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Hepatoprotective effects of linalool against liver ischemia-reperfusion: the role of Nrf2/HO-1/NQO1 and TLR4/RAGE/NFκB pathways

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Abstract. – OBJECTIVE: Hepatic ischemia-reperfusion (H I/R) injury arises due to a temporary obstruction followed by the re-establishment of blood supply to the liver. Linalool (LIN), a main volatile constituent of essential oils in numerous aromatic plant species, exhibited various medicinal and pharmacological actions. This study investigated the protective effect of LIN on the status of H I/R, with the study of the possible mechanisms. In addition, linalool's antagonistic effects were tested against several metabolic targets using *in silico* molecular docking technique.

MATERIALS AND METHODS: Wistar rats were allocated into five groups. Sham and LIN + Sham groups in which animals were administered either vehicle (1% CMC) or LIN (200 mg/kg/day) orally for two weeks. H I/R group in which rats were administered 1% CMC for two weeks and then experienced hepatic ischemia for 60 min followed by 6 hours of reperfusion. LIN 100 + H I/R and LIN 200 + H I/R groups in which rats were pretreated with LIN (100, 200 mg/kg/day) respectively for two weeks, then subjected to H I/R.

RESULTS: H I/R-induced injury resulted in impaired liver function and activated Keap1/Nrf2/HO-1/NQO1 and HMGB1/TLR4/RAGE/NFκB pathways with subsequent oxidative stress, inflammation, and apoptosis. LIN pretreatment alleviated I/R-induced impairment in liver function, promoted Keap1/Nrf2/HO-1/NQO1, and mitigated the HMGB1/TLR4/RAGE/NFκB pathway. LIN pre-administration deterred adhesion molecule, neutrophils infiltration, RAGE, IL-1β, IL-6, TNF-α, and apoptosis.

CONCLUSIONS: LIN demonstrated hepato-protective effects against H I/R *via* instigation Keap1/Nrf2/HO-1/NQO1 and mitigating the HMGB1/TLR4/RAGE/NFκB pathways with subsequent deterring oxidative stress, inflammation, and apoptosis.

Key Words:
Linalool, Apoptosis, Hepatic ischemia-reperfusion; In silico docking, Inflammation.

Introduction

Hepatic ischemia-reperfusion (H I/R) injury is a syndrome that happens quite often during several hepatic clinical situations, resulting in liver dysfunction and failure[1,2]. H I/R injury arises due to a temporary obstruction of blood supply tailed with the reestablishment of the blood supply. Extensive damage happens during the blood reperfusion rather than during ischemia[3]. Numerous aspects are concomitant in the H I/R-induced damage, including mitochondrial dysfunction, oxidative stress, inflammation, calcium overload, microcirculation disruption, apoptosis, and autophagy, among other aspects[4]. Reactive oxygen species (ROS) is the main initiator for different signaling leading to hepatocyte injury and, subsequently, inflammation and necrosis/apoptosis[5,6].

The Kelch-like ECH-Associated protein 1/ nuclear factor erythroid 2 related factor 2/antioxidant response element (Keap1/Nrf2/ARE) has a vital part in the defense against H I/R induced damage[1,4,7]. Under unstressed conditions, Nrf2 degenerates continually *via* the ubiquitin-proteasome pathway when associated with Keap1. Whereas under stress, Nrf2 degradation terminates as it separates from Keap1 to translocate into the nucleus[8]. Nrf2 translocating triggers and releases numerous downstream target genes comprising antioxidant enzymes [such as Heme Oxygenase-1 (HO-1), peroxidase-1, superoxide dismutase (SOD), Glutathione (GSH)] and phase II metabolizing enzymes [containing mainly glutathione-S-transferase, and NAD(P)H quinone dehydrogenase 1 (NQO1)] to eradicate ROS and diminish the whole redox reaction[7]. Triggering the nuclear Nrf2 pathway regulates both the oxidative

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stress as well as the inflammation process[9]. Furthermore, Nrf2 is capable of modulating other signal pathways, for instance, Nuclear Factor kappa B (NFκB)/Toll-like receptor-4 (TLR-4)[10]. NFκB is another key regulator in the inflammation and cell death involved in hepatocellular injury. Some previous studies[11] recognized the role of TLR-4/NFκB-p65 pathway activation in diverse hepatic injury models, including liver fibrosis, encephalopathy[12], and ischemia-reperfusion[10].

High-mobility group box 1 (HMGB1) is a non-histone nuclear protein that acts as an essential damage-associated molecular pattern (DAMP) molecule[13]. HMGB1 activates proinflammatory signaling by interacting with certain pattern recognition receptors, such as TLR4, and the receptor for advanced glycation end-products (RAGE)[14,15]. HMGB1 is an early moderator of damage and inflammation subsequent to I/R of the liver[16], kidney[17], brain[18], and heart[19].

Linalool (LIN), a major volatile constituent of the essential oils in numerous plant species such as lavender oil, has been reported[20-24] to exhibit cytotoxic[20], antibacterial[21], antinociceptive[22], anti-inflammatory[23], and antioxidant[24] activities. LIN protected against acetic acid-induced ulcerative colitis[25], ovalbumin-associated lung inflammation[26], cigarette smoke (CS) prompted acute pulmonary inflammation[23], and cisplatin-induced nephrotoxicity[27]. As for neuroprotective actions, LIN alleviated A β 42-induced Alzheimer's disease[24], oxygen-glucose deprivation-induced neuronal injury[28], and acrylamide-induced neurotoxicity[29]. LIN demonstrated hepatoprotective actions against carbon tetrachloride CCl_4[30,31], and lipopolysaccharide/D-galactosamine[32] persuaded liver damage.

However, LIN actions on hepatic ischemia-reperfusion have not been studied before. Therefore, whether the Keap1/Nrf2/HO-1/NQO1 and HMGB1/TLR4/RAGE/NFκB pathways contribute to the protection provided by LIN against H I/R-induced injury remains unclear. Accordingly, the present investigation investigated the hepatoprotective effect of LIN against H I/R-induced injury. Moreover, the study inspected the role of Keap1/Nrf2/HO-1/NQO1 and HMGB1/TLR4/RAGE/NFκB signaling pathways in the LIN effect in H I/R.

Materials and Methods

Animals Acquisition

Wistar male rats were obtained from the Experimental Animal Research Centre, King Saud University. The animals were kept with standard laboratory food and water *ad libitum* in a ventilated cage system (12 h light/dark cycles, 20.3-23.1°C). After one week of acclimatization, the rats were used for the current study.

Animals Ethical Statement

The Institutional Animal Care and Use Committee at King Faisal University (KFU) permitted the study with the protocol (KFU-REC-2022-FEB-EA000418). Different experiments were performed in harmony with the measures and guidelines of the Ethical Conduct for the Use of Animals in Research at KFU.

H I/R Model Surgical Procedures

Rats were anesthetized with isoflurane with oxygen (2%, 0.5 L/h) throughout the whole surgery. Animals' abdomens were shaved, and a surgical incision (about 3 cm) from the pubic to the sternum was executed. The portal triad, comprising the hepatic artery, portal vein, and common bile duct, was clamped using a micro-vascular clamp for 60 min to prompt ischemia (temporary obstruction of blood supply, hepatic deoxygenation). Hepatic reperfusion (re-establishment of the blood supply, reoxygenation) was accompanied by releasing the micro-vascular clamp[10,33] and permitted reperfusion for six hours. The sham animals were subjected to all the techniques that occurred during H I/R, and deprived of blood flow blockage.

Investigational Design

LIN was isolated from the essential oil of lavender, as stated by Mohamed et al[34]. LIN was administered orally by gavage after being suspended in 1% carboxymethyl cellulose (CMC). LIN dose (100 and 200 mg/kg) selection was based on previous studies[30,34]. Thirty Wistar rats were haphazardly divided into four groups (n=6). The sham group was given a vehicle (1% CMC) orally for two weeks and subjected to sham steps. LIN + Sham group was administered LIN (200 mg/kg/day) for two weeks and then subjected to sham steps. The third group, the H I/R group, in which rats were administered 1% CMC for two weeks and then hepatic ischemia for 60 min followed by 6 hours reperfusion was implemented. Finally, LIN 100 + H I/R and LIN 100 + H I/R groups in which rats were administered LIN (100, 200 mg/kg/day) respectively for two weeks orally, then subjected to hepatic I/R surgical procedure.

Hepatic Tissue and Serum Isolation

Once the reperfusion period was accomplished, animals' blood was obtained through the hearts, centrifuged at 4,000 ×g for 15 min at 4°C, and stored at -20°C to be utilized in the biochemical estimation of liver function tests, including ALT, AST, ALP, and LDH. Additionally, hepatic tissues were isolated, washed in cool PBS, and distributed in three portions. The first part was homogenized with PBS, centrifuged at 10,000 ×g for 15 min at 4°C, and consumed to evaluate oxidative stress. Another portion was kept at -20°C for subsequent western blot analysis and qRT-PCR. The third amount was used to estimate the histopathological variations as well as the immunohistochemical manifestation after being fixed with 10% formalin-buffered saline.

Assessment of Hepatic Function Biomarkers

Rat Alanine Aminotransferase (ALT) ELISA kit (ab285264), Aspartate Aminotransferase (AST) ELISA kit (ab263883), and Alkaline Phosphatase (ALP) colorimetric assay kit (ab83369), Lactate Dehydrogenase (LDH) colorimetric assay kit (ab102526) were attained from Abcam (Boston, MA, USA).

Assessment of Oxidative Stress

Antioxidant and lipid peroxidation were monitored in the hepatic homogenate. Malondialdehyde (MDA, ab238537), glutathione (GSH, ab65322), glutathione-s- transferase (GST, ab65326), nitric oxide (NO, ab272517) assay kits were purchased from Abcam Inc. (Cambridge, UK). Superoxide dismutase (SOD; MBS036924) and catalase (MBS726781) ELISA kits were obtained from My BioSource (San Diego, CA, USA).

Assessment of Histopathological, Immunohistochemistry and Immunofluorescence Analysis

Part of the ischemic hepatic fraction was fixed with 10% formalin, embedded in paraffin, cut into 5 µm thick paraffin slices, and stained with hematoxylin & eosin (H&E). Ischemic hepatic damage was estimated by two pathologists blinded to the investigational design under a light microscope (magnification, x400) using the Suzuki grading system[35]. Suzuki grading standard includes five scores (04) related to hepatocyte cytoplasm vacuolization, necrosis, and tissue congestion.

For the immunohistochemistry (IHC) procedure, the expression of NFκB was determined, as shown formerly[27]. Liver sections were blocked, incubated with NFκB antibody (1:100, Thermo Fisher Scientific, Cambridge, UK) overnight at 4°C, followed by goat anti-rabbit-horseradish peroxidase (HRP) conjugated IgG antibody (1:1,000; Cat. no. ab6721; Abcam, Eugene, OR, USA) for 1 hour at 37°C. NIS-Elements software (Nikon Instruments Inc., Melville, NY, USA) was used for quantitative analysis. Firstly, the area of IHC reaction in the picture was selected, and then the average optical density in the designated area of each photograph was assessed. Positive cells were counted under x400 magnification, observing 10 consecutive non-overlapping fields per animal in a blinded manner.

