Acute Inflammation and Oxidative Stress Induced by Lipopolysaccharide and the Ameliorative Effect of Stingless Bee Honey

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Abstract: ***Background*:** Systemic acute inflammation is the hallmark of sepsis and is associated with multiple organ dysfunction.

***Objective*:** This study investigated the potential of stingless bee honey (SBH) to suppress lipopolysaccharide (LPS)-induced systemic acute inflammation in rats and to reveal the probable mechanism of action.

***Methods*:** Rats received 4.6 and 9.2 g/kg SBH for 7 days followed by a single injection of LPS after which blood samples were taken 6h later.

***Results*:** LPS induced liver, kidney, heart, and lung injury, were manifested by increased serum transaminases, alkaline phosphatase, creatine kinase, creatinine, and urea, along with multiple histological alterations, particularly leukocyte infiltration. Pro-inflammatory cytokines were elevated in the serum, and NF-κB p65, p38 MAPK, and HMGB-1 were significantly increased in different tissues of LPS-challenged rats. SBH prevented tissue injury, ameliorated pro-inflammatory cytokines, and suppressed NF-κB p65, p38 MAPK, and HMGB-1 in rats that had received LPS. In addition, SBH diminished reactive oxygen species (ROS) production, lipid peroxidation, and oxidative DNA damage, and enhanced glutathione and Nrf2 in LPS-treated rats.

***Conclusion*:** SBH prevents systemic acute inflammation by suppressing NF-κB, p38 MAPK, HMGB-1, oxidative stress, and tissue injury in rats. Thus, SBH may represent an effective anti-inflammatory nutraceutical, pending further mechanistic studies.

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1. INTRODUCTION

Systemic acute inflammation is the hallmark of sepsis, which is a life-threatening complication associated with single or multiple organ dysfunction [1]. Although antibiotic therapy, along with critical care management, has been improved recently in intensive care units, the number of victims due to sepsis has been estimated to be approximately 225,000 in the U.S. alone [2]. The onset of sepsis among intensive care units' patients is predicted to be elevated, suggesting a high mortality rate in the next twenty years [3]. One of the main contributors to systemic acute inflammation accompanied by sepsis is bacterial endotoxemia which translocates from the compromised gut lumen into the bloodstream. Since a normal colon contains a large population of microorganisms, lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, exists in high levels in the gut lumen [4]. The translocation of LPS, together with LPS binding protein (LBP) and CD14, into the circulation, forms a complex with toll-like receptor 4 (TLR-4). This complex triggers the influx of pro-inflammatory cytokines and mediators via the downstream signaling pathway of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase

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(MAPK). Interestingly, the expression of TLR-4 exists not only in innate immune cells but also in various cell systems, including cardiomyocytes, hepatocytes, alveolar cells, and glomeruli [5-8]. Thus, the production of pro-inflammatory cytokines and mediators, including TNF-α, IL-1β, IL-6, IL-8, and MCP-1 is possible in the heart, liver, lung, and kidney. Furthermore, the unregulated production of pro-inflammatory mediators and cytokines results in injury to the host cells and organs. At the same time, there is an implication that oxidative stress, mediated by reactive oxygen species (ROS) in the systemic inflammatory injury that has been induced by LPS regulates the levels of NF-κB activity [9]. Ultimately, anti-LPS drugs have been thought to be specific for a single class of bacteria with limited results, therefore, the inhibition of pro-inflammatory cytokines and mediators remains a possible measurement to ameliorate systemic acute inflammation induced by endotoxin [10]. Hence, identifying a nutritional therapeutic agent that would ameliorate excessive production of pro-inflammatory mediators post systemic inflammation, improve the clearance of endotoxin, protect tissues and organs from oxidative injury, preserve hemostasis and increase survival is required.

Honey has been utilized in ancient and modern civilizations as a natural product with nutritional and functional applications. Besides its sweetening function, honey is considered as a source of valuable nutrients with medicinal benefits. For this reason, honey has been implicated in various social roles and religious ceremonies as oblations for deities [11]. The preferability of using honey as a natural therapy for a wide range of maladies is termed as apitherapy [12]. With this intention, honey has been thought of like a wagon for transporting medicinal plants' compounds. The therapeutic actions of honey have been attributed to its bioactive compounds, including phenolic acids and flavonoids [13], which possess potent antioxidant and anti-inflammatory activities [14-18]. However, the botanical and geographical areas along with the type of bees contribute mainly in determining the biological composition which subsequently affects the pharmacological activities [19]. In this sense, the presence of various flowers in Southeast Asia, Australia, and South America all year round is considered to be the main reason behind the presence of different kinds of honeybee. One of those is the stingless bee, which belongs to the tribe of *Meliponini*. Since tropical and sub-tropical areas are the native place of stingless bees, the *Trigona* spp. is found in Malaysia and its common name is the "*Kelulut*" [20].

Stingless bee honey (SBH) has been traditionally consumed for various medical purposes. SBH has a distinct viscosity, clearer color, and a sour-like taste [13]. Recently, analytical food studies have shown that SBH contains higher concentrations of phenolic and flavonoid contents than other types of honey [11, 21]. Thus, SBH is gaining more attention globally as a potential therapy for clinical use. Previously, SBH has been proven to act as an antibacterial, antioxidant, and anti-cancer agent [22-24]. *In vivo*, SBH reduces the levels of ROS and myeloperoxidase (MPO) in inflammatory ear tissue of mice [25]. We have recently reported the anti-inflammatory activity of SBH in a murine model of chronic systemic inflammation [26]. The present study aimed to investigate the preventive effect of SBH on systemic acute inflammation and oxidative stress induced by LPS in an *in vivo* rat model.

2. MATERIALS AND METHODS

2.1. Reagents and Kits

LPS (derived from *Escherichia coli* 055:B5) was supplied by Sigma-Aldrich (St. Louis, USA). TNF-α, NO, IL-6, IL-1β, GSH, IL-8, GST, GPx, 8-OHdG, ROS, and HMGB-1 ELISA kits were supplied by Melsin Medical Co. (China). ELISA kits for MCP-1, Nrf2, NF-κB p65, and p38 MAPK were supplied by Fine Biotech (China).

2.2. Experimental Animals and Treatments

Forty-two pathogen-free Sprague-Dawley (SD) rats, weighing 250-270 g, were housed under standard laboratory conditions. The animals were given standard rodent chow and water *ad libtium*. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University Putra Malaysia (UPM/IACUC/AUP-R007/2017).

