USA) and reverse transcription-polymerase chain reaction (RT-PCR) kit (TaKaRa, (Shiga, Japan), Cat. No. RR037A) were used to cleanse total RNA and inverse transcription reaction, respectively. In total, 20 µL of the reaction volume was mixed with 1 µL total RNA (1 µg/µL), incubated at 42°C for 15 min, followed by 95°C for 2 min, and the generated cDNA was stored at -20°C. In total, 50 µL of PCR reaction mixture enclosed × 50 ROX Reference Dye (1 µL), sense and antisense primers (1 µL each, primers are mentioned in Table I), × 2 SYBR Green PCR Master Mix (25 µL), cDNA template (4 µL) and sterilized distilled H2O (18 µL). The PCR reaction condition incorporated pre-denaturing at 95°C for 10 s, then 40 cycles of 95°C/5 s and 60°C/30 s, and 72°C/1 min. Quantification analyses were completed via Opticon-2 Real-Time PCR reactor (MJ Research, Reno, NV, USA). Step PE Applied Biosystems (Perkin Elmer, Waltham, MA, USA) analyzed real-time PCR results. Expression of the target gene was measured and correlated to the reference gene (β-actin). β-actin expression was used for sample normalization, where the $2^{-\Delta\Delta CT}$ equation was used for relative expression determination.

Determination of Inflammation and Apoptotic Signaling Markers

Inflammation markers including TNF-α (ab46070), IL-1β (ab100768), IL-6 (ab100772) and IL-10 (ab133112), ICAM-1 (CD54) (ab100763) ELISA kits were obtained from Abcam Co., Eugene, OR, USA. As for the apoptotic signaling markers, cleaved caspase-3 (KHO1091) was purchased from Thermo Fisher Scientific Inc. Waltham, MA, USA, whereas caspase-9 (LS-F4141) was acquired from Biocompare, San Francisco, CA, USA. These markers were measured according to the manufacturers' instructions using a microplate reader SpectraMax i3X (Molecular devices San Jose, CA, USA).

Statistical Analysis

Data are presented as mean ± SD. For multiple comparisons, one-way ANOVA followed by Tukey-Kramer as a post-hoc test was performed. The 0.05 level of probability was used as the significance level ($p < 0.05$). All statistical analyses were performed using Graph Pad software (version 5, San Diego, CA, USA).

Results

BCP Mitigates H I/R Induced Hepatic Dysfunction

Both serum levels of ALT and AST activities and histological inspection evaluated H I/R-induced injury. ALT and AST serum levels in the H I/R group were significantly amplified, demonstrating impaired liver function (Figure 1 A-B). On the other hand, pretreatment with BCP before H I/R resulted in diminished ALT and AST levels significantly by 26.65% and 43.79% with BCP (100 mg/kg) and 21.62% and 41.44% with BCP (200 mg/kg), respectively, which indicated that BCP might exert a protective effect on hepatic function. Consistent with the liver function tests, ALT, and AST variations, liver tissue from sham exhibited normal histology architecture, while the H I/ R group exhibited several histopathological alterations. The hepatocytes displayed diffuse

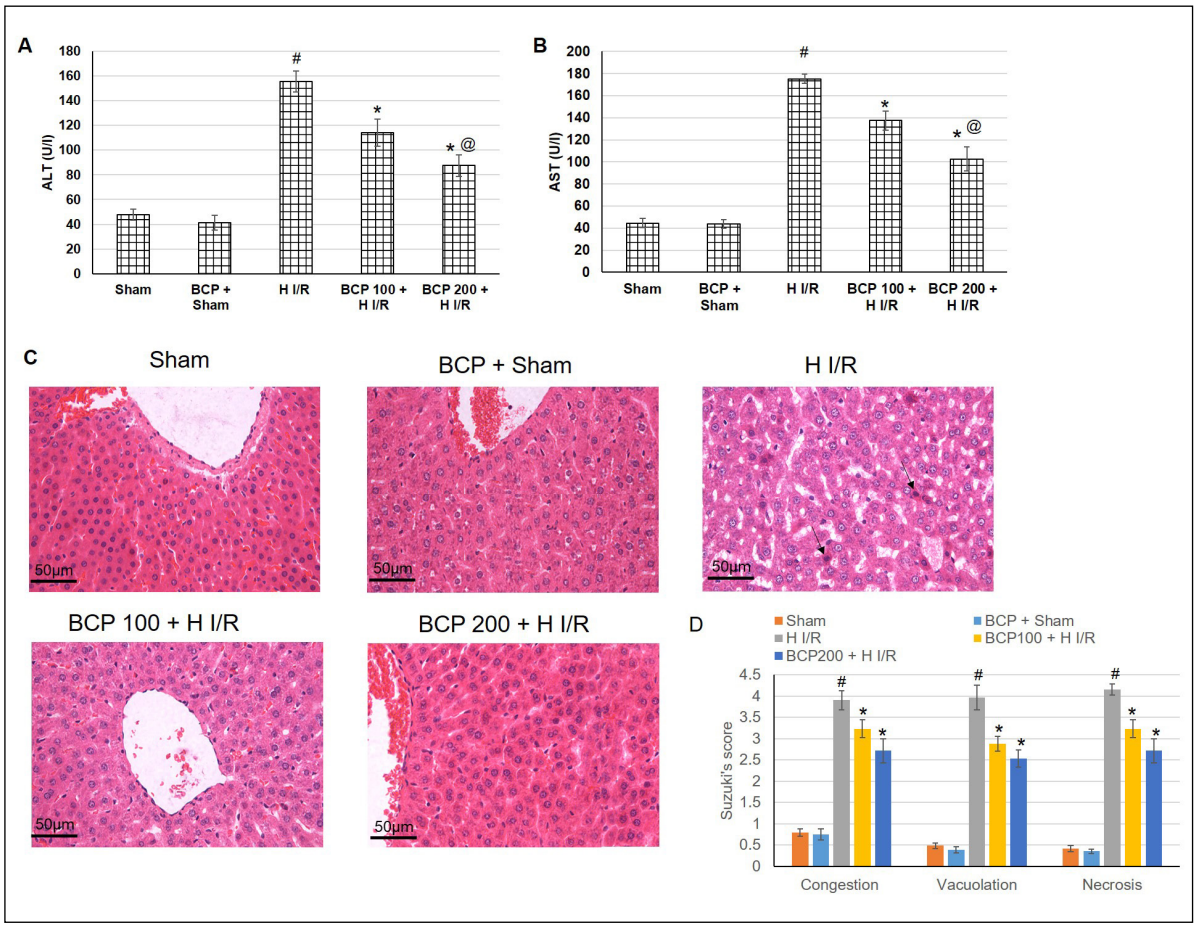

Figure 1. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days prior to hepatic ischemia/perfusion (H I/R) on the hepatic function tests, including (**A**) ALT and (**B**) AST, and on (**C**) histopathological hepatic sections stained with hematoxylin and eosin (H&E) and (**D**) Suzuki's score in H I/R-induced injury. All values are stated as mean± SD. # designates statistically significant compared to the sham group and * designates statistically significant compared to the H I/R group ($p < 0.05$) using one-way ANOVA followed by Tukey's post-hoc test.

