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Berberine protects against sepsis-related acute lung injury in rats via PPAR-γ signaling pathway upregulation and improvement at the cellular level: Functional, biochemical, and immunohistochemistry study

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Abstract. This study aimed to assess the prophylactic effects of Berberine on experimentally induced lung sepsis and examine its effects on selected cytokines, genes, and protein expression besides the histopathological evaluation. Berberine significantly reduced the wet/dry lung ratio, the broncho-alveolar lavage fluid (BALF) protein, cells, neutrophils percentage, and cytokines levels. In addition, pretreatment with Berberine decreased the myeloperoxidase (MPO) and malondialdehyde (MDA) levels and decreased gene expression of nuclear factor kappa B (NF-κB), monocyte chemoattractant protein-1 (MCP-1), and the intracellular adhesion molecule 1 (ICAM-1) by RT-qPCR analysis, revealing Berberine's antioxidant and anti-inflammatory mode of action. Western blot analysis revealed increased peroxisome proliferator-activated receptor gamma (PPAR-γ) expression in the Berberine pretreated group compared to the cecal ligation and puncture (CLP) group, in which the histopathological examination evidenced this improvement. In conclusion, Berberine improved lung sepsis *via* its PPAR-γ mediated antioxidant and anti-inflammatory effects.

Key words: Berberine — Septic lung injury — CLP — Peroxisome proliferator-activated receptor gamma (PPAR-γ)

Abbreviations: α-SMA, alpha-smooth muscle actin; ALI, acute lung injury; Anti-Cox-2, anti-cyclooxygenase 2; BALF, broncho-alveolar lavage fluid; cDNA, complementary deoxyribonucleic acid; CLP, cecal ligation and puncture; COX, cyclooxygenase; ICAM-1, intracellular adhesion molecule 1; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MMP-9, matrix metallopeptidase 9; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; PBS, phosphate-buffered saline; PPAR-γ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; SPSS, statistical package for the social sciences; TGF, transforming growth factor; TIMP1, tissue inhibitor of metalloproteinases; TNF-α, tumor necrosis factor-α.

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Introduction

Berberine, the active component of Rhizoma, is an herbal alkaloid that is widely used in Chinese natural medicine (Jin et al. 2016). Several beneficial effects of Berberine have been recently identified against obesity, malignant tumors, heart failure (Chang et al. 2016), chronic neurological disorders, inflammatory conditions, atherosclerosis, autoimmune diseases (Jiang et al. 2015), gastrointestinal infections (Yin et al. 2008) and metabolic disorders, such as dyslipidemia and insulin resistance (IR) (Caliceti et al. 2016). Also, recent data suggests its potentially beneficial effects in the case of cerebrovascular accidents both in human and animal models (Zhou et al. 2008). Although Berberine exerts various therapeutic effects, its exact effector mechanisms still require further investigation. However, literature reported Berberine's potent anti-inflammatory (Zhang and Chen 2012) and antioxidant properties (Ehteshamfar et al. 2020; Rajasekhar et al. 2020).

Acute lung injury (ALI) is a serious life-threatening condition, along with its more severe form, acute respiratory distress syndrome, causing acute respiratory failure, pulmonary edema, and inflammatory insult. The mortality rate of patients with ALI remains high despite the advances in critical care and mechanical ventilation methods (Matthay et al. 2020). Noteworthy, the association between peroxisome proliferator-activated receptor-γ (PPAR-γ) and ALI has been reported by prior studies (Lin et al. 2015; Ning et al. 2018).

As the most common cause of ALI in clinical medicine is sepsis. Several animal models protocols, notably cecal ligation, and puncture (CLP) or lipopolysaccharide (LPS) injection, are used as septic pulmonary injury models (Rojas et al. 2005), causing widespread inflammation, pulmonary edema, altered pulmonary function, in addition to abnormal depositions of the extracellular matrix (Bucher and Taeger 2002).

CLP is a widely used animal model protocol to induce generalized systemic septic condition (Altemeier et al. 2005), hence it is frequently used in ALI animal models (Gharib et al. 2006). Exposure to a generalized septic condition activates macrophages and other immune cells leading to a widespread inflammatory response with the activation of various immune cells and signaling mechanisms (Lentsch et al. 1999).

This study aimed to explore the role of Berberine as a protective agent against acute septic lung injury, along with investigating the possible proposed mechanisms for the anticipated protection with an emphasis on Berberine as a potential anti-inflammatory and antioxidant agent, as well as investigating the potential contribution of PPAR-γ to such protective effects of Berberine.

Materials and Methods

Ethical approval

All study procedures were conducted according to the guidelines for the care and use of experimental animals of Cairo University and approved by the Institutional Animal Care and Use Committee with ethical committee approval number: CU-III-F-4-22.

Animals

Thirty male albino rats aged 6–8 weeks (body weight of 150–200 g) were obtained from the animal house of the Faculty of Medicine, Cairo University, Egypt. The animals were housed individually in suitable chip-bedded cages, kept at a room temperature of 25 ± 2°C with 50% relative humidity, and exposed to a 12:12-h daylight/darkness cycle. All rats in the current research were provided with free access to unrestricted tap water and standard pellet food (306.2 kcal/100 g) from El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt, which contained 48.8% carbohydrates, 21% proteins, 3% fats, 0.8% calcium, 0.4% phosphorus, 5% fiber, 13% moisture, and 8% ash (Abbas et al. 2022). Before the start of the experiment, rats were acclimated for two weeks to their local environment.

Water and diet were offered ad libitum throughout the study period with regular weighing to ensure stable body weight. After acclimation, rats were arbitrarily assigned into distinct groups.

Experimental protocol

The rats were randomly assigned into the following groups: Control (Sham group) where the animals underwent the same surgical procedure, but the cecum was neither ligated nor punctured, received oral saline throughout the study duration (Dear et al. 2006). CLP (CLP-induced ALI group) and CLP+BER (Berberine-pre-treated group), at a dose of 380 mg/kg/day by gavage for two weeks before the surgical procedure and continued for 5 days after surgery (Lee et al. 2006; Hu et al. 2012). All procedures were performed under anesthesia using both ketamine (50 mg/kg/i.p.) and xylazine (10 mg/kg/i.p.), obtained from Sigma Pharmaceuticals (Cairo; Egypt).