After acclimatization for two weeks, the rats were randomized into seven groups, each comprising 6 animals (n = 6). Groups 1 and 4 rats were treated orally with 1 ml of distilled water, while groups 2,3,5 and 6 received one of the two different concentrations of SBH: 4.6 g/kg/day or 9.3 g/kg/day by gavage in a 1 ml volume. Group 7 rats were injected through the i.p route with 5 mg/kg dexamethasone diluted in phosphate- buffered saline (PBS) 18 h before LPS injection [27]. All the groups, except for group 7, received treatment at 10:00 am for seven successive days by oral gavage. LPS was dissolved in sterile PBS. On the seventh day and 1 h after the last treatment, groups 4, 5, 6, and 7 were injected intraperitoneally with LPS (5 mg/kg) in a volume of 2 ml while groups 1, 2, and 3 were injected with the exact volume of saline that was used for the LPS injections.

Fresh Malaysian SBH, produced by *Trigona* bees, was obtained from local honeybee collectors from the forest of Johor Bahru state. The honey sample was stored at 15°C in sterile air-tight glass bottles. The composition of the honey has been described in our previous research [28]. SBH has ameliorated chronic subclinical systemic inflammation [26]. Based on that, the same dosage of SBH was used in the current research. Therefore, SBH was given to the rats at two doses: 9.3 g/kg/day or 4.6 g/kg/day by oral gavage. Body weight and water and food intake were recorded daily thorough the experiment.

2.3. Sample Collection and Preparation

6 h after LPS injection, the rats were euthanized by sodium pentobarbital (75 mg/kg) to obtain blood via cardiac puncture and the serum was isolated and stored at -80°C for analysis. Lung, heart, kidney and liver tissues were homogenized (10%, w/v) in 50 mM NaH_2PO_4 buffer containing 1mM EDTA and 1% Triton-X (pH = 7.5) on ice. The homo-

genates of the organs were centrifuged at 10,000 g for 20 min at 4°C. The supernatants were collected and stored at -80°C until used for biochemical assays. The protein content in the tissue homogenates was estimated according to the method of Lowry *et al.* [29]. Samples from the collected tissues were immediately placed in 10% buffered formalin.

2.4. Biochemical Assays

Creatinine, urea, creatine kinase (CK), ALT, AST, and ALP were assayed in serum using a Hitachi 900 Auto Analyzer (Roche Diagnostics, Switzerland). TNF-α, IL-1β, IL-6, IL-8, NO, GSH, GST, GPx, 8-OHdG, MDA, MCP-1, and ROS levels in serum were measured using ELISA kits following the manufacturers' instructions. Nrf2, HMGB-1, NF-κB p65, and p38 MAPK were assayed in tissue homogenates using specific ELISA kits.

2.5. Histological Evaluation

Lung, heart, kidney, and liver tissue samples were fixed in 10% buffered formalin for 24 h and then processed for paraffin embedding. 5 μm sections were cut and processed for hematoxylin and eosin (H&E) staining. The pathological changes were examined using an Olympus light microscope BX40 (Olympus Optical Co., Japan). Inflammatory scoring was conducted based on the following criteria; score 0 = normal tissue and scores 1, 2, 3, and 4 showed that inflammation was present in less than 25%, 50%, 75%, and more than 75% of the field of view, respectively.

2.6. Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). All data were analyzed using one-way ANOVA followed by Tukey's test on GraphPad Prism 7 (GraphPad Software Inc., CA, USA). The level of significance was considered when $P < 0.05$ for all statistical tests.

3. Results

3.1. SBH Ameliorates Liver, Heart, and Kidney Functions in LPS-Treated Rats

The ability of SBH to improve liver, heart, and kidney function in LPS-treated rats was evaluated via determination of serum ALT, AST, ALP, CK, creatinine, and urea levels. As depicted in Figs. (**1A-1C**), the circulating levels of ALT, AST, and ALP were increased ($P<0.05$) in LPS-treated rats. Pre-treatment of LPS-challenged rats with SBH decreased ALT, AST, and ALP. Serum CK was increased in LPS-treated rats, an effect that was reversed in SBH treated groups (Fig. **1D**). Creatinine (Fig. **1E**) and urea (Fig. **1F**) were elevated in LPS-treated rats, whereas SBH treatment prevented the effect of LPS on these kidney function markers. DEX (5 mg) significantly ameliorated liver, heart, and kidney function in LPS-treated rats. Of note is that control rats receiving SBH alone showed no changes in serum ALT, AST, ALP, CK, creatinine, and urea levels.

3.2. SBH Prevents LPS-Induced Histopathological Changes

To assess the anti-inflammatory potential of SBH histologically, liver, kidney, heart, and lung H&E-stained sections from each experimental group were examined. Investigation of the liver of the control (Fig. **2A**) and 9.3 g/kg SBH-treated rats (Fig. **2B**) showed normal hepatic lobules consisting of central veins and radiating hepatic cells separated by regular blood sinusoids. In comparison, the liver of rats inoculated with LPS had various pronounced histopathological changes, including congestion, areas of necrosis, and a macro-abscess involving hepatocytes and inflammatory cells with necrotic debris (Fig. **2C**). Significant reduction in the hepatotoxicity score, including minimal inflammatory foci found in two regions of the field along with small, congested blood vessels was observed in LPS-challenged rats treated with 4.6 g/kg SBH (Fig. **2D**). In addition, treatment with 9.3 g/kg SBH (Fig. **2E**) or DEX (5mg) (Fig. **2F**) markedly reduced LPS-induced liver injury.

The renal histological micrographs in control (Fig. **3A**) and 9.3 g/kg SBH-treated rats (Fig. **3B**) demonstrated normal glomeruli, healthy proximal tubules, and distinct capsular space. Modest glomerular atrophy, signs of necrosis, vacuolar damage of tubular cells, interstitial red blood cells (RBCs), and tubulointerstitial inflammatory cells infiltration were found in many regions of kidney tissue of LPS-treated rats (Fig. **3C**). Treatment with 4.6 g/kg (Fig. **3D**) and 9.3 g/kg SBH (Fig. **3E**) or DEX (5 mg) (Fig. **3E**) ameliorated LPS-induced kidney injury.