hepatocellular necrosis with disorganized hepatic parenchyma and dilated hepatic sinusoids. BCP pretreated animals presented better hepatic parenchyma, declined focal areas of hepatocellular necrosis, slightly congested hepatic sinusoids, and portal vasculatures with mild hepatocellular vacuolation, Figure 1C.

Regarding liver damage scores, Suzuki scores, congestion, hepatocellular vacuolation, and necrosis were significantly increased in the H I/R group. At the same time, BCP pretreated animals showed a substantial decline in liver congestion and hepatocellular vacuolation and a marked reduction in the percentage of necrotic cells compared to the H I/R group. However, a significant difference was not observed between the tested two doses of BCP in the congestion, vacuolation, and necrosis, as shown in Figure 1D.

In Silico Docking Study of BCP

BCP docking was previously performed in different protein targets involved in the TLR4/NF-κB/NLRP3 pathway, especially NLRP3, TLR4, and MyD88[17]. However, the compound *in silico* docking on the Keap1/Nrf2/HO1/NQO1 was not investigated. Keap1 association with Nrf2 leads to the degradation of Nrf2. Therefore, the ability of BCP to prevent this association through association with Keap1 in the binding site of Nrf2 was investigated in the hereby study. Accordingly, BCP was docked to the Nrf2 Keap1 active site (PDB: 5FNU), taking the co-crystalized ligand as the reference structure[32]. BCP was able to block the binding site of Nrf2 on Keap1 protein with a docking score of -5.8 Kcal/mol, which is a good score compared to the co-crystalized ligand reference compound (-7.06 Kcal/mol). BCP exerted

this action by forming van der Waal interactions among the key amino acids in the active site, such as Arg415, Gly603, and pi-alkyl interaction with the Ala556, Figure 2.

BCP Mitigates Hepatic I/R-Induced Oxidative Stress

Hepatic tissue samples were used to detect ROS content to evaluate BCP actions on hepatic oxidative damage. Compared with the sham groups, hepatic I/R tissue samples showed markedly greater levels of MDA content, indicating lipid peroxidation elevation, which was down-regulated with BCP (100 and 200 mg/kg) pretreatment; Figure 3 A. In contrast to MDA, hepatic I/R tissue samples exhibited lowered levels of antioxidant enzyme activities, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-R). Whereas pretreatment with BCP amplified the activities of SOD,

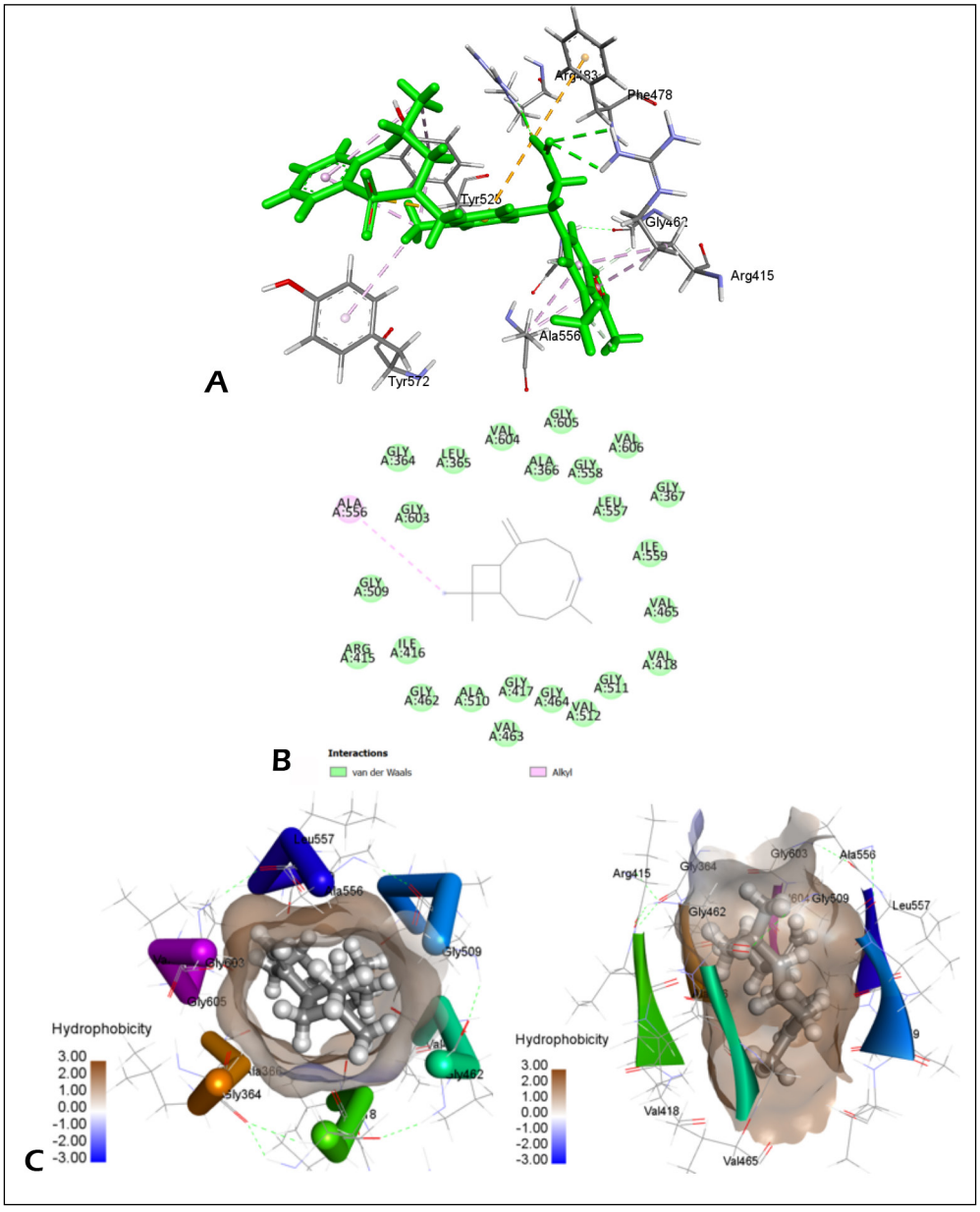

Figure 2. A, 3D interaction diagram of the reference co-crystallized ligand in the active site of Nrf2 on Keap; **B**, 2D interaction diagrams of BCP (compound under investigation) and **C,** 3D interaction diagrams of BCP with different poses blocking the same active site.