At the end of the experimental procedure (day 20), all the groups' rats underwent euthanasia under anesthesia and the lungs were dissected for further biochemical, molecular, histological and immunohistochemistry analysis.

Cecal ligation and puncture

The procedure was performed through a small linea alba incision. After cecal exposure, a 5-0 silk ligature was placed

1 cm from the cecal tip followed by puncturing the cecum twice using a 21-gauge needle. Then, repositioning of the cecum followed by fecal extrusion. Finally, closure of the abdominal incision followed by subcutaneous saline fluid injection to replace fluids loss.

Certain precautions were undertaken to control the severity of bacterial dissemination such as avoiding the necrosis of the cecum with careful surgical procedure, proper maintenance of the body temperature as well as careful extrusion of the gas bubbles. Besides, continuous stool consistency monitoring in which three rats were excluded from the experiment due to overhydrated stool (Su et al. 2023).

Broncho-alveolar lavage fluid (BALF) and lung water content estimation

After anesthesia and euthanasia, a thoracotomy incision was performed, and a cannula was inserted into the trachea, then secured by a silk suture. Next, phosphate-buffered saline (PBS) solution was administered *via* a syringe and the tracheal cannula, left there for 30 s, then retrieved and reinstalled for three washes (Mei et al. 2007). To obtain the supernatant, BALF was collected, and centrifuged at 800 × *g*, the supernatant was stored at –70°C immediately and used subsequently for protein detection. Both lungs were extracted and carefully dissected from the surrounding tissues. The left lung was preserved in formalin for histological studies. For estimation of lung water content, the inferior lobe of right lung was dissected and weighed immediately to obtain the wet lung weight, left to dry at 80°C for twelve hours. Finally, we calculated the lung wet/dry (W/D) ratio (Lam et al. 2008). The remaining lung tissue was sent for biochemical analysis.

Biochemical measurements

BALF protein concentration and cellular count

BALF protein concentration was measured as a cell permeability marker using the Bio-Rad Protein Assay Kit. The total cell counts were then determined using a hemocytometer. The differential cell counts were calculated as the ratio of neutrophils ×100 divided by the total number of cells determined in the BALF.

BALF pro-inflammatory biomarkers

The pro-inflammatory cytokine interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels in the BALF were measured using the enzyme-linked immunosorbent assay (ELISA) method (R&D, Minneapolis, MN, USA), according to the manufacturer's instructions.

Myeloperoxidase (MPO) activity determination in the rat lung tissue

The lung tissue was homogenized to measure the MPO activity using the spectrophotometric method described by Mizutani and colleagues. The results were expressed as MPO relative units/100 mg lung tissue. One MPO activity unit was defined as the quantity of enzyme degrading one mmol peroxide at 25°C. Purified known human neutrophil MPO activity was used as a standard (Mizutani et al. 2003).

Malondialdehyde (MDA) measurement

We homogenized 100 mg of the lung tissue in 1 ml PBS (pH 7.0) with a micro pestle for the MDA concentration measurement. Next, 20% TCA was added to the lung tissue homogenate to precipitate the protein and then centrifuged. Afterwards, thiobarbituric acid solution was added to the gathered supernatants. After boiling in a water bath for 10 min, we evaluated the absorbance. The MDA concentration in lung tissue was calculated using the standard curve (Khalifa et al. 2022).

RNA extraction and RT-qPCR analysis

For real-time reverse transcription polymerase chain reaction, liquid nitrogen was used to snap-freeze lung tissues kept in a 1.8 cc cryotube. Tissue Lyser LT (Qiagen, Hilden, Germany) was used to disrupt extracted lung tissue according to the manufacturer's instructions by one 500 mm diameter stainless steel bead and 700 µl of QIAzol as the lysis reagent. With the miRNeasy mini kit (Qiagen, Hilden, Germany), the total RNA has been extracted according to the guidelines provided by the manufacturer. Each RNA sample was evaluated for purity and concentration using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). The 2100 Bioanalyzer Instrument and the RNA 6000 Nano assay were used to determine the RNA integrity score (Agilent Technologies, Wilmington, DE). Until analysis, purified RNA samples were kept in 5-µl aliquots at –80°C. By applying the SensiFAST cDNA Synthesis Kit (Bioline) and following the supplier's instructions, cDNA was generated from 100 ng of total RNA. Using 4 µl of buffer and 1 µl of reverse transcriptase from the supplier, the reactions were carried out in a final amount of 20 µl. In a Bio-Rad T100 Thermal Cycler, reverse transcription processes were carried out as follows: Ten minutes at 25°C are used for primer annealing, fifteen minutes at 42°C are used for reverse transcription, and five minutes are spent at 85°C for enzyme inactivation. β-actin amplification was used as a control. The primer sequences for nuclear factor kappa B (NF-κB), monocyte chemoattractant protein-1 (MCP-1), and the intracellular adhesion molecule 1 (ICAM-1) and β-actin were mentioned

Table 1. Primer sequences of NF-κB, MCP-1, and ICAM-1 genes

Gene	Primer sequence
NF-κB	F: GACGACACCTCTACACATAGCA R: CCTCATCTTCTCCAGCCTTCTC
MCP-1	F: TGT TCA CAG TTG CCG GCT GGA G R: AGC TTC TTT GGG ACA CCT GCT GC
ICAM-1	F: TCC GCT GTG CTT TGA GAA CT R: AGG GTG AGG TCC TTG CCT AC
β-actin	F: ACAGGATGCAGAAGGAGATTAC R: ACAGTGAGGCCAGGATAGA

NF-κB, nuclear factor kappa B; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intracellular adhesion molecule 1; F, forward; R, reverse.

in Table 1. The gene expression was measured by generating densitometry data for band intensities in different sets of experiments by semi-quantitative gel image analysis. The resulting band intensities were compared with that of constitutively expressed β-actin (Khalifa et al. 2019; Esteva-Socias et al. 2020).

Western blot analysis

Proteins were extracted from the lung tissues. 100 mg of frozen lungs were homogenized in a lysis buffer on ice for 30 min in a shaker, followed by centrifugation at 16,000 × *g* for 30 min at 4°C. The supernatant was transferred into a new tube for further analysis. The quantitative protein analysis was performed using a commercial Bradford Protein Assay Kit (SK3041, BIO BASIC INC., Markham, Ontario, Canada).