For the ROS fluorescent protocol, frozen hepatic sections were used for ROS content determination by a fluorescent dye dihydroethidium (DHE) (D11347, Thermo Fisher Scientific, Cambridge, UK). Firstly, the tissue was marked by a liquid blocker pen at room temperature. A spontaneous fluorescence quenching reagent was added for 5 min, then washed with tap water. ROS staining solution (DHE 10 µmol/l) was added to the tissue area at 37°C for 30 min in the dark. Sections were washed three times with PBS (pH 7.4), incubated with DAPI solution (62248, Thermo Fisher Scientific, Cambridge, UK) for 10 min in the dark, and washed with PBS. Sections were mounted with an anti-fade mounting medium, and images were captured using a fluorescence microscope (magnification, x400). ROS-positive cells were labeled by fluorescein and appeared red.

In Silico Docking Study

The molecular docking of LIN on TLR-4/MD-2, IκB kinase (IKK)[36], Keap1, and RAGE proteins was performed on MOE software and visualized by Discovery Studio visualizer (Accelrys, Inc., San Diego, USA). The procedures started by downloading the 3D crystal structure of TLR-4/MD-2 (PDB: 3FXI, available at: https://www.rcsb.org/structure/3FXI), IKK (PDB: 4KIK, available at: https://www.rcsb.org/structure/4KIK), Keap1 (PDB: 5FNU, available at: https://www.rcsb.org/structure/5FNU), and RAGE (PDB: 3O3U, available at: https://www.rcsb.org/structure/3O3U) from the protein data bank, followed by preparing the protein structure, adding protons, removing unnecessary water molecules, and fixing missing chains. The binding sites were defined from the co-crystalized ligand position. A validation step was carried out to ensure the docking procedures. The 2D structure of LIN was drawn on ChemDraw Professional 16.0 (PerkinElmer Informatics, Inc., Waltham, Massachu-

setts, USA), followed by ligand preparation and energy minimization in MOE to be docked inside the binding sites. The best ten docking poses were selected and studied to get the best orientation with the best score compared to the reference ligand.

Gene Expression Determination of Keap1/Nrf-2/HO-1/NQO-1 and HMGB1/TLR4/RAGE /NFĐB Pathways

Real-time PCR was performed according to the technique described elsewhere. The primers used are as follow Nrf2 (NM_031789.2) F: 5'-CATTTGTAGATGACCATGAGTCGC-3', R: 3'-ATCAGGGGTGGTG AAGACTG-5'; HO-1 (NM_012580.2) F: 5'-GTGCACATC-CGTGCAGAGAA-3', R: 3'-GTGCACATCC GTGCAGAGAA-5'; NQO-1 (NM_008706) F: 5′-GTCCATTCCAGCTGACAACCA-3′, R: 3′-GTCCATTCCAGCTGACAACCA-5′ ; TLR4 (NM_019178) F: 5′-GCTGCCAACATCATC-CAGGAAGG-3′, R: 3′-TG ATGCCAGAGCG-GCTACTCAG-5′; RAGE (NM_053336) F: 5′- CTGCCTCTGAACTCAC AGCCAATG -3′, R: 3′-GTGCCTCC TGGT CTCCTCCTTC-5′; HMGB-1 (XM_0391002 70) F: 5′- AGGCTGAC AAGGCTCGTTATG -3′, R: 3′- TGTCATCC-GCAGCAGTGTTG -5′; Bcl-2 (NM_016993.1) F: 5′-CCGGGAGATCGTG ATGAAGT-3′, R: 3′-ATCCCAGCCTCCGTTATCCT-5′; Bax (NM_017059.2) F: 5′-GTGGTTGCCCTCT-TCTACTTTG-3′, R: 3′-CACAAAGATG-GTCACTGTCTGC-5′; β-actin (NM_ 03 144.3) F: 5′-TGACAGGATGCAGAAGGAGA-3′, R: 3′-TAGAGCCACCAATCCACACA-5′. Expression of the target gene was assessed 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.

Protein Expression Experiments of Nrf-2/HO-1/NQO-1

Western blot analysis was carried out as described previously[37]. Nrf2 nucleoprotein was measured in the nuclear lysate portion using a nuclear protein extraction kit (ab113474; Abcam Inc., Cambridge, UK). Total protein was extracted using RIPA lysis buffer. 20 µg protein samples were separated using 10% SDSPAGE and transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk (at room temperature for one hour), and then incubated with primary antibodies at 4°C overnight. Nrf2 (cat. no. ab92946), HO1 (cat. no. ab13248), NQO1 (cat. no. ab2346) were purchased from Abcam Inc., UK. Membranes were washed with Tris-buffered saline containing 0.1% (v/v) Tween®20 (TBST) (Merck KGaA, Darmstadt, Germany) three times and incubated with horseradishconjugated goatanti-rabbit secondary antibody (1:10,000; cat. no. SA000012; ProteinTech Group Inc., Chicago, IL, USA) at room temperature for one hour, then washed again. Band intensities were measured using Image J v1.8.0 software (National Institutes of Health NIH, Bethesda, Maryland, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)Determination of HMGB1/TLR4/RAGE Pathway

RAGE (ab202409, Abcam, UK), HMGB1 (OKCD04073, Aviva Systems Biology, CA, USA), TLR4 (E15822, Cusabio, Wuhan, China) ELISA kits were used for the quantitative measurement of HMGB1, TLR4, and RAGE proteins following the manufactures instructions.

Assessment of Neutrophils Infiltration, Inflammation Mediators, and Apoptotic Markers

Inflammation markers including TNF-α (ab46070), IL-1β (ab100768), IL-6 (ab100772) and IL-10 (ab133112), intercellular adhesion molecule-1 (ICAM-1, CD54) (ab100763), and Myeloperoxidase (MPO) (ab285308) ELISA kits were obtained from Abcam Inc., Cambridge, UK. 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.

Statistical Analysis

Data are demonstrated as mean ± SD. One-way ANOVA test, followed by Tukey-Kramer as a post-hoc test, were executed for multiple comparisons between different experimental groups. p as a level of probability was set lower than 0.05 ($p < 0.05$), indicating the significance level. All statistical analyses were performed using Graph Pad software (version 8.0, CA, USA).

Results

LIN Alleviated Hepatic Function and Histopathological Examination in H I/R-Induced Injury

Liver function was assessed by identifying the serum ALT, AST, ALP, and LDH levels[4]. As di-

splayed in Figure 1, and in comparison, with the sham, the H I/R animals exhibited elevated ALT, AST, ALP, and LDH levels. Alternatively, related to the H I/R group, LIN pretreatment alleviated ischemia-induced impairment in liver function, as confirmed by lowered ALT, AST, ALP, and LDH. LIN (100 and 200 mg/kg) caused a percentage decrease in ALT (22.4% and 40.9%), AST (20.96% and 39.9%), ALP (25.14% and 41.87%), and LDH (27.03% and 50.94%), respectively, when compared to the H I/R (Figure 1a-d).

In regard to the histopathological investigation, sham-operated groups showed regular histology architecture, while the H I/R exhibited plentiful histopathological variations, comprising diffuse necrosis with disorganized parenchyma and dilated sinusoids. LIN pretreated groups revealed re-established liver parenchyma, less necrosis, fewer congested sinusoids, and portal vasculatures with minor vacuolation. The histopathological changes were numerically expressed using Suzuki scores, where all three features, congestion, vacuolation, and necrosis, were extensively amplified in the H I/R (Figure 1f). On the other hand, LIN preconditioned animals demonstrated a considerable reduction in hepatic congestion and hepatocellular vacuolation and necrosis when related to the H I/R animals. Nevertheless, no significant variance was detected between the two doses of LIN in Suzuki scores, as illustrated in Figure 1f.

In Silico Studies

In order to investigate the mechanism of action of LIN, the compound's antagonistic effects were tested against several metabolic targets using *in silico* molecular docking techniques. The preliminary investigation identified four potential targets to continue the study on TLR4, RAGE, Keap1, and IKK, giving rise to the inflammatory HMGB1/TLR4/RAGE/NFκB and the oxidative stress Keap1/Nrf2/HO-1/NQO-1 pathways.

Molecular Docking in TLR4/MD2 Complex (PDB: 3FXI)[38,39]

Figure 2 represents the interaction diagrams that show the insertion of LIN in the hydrophobic region of the LPS binding site in the TLR4/MD2 complex. LIN had strong interactions with the key amino acids Lys109 (H-bonding) and Arg69 (alkyl interaction), with a docking score of -4.55 Kcal/mol.

Molecular Docking in IKK (PDB: 4KIK)[40,41]

IKK is known to be responsible for NF-Kβ activation and nuclear translocation. Diagrams in Figure 3 demonstrated that LIN occupied the main binding site (docking score -4.80 Kcal/mol) of the co-crystalized ligand and interacted with the key amino acids Asp166 (H-bonding), and Phe26 (alkyl interaction).

Molecular Docking for Studying Keap1 Inhibition by LIN

As the Keap1/Nrf2 complex is formed in the cell cytoplasm, the complex will be degraded by cell proteases, preventing Nrf2 from traveling to the nucleus and activating oxidative stress mechanisms. Therefore, the ability of potential drug molecules to inhibit the Keap1/Nrf2 complex is a measure of these drug antioxidant activities. The hereby molecular docking study was carried out in the Keap1/Nrf2 binding site[42] and gave a binding score of -5.61 Kcal/mol. LIN occupied the binding site of Nrf2 on Keap1 and interacted with the amino acids ALA556 (alkyl interaction), ALA510 (H-bonding), and Gly509 (H-bonding), as demonstrated in Figure 4.

Molecular Docking for Studying RAGE Inhibition (PDB: 3O3U)[43]

RAGE is one of the components of the inflammatory HMGB1/TLR4/RAGE/NFκB pathway. Figure 5 shows the docking of LIN in the active site of RAGE. LIN occupied the active site through interaction with the amino acids Arg66 (H bonding), Glu44 (H bonding), and Tyr341 (alkyl interaction). LIN gave a binding score of -5.43 Kcal/mol.

LIN Triggered Nrf2/HO-1/NQO-1 Signaling in H I/R-Induced Damage

Triggering Nrf2 pathway regulates both the oxidative stress as well as the inflammation process[9]. The *in-silico* studies suggested the contribution of the Keap1/Nrf2/HO-1/NQO-1 signaling; therefore, the gene expression of Nrf2, and its downstream target proteins, including HO-1 and NQO-1, were assessed. As shown in Figure 6, the outcomes indicated that H I/R had induced the stimulation of Nrf-2/HO-1, as established by the greater expression of Nrf2, HO-1, and NQO-1 in the H I/R, when related to the sham group. Alternatively, LIN pretreatment even further promoted Nrf-2/HO-1/NQO-1, as displayed by greater expression of Nrf-2, HO-1, and NQO-1 in comparison to the ischemic group ($p < 0.05$).