catalase, GSH-Px, and GSH-R in hepatic tissue samples when related to H I/R (Figure 3 B-E).

BCP and Nrf2/HO1/NQO1 Pathway Gene and Protein Expression

Real-time PCR and western blotting were employed to distinguish the gene and protein expression levels of the Keap1/Nrf2/HO1/NQO1 pathway. Compared to sham groups, Keap1 protein expression was significantly decreased, whereas with BCP, more reduction occurred; nevertheless, there was no substantial difference between the two doses of BCP. Related to the sham and BCP groups, both gene and protein expression levels of Nrf2 nucleoprotein and total HO1 and NQO1 were considerably augmented in the hepatic I/R, as presented in Figure 4. On the other hand, gene and protein expression levels of Nrf2 nucleoprotein and total HO1 and NQO1 were further augmented due to the pretreatment with BCP compared to H I/R animals.

BCP Intensifies TLR4/NF-κB/NLRP3 Pathway Gene and Protein Expression

As illustrated in Figure 5, the H I/R experienced animals displayed an increment in the gene expression and protein content of TLR4, which subsequently augmented NF-κB and NLRP3 compared to sham groups. Interestingly, BCP pretreatment modified these alterations via lowing the gene and protein expression levels of TLR4/NF-κB/NLRP3.

As for the IHC analysis, the results revealed that TLR4 (Figure 6 A) and NF-κB (Figure 6 B) positive cells were infrequently found in the sham group. Conversely, I/R hepatic tissues exhibited strongly TLR4 and NF-κB positive cell expression ($p < 0.05$). TLR4 and NF-κB positive cells were lowered in animal groups pretreated with BCP compared with the H I/R group ($p < 0.05$) as indicated in Figure 6 C and D. These results signifying the hepatoprotective effect of BCP could be via the inhibiting TLR 4/NF-κB/NLRP3 pathway.

BCP Mitigates Hepatic I/R Induced Neutrophils Infiltration

The adhesion molecule ICAM-1 was prompted in the H I/R group, contributing to the neutrophil infiltration as signified by MPO levels augmentation (Figure 7 A-B). However, BCP pre-administration averted the hepatic I/R induced adhesion molecule ICAM-1 and neutrophils infiltration (MPO) elevations, as demonstrated in Figure 7.

BCP Mitigates Hepatic I/R Induced Inflammation and Apoptosis Responses

Figure 7 illustrates the escalation in the inflammatory mediators, including IL-1β, IL-6, and

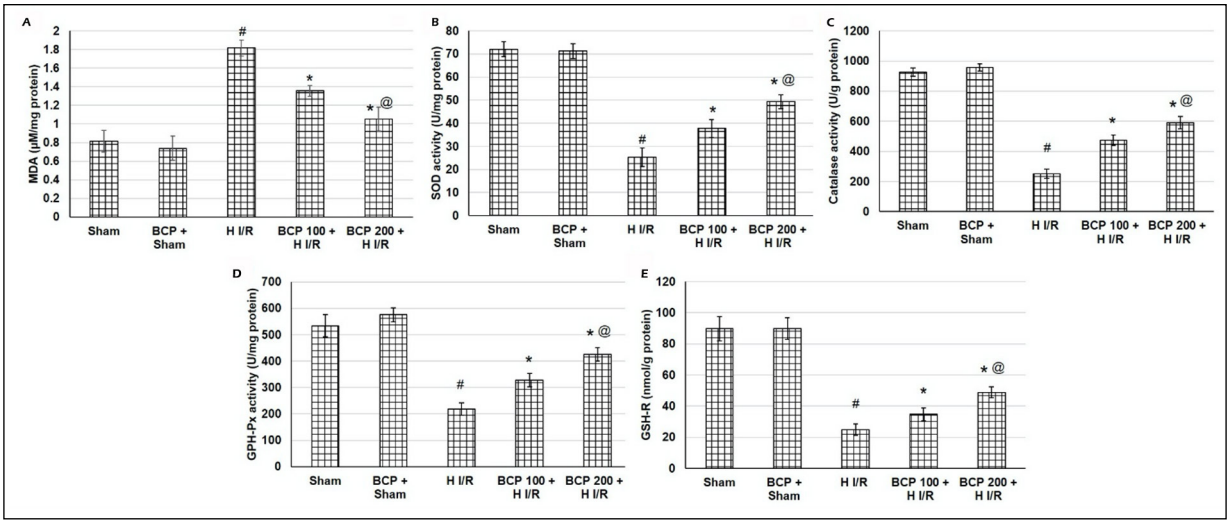

Figure 3. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days prior to hepatic ischemia/perfusion (H I/R) on lipid peroxidation, including **A,** MDA content and antioxidant enzymes activities, including **B,** superoxide dismutase (SOD), **C,** catalase, **D,** glutathione peroxidase (GSH-Px) and **E,** glutathione reductase (GSH-R) activities in H I/R-induced injury. All values are stated as mean± SD. # designates statistically significant compared to the sham group and *designates statistically significant compared to the H I/R group, @ designates statistically significant compared to BCP 100 + H I/R ($p < 0.05$) using one-way ANOVA followed by Tukey's post-hoc test.