We loaded 20 μg of each protein sample with an equal volume of Laemmli buffer, boiled the mixture at 95°C for 5 min to denature, and then loaded it in an individual lane for gel electrophoresis. The membranes were blocked with blocking solution for 1 h at room temperature, then incubated with the primary anti-PPAR-γ antibody (Santa Cruz Biotechnology, Santa Cruz, USA; 1:500 dilution) against the blotted target protein overnight at 4°C. The membranes were then incubated in peroxidase-conjugated secondary antibody solutions against the blotted target proteins for 1 h at room temperature. Protein band visualization was performed using an enhanced chemiluminescence system (Clarity™ Western ECL substrate, BIO-RAD, USA). Signals were captured using a CCD camera-based imager. An image analysis software was used to read the band intensity of the target proteins against the control sample by normalizing to β-actin using the ChemiDoc MP imager (BIO-RAD, USA).

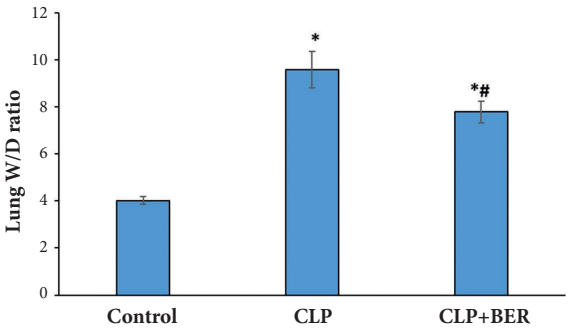

Figure 1. Lung wet/dry (W/D) ratio among all study groups. Cecal ligation and puncture in CLP group caused significant increases of the lung W/D ratio indicating pulmonary edema and inflammation, which was significantly attenuated in the CLP+BER group. Results were represented as mean ± standard deviation. * $p < 0.05$ *vs.* control, # $p < 0.05$ *vs.* CLP. CLP, cecal ligation and puncture-induced acute lung injury group; CLP+BER, Berberine-pre-treated CLP group.

Histological and immunohistochemistry evaluation

The lung specimens were fixed in formol saline. Serial sections of 5-μm thick were cut and subjected to the following stains: hematoxylin and eosin (H&E) to measure the intra-alveolar septal thickness, and Masson's trichrome to determine the collagen fiber deposition mean area % as well as anti-cyclooxygenase 2 (anti-Cox-2) immunohistochemistry using streptavidin-biotin peroxidase. The image analyzer computer system (Leica Qwin 500, Leica Image System Ltd, Cambridge, UK) was used for image analysis.

Statistical analysis

The 26th version of the Statistical Package for the Social Sciences (SPSS) (IBM Corp., Armonk, NY, USA) was employed for data coding and analysis. Data were summarized using the relative frequencies for categorical variables, and the mean and standard deviation for quantitative variables. Analysis of variance (ANOVA) was used for comparison between the groups and the *post-hoc* test was for comparing more than two groups (Chan 2003). Correlations were performed using Pearson's correlation coefficient. *p*-values less than 0.05 were considered statistically significant (Chan 2003).

Results

Lung weight and BALF analysis results

Increased lung W/D ratio in the CLP group compared to the control, with a statistically significant improvement in the CLP+BER group is demonstrated in Figure 1. BALF analysis revealed a statistically significant increase in the BALF protein content, cell count, and neutrophil per-

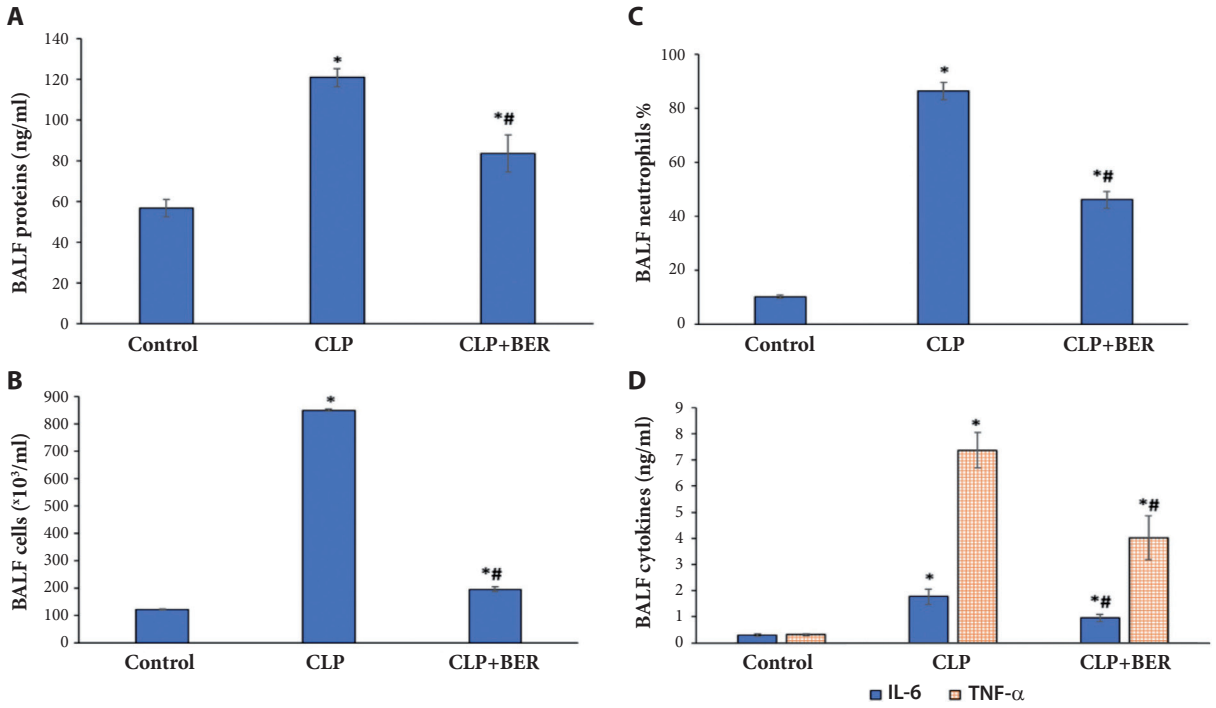

Figure 2. Bronchoalveolar lavage (BALF) measurements. Cecal ligation and puncture in CLP group caused significant increases in the BALF protein concentrations (**A**), cellular count (**B**), neutrophil percentage (**C**), and the cytokine level (**D**) as compared to the control group. The Berberine in the CLP+BER group caused significant improvements in these measurements. Results were represented as mean ± standard deviation. * $p < 0.05$ *vs.* control, # $p < 0.05$ *vs.* CLP. BALF, broncho-alveolar lavage fluid; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α. For other abbreviations see Figure 1.

centage, in the CLP group, with a statistically significant improvement in the CLP+BER group (Fig. 2). Moreover, our results showed increased IL-6 and TNF-α levels in the BALF of the CLP group relative to the control one, however, Berberine could ameliorate these inflammatory markers (Fig. 2D).