LIN Alleviated the Activation of HMGB1/TLR4/RAGE/NFκB Signaling Pathway in H I/R-Induced Injury

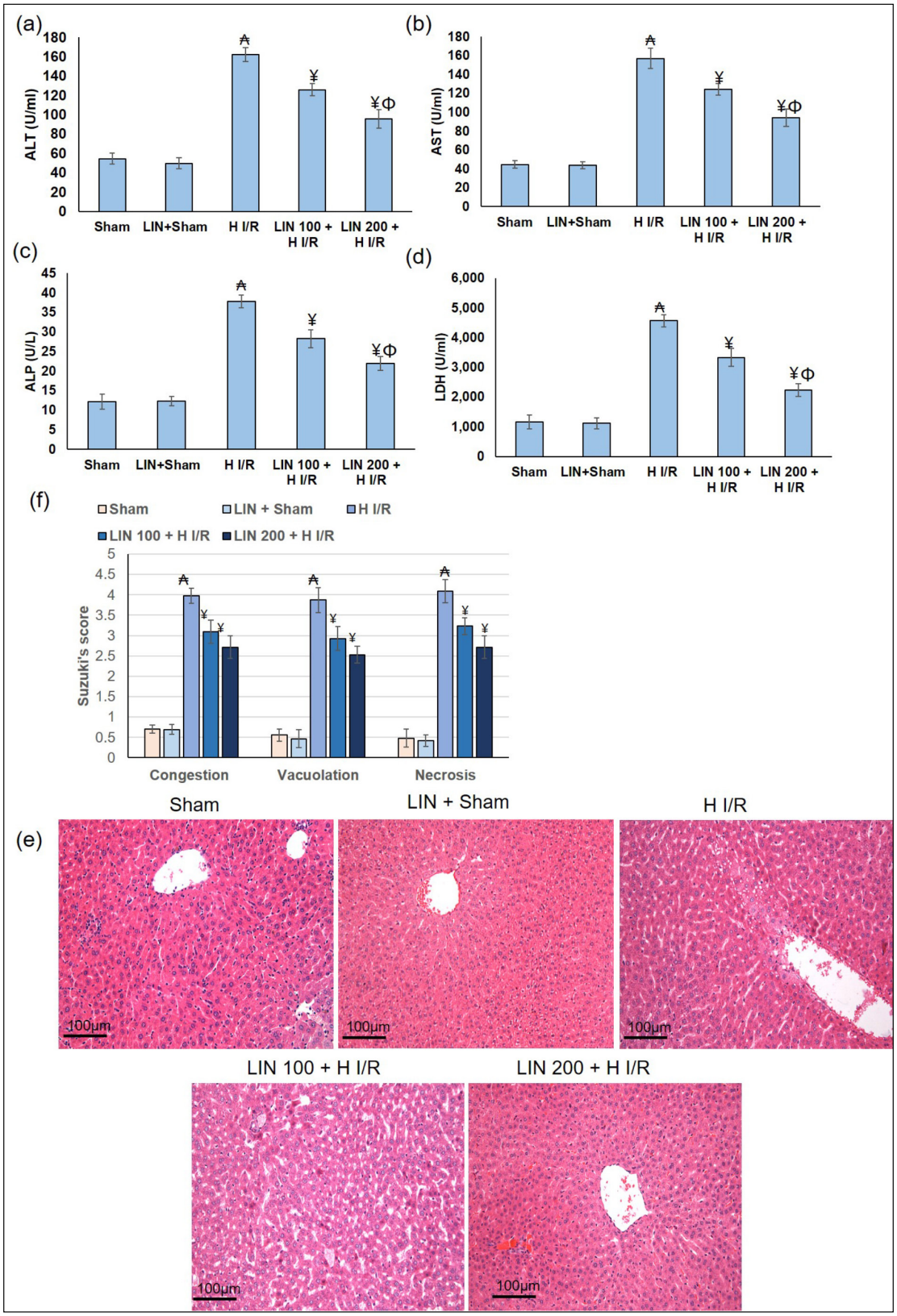

Figure 1. The Effect of linalool (LIN) (100 and 200 mg/kg) preconditioning for 2 weeks prior to liver ischemia/perfusion (H I/R) on the liver function biomarkers, containing ALT (**a**), AST (**b**), ALP (**c**) and LDH (**d**), and on histopathological liver sections (**e**) stained with hematoxylin and eosin (H&E), which was expressed numerically as Suzuki's score (**f**). All values are stated as mean± SD (n=6). ₳ defines statistically signific+ant related to the sham group and ¥ defines statistically significant related to the H I/R group; Φ defines statistically significant related to LIN 100 + H I/R ($p < 0.05$).

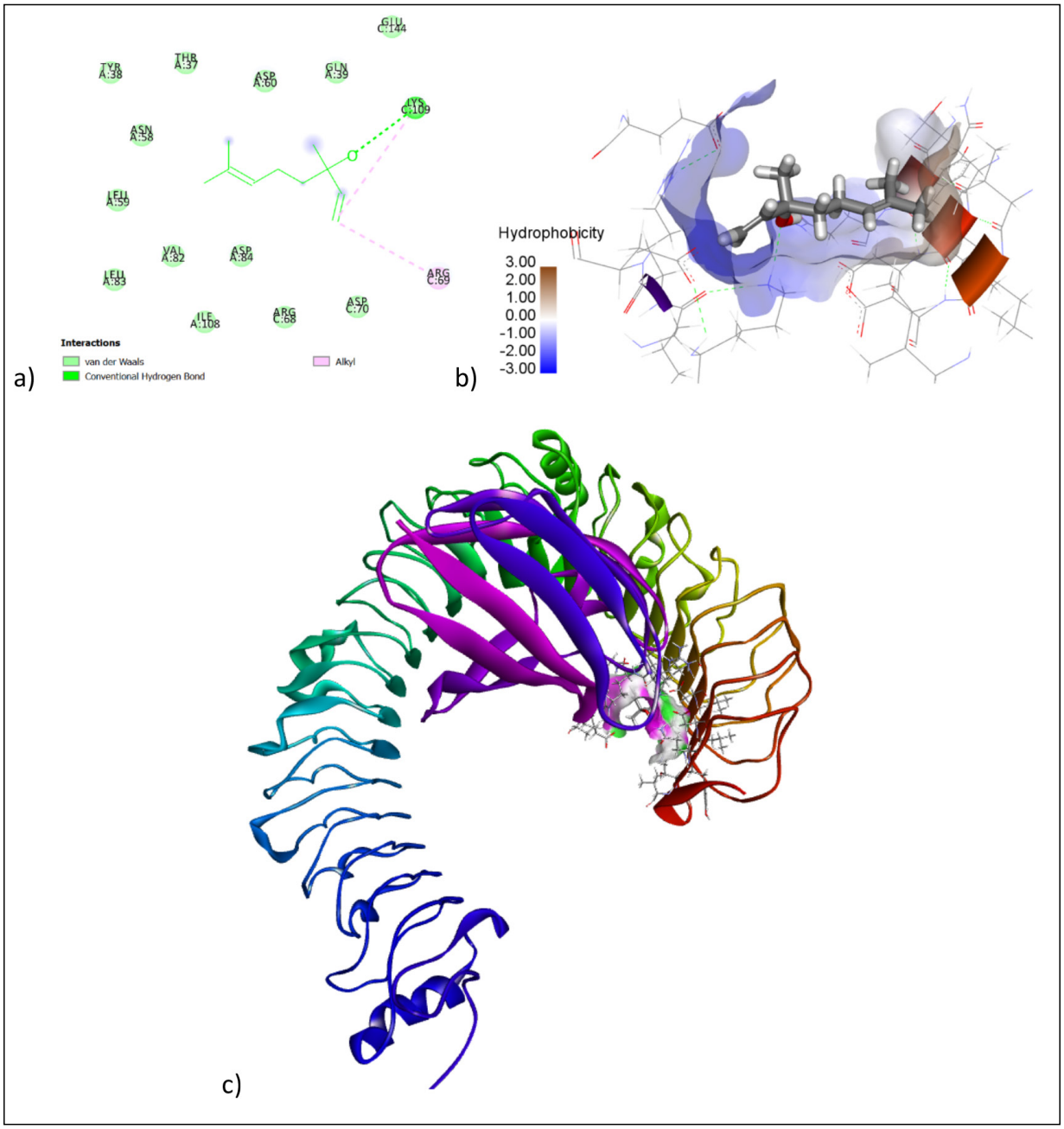

Figure 2. Interaction diagrams of LIN blocking LPS binding site in TLR4/MD2 complex; 2D diagram (**a**); 3D diagram (**b**); Schematic diagram (**c**).

The next pathway we studied is HMGB1, TLR4, and RAGE pathway. The H I/R animals displayed elevated gene and ELISA evaluation of HMGB1, TLR4, and RAGE proteins (Figure 7), signifying stimulated HMGB1/TLR4/RAGE pathway because of ischemia reperfusion-induced injury. Whereas administration of LIN prior to hepatic ischemia resulted in mitigated HMGB1/TLR4/RAGE pathway as shown in Figure 7.

LIN Alleviated Oxidative Stress in H I/R-Induced Damage

The ischemic animals exhibited elevated MDA, NO contents and decreased the antioxidant enzymes (SOD, CAT, and GST) and GSH content, as revealed in Figure 8. Alternatively, pretreatment with LIN diminished MDA, and NO contents and augmented the antioxidant enzymes (SOD, CAT, and GST) and GSH content. These outcomes de-