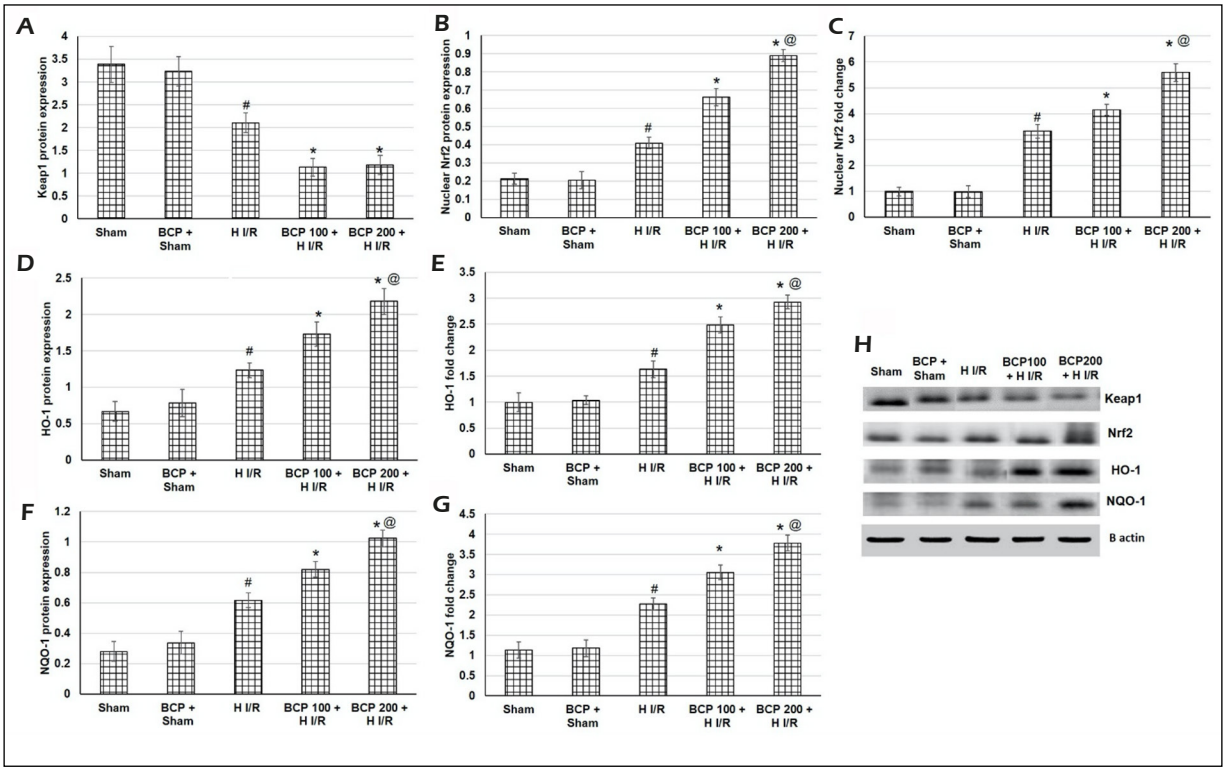

Figure 4. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days prior to hepatic ischemia/perfusion (H I/R) on (**A**) Keap1 protein expression and protein and gene (mRNA) expression levels of (**B**), (**C**) Nrf2, (**D**), (**E**) HO-1 and (**F**), (**G**) NQO-1, respectively and (**H**) Western blot images in H I/R-induced injury. All values are stated as mean± SD. # designates statistically significant compared to the sham group and *designates statistically significant compared to the H I/R group, @ designates statistically significant compared to BCP 100 + HI/R ($p<0.05$) using one-way ANOVA followed by Tukey's post-hoc test.

TNF-α, in the animals that experienced hepatic I/R when related to sham groups. These intensifications were deterred in animals treated with BCP (100 and 200mg/kg), as demonstrated in Figure 7 C-F.

As for the apoptosis response, animals experimented with hepatic I/R surgery exhibited intensified apoptotic markers, including caspase 3 and 9 levels and Bax gene expression, whereas Bcl2 gene expression was diminished, Figure 8. On the other hand, treatment with BCP resulted in apoptosis mitigation as demonstrated by the lowered caspase 3 and 9 and Bax and elevated Bcl2 confirming the anti-apoptotic effect of BCP.

Discussion

H I/R-induced damage is an intricate pathophysiological process associated with poor prognosis and organ failure in patients who undergo complex hepatic surgery and hepatic transplantation. As yet, there is no administered or even suggested drug to treat or protect against such a clinical problem. Repurposing of compounds, especially those originating from food-related or "considered safe" natural products, is a hot spot in scientific societies due to the abundance of those molecules and the broad therapeutic index guaranteeing patient safety. The hereby study demonstrated the protective effect of BCP on liver cells subjected to a status of liver injury through H I/R induction.

Our current investigation displayed that hepatic ischemia for 1 hour, followed by reperfusion for 6 hours, caused a significant elevation in ALT and AST serum levels. At the same time, a series of distinguishing pathological variations were displayed, including hepatocellular necrosis with disorganized hepatic parenchyma and dilat-

ed hepatic sinusoids, which are consistent with the outcomes of preceding reports[2,28,33]. Whereas treatment with BCP considerably lowered transaminase levels and mitigated hepatic histopathological fluctuations following H I/R injury. BCP showed defensive actions in other hepatic models, including in a carbon-tetrachloride-induced liver fibrosis[16], ketoprofen[27], D-galactosamine, LPS[25], and aflatoxin B1 induced liver toxicities[26]. However, the prior studies have not described the effect of BCP on H I/R induced injury, making this study the first concerning the shielding actions of BCP against the H I/R injury model.