Microscopic examination of the heart micrographs of control (Fig. **4A**) and the 9.3 g/kg SBH-treated group (Fig. **4B**) revealed normal cardiomyocytes without vascular or inflammatory changes. LPS-inoculated rats showed tissue injury, infiltration of leukocyte, and congested blood vessels in the heart muscle (Fig. **4C**). Significant improvement of the heart histology with small foci of inflammatory infiltration along with small congested blood vessels was observed in LPS-challenged rats which had received 4.6 g/kg (Fig. **4D**), or 9.3 g/kg SBH (Fig. **4E**) or DEX (Fig. **4F**).

The control (Fig. **5A**) and 9.3 g/kg SBH-treated group (Fig. **5B**) of rats showed healthy and normal morphology of alveolar capillaries. In contrast, LPS-challenged rats showed remarkable damage, leukocyte infiltration into alveolar and interstitial spaces, edema, and increased alveolar wall thickness (Fig. **5C**). These histopathological changes were less pronounced in rats treated with 4.6 g/kg (Fig. 5D), 9.3 g/kg SBH (Fig. **5E**), and DEX (Fig. **5F**).

Analysis of the inflammation score revealed a significant increase in all tissues of LPS-treated rats (Fig. **6**). Treatment with SBH or DEX significantly prevented LPS-induced inflammation in the liver, kidney, heart, and lung of rats ($P<0.05$).

3.3. SBH Reduces Pro-Inflammatory Mediators in LPS-Challenged Rats

CRP, MCP-1, TNF-α, IL-1β, IL-6, and IL-8 were increased in LPS-challenged rats when compared with the con-

trol group (P<0.05; Fig. **7**). In contrast, rats treated with SBH before the LPS injection showed significant decreases in all assayed mediators. However, the lower dose of SBH exerted a non-significant effect on serum MCP-1. The levels of all assayed cytokines and mediators were markedly reduced upon treatment with DEX (5 mg). Meanwhile, control and SBH-treated rats had normal levels of inflammatory mediators and cytokines (Fig.**7**).

3.4. SBH Suppresses NF-κB p65, p38 MAPK and HMGB-1 in LPS-Challenged Rats

To determine the mechanism behind neutralizing the pro-inflammatory cytokines and mediators by SBH, the levels of NF-κB p65, p38 MAPK, and HMGB-1 were measured in LPS-challenged rats. The results showed an increase in NF-κB p65 (Fig. **8B**), p38 MAPK (Fig. **8B**), and HMGB-1 (Fig. **8C**) protein levels in different tissues of LPS-challenged rats (P<0.05). The lower dose of SBH significantly reduced NF-κB p65 levels in the liver, kidney, and lung, but not in the heart of LPS-challenged rats (Fig. **8A**). The same dose ameliorated hepatic p38 MAPK (Fig. **8B**) and HMGB-1 (Fig. **8C**) in both the heart and lung of LPS-treated rats. On the other hand, the higher dose of SBH and DEX significantly ameliorated NF-κB p65, p38 MAPK, and HMGB-1 levels in LPS-treated rats.

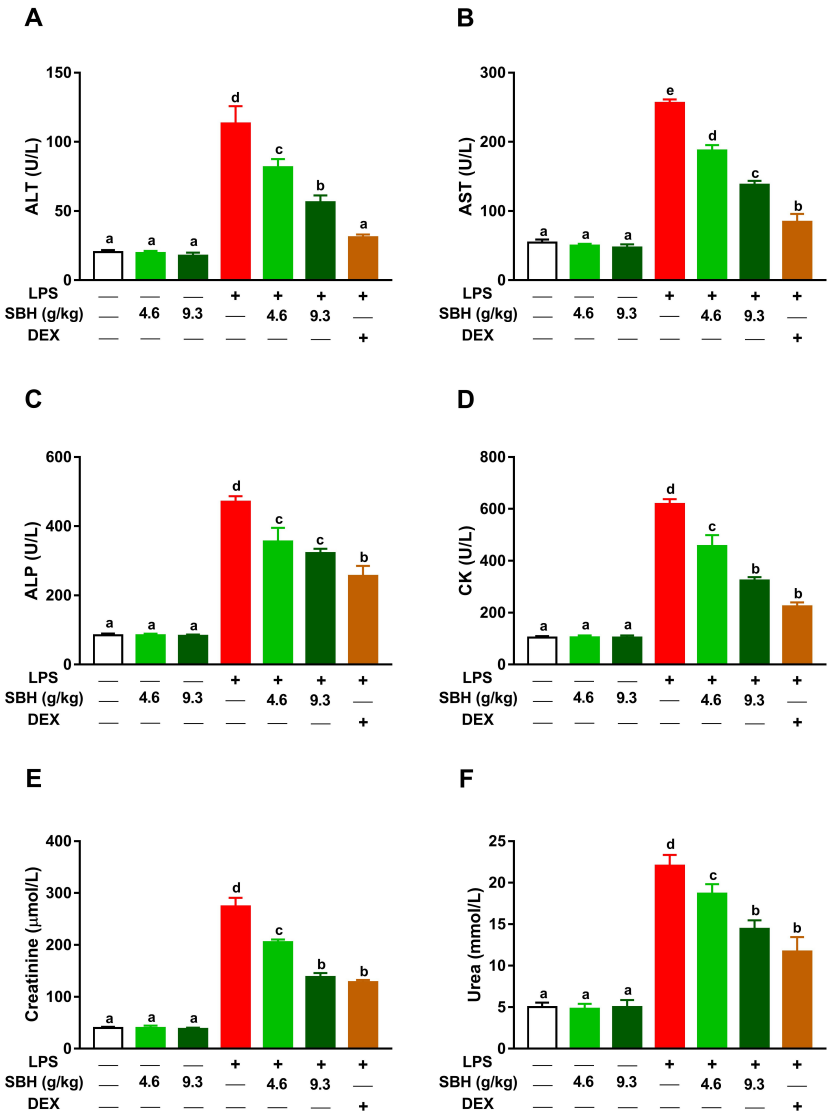

Fig. (1). SBH ameliorates liver, heart, and kidney function disturbances in LPS-treated rats. Treatment with SBH or DEX significantly decreased serum ALT (**A**), AST (**B**), ALP (**C**), CK (**D**), creatinine (**E**), and urea in LPS-induced rats. Data are shown as mean ± SEM (*n*=6). Significantly different values are indicated by different letters. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