Lung tissue MPO activity and MDA level results

Increased MPO and MDA levels in the lung tissue of the CLP group was measured. On the contrary, these effects were partially reversed in the CLP+BER group compared to the control group as displayed in Figure 3.

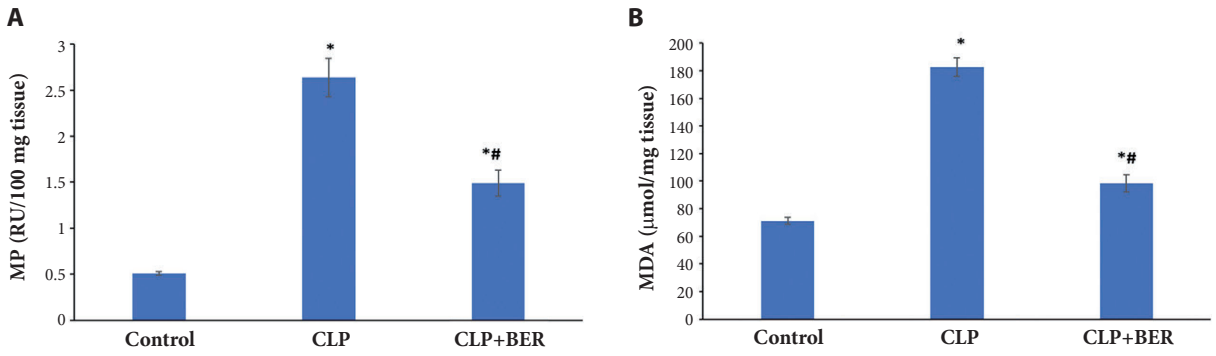

Figure 3. Lung tissue MPO activity (**A**) and MDA level (**B**) results among all study groups. Cecal ligation and puncture resulted in significant increases in MPO activity (A) indicated neutrophil infiltration and MDA level (B) indicated increased oxidative stress in the rat lung tissue in CLP group. Pretreatment with Berberine in the CLP+BER group showed significant ameliorative effects. Results were represented as mean ± standard deviation. * $p < 0.05$ *vs.* control, # $p < 0.05$ *vs.* CLP. MPO, myeloperoxidase; MDA, malondialdehyde; RU, relative unit. For other abbreviations see Figure 1.

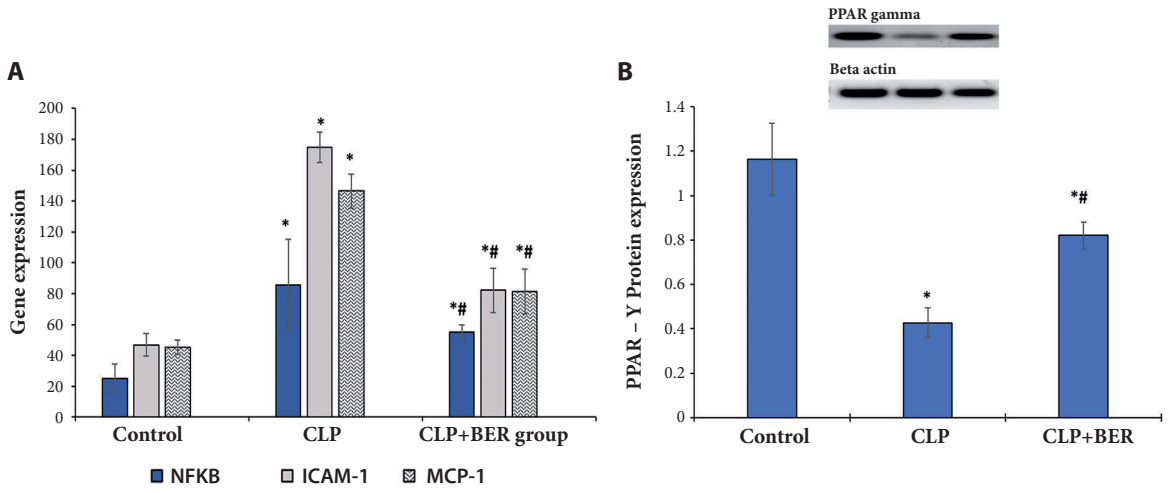

Figure 4. RT-qPCR results of the lung tissue NF-κB, MCP-1, and ICAM-1 gene expression (**A**), and Western blot analysis of PPAR-γ (**B**) among all study groups. Cecal ligation and puncture in CLP group resulted in significant increases in the gene expression of NF-κB, MCP-1, and ICAM-1 and reduction in the PPAR-γ levels in the rat lung tissue as compared to the control group. Berberine in the CLP+BER group significantly improve these measurements. Results were represented as mean ± standard deviation. * $p < 0.05$ *vs.* control, # $p < 0.05$ *vs*. CLP. RT-qPCR, real-time reverse transcription polymerase chain reaction; NF-κB, nuclear factor kappa B; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intracellular adhesion molecule 1. PPAR-γ, peroxisome proliferator-activated receptor gamma. For other abbreviations see Figure 1.

RT-qPCR results of NF-κB, MCP-1 and ICAM-1

Increased lung tissue expression of *NF-κB, MCP-1,* and *ICAM-1* in the CLP group compared to the control group was measured. These effects were partially ameliorated in the CLP+BER group, compared to the control group, with a statistically significant difference (Fig. 4A).

Western blot analysis results

Statistically significant reduction in PPAR-γ protein expression by the Western blotting in the CLP group compared to the control, while a statistically significant improvement in the CLP+BER group compared to the CLP group was detected (Fig. 4B). There was reduced PPAR-γ expression in the CLP group, compared to the control group ($p < 0.001$), increased expression in the CLP+BER group compared to the CLP group ($p < 0.001$), and decreased expression compared to the control group ($p < 0.001$).