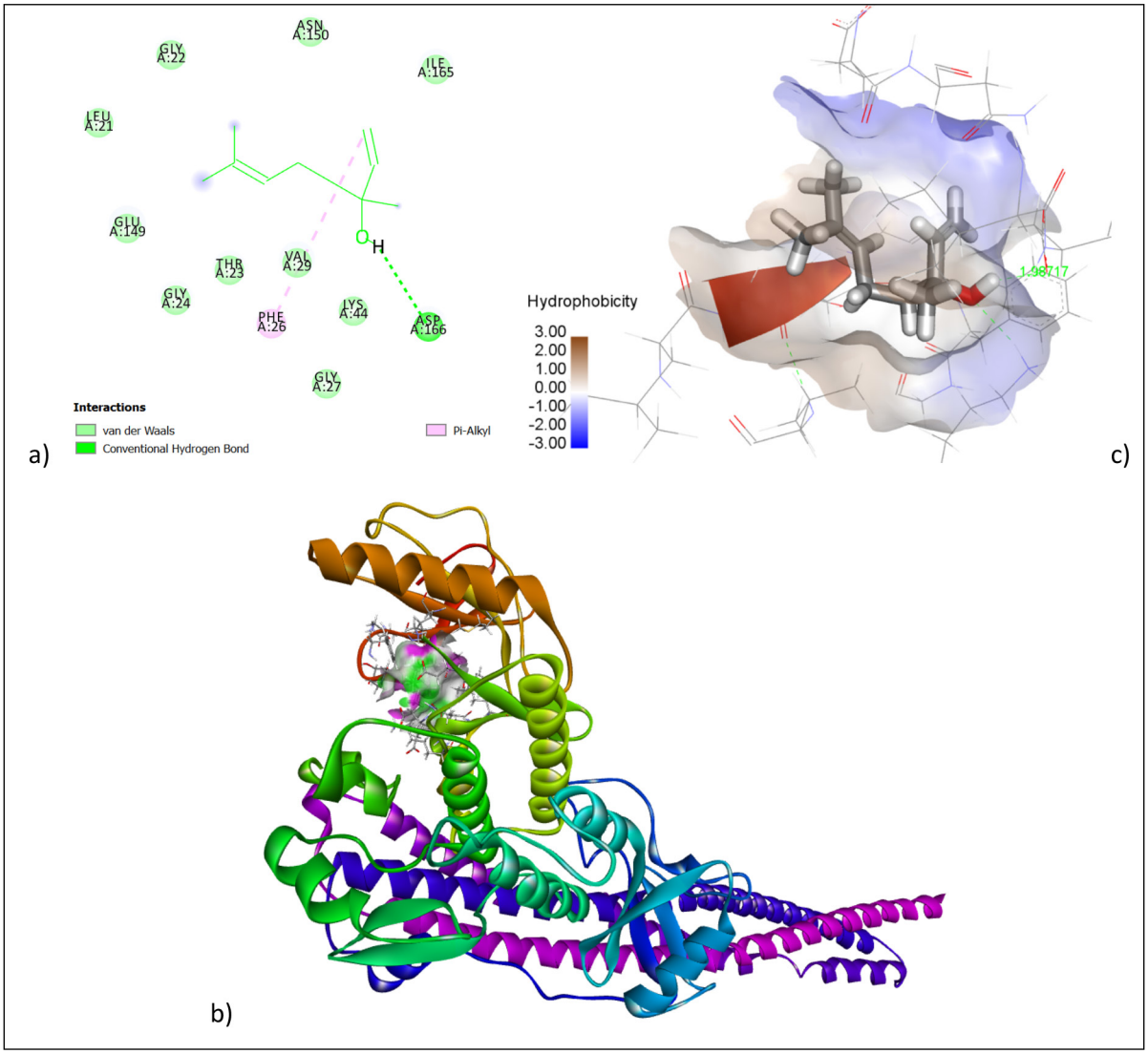

Figure 3. 2D and 3D interaction diagrams of LIN blocking IKK. Co-crystalized inhibitor (**a**); LIN interactions (**b**); schematic diagram of IKK inhibited by LIN (**c**).

monstrated that Keap1/Nrf-2/HO-1/NQO-1 activation may mitigate the oxidative stress status arising within the hepatic tissue (Figure 8). In addition, compared with the Sham and LIN + Sham groups, H I/R displayed markedly amplified intracellular ROS levels (Figure 8j). At the same time, management with LIN deterred ROS levels.

LIN Alleviated Neutrophils Infiltration and Inflammatory Indicators in H I/R-Induced Damage

Hepatic ischemic animals exhibited prompted ICAM-1, which contributed to the neutrophil infiltration as indicated by amplified MPO levels (Figure 9a-b). Meanwhile, LIN administration deterred the H I/R-induced ICAM-1 and MPO elevations.

H I/R animals experienced an escalation in diverse inflammatory mediators, including TNF-α, IL-6, IL-1β, and NFκB, whereas IL-10 was depressed, as illustrated in Figure 9c-g. These intensifications were deterred significantly ($p < 0.05$) in animals pretreated with LIN (100 and 200 mg/kg), while IL-10 was elevated ($p < 0.05$).

The IHC analysis (Figure 9h-i) outcomes exposed that NFκB positive cells were hardly displayed in the sham group. On the other hand, H I/R sections revealed intense NFκB positive cell. NFκB positive cells were depressed in the groups pretreated with LIN when related to the H I/R

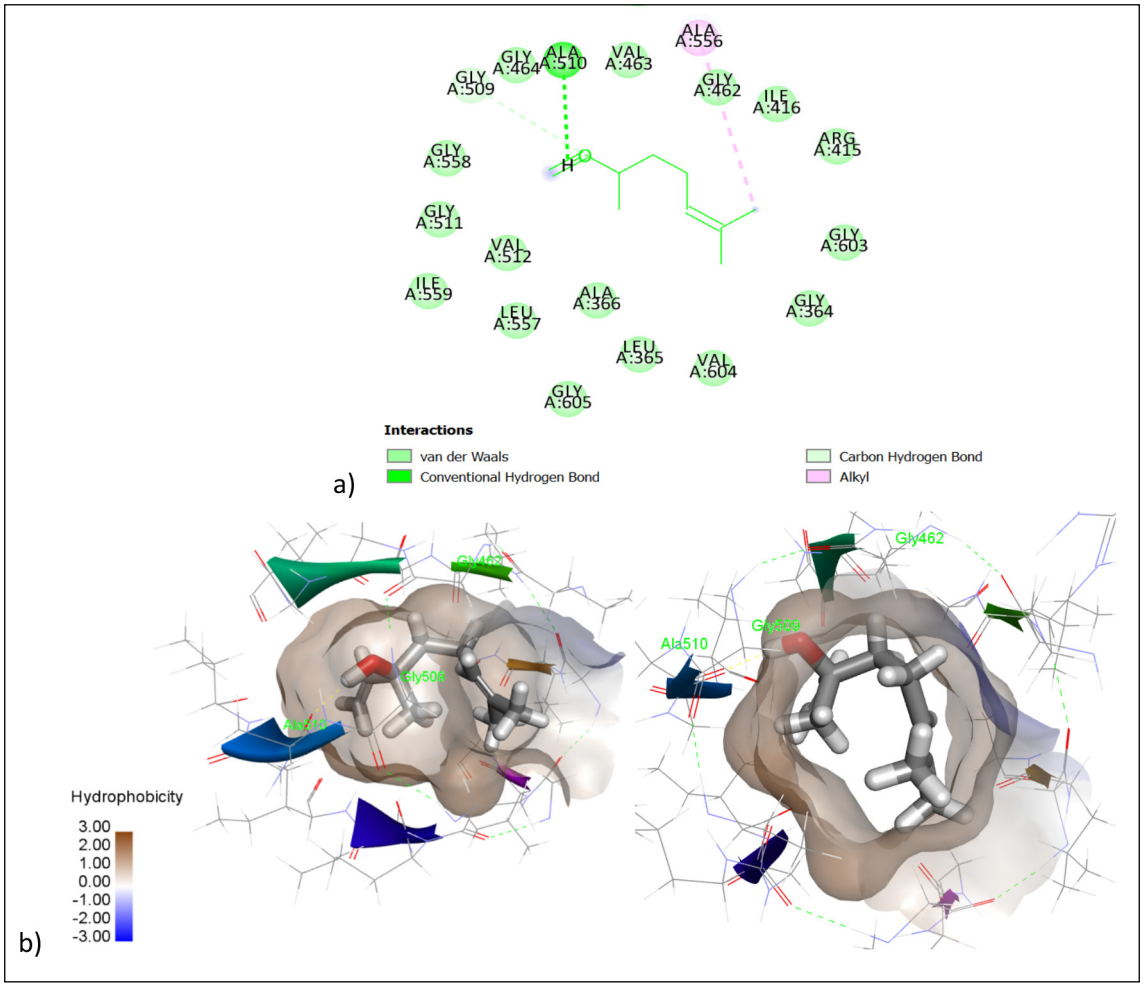

Figure 4. The *in-silico* interaction diagram of LIN in the active site of Nrf2 on Keap1. **a**, A 2D interaction diagram of LIN, forming an H-bond with the key amino acid Ala510 and an alkyl interaction with Ala556. **b**, 3D interaction diagrams at different angles.

group. Altogether, these outcomes signify that the hepatoprotective action of LIN might be by the deterring of HMGB1/TLR4/RAGE/NFκB signaling pathway.

LIN Alleviated Apoptotic Indicators in H I/R-Induced Damage

Animals experimented with H I/R surgery displayed significantly exacerbated apoptotic elements, containing caspase 3 and 9 levels and Bax gene expression, while Bcl-2 gene expression was lessened, as displayed in Figure 10. Management with LIN moderated the apoptosis as established by the depressed caspase 3 and 9 and Bax and amplified Bcl2, signifying the anti-apoptotic influence of LIN.

Discussion

The main objective was to prove the capability of LIN to defend the liver cell tissues from the manifestation and symptoms resulting from the H I/R status. The study was initiated with the induction of H I/R in experimental animals, priory administered LIN in different doses. Injury markers were the first to be determined in order to identify the protective influence of LIN on ischemic liver cells.

H I/R surgery surely induced injury in liver cells, which was evidenced by the upregulation of AST, ALT, ALP, and LDH, released from damaged hepatocytes as hepatic injury markers. Moreover, H I/R caused severe histopathological

alterations. These consequences are consistent with previous reports[4,7,44]. Conversely, LIN pretreatment deterred ischemia-induced impairment in liver function and nearly restored histopathological architectures. Previously, LIN demonstrated[32] a defensive outcome against LPS/GalN-promoted acute liver damage, accompanied by pathological damage restrictive and ALT and AST levels reduction. Furthermore, LIN significantly reduced AST and ALT in CCl_4-induced hepatotoxicity[30].

The next step was to suggest the involved mechanisms that could be responsible for this protective effect of LIN. We implemented *in-silico* molecular docking approaches to predict the inhibitory effect of LIN on different molecular targets representing different signaling pathways. LIN produced promising antagonistic actions with mainly four targets: TLR4, RAGE, Keap1, and IKK, suggesting the involvement of the oxidative stress Keap1/Nrf2/HO-1/NQO-1 and the inflammatory HMGB1/TLR4/RAGE/NFκB pathways.