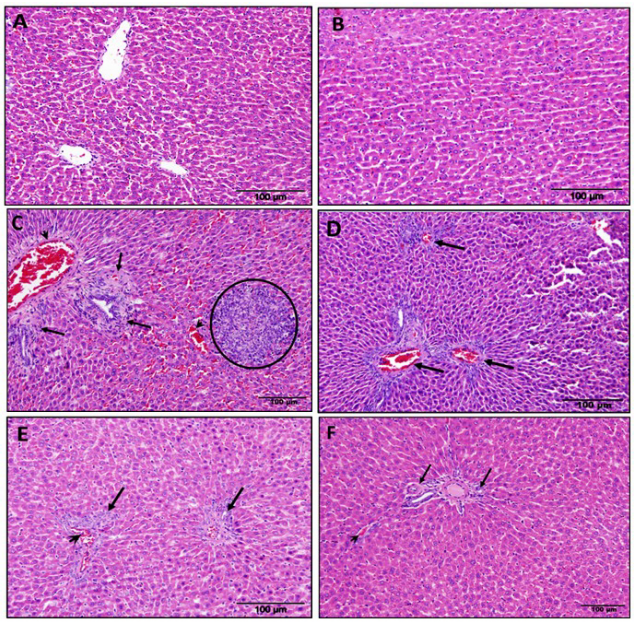

Fig. (2). SBH attenuates LPS-induced liver injury in rats. Photomicrographs of liver from (**A**) control and (**B**) 9.3 g/kg SBH-treated rats showing normal hepatic lobules consisting of central vein and radiating hepatic cells separated by regular blood sinusoids, (**C**) LPS-treated rats' liver showing congestion (arrowheads), necrosis (arrows), and a macro-abscess (circle) involving hepatocyte and inflammatory cells with necrotic debris, (**D**) LPS-induced liver damage in rats treated with 4.6 g/Kg SBH showing three inflammatory foci of leukocytes with vascular congestion in the central veins (arrows), (**E**) LPS-challenged rats treated with 9.3 g/Kg SBH showing minimal inflammatory foci in two regions of the field (arrows), and (**F**) LPS-induced damage in rats treated with DEX (5 mg) showing a minimal infiltration of inflammatory leukocytes (arrows). (H&E; x200). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

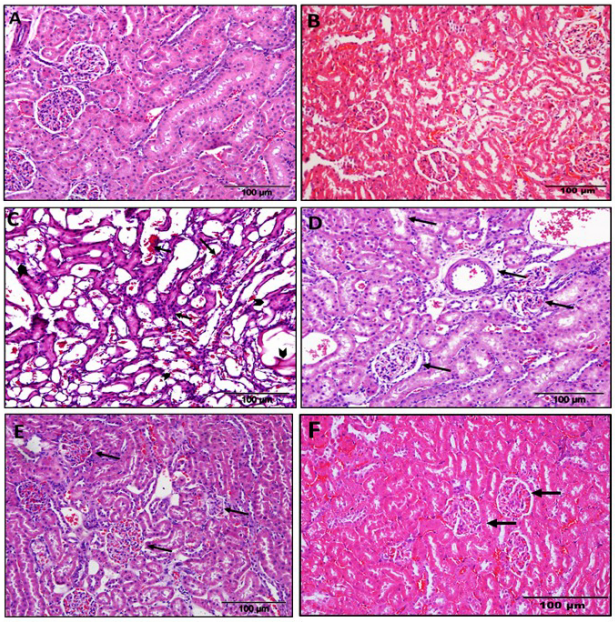

Fig. (3). SBH prevents LPS-induced kidney injury in rats. Photomicrographs of the kidney from (**A**) control and (**B**) 9.3 g/kg-supplemented rats showing normal structures, (**C**) LPS-induced kidney changes in rats showing vacuolar damage, necrosis (arrowheads), RBCs extravasating into the interstitum and tubulointerstitial inflammatory cell infiltration (arrows), and (D-F) LPS-challenged rats which had received 4.6 g/Kg SBH (**D**), 9.3 g/Kg SBH (**E**) or DEX (5 mg) (**F**) showing great improvement in kidney histology with minimal infiltration of inflammatory leukocytes. (H&E; x200). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

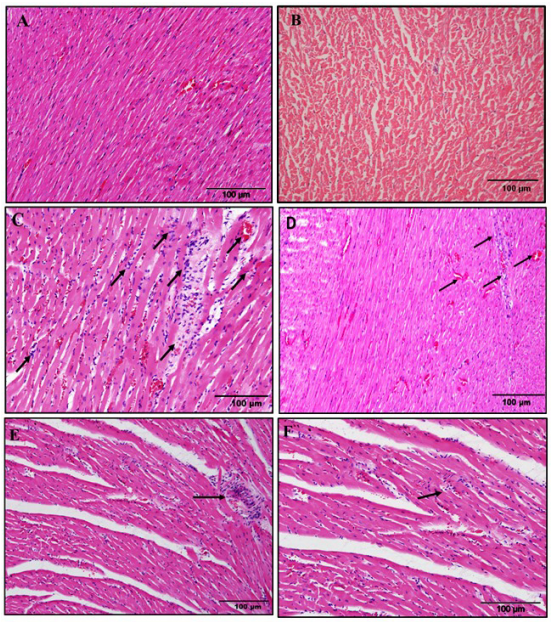

Fig. (4). SBH mitigates LPS-induced heart injury in rats. Photomicrographs of heart from (**A**) control and (**B**) 9.3 g/kg-supplemented rats showing normal cardiomyocytes without vascular or inflammatory changes, (**C**) LPS-treated rat tissue showing infiltration of leukocytes and congested blood vessels in the heart muscle (arrows), (**D**) LPS-challenged rats treated with 4.6 g/Kg SBH showing several small foci of inflammatory infiltration along with small, congested blood vessels (arrows), (**E**) LPS-challenged rats that had received 9.3 g/Kg SBH showing small, inflammatory foci (arrows), and (**F**) LPS-induced changes in rats treated with DEX showing a minimal infiltration of inflammatory leukocytes (arrows). (H&E; x200). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