Histological and immunohistochemistry results

H&E results

The lung tissue in the control group showed normal alveolar structures, interalveolar septa, and bronchioles as shown in Figure 5a and 5b. However, lung tissues in CLP group displayed a distorted structure with marked thickening of the interalveolar septa, massive cellular infiltration, and bronchiolar damage (Fig. 5c,d). The CLP+BER group showed an approximately normal lung architecture with slight interalveolar septal thickening and minimal cellular infiltration (Fig. 5e,f).

Masson's trichrome results

The lung tissue in the control group exhibited standard collagen fibers within the interstitium (Fig. 6a) while those of the CLP group displayed extensive collagen deposition (Fig. 6b). Oppositely, the CLP+BER group showed minimal collagen deposition (Fig. 6c).

Anti-Cox-2 immunohistochemical staining results.

The alveolar cells of the control group showed weak a*nti-Cox-2* immunoreaction (Fig. 7a), while those of the CLP group displayed a strong *anti-Cox-2* immunoreaction (Fig. 7b) which was significantly reversed in the CLP+BER group (Fig. 7c) and slides were scored with respect to IHC as negative (−), mild (+), moderate (++), severe (+++), or very severe (++++) (Schafer et al. 2018) as displayed in Table 2.

Histomorphometry results

Compared to the control group, a significant increase in the area % of anti-Cox-2 immunoreactivity and collagen fibers recorded in the CLP group was shown, however, these

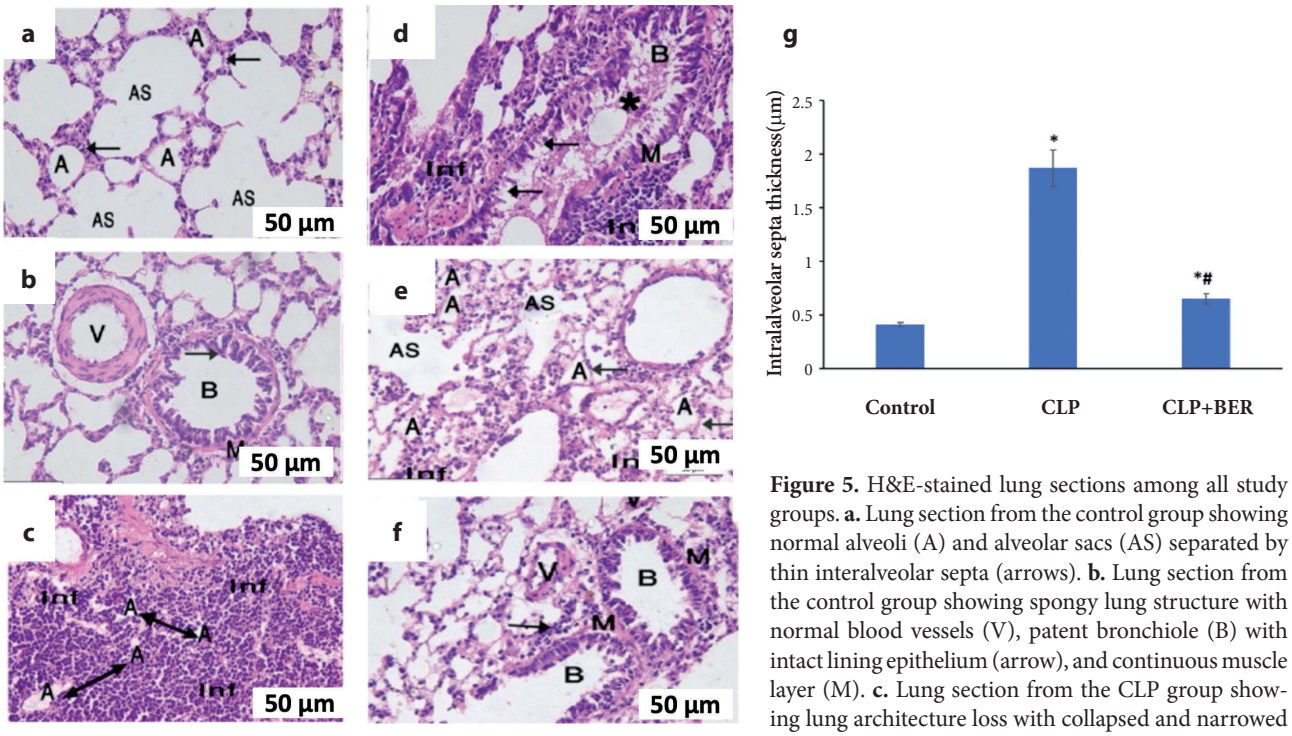

Figure 5. H&E-stained lung sections among all study groups. **a.** Lung section from the control group showing normal alveoli (A) and alveolar sacs (AS) separated by thin interalveolar septa (arrows). **b.** Lung section from the control group showing spongy lung structure with normal blood vessels (V), patent bronchiole (B) with intact lining epithelium (arrow), and continuous muscle layer (M). **c.** Lung section from the CLP group showing lung architecture loss with collapsed and narrowed alveoli (A). Marked thickening of the interalveolar septa (arrows) and diffused cellular infiltration with eosinophilic exudates (Inf). **d.** Lung section from the CLP group showing obliterated bronchiole (B) filled with eosinophilic material and desquamated epithelium (star). Area of lost bronchial epithelium (arrows) and interrupted muscle layer (M). Cellular infiltration (Inf). **e.** Lung section from the CLP+BER group showing lung architecture restoration with patent alveoli (A) and alveolar sacs (AS) separated by thin interalveolar septa (arrows). Moderate cellular infiltration (Inf). **f.** Lung section from the CLP+BER group showing normal blood vessels (V) and patent bronchioles (B) with an intact muscle layer (M). Minimal cellular infiltration (arrow). **g.** Statistical analysis of the thickness of alveolar septa. H&E (hematoxylin and eosin), ×200. For abbreviations see Figure 1.

disturbances were significantly ameliorated in CLP+BER group but did not yet attain the control values (Fig. 8).

Discussion

Sepsis is a severe life-threatening pathological condition that is characterized by a significant deterioration in general body condition with the possible progression to multisystem failure. Sepsis is well known to be a leading mortality cause, especially in the elderly population, despite the widespread antibiotics used in critical care settings (Prescott and Angus 2018). The outcome of sepsis conditions strongly correlates with the premorbid general status and the integrity of the immune system along with the other physiological adaptive mechanisms (Prescott et al. 2019).