Keap1/Nrf2/HO-1/NQO-1 pathway is able to defend against oxidative stress. Keap1/Nrf2/HO-1/NQO-1 pathway activation motivates numerous antioxidant and anti-inflammatory factor expressions. Keap1 complex with, and thus destroy Nrf2 in the cytoplasm through the ubiquitination and proteasome pathway[7]. With motivated

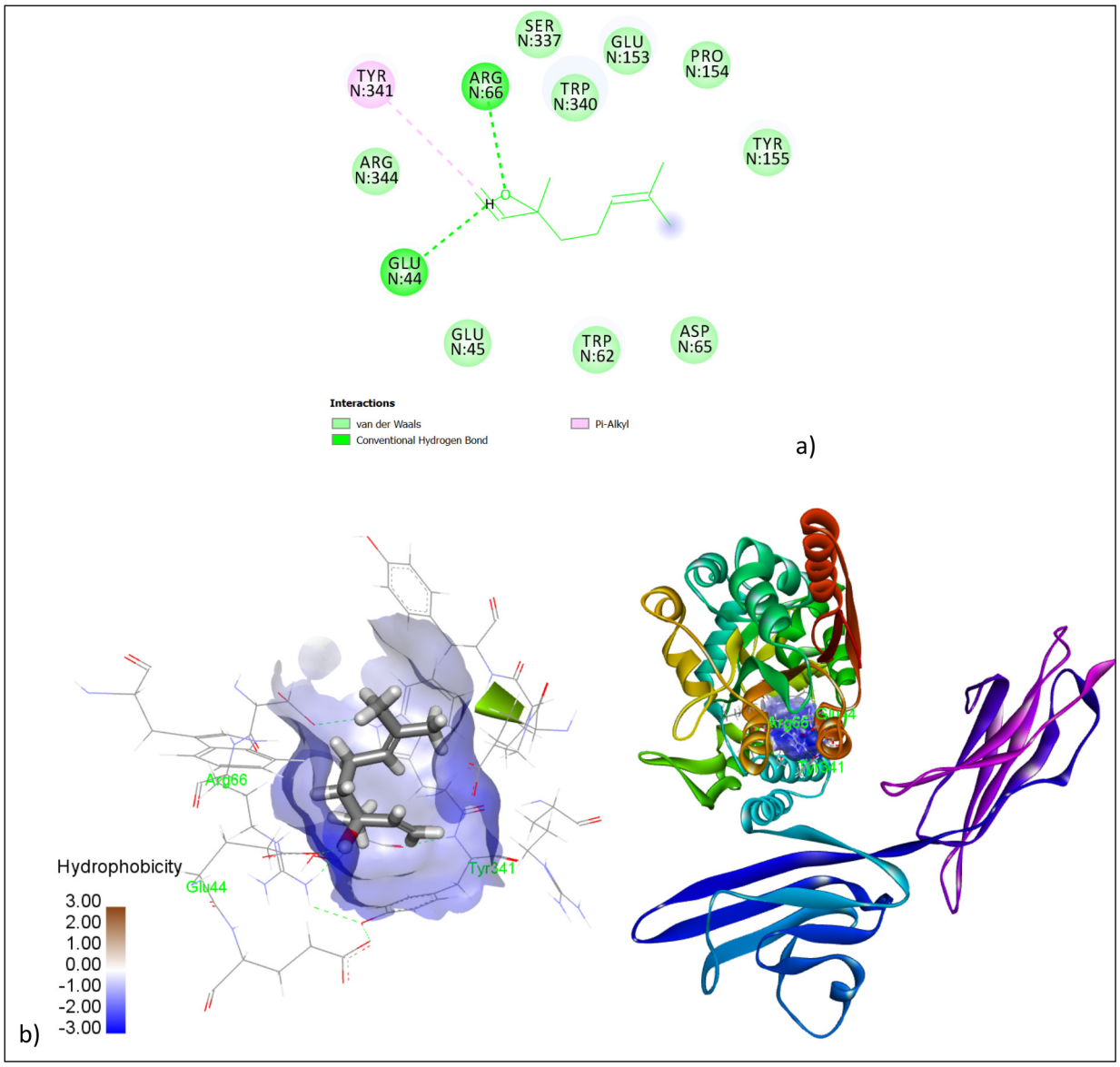

Figure 5. The *in-silico* docking diagrams of LIN in RAGE. **a**, 2D interaction diagram of LIN in RAGE active site forming an H-bond with the key amino acid Arg66, Glu44, and an alkyl interaction with Tyr341. **b**, 3D interaction diagrams.

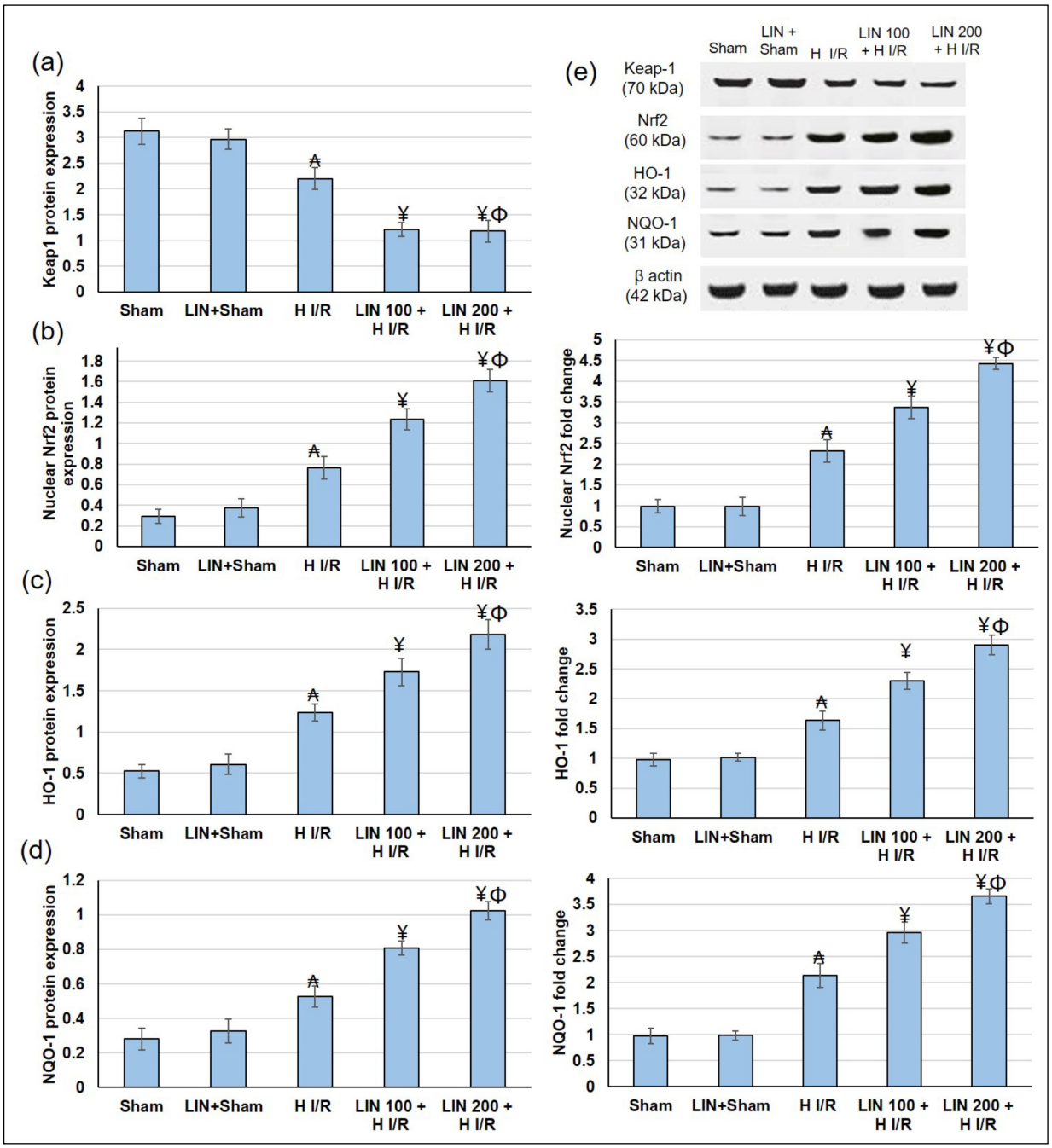

Figure 6. The actions of LIN (100 and 200 mg/kg), preconditioning for 2 weeks prior to H I/R, on the protein expression keap-1 (**a**) and the gene and protein expression levels of Nrf2 (**b**), HO-1 (**c**), and NQO-1 (**d**) in H I/R-induced injury. **e**, The gel bands of the Western blot analyses. All values are stated as mean± SD (n=6). ₳ defines statistically significant related to the sham group and ¥ defines statistically significant related to the H I/R group; Φ defines statistically significant related to LIN 100 + H I/R ($p < 0.05$).

by any oxidative stress situation, Nrf2 translocates into the nucleus to activate and release numerous downstream target genes comprising antioxidant enzymes, including HO-1, peroxidase-1, SOD, GSH, γ - glutamylcysteine synthetase, and phase II metabolizing enzymes. These measures eradicate ROS, lessen MDA and diminish the whole redox reaction[7]. In this investigation, H I/R injury resulted in amplified the gene expressions of Nrf2, HO-1, and NQO1 and lowered Keap1, implying that H I/R injury promoted the Keap1/Nrf2/HO-1/NQO1 signaling to augment the antioxidant capa-

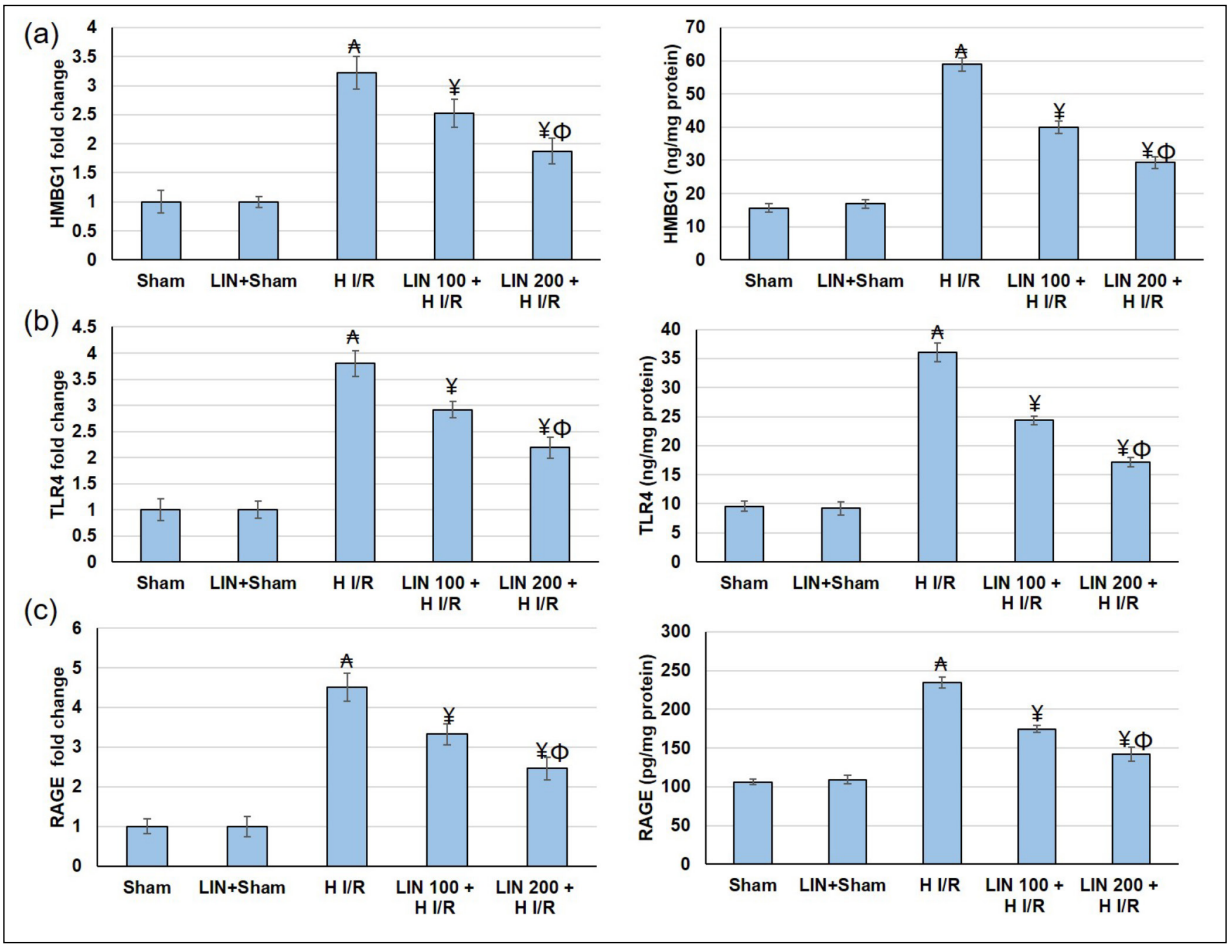

Figure 7. The actions of LIN (100 and 200 mg/kg) preconditioning, for 2 weeks prior to H I/R, on gene (mRNA) expression and ELISA results: HMGB1 (**a**), TLR4 (**b**), and RAGE (**c**) in H I/R-induced injury. All values are stated as mean± SD (n=6). ₳ defines statistically significant related to the sham group and ¥ defines statistically significant related to the H I/R group; Φ defines statistically significant related to LIN 100 + H I/R ($p < 0.05$).