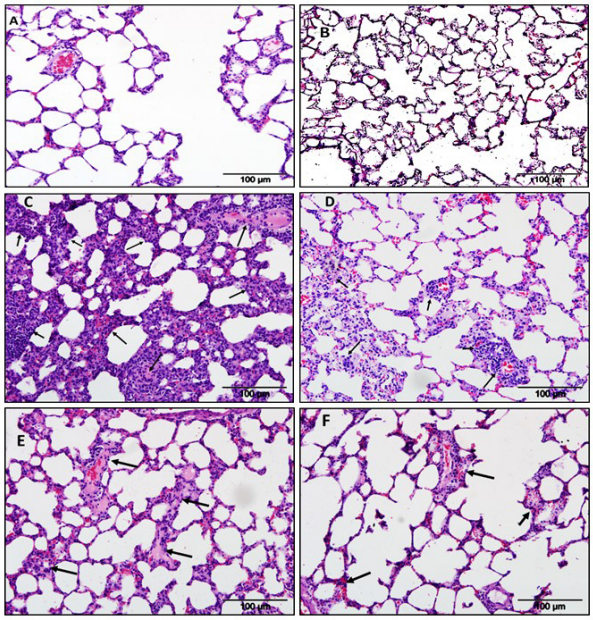

Fig. (5). SBH prevents lung injury in LPS-challenged rats. Photomicrographs of lungs from (**A**) control and (**B**) 9.3 g/kg-treated rats showing normal structures with no pathological changes, (**C**) LPS-induced damage showing leukocyte infiltration, edema, and increased alveolar wall thickness (arrows), and (**D-F**) LPS-challenged rats that had received 4.6 g/kg SBH (D), 9.3 g/kg SBH (**E**) or DEX (**F**) showing great improvement in lung histology with minimal infiltration of inflammatory leukocytes. (H&E; x200). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

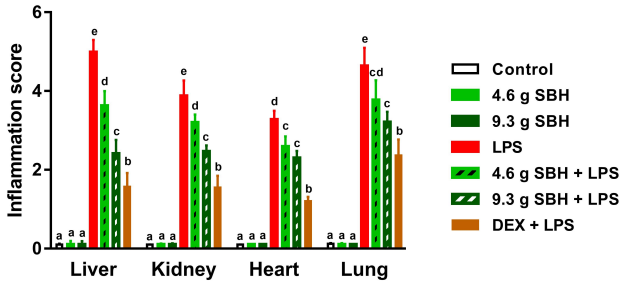

Fig. (6). Inflammation score of different tissues of control and LPS-challenged rats treated with SBH or DEX. Data are mean ± SEM (n=6). Different letters indicate significant differences. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

3.5. SBH Attenuates Oxidative Stress and DNA Damage and Enhances Nrf2 in LPS-Treated Rats

The ameliorative effect of SBH on oxidative stress in LPS-treated rats was investigated by assaying ROS, MDA, NO, 8-Oxo-dG, and GSH in the serum of these rats. The administration of LPS resulted in a significant elevation of ROS (Fig. **9A**), MDA (Fig. **9B**), NO (Fig. **9C**), and 8-Oxo-dG (Fig. **9D**) with a concomitant decline in GSH (Fig. **9E**). Treatment with SBH or DEX reduced ROS, MDA, NO, and 8-Oxo-dG, and boosted GSH levels in LPS-treated rats. SBH exerted a non-significant effect on redox parameters and GSH in normal rats (Fig. **9E**).

To determine the potential involvement of Nrf2 in the ameliorative efficacy of SBH in LPS-challenged rats, Nrf2 was quantified using ELISA (Fig. **9F**). LPS-challenged rats

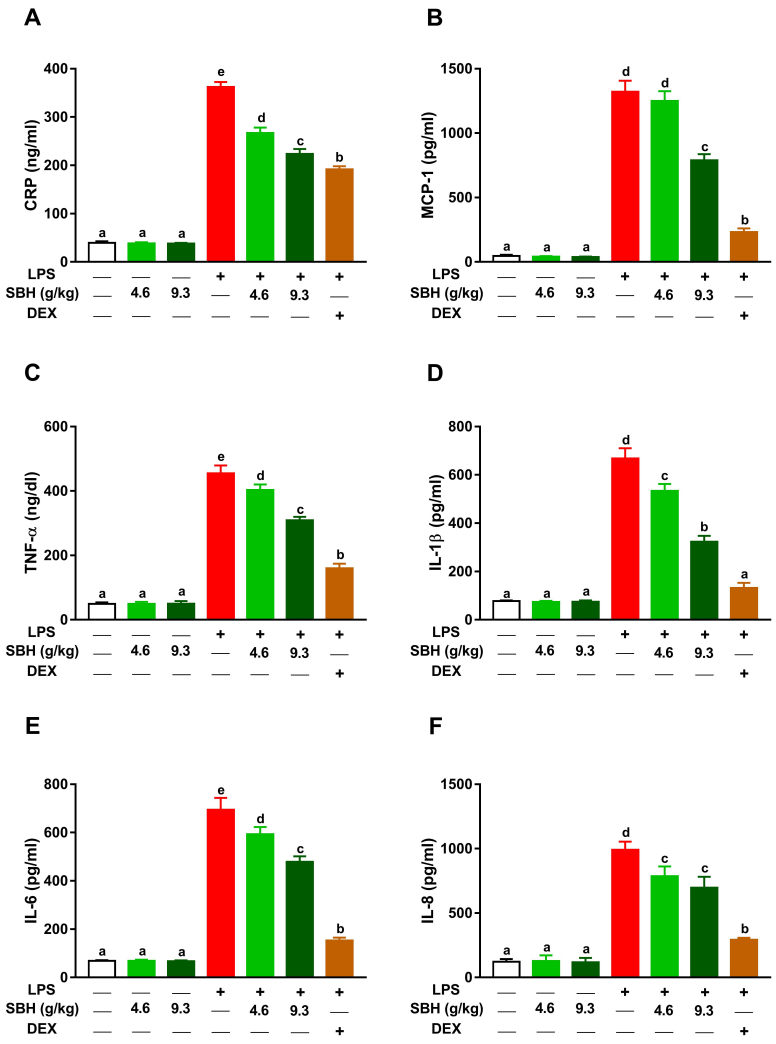

Fig. (7). SBH reduces pro-inflammatory mediators in LPS-challenged rats. Treatment with SBH suppressed serum CRP (**A**), MCP-1 (**B**), TNF-α (**C**), IL-1β (**D**), IL-6 (**E**), and IL-8 (**F**) in LPS-challenged rats. Data are mean ± SEM (n = 6). Different letters indicate significant differences. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