Table 2. Immunohistochemistry scoring of lung tissue

	Control group	CLP group	CLP + BER group
Thickening of the interalveolar septa	–	++++	++
Cellular infiltration	–	++++	++
Desquamation of alveolar epithelium	–	++++	++
Bronchial and bronchiolar degeneration	–	++++	+
Disorganization of lung architecture	–	++++	+
Collagen deposition	+	+++	++
Anti-Cox-2 immunoreactivity	+	++++	++

CLP, cecal ligation and puncture; CLP+BER, Berberine pretreated CLP group; Anti-Cox-2, anti-cyclooxygenase; – none; + mild; ++ moderate; +++ severe; ++++ very severe.

Given that the outcome strongly depends on the premorbid conditions, several recent studies shed light on encouraging results that nutrients and vitamin supplementations could modify the sepsis outcome (Kuhn et al. 2018; Waele et al. 2020). In the current study, we investigated the potential capability of Berberine supplementation to attenuate lung sepsis outcomes.

Berberine is well-known for its hypoglycemic, antifibrotic, and immuno-modulatory effects. However, its role as an effective antiseptic agent has not yet been confirmed (Wang et al. 2017). Moreover, Berberine has been used in traditional medicine for a long time as an anti-infective agent, however, these effects have not yet been adequately confirmed by appropriate studies. The exact impact on different organs, pathogenic species, and mechanisms of such antiseptic effects, if present, are not yet precisely known (Aswathanarayan and Vittal 2018).

Our results revealed the protective effects of the Berberine pretreatment against pulmonary sepsis. These results were consistent with those of (Pierpaoli et al. 2021), who found that Berberine beneficially affected the response against systemic *E. coli* infection (Piperaoli et al. 2021). Several inflammatory, and antioxidant stress downstream signaling cascades are involved in the adaptive responses to septic conditions. The complexity of such interactions and the importance of adequate premorbid anti-inflammatory and antioxidant agents highlight the importance of studying inflammatory and oxidative stress biomarkers and their relations to any investigated potential therapeutic modality (Gibbs et al. 2021).

Our findings suggested that Berberine could potentially directly affect the anti-inflammatory and oxidative stress signaling pathways in septic rats. These results were in agreement with (Shi et al. 2018).

However, the cytokine level modulatory ability of Berberine was considered by certain authors a double-edged sword as the potential Berberine-induced immunological changes could unexpectedly influence the infection outcome (Piperaoli et al. 2021). Therefore, further studies are still required to clarify the exact Berberine interactions with different cytokines and immune pathways in humans.

MPO catalyzes the formation of reactive oxygen intermediates, including hypochlorous acid (HOCl), and is also considered to be a crucial molecule involved in the microbial scavenging processes of the immune system. In addition to its functions in immune regulation and cytokine production control, it displays diagnostic functions, used as a biomarker for acute infections or a local mediator in cases of tissue damage. MPO levels gain utmost importance as a diagnostic and prognostic parameter in research (Aratani 2018). Our results showed increased lung tissue MPO activity in the CLP group, confirming the CLP-acute inflammatory effect contributing to tissue damage. On the contrary, MPO activity decreased in the CLP+BER group, reflecting protective, anti-inflammatory, and tissue damage-preventive effects. These results were consistent with other research results, concluding that MPO instigates an immune response in the case of sepsis (Yu et al. 2020).

Moreover, our results revealed a significant increase in the expression of inflammatory mediators such as *NF-κB, MCP-1,* and *ICAM-1* in the lung tissue and IL-6 and TNF-α concentration in the BALF of the CLP group confirming a CLP-induced acute inflammation (Schrijver et al. 2017). Oppositely, the CLP+BER group showed a significant reduction in these inflammatory cytokines reflecting the significant anti-inflammatory effect of Berberine and its ability to affect cytokine levels *via* its modulatory effects affecting various limbs of the immune system (Mittal and Sanyal 2011). Although it was recently proven that Berberine is effective in the NF-κB pathway modulation, the exact role of such modulation in sepsis, particularly in lung sepsis, still requires further clarification (He et al. 2017; Wu et al. 2018).

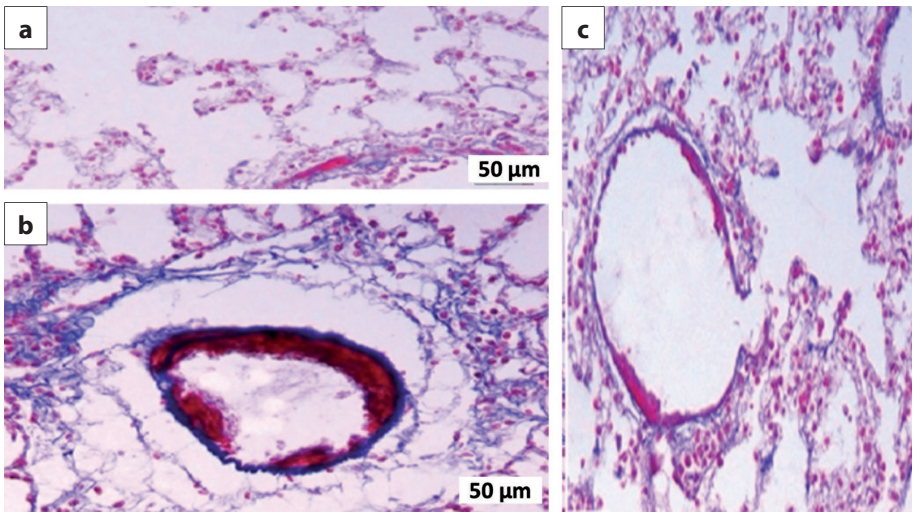

Figure 6. Masson's trichrome staining of lung sections among all study groups. **a.** The lung section from the control group shows fine collagen fibers within the lung interstitium. **b.** Lung section from the CLP group showing marked deposition of collagen fibers in the lung interstitium. **c.** Lung section from the CLP+BER group showing mild deposition of collagen fibers in the lung interstitium. Masson's trichrome, ×200. For abbreviations see Figure 1.