BCP exhibited good *in silico* interaction with the TLR4/NF-κB/NLRP3 pathway proteins, especially NLRP3, TLR4, and MyD88, as demonstrated in previous studies[17]. However, the Keap1/Nrf2/HO1/NQO1 pathway was not investigated before, maybe because most of the proteins belonging to the pathway are of an agonist nature, and any ligand interaction would diminish their antioxidant activity. However, this is not the case with one component of the pathway, Keap1, in which the ligand interaction may lead to further activation of the whole pathway by freeing more of the Nrf2 protein and reducing the degradation of the Keap1-Nrf2 complex[34,35]. Nrf2 interacts with Keap1 in a specific active binding site on Keap1, forming a complex which directly undergoes degradation by proteolytic enzymes. Therefore, the Nrf2 active binding site on Keap1 could be a potential target for antioxidant drugs. It would increase the concentration of free, undegraded Nrf2, increasing gene expression of many antioxidant portions[8,36]. In the herewith study, the affinity of BCP to the Nrf2 active binding site on Keap1 was predicted through *in silico* docking, and the compound exhibited good affinity in relation to the reference inhibitor. These results indicate that PCB could potentially activate the Keap1/Nrf2/HO1/NQO1 pathway by increasing the concentration of the free cytosolic (therefore,

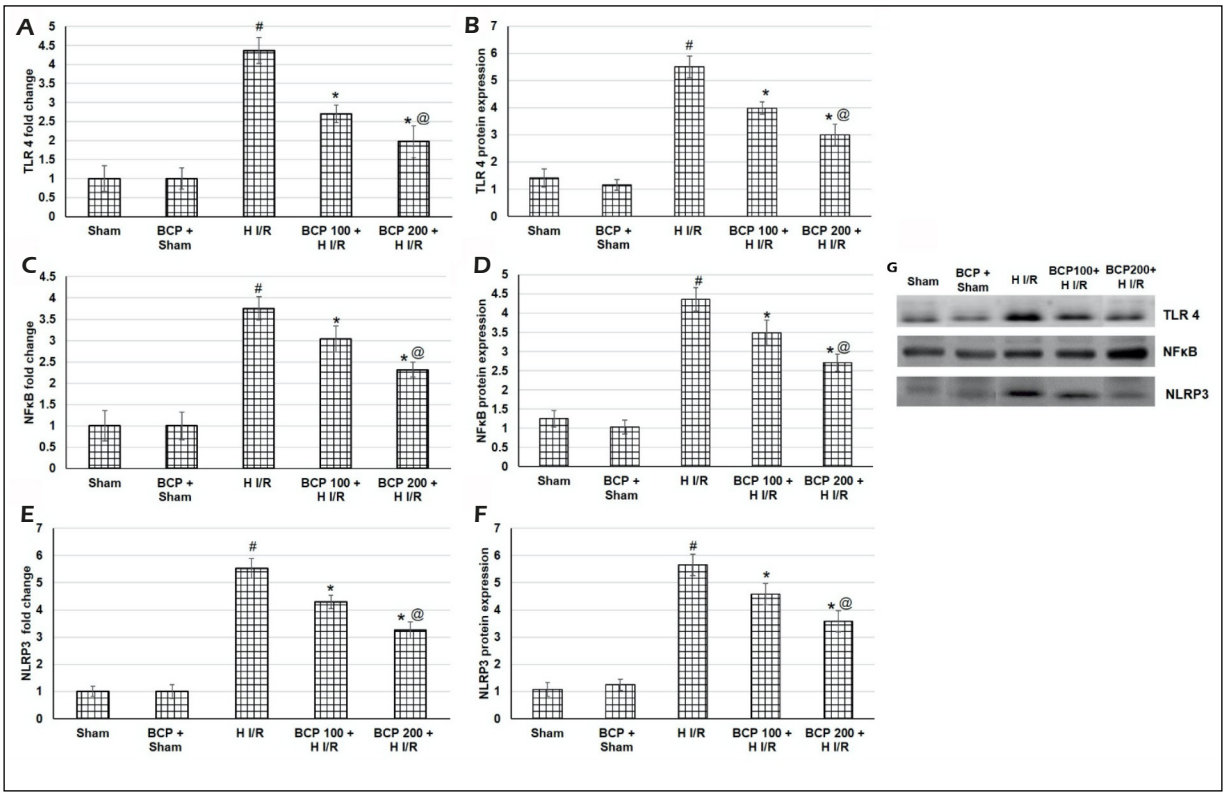

Figure 5. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days prior to hepatic ischemia/perfusion (H I/R) on gene (mRNA) and protein expression levels of (**A**), (**B**) TLR4, (**C**), (**D**) NF-κB (**E**), (**F**) NLRP3 respectively and (**G**) Western blot images in H I/R-induced injury. All values are stated as mean± SD. # designates statistically significant compared to the sham group and *designates statistically significant compared to the H I/R group, @ designates statistically significant compared to BCP 100 + H I/R ($p < 0.05$) using one-way ANOVA followed by Tukey's post-hoc test.