city of hepatocytes. Similarly, Chen et al[4] showed that H I/R activated Nrf2/HO-1. Preconditioning the animals with LIN resulted in even further promotion of Keap1/Nrf2/HO-1/NQO1 activation, as displayed by greater expression of Nrf2, HO-1, and NQO-1 and lower expression of Keap1. Prior studies showed that LIN increased the Nrf2 and HO-1 expression up-regulation by LPS/GalN-induced liver injury[32]. In addition, LIN induced nuclear translocation of Nrf2 and expression of HO-1 in LPS-stimulated BV2 microglia cells, suggesting that LIN reserved LPS prompted inflammation in BV2 microglia cells by stimulating Keap1/Nrf2/HO-1/NQO1signaling[45].

H I/R triggers the overproduction of ROS, which promotes lipid peroxidation and the manufacture of exceptionally destructive free radicals[4]. These free radicals exceed the hepatocytes' capability to eliminate, causing depletion of antioxidant enzymes such as CAT and GSH[5]. Hence, in the current study, ischemic animals exhibited elevated MDA, NO contents and decreased SOD, CAT, GST, and GSH content. Whereas pretreatment with LIN diminished MDA, NO contents and augmented SOD, CAT, GST, and GSH content. Previous reports[30,46-48] showed that LIN possesses an influential antioxidant activity. For instance, LIN attenuated oxidative stress facilitated by glutamate and NMDA toxicity[46], benzene-induced oxidative stress in human lymphocytes[47], and doxorubicin-induced kidney injury[48]. In another study[30], LIN significantly lowered MDA and increased GSH levels and catalase activity in the CCl_4-induced hepatotoxicity. Besides, LIN decreased MDA content and MPO activity in LPS/GalN-induced liver damage[32].

As suggested by the *in-silico* studies in literature, LIN could have an effect on the inflammatory HMGB1/TLR4/RAGE/NFκB pathway, which was proved *in vivo*. Growing evidence[16,49,50] has demonstrated that HMGB1/TLR4/RAGE/NFκB pathway is a key signaling pathway in ischemic injuries. HMGB1 is a promising therapeutic target in disease[51]. A previous study by Chen et al[52] has shown that HMGB1 exacerbated CCl_4-induced acute liver injury and that blocking or knocking down HMGB1 significantly attenuated CCl_4-induced hepatic injury. HMGB1 binds to multiple

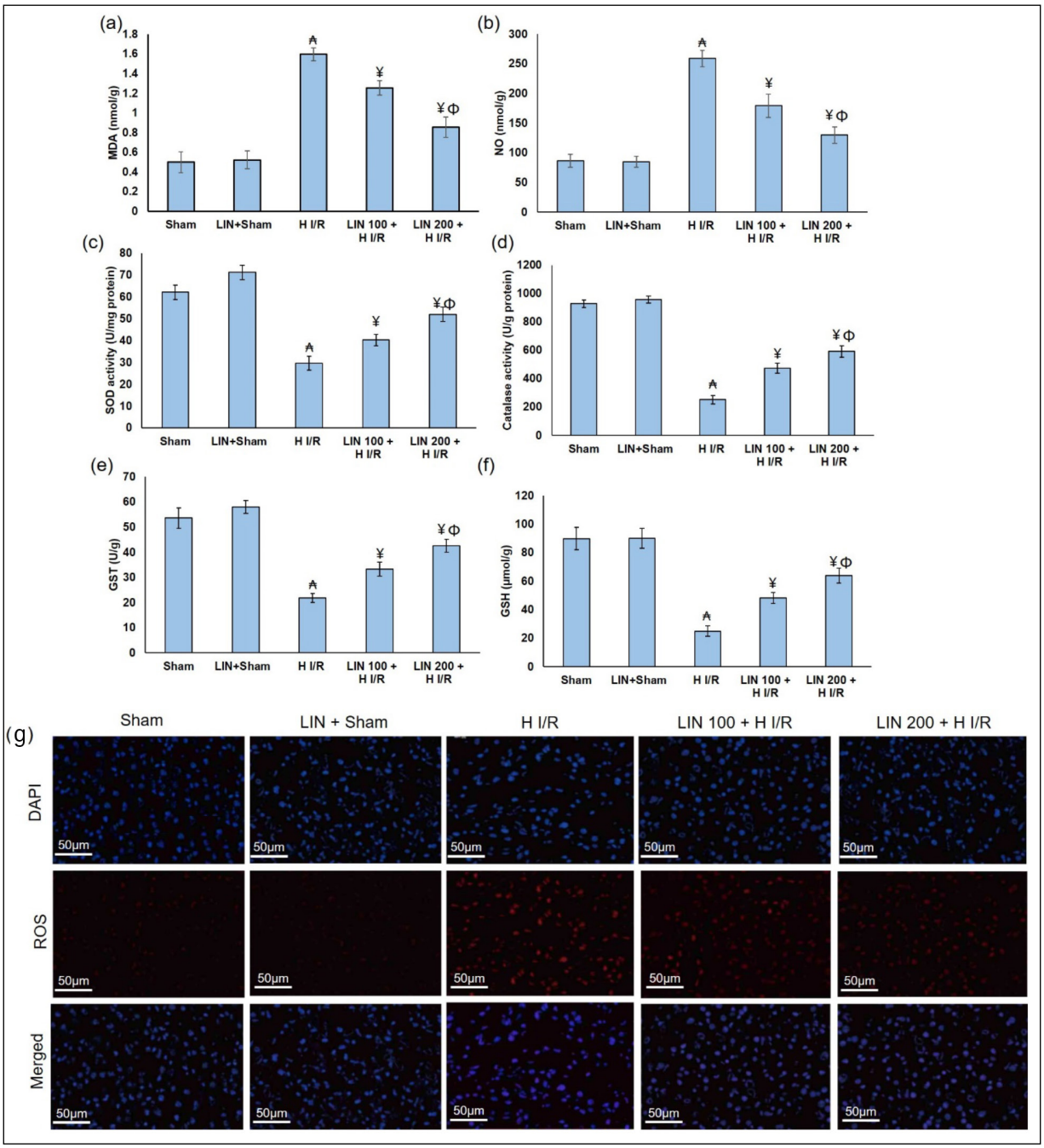

Figure 8. The actions of LIN (100 and 200 mg/kg) preconditioning for 2 weeks prior to H I/R on MDA (**a**), NO (**b**), SOD (**c**), CAT (**d**), GST (**e**), GHS (**f**) and ROS (**g**) in hepatic tissue. All values are stated as mean ± SD (n=6). ₳ defines statistically significant related to the sham group, and ¥ defines statistically significant related to the H I/R group; Φ defines statistically significant related to LIN 100 + H I/R ($p < 0.05$).

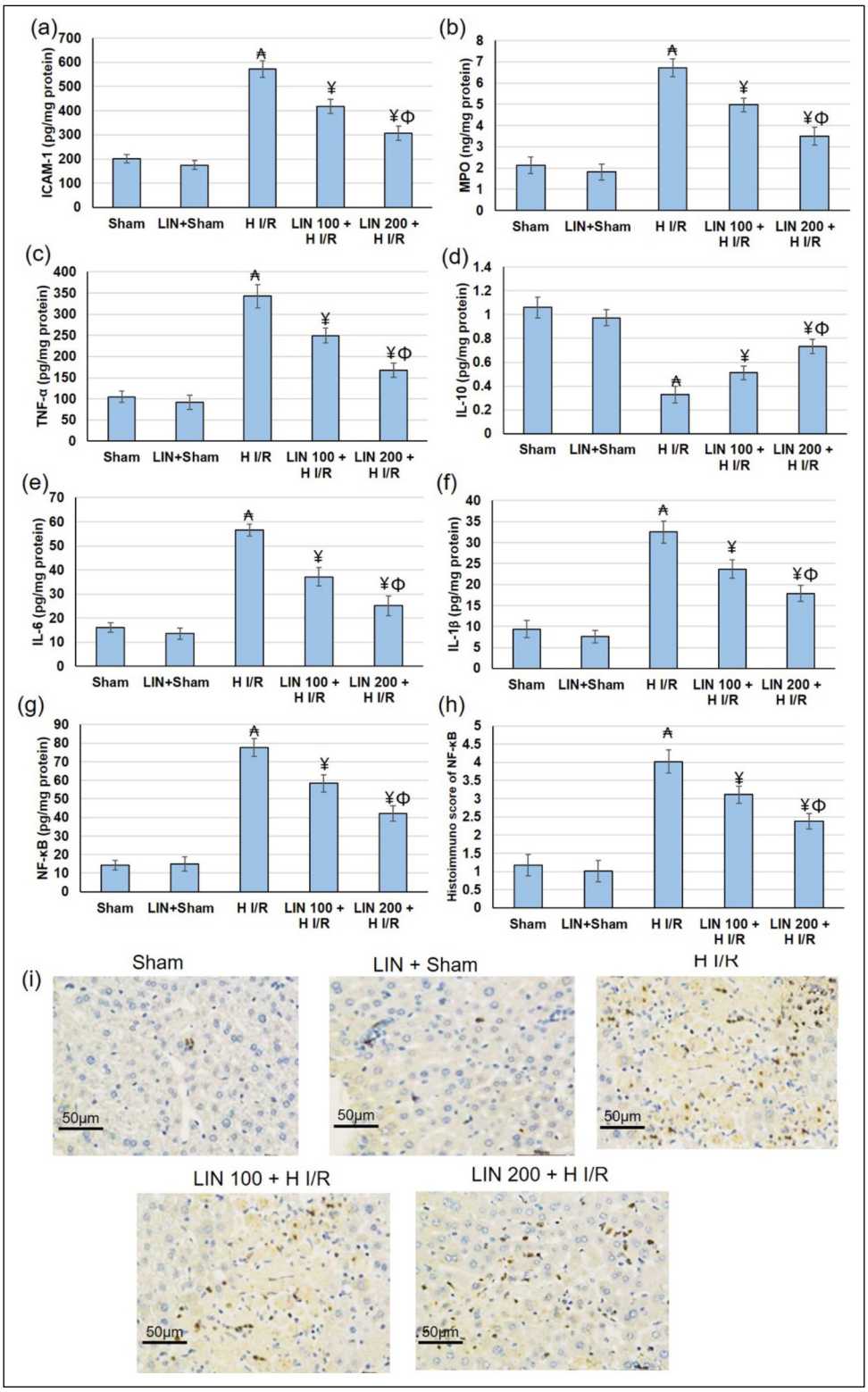

Figure 9. The actions of LIN (100 and 200 mg/kg) preconditioning for 2 weeks prior to hepatic H I/R on the levels of ICAM-1 (**a**), MPO (**b**), TNF-α (**c**), IL-10 (**d**), IL-6 (**e**), IL-1β (**f**), NFκB (**g**) and NFκB scoring from IHC (**h**). **i,** IHC photos showing NFκB positive cells. All values are stated as mean ± SD (n=6). ₳ defines statistically significant related to the sham group and ¥ defines statistically significant related to the H I/R group; Φ defines statistically significant related to LIN 100 + H I/R ($p < 0.05$).