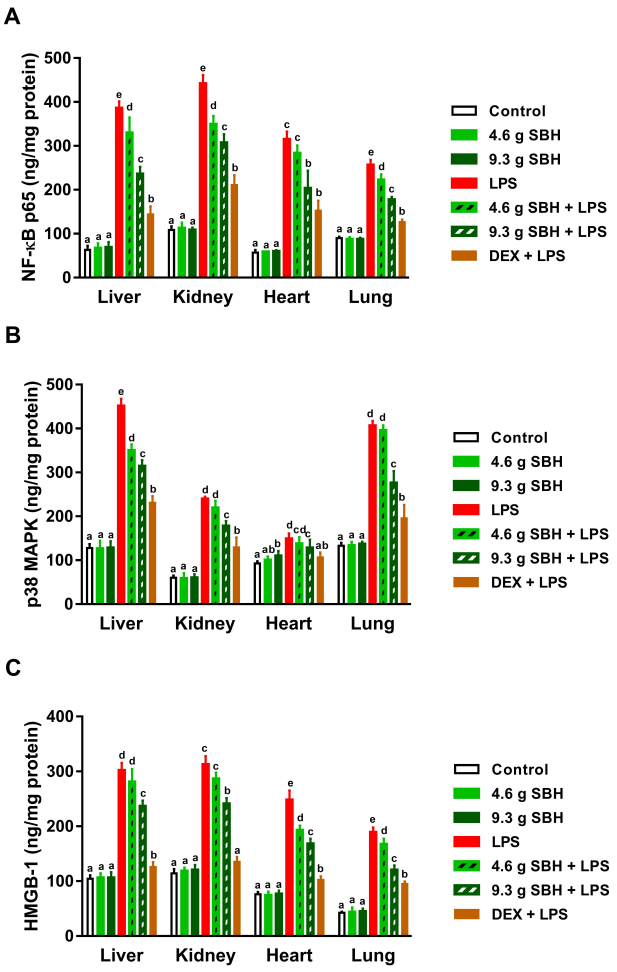

Fig. (8). SBH suppresses NF-κB p65, p38 MAPK, and HMGB-1 in LPS-challenged rats. Data are mean ± SEM (n = 6). Different letters indicate significant differences. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

exhibited a significant reduction in Nrf2 in all tissues. The lower SBH dose enhanced hepatic and pulmonary Nrf2 levels in LPS-treated rats, whereas it failed to increase it significantly in the kidney and heart. The higher SBH dose significantly improved Nrf2 levels in the liver, kidney, and lung of LPS-challenged rats, and in the lung of normal rats (Fig. **9F**).

4. DISCUSSION

Systemic acute inflammation is a serious sickly condition characterized by the overproduction of pro-inflammatory cytokines and mediators that cause organ dysfunction and culminate in death [30]. The cell wall of Gram-negative bacteria contains LPS which is implicated in triggering systemic acute inflammation and oxidative stress simultaneously by activating MAPK and NF-κB signaling [10]. The LPS-induced systemic acute inflammation model is frequently and widely used to resemble sepsis in order to identify novel interventions prior to commencing their clinical application [31].

Upon exposure to LPS, leukocytes along with other immune cells release a variety of pro-inflammatory mediators. During LPS-induced systemic acute inflammation, the up-regulation of pro-inflammatory mediators is a well-known phenomenon called a “cytokine storm”. TNF-α and IL-1β, the key regulators of the inflammatory process, contribute primarily to the amplification of the inflammatory cascade through activating macrophages to release other pro-inflammatory cytokines and ROS [32]. The current research confirmed that LPS promotes the elevation of pro-inflammatory mediators. LPS-challenged rats exhibited an increase in circulating CRP, MCP-1, TNF-α, IL-1β, IL-6, and IL-8, demonstrating an inflammatory response. Pre-treatment with SBH for 7 days was able to attenuate the release of inflammatory mediators. In other studies, the intravenous injection of Gelam honey one hour before LPS injection reduced TNF-α and IL-1β as well as IL-6 after 4 h but not after 24 h. However, IL-10, a potent anti-inflammatory cytokine, was not elevated [33]. Pre-treatment of animals with Gelam honey orally for seven days reduced TNF-α expression in inflammatory paw edema [34]. Here, the oral administration of SBH was selected to mimic human consumption. This route of administration could expose SBH to pre-systemic metabolism in which the bioactive compounds of SBH might have been broken down by gastrointestinal tract enzymes or by gut microbiota. The anti-inflammatory effect of SBH was further supported by a previous study showing suppressed acute inflammation in rats pre-treated with 10 g/kg honey [35]. *In vitro*, honey flavonoids improved neuroinflammation via downregulating the mRNA expression of TNF-α and IL-1β in microglia challenged with LPS [36].

The protective efficacy of SBH against LPS-induced injury was evidenced by the biochemical and histological findings. LPS provoked tissue injury as manifested by increased markers of liver, kidney, and heart function, as well as histological alterations, particularly leukocyte infiltration in the lung, kidney, liver, and heart of rats. SBH supplementation prevented LPS-induced tissue injury and dysfunction, and reduced leukocyte infiltration and the inflammation score in these different tissues of rats.

To explore the mechanism underlying the anti-inflammatory efficacy of SBH, we investigated its effect on NF- κB p65 and p38 MAPK. The ligation of the LPS-LPB-CD14 complex with TLR4 and MD-2 stimulates the NF-κB and MAPK pathways, leading to the release of pro-inflammatory cytokines [10]. NF-κB p65 and p38 MAPK play a key role in regulating the immune response by controlling the release of inflammatory cytokines [37]. When LPS activates receptor-associated proteins, the phosphorylation of IκB disassociates NF-κB p65, while p38 MAPK is activated after transducing the activation signals into cellular responses. NF-κB p65 triggers the transcription of cytokines genes directly, while p38 MAPK regulates the production of inflammatory mediators at the post-transcriptional level [7, 37]. The