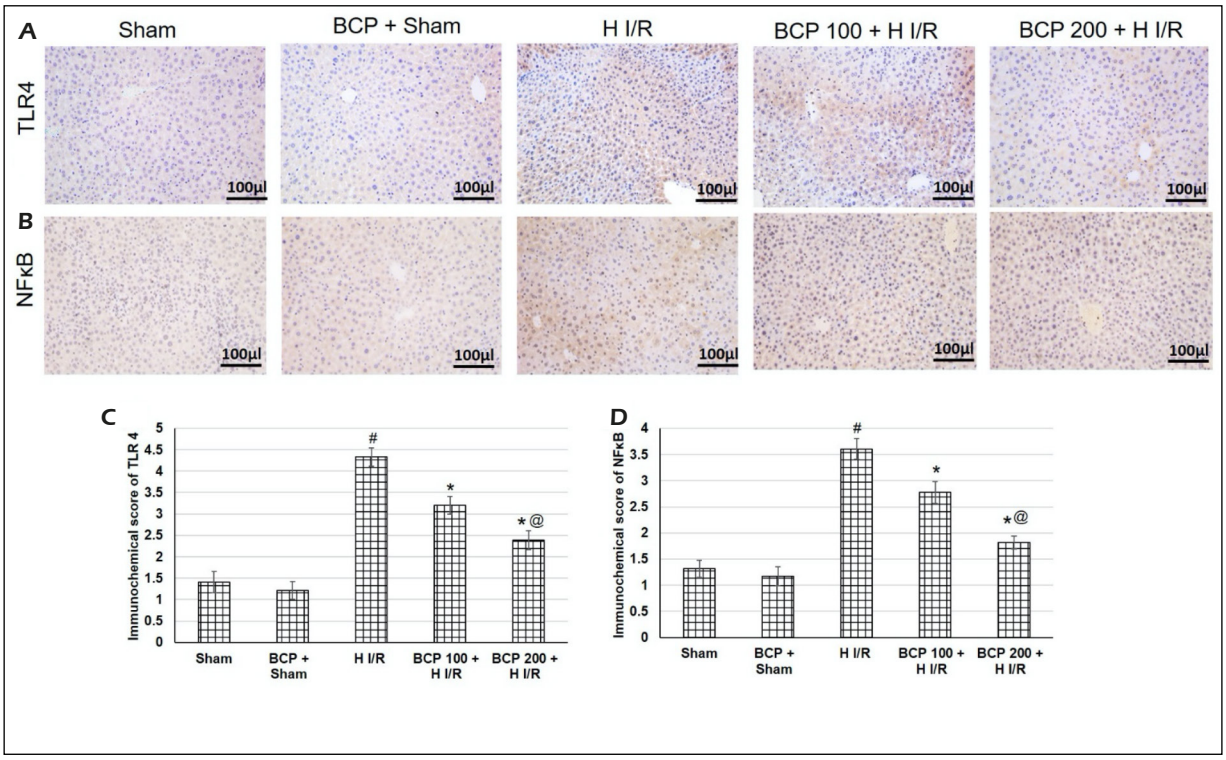

Figure 6. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days before hepatic ischemia/perfusion (H I/R) on the hepatic immunohistochemical assay of (**A**) TLR4 (**B**), NF-κB, (**C**) TLR4 scoring, and (**D**) NF-κB scoring in H I/R-induced injury. All values are stated as mean± SD. #designates statistically significant compared to the sham group and *designates statistically significant compared to the H I/R group, @designates statistically significant compared to BCP 100 + H I/R ($p < 0.05$) using one-way ANOVA followed by Tukey's post hoc test.

nucleus Nrf2). This approach of *in silico* investigation of the Keap1/Nrf2/HO1/NQO1 pathway was used in a previous study by Dare et al[37] to investigate the antioxidant machinery of L-ergothioneine type-2 diabetic rats. The approach to exploring the Keap1-Nef2 complex inhibitors is more and more emerging as a potential approach to study the pathway and thus the antioxidant capacity for many small molecules[36].

Multiple influences are elaborated in the manifestation of hepatic I/R-induced injury. One cornerstone is the excessive oxygen free radical generation with subsequent inflammatory response and hepatocyte apoptosis[5,38]. Extreme ROS concentrations are generated during the H I/R process beyond the hepatic tissue's antioxidant elimination capability, thus damaging the lipids and protein structures, and leading to the death of hepatocytes. MDA is the most crucial metabolite of membrane lipid peroxidation, which can aggravate cell membrane damage and alter the activity of critical enzymes in the mitochondria. Meanwhile, SOD is a vitally important member of the antioxidant metalloenzymes[28]. The present study revealed that hepatic MDA was significantly increased. In contrast, SOD, catalase, GSH-Px, and GSH-R levels were considerably diminished in the H I/R group compared with the sham group. Hepatic I/R-induced injury may be related to the development of lipid peroxides by a combination of extreme ROS and lipids within the hepatocytes' cell membrane, triggering the destruction of the hepatocyte membrane. The lipid peroxides end product is MDA, which imitates the cell peroxidation degree. Similarly, earlier studies[4,28,38] reported the high oxidative stress and lipid peroxidation accompanying H I/R induced injury. Compared with the H I/R group, pretreatment with BCP depressed MDA levels while increasing the levels of SOD, catalase, GSH-Px, and GSH-R, signifying the efficacious antioxidant activity of BCP in the H I/R injury condition. BCP has been revealed to have a powerful antioxidant capability in different animal models, e.g., MPTP model

of Parkinson's disease[21], hyperoxaluria-induced kidney dysfunction[22], doxorubicin-induced acute cardiotoxicity[20], and hypercholesterolemic[39] animal models.

Plentiful endogenous antioxidant defense genes, such as SOD and phase II detoxification enzymes, including HO1, are dominated via the Keap1/Nrf2/HO1/NQO1 pathway[34]. Previous studies[2,5,6] demonstrated that during H I/R, an escalation in Nrf2 nucleoprotein and subsequent HO1 and NQO1, signifying that Keap1/Nrf2/HO1/NQO1 pathway plays an imperative role in the defense against H I/R induced injury. In the current study, pretreatment with BCP further augmented Nrf2 nucleoprotein expression levels with subsequent elevation in downstream antioxidative enzymes, such as HO1 and NQO1. These outcomes indicated that BCP stimulated Nrf2 nuclear translocation, amplifying the hepatic tissue's antioxidant capability. These results are in accordance with those of the *in silico* part of this study and matched with previous studies, which verified that BCP activated the Keap1/Nrf2/HO1/NQO1 pathway in C57BL/6 mice model of obesity-associated airway hyperresponsiveness (AHR)[40], focal cerebral ischemia-reperfusion (I-R) injury[24], LPS -induced oligodendrocyte toxicity[41],