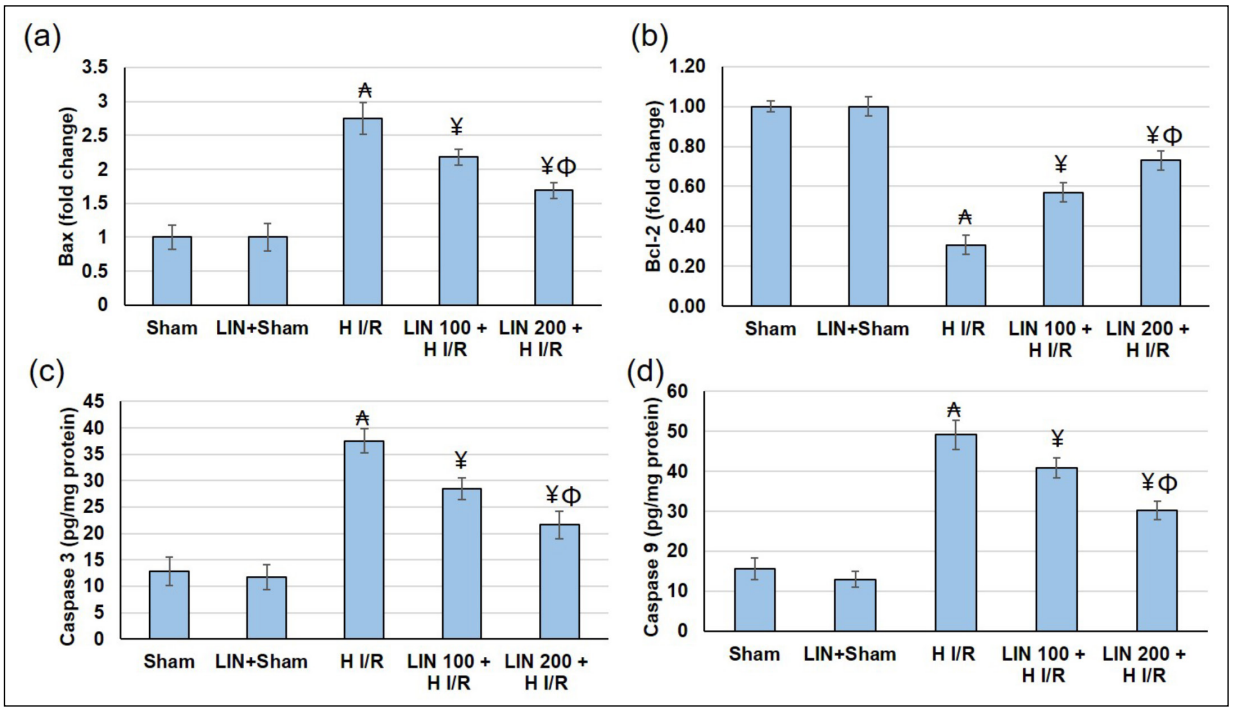

Figure 10. The actions of LIN (100 and 200 mg/kg) preconditioning for 2 weeks prior to H I/R on the gene expression levels of Bax (**a**), Bcl2 (**b**), and protein contents of caspase 3 (**c**), caspase 9 (**d**). All values are stated as mean± SD (*n=6*). ₳ defines statistically significant related to the sham group and ¥ defines statistically significant related to the H I/R group; Φ defines statistically significant related to LIN 100 + H I/R ($p < 0.05$).

receptors, such as TLRs and RAGE, which triggers NFκB signaling pathway. The NFκB stimulates the inflammatory cytokines. Hence, blocking this pathway may be used to protect against I/R damage. The H I/R animals, in the hereby study, displayed elevated gene expression of HMGB1, TLR4, and RAGE. Indeed, preconditioning the animals with LIN earlier than the hepatic ischemia diminished the HMGB1/TLR4/RAGE/NFκB pathway as demonstrated by the depressed gene expression of HMGB1, TLR4, and RAGE and lowered activity of NFκB. LIN limited endotoxin-associated weight loss by mitigating HMGB-1, TLR4, and RAGE, with subsequent minimized inflammatory cytokines[53].

H I/R animals exhibited prompted ICAM-1, which contributed to the neutrophil infiltration as indicated by amplified MPO levels. Besides, H I/R animals experienced an intensification in diverse inflammatory mediators, containing IL-1β, IL-6, TNF-α, and NFκB, and depressed IL-10. At the same time, LIN pre-administration prevented the H I/R-induced ICAM-1, MPO, IL-1β, IL-6, TNF-α, and NFκB elevations and elevated IL-10. Numerous studies in literature have demonstrated the anti-inflammatory actions of LIN. For instance, LIN lessened TNF-α and IL-6 and iNOS and COX-2 expression, by deterring NFκB in LPS/GalN-associated hepatic injury[32]. Likewise, LIN attenuated cigarette smoking-associated pulmonary inflammation, inhibited inflammatory cell infiltration and MCP-1 production, and suppressed NFκB activation[23]. Furthermore, LIN inhibited UVB- induced skin damage by deterring the phosphorylation of ERK1, JNK, and p38 proteins of the MAPK family, inhibiting NFκB[54]. LIN inhibited LPS-prompted TNF-α, IL-1β, NO, and PGE2 production and NFκB activation in LPS-stimulated BV2 microglia cells[45].

As a result of augmenting Keap1/Nrf2/HO-1/NQO1 and deterring HMGB1/TLR4/RAGE/NFκB signaling pathway, LIN caused apoptosis moderation as established by the depressed caspase 3 and 9 and Bax and amplified Bcl2. Similarly, management of LIN amplified Bcl-2 expression and reserved caspase-3 and caspase-8 in LPS/GalN-associated liver damage[32].

Conclusions

This study demonstrated the possible efficacy of LIN as a protective compound against

H I/R-induced liver damage. LIN protected the hepatocytes against H I/R by activating Keap1/Nrf2/HO-1/NQO-1 pathway as well as mitigating the HMGB1/TLR4/RAGE/NFκB pathway. Subsequently, LIN alleviated liver function enzyme augmentation and ameliorated oxidative stress status, mitigated the inflammatory, and apoptotic markers. This study adds to the scientific pool of the pharmacological actions of the natural monoterpene LIN, showing LIN as a protective agent in liver ischemic/reperfusion and associated conditions.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Ethics Approval

The Institutional Animal Care and Use Committee at King Faisal University (KFU) permitted the study protocol (KFU-REC-2022-FEB-EA000418). Different experiments were performed in harmony with the measures and guidelines of the Ethical Conduct for the Use of Animals in Research at KFU.

Informed Consent

Not applicable as no humans or any organizations were involved in this study.

Availability of Data and Materials

All the data generated in this study is available on request from the corresponding author.

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Authors' Contributions

Conceptualization was carried out by all authors, M.E. Mohamed, R.M. Abdelnaby, N.S. Younis; methodology, N.S. Younis, M.E. Mohamed; software, R.M. Abdelnaby; validation, N.S. Younis, M.E. Mohamed; formal analysis, N.S. Younis; resources, M.E. Mohamed; data curation, N.S. Younis, M.E. Mohamed; 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 acquisition, M.E. Mohamed. All authors have read and agreed to the published version of the manuscript.

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References

1) Zhou Y, Tan Z, Huang H, Zeng Y, Chen S, Wei J, Huang G, Qian C, Yuan G, He S. Baicalein pre-treatment alleviates hepatic ischemia/reperfusion injury in mice by regulating the Nrf2/ARE pathway. Exp Ther Med 2021; 22: 1380.
2) Xiong L, Yu KH, Zhen SQ. MiR-93 blocks STAT3 to alleviate hepatic injury after ischemia-reperfusion. Eur Rev Med Pharmacol Sci 2018; 22: 5295-5304.
3) Aydin I, Sehitoglu I, Ozer E, Kalkan Y, Tumkaya L, Cure MC, Cure E. High dose zoledronic acid increases ischemia-reperfusion damage of the liver. Eur Rev Med Pharmacol Sci 2021; 25: 3567-3575.
4) Chen L, Zhang WL, Xie DQ, Jia W. Sulforaphane alleviates hepatic ischemia-reperfusion injury through promoting the activation of Nrf-2/HO-1 signaling. Transpl Immunol 2021; 68: 101439.
5) Elias-Miró M, Jiménez-Castro MB, Rodés J, Peralta C. Current knowledge on oxidative stress in hepatic ischemia/reperfusion. Free Radic Res 2013; 47: 555-568.
6) Bilecik T, Karateke F, Elkan H, Gokce H. The effects of TRPM2, TRPM6, TRPM7 and TRPM8 gene expression in hepatic ischemia reperfusion injury. Eur Rev Med Pharmacol Sci 2019; 23: 3088-3095.
7) Xue R, Qiu J, Wei S, Liu M, Wang Q, Wang P, Sha B, Wang H, Shi Y, Zhou J, Rao J, Lu L. Lycopene alleviates hepatic ischemia reperfusion injury via the Nrf2/HO-1 pathway mediated NLRP3 inflammasome inhibition in Kupffer cells. Ann Transl Med 2021; 9: 631.
8) Ke B, Shen XD, Zhang Y, Ji H, Gao F, Yue S, Kamo N, Zhai Y, Yamamoto M, Busuttil RW, Kupiec-Weglinski JW. KEAP1-NRF2 complex in ischemia-induced hepatocellular damage of mouse liver transplants. J Hepatol 2013; 59: 1200-1207.
9) R GB, Panisello-Roselló A, Sanchez-Nuno S, Alva N, Roselló-Catafau J, Carbonell T. Nrf2 and oxidative stress in liver ischemia/reperfusion injury. Febs j 2021.

10) Hassanein EHM, Khader HF, Elmansy RA, Seleem HS, Elfiky M, Mohammedsaleh ZM, Ali FEM, Abd-Elhamid TH. Umbelliferone alleviates hepatic ischemia/reperfusion-induced oxidative stress injury via targeting Keap-1/Nrf-2/ARE and TLR4/NF-κB-p65 signaling pathway. Environ Sci Pollut Res Int 2021; 28: 67863-67879.