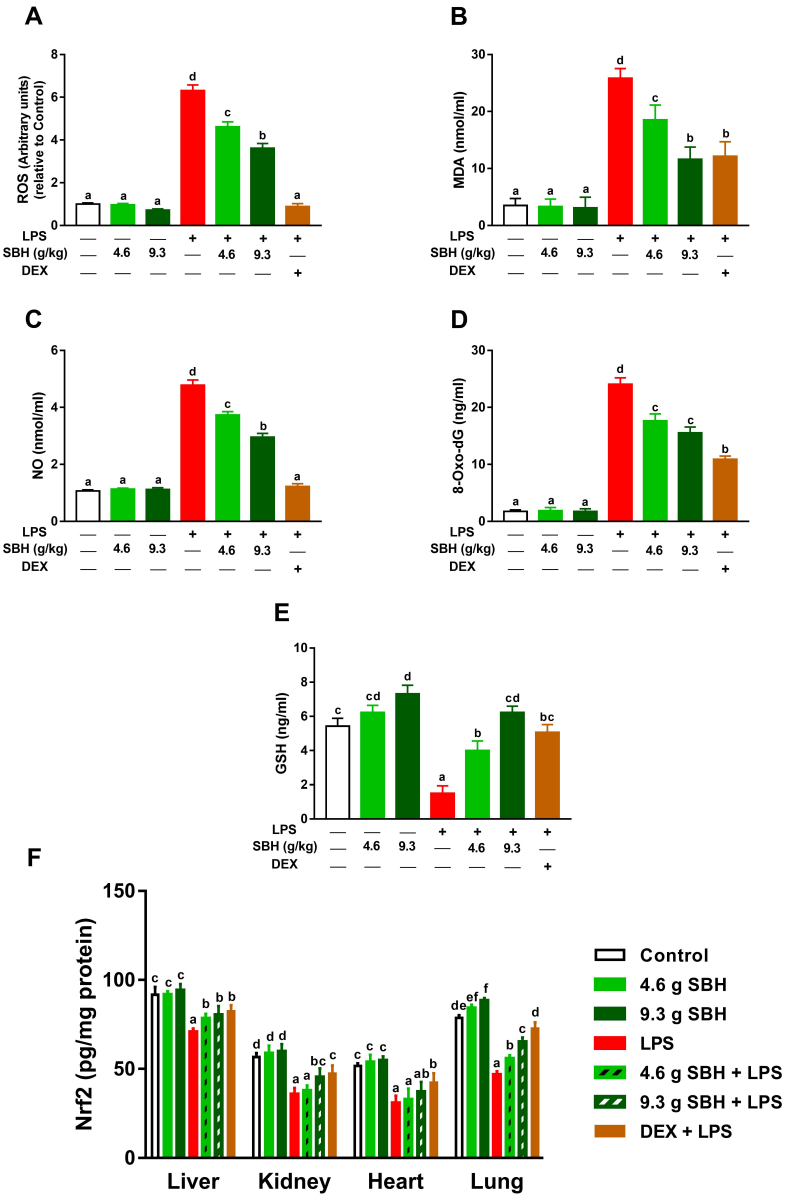

Fig. (9). SBH reduced serum ROS (**A**), MDA (**B**), NO (**C**), and 8-Oxo-dG (**D**), boosted GSH (**E**), and increased Nrf2 in different tissues of LPS-treated rats. Data are mean ± SEM (n = 6). Different letters indicate significant differences. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

results showed that SBH diminished NF-κB p65 and p38 MAPK levels in different tissues of LPS-challenged rats. These findings demonstrated that the suppression of NF-κB and p38 MAPK mediated, at least in part, the anti-inflammatory mechanism of SBH. Accordingly, the results were consistent with other studies where Gelam honey suppressed NF-κB nuclear translocation and protected IκB from phosphorylation [34].

Next, we evaluated the effect of SBH on HMGB-1, a chromatin-binding protein that has been listed recently as an inflammatory mediator released by macrophages and/or monocytes during systemic acute inflammation [38]. Functionally, HMGB-1 contributes to the maintenance of the nucleosome structure as well as in the regulation of gene transcription [39]. Once released, HMGB-1 initiates chemotaxis and downstream NF-κB signaling pathways through ligation with TLR-4 and the receptor of advanced glycation end products (RAGE) [40]. Circulating HMGB-1 has been detected at high concentrations in endotoxic animals and septic patients with clinical signs, such as fever, tissue injury, and acute respiratory stress [41]. At the same time, HMGB-1 per

se triggers redox modification by boosting the cellular generation of ROS and reactive nitrogen species [39]. In the current study, HMGB-1 was observed to be at high levels in different tissues of the rats challenged with LPS; an effect that was reversed in rats pre-treated with SBH. These results are similar to another observation where Gelam honey improved the survival of LPS-induced endotoxic animals by reducing HMGB-1 levels [33]. It is worth mentioning that some phenolic compounds, but not all, promote the inhibition of HMGB-1. However, the mechanism mediating HMGB-1 suppression is still controversial [42]. Thus, the ameliorative effect of SBH on HMGB-1 could be connected to its phenolic constituent.