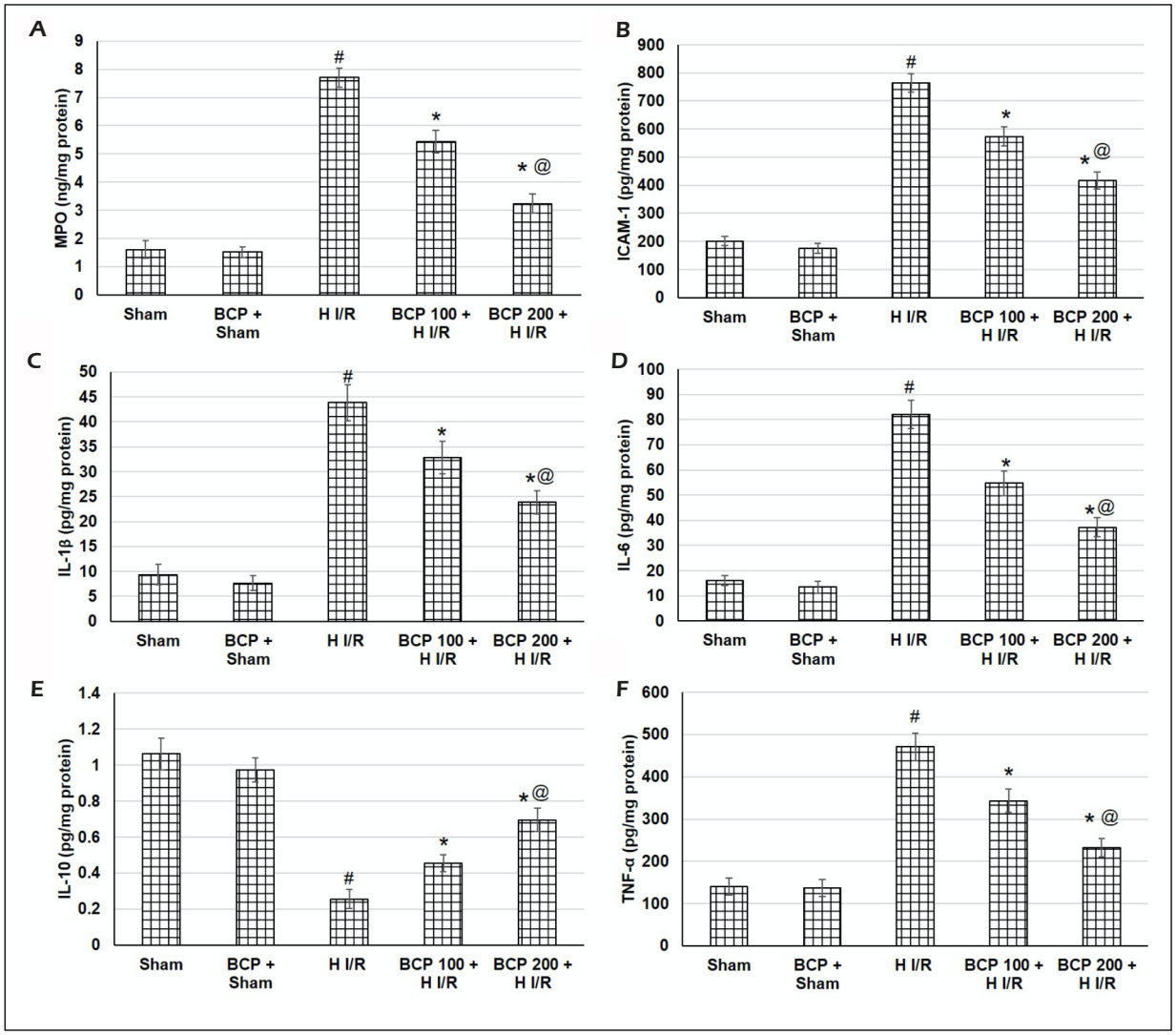

Figure 7. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days before hepatic ischemia/perfusion (H I/R) on the levels of (**A**) MPO, (**B**) ICAM-1, (**C**) IL-1β, (**D**) IL-6, (**E**) IL-10, (**F**) TNF-α in H I/R-induced injury. All values are stated as mean± SD. # designates statistically significant compared to the sham group and * designates statistically significant compared to the H I/R group, @ designates statistically significant compared to BCP 100 + H I/R ($p < 0.05$) using one-way ANOVA followed by Tukey's post hoc test.

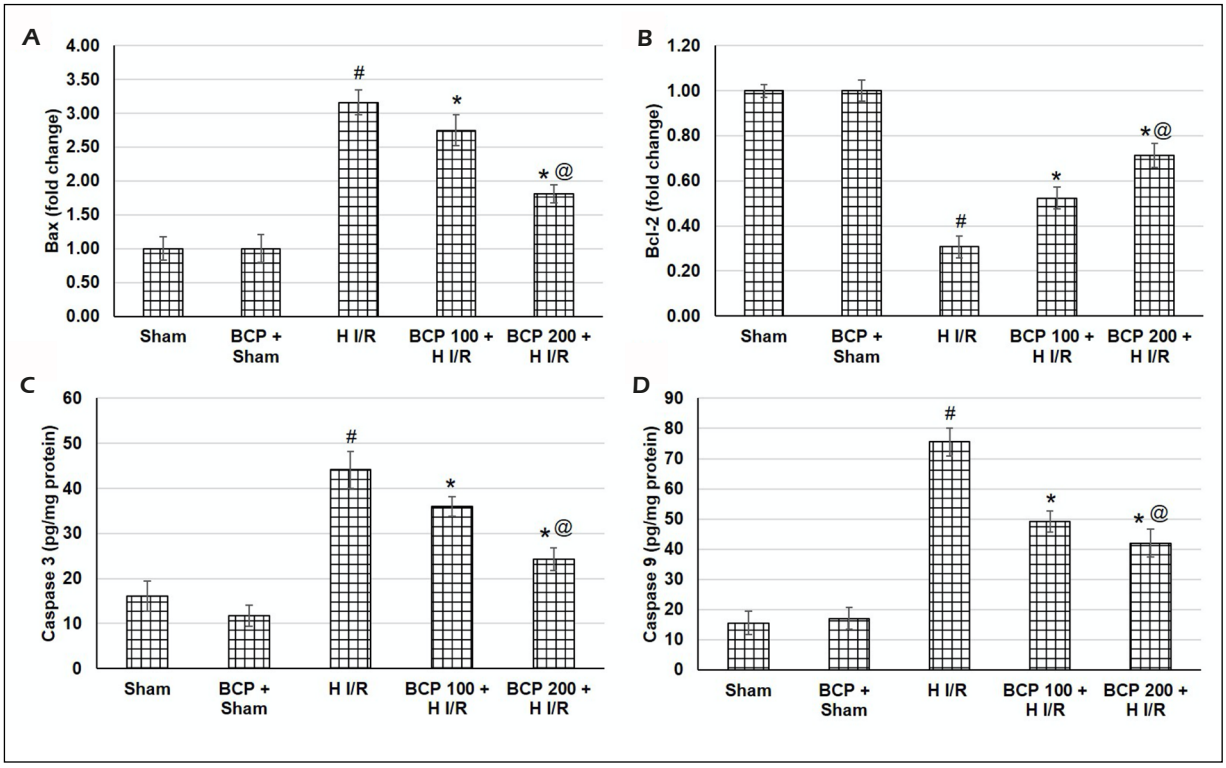

Figure 8. Effects of β-caryophyllene (BCP) (100 and 200 mg/kg) administration for 14 days before hepatic ischemia/perfusion (H I/R) on the gene expression levels of (**A**) Bax, (**B**) Bcl2, and protein expression of (**C**) caspase 3, (**D**) caspase 9 in H I/R-induced injury. All values are stated as mean ± SD. # designates statistically significant compared to the sham group and *designates statistically significant compared to the H I/R group, @ designates statistically significant compared to BCP 100 + H I/R ($p < 0.05$) using one-way ANOVA followed by Tukey's post hoc test.

glutamate (Glu)-induced cytotoxicity in the C6 glioma cell line[42], aflatoxin B1-induced liver toxicity[26] and sulfasalazine-induced nephrotoxicity[43].