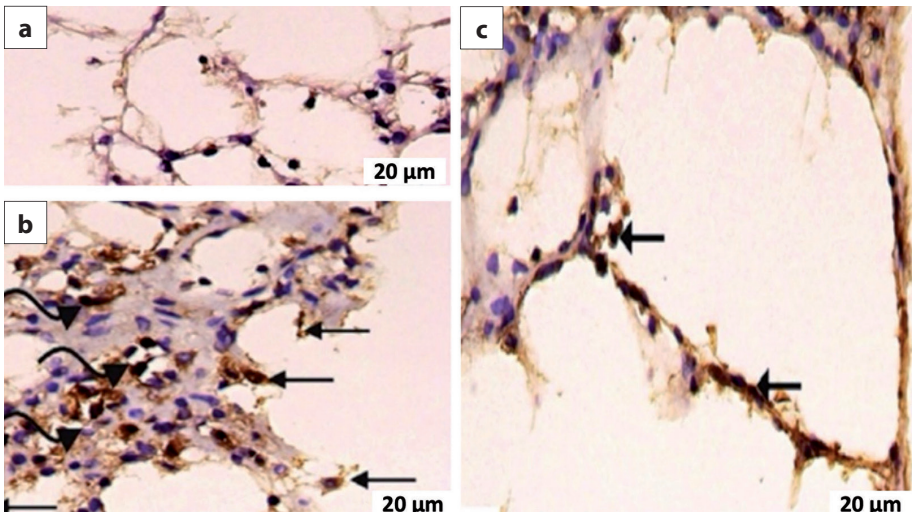

Figure 7. Immunohistochemistry reactions in the alveolar cells among all study groups. **a.** Lung section from the control group showing faint immunohistochemical reaction in a few alveolar cells (arrows). **b.** Lung section from the CLP group showing strong immunohistochemical reaction in numerous alveolar cells (arrows) and interalveolar cells (curved arrows). **c.** Lung section from the CLP+BER group showing mild immunohistochemical reaction in certain alveolar cells (arrows). Anti-Cox-2 (anti-cyclooxygenase 2), ×400. For abbreviations see Figure 1.

Furthermore, the Berberine's potent antioxidant properties were well-proven by several recent studies (Ehteshamfar et al. 2020; Rajasekhar et al. 2020) in accordance with our results, confirmed by the improvement in the lipid peroxidation biomarker MDA which was triggered by CLP. In support of our results, a recent study proved that the administration of a natural antioxidant-promoting factor, such as selenium, was associated with clinical improvement of sepsis and attenuated systemic inflammatory response syndrome (SIRS) response, suggesting a potential therapeutic link between reactive oxygen species (ROS) and SIRS (Andrades et al. 2011).

In addition, a significant increase in Cox-2 immunoreactivity mean area % was observed in lung sepsis (Kong et al. 2020; Chen et al. 2021). However, the Berberine pretreatment in this study markedly reduced lung inflammation, and interalveolar thickening, and preserved lung architecture close to the control level. Our results were consistent with others (Yang et al. 2020; Bormann et al. 2021). Also, Liang et al. reported that Berberine improved lung morphology and protected against lipopolysaccharide (LPS)-induced ALI (Liang et al. 2019). This could be explained by previous studies reporting that Berberine might reverse the NF-κB signaling upregulation during its translocation from the cytoplasm to the nucleus and attenuate inflammatory reactions in the lung and other organs (Sadraie et al. 2019; Zhai et al. 2020; Xu et al. 2021). Also, Zhao et al. reported that Berberine treatment, prior to lung injury induction, inhibited the production of various proinflammatory cytokines and significantly reduced the inflammatory cells in the lung tissues compared to the non-treated group. This might contribute to the vital role of Berberine in mitophagy attenuation (Zhao et al. 2021).

Although adequate knowledge regarding the molecular mechanisms underlying septic complications and fatalities is still missing, the loss of the normal redox balance is a reasonable explanation for the rapidly progressing complications and multisystem failure in sepsis (Toro-Perez and Rodrigo 2021), associated with toll-like receptors (TLR) activation leading to inflammation, increasing adhesion molecule expression on endothelial and blood cells (Nucci et al. 2017), with an excessive release of various cytokines, nitric oxide synthase (NOS) pathway upregulation, and concomitant increase in nitric oxide (NO) production, therefore, the cross-talk between oxidative stress and inflammation may worsen the sepsis outcome (Chousterman et al. 2017; Spiller et al. 2019).

In addition, the current study aimed to investigate the possible involvement of PPAR-γ in experimentally induced lung sepsis and explore its potential contribution to the protective

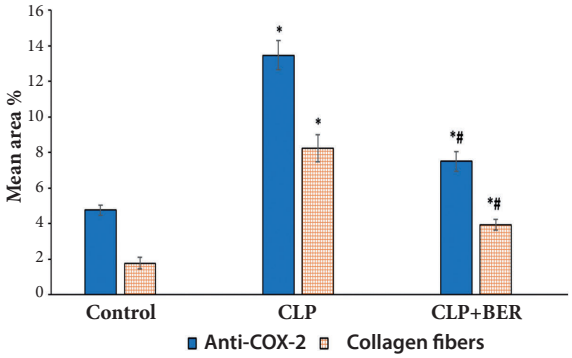

Figure 8. Histomorphometry results of area % of anti-Cox-2 immunoreactivity and collagen fibers. A significant increase in the area % of anti-Cox-2 immunoreactivity and collagen fibers was observed in the CLP group. However, these disturbances were significantly ameliorated by Berberine in CLP+BER group. Results were represented as mean ± standard deviation. * $p < 0.05$ *vs.* control, # $p < 0.05$ *vs.* CLP. Anti-Cox-2, anti-cyclooxygenase 2. For other abbreviations see Figure 1.

Berberine effect. PPARs are nuclear hormone receptors of ligand-activated transcription factors, and they play a key role in the regulation of various physiological functions, with different types of receptors and variable expressions among various tissues such as lipid and carbohydrate metabolism (Tutunchi et al. 2020; Zhao et al. 2020). In addition, PPAR agonists of different types are either known or potential therapeutic modalities for different pathological conditions such as insulin resistance (Diniz et al. 2021), atherosclerosis (Montaigne et al. 2021) and Alzheimer's disease (Wojtowicz et al. 2020).