Recently, it has been proposed that upregulating Nrf2 is associated with HMGB-1 deactivation along with TNF-α, IL-β, and NO *via* heme oxygenase-1 (HO-1) [43]. Since oxidative stress is involved in systemic acute inflammation, Nrf2 is thought to be a therapeutic target for attenuating endotoxemia-induced by LPS. The genetic ablation of Nrf-2 in rodent models has caused lupus-like autoimmune nephritis and diabetes-induced inflammation and oxidative stress [44]. Under constitutive conditions, Nrf-2 is kept inactive in the cytoplasm by Keap1. The phosphorylation of Keap1 results in releasing Nrf-2 and its translocation to the nucleus [45]. In this context, various natural compounds including polyphenols have been reported to disrupt the Nrf2-Keap1 complex which automatically increases the expression of antioxidants agents [46-48]. In the present study, Nrf2 levels were increased in SBH-treated groups despite the LPS challenge, revealing the fact that SBH has the ability to protect the cells against oxidative damage and to maintain cellular homeostasis. These results were supported by our previous research which demonstrated that SBH possesses high antioxidant activities [28]. In addition, Manuka honey has similarly boosted the protein expression of Nrf2 levels in fibroblast cells-challenged with 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) [49]. Furthermore, glutathione enzymes including, GST, GSH, and GPx have been implicated in protecting the hemostasis of cells from oxidative stress by several mechanisms. H_2O_2 which is a strong pro-oxidant is removed by GPx through oxidizing two molecules of GSH to produce H_2O and GSSH. Similarly, GST which constitutes a family of enzymes detoxifies xenobiotics by catalyzing the nucleophilic attack of GSH to prevent the interaction of xenobiotics with crucial cellular components [50]. In the present study, the SBH administration promoted the levels of GSH in rat serum even after the LPS challenge. These results account for the reduction of ROS, 8-OHdG, and MDA in SBH-treated groups with LPS-challenge. Recent studies have reported that ROS generated by the endoplasmic reticulum, NADPH oxidase, and mitochondria play a critical role in activating MAPK and NF-κB [43, 51]. At the same time, ROS has been deeply implicated in tumorigenesis through oxidizing DNA into 8-OHdG. The results of this study demonstrated that SBH treatment reduced the level of DNA oxidation in LPS-challenged rats, as observed in 8-OHdG levels. Numerous natural products rich with polyphenols and powerful antioxidant agents have been proposed to be consumed as a preventive measure against oxidative stress and as an endogenous antioxidant system promotor [52]. In this context, oral administration of pine honey has enhanced GPx, CAT, and SOD enzymes in BALB/c mice-challenged with trichlorfon [53]. Furthermore, consuming 1.5 g/kg natural honey increased the plasma antioxidant capacity in healthy subjects due to the bioavailability of honey polyphenol [54]. To ensure that honey polyphenols can exert beneficial activities, renal tissues of diabetic rats-treated with honey showed high levels of GST, GPx, and SOD along with low levels of MDA [55]. In this context, it is postulated that SBH has potential antioxidant properties which could be manifested by promoting the endogenous antioxidant system or quenching the ROS which collectively attenuate oxidative stress and inflammation simultaneously in the heart, lung, kidney, and liver. Therefore, the observed pathological changes in the organs, including infiltration of leukocytes [55], congested blood vessels, tissue damage, and necrosis due to LPS-challenge were attenuated by SBH pretreatment. These results were consistent with lung, cardiac, renal, and hepatic function parameters.

The beneficial effects of SBH, such as antioxidant and anti-inflammatory efficacies, could be attributed to its bioactive constituents. In our previous research, 8 phenolic acid and 5 flavonoids, namely, gallic acid, caffeic acid, caffeic acid phenethyl ester, syringic acid, catechin, apigenin, chrysin, cinnamic acid, 4-hydroxybenzoic acid, 2-hydroxycinnamic acid, kaempferol, *P*-coumaric acid, and quercetin-3-O-rutinosid were identified in SBH [28]. This diversity of polyphenols in SBH creates a bioactive environment with therapeutic potency [56]. Despite being introduced orally into the animals, SBH polyphenols and their primary metabolites could be synergistically responsible for their biological efficacy. Probably, the presence of SBH-bioactive compounds in the gut lumen establishes close contact with the intestinal epithelium and/or with the intestinal microbiota. Preserving the homeostasis of an intestinal epithelium with polyphenols may promote intestinal alkaline phosphatase which exerts a protective role in intestinal and systemic inflammation [57]. At the cellular level, the possible underlying mechanism of SBH-mediated attenuation of systemic acute inflammation induced by LPS is probably due to inhibition of LPS-induced cytoplasmic translocation, suppression of NF-κB and MAPK phosphorylation, activation of Nrf2 translocation through Keap-1 dissociation to enhance the genetic expression of antioxidant enzymes, deactivation of pro-inflammatory mediators through binding with their active sites, and neutralizing ROS through donating electrons (Fig. **10**). These pharmacological actions mediated by SBH may reduce the adhesion of leukocyte into endothelium cells, leukocyte activation, tissue invasion, and parenchymal damages in the organs.

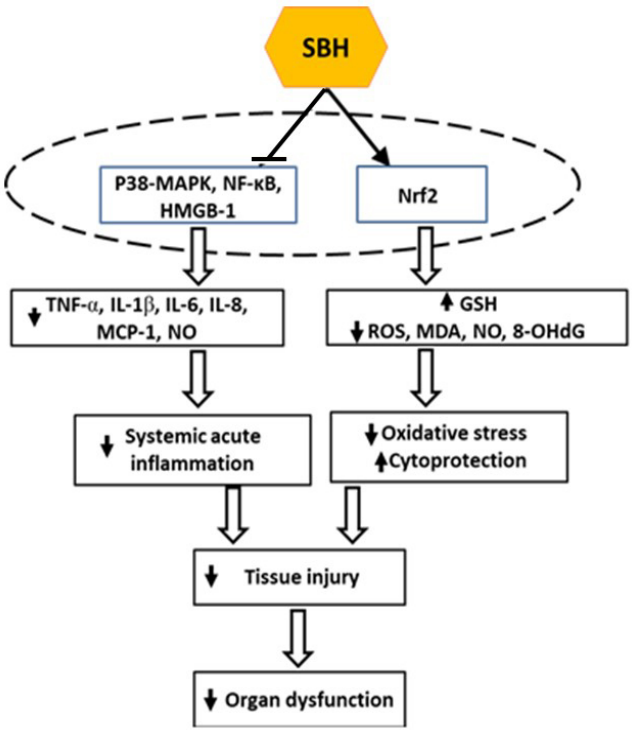

Fig. (10). The proposed protective mechanism of SBH against LPS-induced systemic acute inflammation. Binding of LPS with TLR-4 up-regulates NF-κB, and p38 MAPK and subsequently HMGB-1, leading to the release of pro-inflammatory mediators and tissue injury. SBH suppressed LPS-mediated activation of NF-κB p65, p38 MAPK, and HMGB-1, and decreased pro-inflammatory cytokines. In addition, SBH attenuated oxidative stress and upregulated Nrf2 in different tissues of LPS-challenged rats. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

CONCLUSION

This study indicates that SBH has displayed a therapeutic role as having anti-inflammatory and antioxidant properties, demonstrating a variety of pharmacological effects. Supplementing SBH rats with LPS-induced systemic acute inflammation attenuated the release of pro-inflammatory cytokines and oxidative stress markers by inhibiting NF-κB p65 and p38 MAPK and activating Nrf-2 signaling pathways. These findings demonstrate the potential of SBH as a promising nutritional approach to attenuate systemic acute inflammation in septic patients. Therefore, establishing a convenient therapeutic strategy based on the findings of this study is worth further investigated.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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