The second cornerstone the hereby study focused on is TLR4/ NF-κB/ NLRP3 signaling pathway, which is a detrimental pathway implicated in inflammation and apoptosis associated with H I/R induced injury. TLRs are transmembrane protein receptors recognized as the first line of protection against pathogen attack, with a crucial role in inflammation, immune cell regulation, and apoptosis. The TLR-4/NF-κB signaling pathway plays an imperative role in ischemic damage of several organs, including heart[44], brain[45], liver[28,46], and kidneys[47]. During H I/R, ROS stimulates the early response transcription expression, which activates TLR-4, which enables the nuclear transcription factor NF-κB P65, inhibits IκB-a kinase activity, and mediates the release of several inflammatory factors, including TNF-α, IL-1β, and IL-6[1,4]. The scrutiny results demonstrated that H I/R was associated with augmented TLR4/ NF-κB/ NLRP3 signaling pathway with subsequent elevation in pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), adhesion molecule ICAM-1, and leukocytes infiltration. In the existing work, treatment with BCP depressed gene and protein expression of TLR4, which could be related to enhancing antioxidant enzymes activities, where TLR4 inflammatory reaction is prompted by different stressors including ROS[28]. Previously, BCP alleviated D-galactosamine and LPS-induced hepatic injury by suppressing the TLR4 and RAGE signaling pathways[25].

Additionally, pretreatment with BCP inhibited gene and protein expression of NF-κB, which may be attributed to Keap1/Nrf2/HO1/NQO1pathway intensification. Keap1/Nrf2/HO1/NQO1 pathway amplification has been described to reduce NF-κB inflammatory response via transferring the cells' conditions to a more reducing state, causing inhibition of NF-κB activation[35]. In addition to inhibiting NF-κB, the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β were successfully dimin-

ished by BCP administration in accordance with previous results revealing the anti-inflammatory activity of BCP[13,20,44]. These effects point to the robust anti-inflammatory effects of BCP against hepatic I/R-induced injury, taking into account the vital part of NF-κB and, consequently, its potent capability to provoke inflammatory mediators.

Elevated ICAM-1, following the H I/R status, specifies the adherence and the leukocyte infiltration of the hepatic endothelial cells, which can be justified by the inflammatory cytokines' intensification[2,33]. BCP treatment declined the elevated ICAM-1 level and deterred neutrophils infiltration as verified by ICAM-1 and MPO levels reduction after H I/R, which is in harmony with the preceding report establishing BCP repressive effects on adhesion molecule ICAM-1, and neutrophil and macrophage infiltration in cisplatin-induced nephrotoxicity[48], alcoholic steatohepatitis[49] and LPS-induced acute lung injury[50].

NLRP3 is one of the vital inflammasomes contributing to the H I/R injury, lowering inflammation and regulating infiltration of neutrophils. Treatment with BCP in the current investigation caused a significant NLRP3 suppression, which is an imperative regulator of neutrophils function in the H I/R-induced insult[51]. The merit of BCP to inhibit NLRP3 was previously exhibited in various ailments, where BCP ameliorated gouty arthritis[17] and rheumatoid arthritis[52] by inhibiting NLRP3 and NF-κB signal pathway. Remarkably, Nrf2 activators negatively control NLRP3 activity by inhibiting the ROS-induced NLRP3 priming[53]. Noteworthy, TLR4 or NF-κB inhibition interrupts NLRP3 inflammasome stimulation, which highlights the complicated significance of NLRP3 suppression as a BCP mechanism of action. Therefore, it may be reasonable to suppose that treatment with BCP, Nrf2 up-regulation restrained ROS induced TLR4/NF-κB/NLRP3 activation.

Apoptosis was evident in the current study by enlarged Bax and caspase-3,9 levels and lessened Bcl2, implying that an apoptotic status is associated with H I/R. Pretreatment with BCP lowered caspase 3 and 9 and Bax and elevated Bcl2 confirming the anti-apoptotic effect of BCP. Previously, BCP has been demonstrated for its anti-apoptotic action in experimental myocardial infarction[54], focal cerebral ischemia-reperfusion injury[24], and doxorubicin-induced chronic cardiotoxicity[55], signifying the prominent anti-apoptotic action of BCP.

Conclusions

BCP, a natural sesquiterpene, was confirmed to possess a hepatocyte protective effect in the H I/R status. The *in silico* molecular docking suggested two pathways for this protective action, Keap1/Nrf2/HO1/NQOone and TLR4/NF-κB/ NLRP3. The existing investigation outcomes revealed that mitigation of oxidative stress by regulating the Keap1/Nrf2/HO1/NQO1 pathway might participate in the proven protective effect of BCP against H I/R induced injury. Additionally, pretreatment with this natural sesquiterpene inhibited TLR4/NF-κB/ NLRP3, which is another mechanism that might elucidate the compound's beneficial protective effect. These actions were reflected by multiple inflammatory markers reduction such as TNF-α, IL-6, and IL-1β, and reduced neutrophil recruitment and adhesion molecules. Bringing together all these outcomes, BCP might be a promising therapeutic medication to prevent the H I/R-induced injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

Conceptualization was carried out by all authors, M.E. Mohamed, R.M. Abdelnaby, N.S. Younis; meth-odology, N.S. Younis, M.E. Mohamed; software, R.M. Abdelnaby; validation, N.S. Younis, M.E. Mo-hamed; formal analysis, N.S. Younis; resources, M.E. Mohamed; data curation, N.S. Younis, M.E. Mo-hamed; writing-original draft preparation, N.S. Younis, M.E. Mohamed; writing-review and editing, N.S. Younis, M.E. Mohamed; supervision, M.E. Mohamed; project administration, N.S. Younis; funding ac-quisition, M.E. Mohamed. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by Al Bilad Bank Scholarly Chair for Food Security, the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia, Grant No. CHAIR28.

Acknowledgements

The authors are grateful to Al Bilad Bank, Scholarly Chair for Food Security, the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, and King Faisal University for financially supporting this study (Grant No. CHAIR28).

Ethical Statement

The experimental protocol (KFU-REC-2022-FEB-EA000418) was permitted by the Institutional Animal Care and Use Committee of King Faisal University. All the experiments were executed in harmony with the relevant procedures and regulations of the Ethical Conduct for the Use of Animals in Research at King Faisal University.

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