The effect of sepsis on the PPAR signaling pathway is controversial. Our study showed a statistically significant PPAR-γ protein expression reduction detected by the Western blotting in the CLP group compared to the control rats. These results were consistent with those of several recent publications that showed significant PPAR pathway downregulation under septic conditions both in animal model- and human-based studies (Wang et al. 2017; Liu et al. 2020; Van Wyngene et al. 2020).

Oppositely, our findings revealed a statistically significant improvement in the lung PPAR-γ protein expression among the Berberine-treated rats compared to the CLP group. In concordance with our findings, literature demonstrated that the antimicrobial and herbal remedy-related antiseptic effects are achieved *via* the activation of the PPAR-γ pathway (Siddiqui et al. 2006). PPAR-γ protein expression upregulation could be a reasonable explanation for the wide range of Berberine-induced effects. This is consistent with several recent study results that confirmed increased PPAR-γ protein expression upon Berberine administration (Zhou et al. 2019; Sahraei et al. 2020; Xu et al. 2021). Whether the protective effects of Berberine, mediated *via* PPAR pathway upregulation, are exerted by direct antimicrobial effects, or related to general antioxidant and antiapoptotic mechanisms remains unclear, although it was strongly postulated that both mechanisms are partially involved. However, confirmation of the exact role of each mechanism in septic conditions, particularly in humans, still needs further research.

Nevertheless, other research revealed sepsis-induced PPAR-γ pathway upregulation (Reddy et al. 2008). This contradiction could be related to the type of induced sepsis in the study, the stage of the septic complications, or the considerable difference between the animal models and human patients. Nevertheless, this aspect still requires further research and clarification.

There are insufficient studies examining the protective effects of Berberine at the cellular level, either generally or under sepsis. We hypothesized that Berberine has a therapeutic impact not only on the functional and biochemical, but also on other histological and cellular levels. The histopathological lung tissue findings in this study were consistent with those of previous studies, which proved the CLP-induced alteration of the typical lung architecture such as collapsed alveoli, thickened interalveolar septa, extravasated red blood cells (RBCs), degenerated bronchioles, in parallel with massive inflammatory cellular infiltration. In addition, a significant increase in collagen fiber deposition was observed in lung sepsis (Kong et al. 2020; Chen et al. 2021). Also, our results were in alignment with Yue and his colleagues who reported that CLP induced pulmonary fibrosis at day 5 and day 10 timepoints evidenced with the elevation in the fibrogenic cytokines TGF-β, matrix metallopeptidase 9 (MMP-9), and TIMP1, a tissue inhibitor of metalloproteinases in lung tissues in parallel with enhanced immunohistochemical staining with both alpha-smooth muscle actin (α-SMA) and fibronectin (Yue et al. 2016).

However, the Berberine pretreatment in this study markedly reduced it. The Masson's trichrome stained lung sections showed a reduced collagen fiber mean area % in the CLP+BER group compared to the CLP group. This result was in line with those of Guan et al. showing that Berberine markedly inhibited collagen deposition and protected against lung fibrosis (Guan et al. 2018). Moreover, Li et al. discovered that Berberine displayed a synergistic protective effect on dexamethasone treatment for lung fibrosis, proven by a significant reduction in collagen deposition. The authors mentioned that Berberine inhibited fibrosis *via* the Smad2/3 (suppressor of mothers against decapentaplegic) transcription factors signaling pathway (Li et al. 2019). This also could be explained by the influential role of Berberine in preventing extracellular matrix deposition, collagen downregulation, fibronectin expression, transforming growth factor (TGF) signaling inhibition, and macrophage polarization (Bansod et al. 2020).

Cyclooxygenase-2 (Cox-2) is a ROS- and peroxide-producing enzyme that activates proinflammatory cytokines and causes oxidative stress-induced tissue damage (Zaki et al. 2021). Cox-2 upregulation occurs in different cells, including fibroblasts, as a result of transforming growth factor-β (TGF-β) activation that stimulates fibroblasts and ROS release, enhancing fibrogenesis (Chen et al. 2021).

This study could provide an explanation of how the Berberine pretreatment could reduce inflammation and fibrosis, and improve the functional, biochemical, and structural parameters *via* PPAR-γ upregulation and Cox-2 signaling pathway inhibition. However, further investigations are required concerning the impact of these findings and their application in humans and under different clinical conditions.

There are some limitations to our study including the absence of a positive control group (Berberine) to record any toxic signs such as >20% body weight loss, inappetence, prolonged diarrhea, motor abnormalities, hypothermia, dehydration, vocalizations/respiratory difficulty, discharge from eyes, nose, mouth and perianal area. However, the dose was selected based on the research of Lee et al. (2006) and Hu

et al. (2012) in which Berberine intake showed a protective effect without any reported adverse effects as well and our rats were continuously monitored throughout the experiment without recording any dangerous signs otherwise the study would be terminated. However, adding a Berberine to the study would ultimately add to the validity of the results. Secondly, comparing the efficacy of various doses of Berberine in different experimental models using various ways of sepsis induction to determine the most effective dose regimen with the highest bioavailability and the least adverse effect needs to be further investigated. Thirdly, comparing the prophylactic *versus* the therapeutic effects of Berberine is still needed. Fourth, the small sample size of our study could be a limiting factor, however, ongoing experiments with a greater sample size will give more added power to detect significant differences in the sepsis outcomes.

Further animal and human research is still required to understand the underlying pathophysiology of sepsis and to explore other mechanisms mediating the protective mechanisms of Berberine against lung sepsis as well as the safe dose and duration of administration.

Conclusion

Berberine pretreatment mitigated the outcome of CLP-induced lung sepsis *via* various mechanisms, including antioxidant and anti-inflammatory effects, as well as the upregulation of PPAR-γ protein expression, which was emphasized by the histological improvements at the cellular level. Therefore, our results suggested that Berberine could be an adjuvant anti-septic remedy in addition to the conventionally used regimens.

Ethical approval. The current was approved by the Institutional Animal Care and Use Committee "IACUC" with ethical committee approval number: CU-III-F-4-22.

Authors' contribution statement. Mohamed M. Khalifa, Nermeen A. Bastawy, Laila A. Rashed, Hanan A. Hassan, Omnia M. Abdel-Maksoud, and Fatma E. Hassan conceptualized, designed, carried out, and supervised the experiment, analyzed, and interpreted the data; and participated in writing the manuscript draft and approved the final version.

Data availability statement. The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest. The authors declare no conflict of interest.

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