11) Liu X, Huang K, Zhang RJ, Mei D, Zhang B. Isochlorogenic Acid A Attenuates the Progression of Liver Fibrosis Through Regulating HMGB1/TLR4/NF-κB Signaling Pathway. Front Pharmacol 2020; 11: 582.

12) El-Marasy SA, El Awdan SA, Abd-Elsalam RM. Protective role of chrysin on thioacetamide-induced hepatic encephalopathy in rats. Chem Biol Interact 2019; 299: 111-119.

13) Javaherian K, Liu JF, Wang JC. Nonhistone proteins HMG1 and HMG2 change the DNA helical structure. Science 1978; 199: 1345-1346.

14) Tsung A, Hoffman RA, Izuishi K, Critchlow ND, Nakao A, Chan MH, Lotze MT, Geller DA, Billiar TR. Hepatic ischemia/reperfusion injury involves functional TLR4 signaling in nonparenchymal cells. J Immunol 2005; 175: 7661-7668.

15) Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. J Biol Chem 1995; 270: 25752-25761.

16) Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005; 201: 1135-1143.

17) Wu H, Ma J, Wang P, Corpuz TM, Panchapakesan U, Wyburn KR, Chadban SJ. HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol 2010; 21: 1878-1890.

18) Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke T, Sato Y, Hiraga N, Adachi N, Yoshino T, Nishibori M. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. Faseb j 2007; 21: 3904-3916.

19) Andrassy M, Volz HC, Igwe JC, Funke B, Eichberger SN, Kaya Z, Buss S, Autschbach F, Pleger ST, Lukic IK, Bea F, Hardt SE, Humpert PM, Bianchi ME, Mairbäurl H, Nawroth PP, Remppis A, Katus HA, Bierhaus A. High-mobility group box-1 in ischemia-reperfusion injury of the heart. Circulation 2008; 117: 3216-3226.

20) Chang MY, Shen YL. Linalool exhibits cytotoxic effects by activating antitumor immunity. Molecules 2014; 19: 6694-6706.

21) Liu X, Cai J, Chen H, Zhong Q, Hou Y, Chen W, Chen W. Antibacterial activity and mechanism of linalool against Pseudomonas aeruginosa. Microb Pathog 2020; 141: 103980.

22) Batista PA, Werner MF, Oliveira EC, Burgos L, Pereira P, Brum LF, Story GM, Santos AR. The antinociceptive effect of (-)-linalool in models of chronic inflammatory and neuropathic hypersensitivity in mice. J Pain 2010; 11: 1222-1229.

23) Ma J, Xu H, Wu J, Qu C, Sun F, Xu S. Linalool inhibits cigarette smoke-induced lung inflammation by inhibiting NF-κB activation. Int Immunopharmacol 2015; 29: 708-713.

24) Yuan C, Shin M, Park Y, Choi B, Jang S, Lim C, Yun HS, Lee IS, Won SY, Cho KS. Linalool Alleviates Aβ42-Induced Neurodegeneration via Suppressing ROS Production and Inflammation in Fly and Rat Models of Alzheimer's Disease. Oxid Med Cell Longev 2021; 2021: 8887716.

25) Tekeli İ O, Ateşşahin A, Sakin F, Aslan A, Çeribaşı S, Yipel M. Protective effects of conventional and colon-targeted lycopene and linalool on ulcerative colitis induced by acetic acid in rats. Inflammopharmacology 2018.

26) Kim MG, Kim SM, Min JH, Kwon OK, Park MH, Park JW, Ahn HI, Hwang JY, Oh SR, Lee JW, Ahn KS. Anti-inflammatory effects of linalool on ovalbumin-induced pulmonary inflammation. Int Immunopharmacol 2019; 74: 105706.

27) Mohamed ME, Abduldaium YS, Younis NS. Ameliorative Effect of Linalool in Cisplatin-Induced Nephrotoxicity: The Role of HMGB1/TLR4/NF-κB and Nrf2/HO1 Pathways. Biomolecules 2020; 10.

28) Park H, Seol GH, Ryu S, Choi IY. Neuroprotective effects of (-)-linalool against oxygen-glucose deprivation-induced neuronal injury. Arch Pharm Res 2016; 39: 555-564.

29) Mehri S, Meshki MA, Hosseinzadeh H. Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats. Drug Chem Toxicol 2015; 38: 162-166.

30) Altınok-Yipel F, Ozan Tekeli İ, Özsoy Ş Y, Güvenç M, Kaya A, Yipel M. Hepatoprotective Activity of Linalool in Rats Against Liver Injury Induced by Carbon Tetrachloride. Int J Vitam Nutr Res 2020; 90: 302-308.

31) Mazani M, Rezagholizadeh L, Shamsi S, Mahdavifard S, Ojarudi M, Salimnejad R, Salimi A. Protection of CCl(4)-induced hepatic and renal damage by linalool. Drug Chem Toxicol 2022; 45: 963-971.

32) Li J, Zhang X, Huang H. Protective effect of linalool against lipopolysaccharide/D-galactosamine-induced liver injury in mice. Int Immunopharmacol 2014; 23: 523-529.

33) Mohamed ME, Abdelnaby RM, Younis NS. β-caryophyllene ameliorates hepatic ischemia reperfusion-induced injury: the involvement of Keap1/Nrf2/HO 1/NQO 1 and TLR4/NF-κB/NLRP3 signaling pathways. Eur Rev Med Pharmacol Sci 2022; 26: 8551-8566.

34) Mohamed ME, Abduldaium MS, Younis NS. Cardioprotective Effect of Linalool against Isoproterenol-Induced Myocardial Infarction. Life (Basel) 2021; 11.

35) Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation 1993; 55: 1265-1272.

36) Perkins ND. Integrating cell-signalling pathways with NF-κB and IKK function. Nature Reviews Molecular Cell Biology 2007; 8: 49-62.

37) Sahu BD, Tatireddy S, Koneru M, Borkar RM, Kumar JM, Kuncha M, R S, R SS, Sistla R. Naringin ameliorates gentamicin-induced nephrotoxicity and associated mitochondrial dysfunction, apoptosis and inflammation in rats: Possible mechanism of nephroprotection. Toxicology and Applied Pharmacology 2014; 277: 8-20.

38) Billod J-M, Lacetera A, Guzmán-Caldentey J, Martín-Santamaría S. Computational Approaches to Toll-Like Receptor 4 Modulation. Molecules 2016; 21: 994.

39) Padhi S, Sanjukta S, Chourasia R, Labala RK, Singh SP, Rai AK. A Multifunctional Peptide From Bacillus Fermented Soybean for Effective Inhibition of SARS-CoV-2 S1 Receptor Binding Domain and Modulation of Toll Like Receptor 4: A Molecular Docking Study. Front Mol Biosci 2021; 8: 636647.

40) Yadav D, Nath Mishra B, Khan F. 3D-QSAR and docking studies on ursolic acid derivatives for anticancer activity based on bladder cell line T24 targeting NF-kB pathway inhibition. J Biomol Struct Dyn 2019; 37: 3822-3837.

41) Purawarga Matada GS, Dhiwar PS, Abbas N, Singh E, Ghara A, Das A, Bhargava SV. Molecular docking and molecular dynamic studies: screening of phytochemicals against EGFR, HER2, estrogen and NF-KB receptors for their potential use in breast cancer. J Biomol Struct Dyn 2022; 40: 6183-6192.

42) Davies TG, Wixted WE, Coyle JE, Griffiths-Jones C, Hearn K, McMenamin R, Norton D, Rich SJ, Richardson C, Saxty G, Willems HM, Woolford AJ, Cottom JE, Kou JP, Yonchuk JG, Feldser HG, Sanchez Y, Foley JP, Bolognese BJ, Logan G, Podolin PL, Yan H, Callahan JF, Heightman TD, Kerns JK. Monoacidic Inhibitors of the Kelch-like ECH-Associated Protein 1: Nuclear Factor Erythroid 2-Related Factor 2 (KEAP1:NRF2) Protein-Protein Interaction with High Cell Potency Identified by Fragment-Based Discovery. J Med Chem 2016; 59: 3991-4006.

43) Agustin AT, Safitri A, Fatchiyah F. An in Silico Approach Reveals the Potential Function of Cyanidin-3-o-glucoside of Red Rice in Inhibiting the Advanced Glycation End Products (AGES)-Receptor (RAGE) Signaling Pathway. Acta Inform Med 2020; 28: 170-179.

44) Sima LJ, Ma XW. Effect of sevoflurane on hepatic ischemia-reperfusion injury in rats via JAK2-STAT3 pathway. Eur Rev Med Pharmacol Sci 2019; 23: 1350-1356.

45) Li Y, Lv O, Zhou F, Li Q, Wu Z, Zheng Y. Linalool Inhibits LPS-Induced Inflammation in BV2 Microglia Cells by Activating Nrf2. Neurochem Res 2015; 40: 1520-1525.

46) Sabogal-Guáqueta AM, Hobbie F, Keerthi A, Oun A, Kortholt A, Boddeke E, Dolga A. Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate and NMDA toxicity. Biomed Pharmacother 2019; 118: 109295.

47) Salimi A, Khodaparast F, Bohlooli S, Hashemidanesh N, Baghal E, Rezagholizadeh L. Linalool reverses benzene-induced cytotoxicity, oxidative stress and lysosomal/mitochondrial damages in human lymphocytes. Drug Chem Toxicol 2021: 1-9.

48) Altinoz E, Oner Z, Elbe H, Uremis N, Uremis M. Linalool exhibits therapeutic and protective effects in a rat model of doxorubicin-induced kidney injury by modulating oxidative stress. Drug Chem Toxicol 2021: 1-7.

49) Arumugam TV, Okun E, Tang SC, Thundyil J, Taylor SM, Woodruff TM. Toll-like receptors in ischemia-reperfusion injury. Shock 2009; 32: 4-16.

50) Chang WJ, Toledo-Pereyra LH. Toll-like receptor signaling in liver ischemia and reperfusion. J Invest Surg 2012; 25: 271-277.

51) Xue J, Suarez JS, Minaai M, Li S, Gaudino G, Pass HI, Carbone M, Yang H. HMGB1 as a therapeutic target in disease. J Cell Physiol 2021; 236: 3406-3419.

52) Chen M, Huang W, Wang C, Nie H, Li G, Sun T, Yang F, Zhang Y, Shu K, Wang C, Gong Q. High-mobility group box 1 exacerbates CCl_4-induced acute liver injury in mice. Clin Immunol 2014; 153: 56-63.

53) Lee SC, Wang SY, Li CC, Liu CT. Anti-inflammatory effect of cinnamaldehyde and linalool from the leaf essential oil of Cinnamomum osmophloeum Kanehira in endotoxin-induced mice. J Food Drug Anal 2018; 26: 211-220.

54) Gunaseelan S, Balupillai A, Govindasamy K, Ramasamy K, Muthusamy G, Shanmugam M, Thangaiyan R, Robert BM, Prasad Nagarajan R, Ponniresan VK, Rathinaraj P. Linalool prevents oxidative stress activated protein kinases in single UVB-exposed human skin cells. PLoS One 2017; 12: e0176699.