Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Bilobalide, A Bioactive Compound on Sepsis-Induced Acute Lung Injury through its Anti-Inflammatory and Antioxidative Activity

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Submitted: 25-Sep-2020 Revised: 23-Nov-2020 Accepted: 13-Jan-2021 Published: 15-Apr-2021

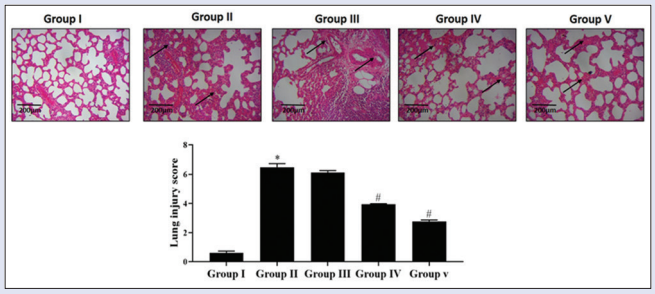
ABSTRACT

Background: Sepsis, one of the major life-threatening conditions and responsible for approximately 40% of clinical Acute Lung Injury (ALI) patients. Nevertheless, there are no specific medications available to reduce mortality. Bilobalide, a natural bioactive component present in *Ginkgo biloba,* has several medicinal properties. However, the effect of Bilobalide against ALI residues unknown. And so, the therapeutic property and underlying molecular mechanism of Bilobalide was investigated against sepsis-prompted ALI in the murine model. **Materials and Methods:** In C57BL/6J mice model, sepsis introduced by cecal ligation and puncture (CLP) to induce lung injury. **Results:** Our results showed that Bilobalide treatment increased the survival rate of CLP-induced sepsis mice. Pretreatment with Bilobalide substantially inhibits the sepsis-induced lung exudation, which is exposed by measuring the wet/dry weight of lung and lung permeability of the mice. Also, Bilobalide attenuated the histopathological alterations alike alveolar hemorrhage and infiltration of inflammatory cells are assessed by hematoxylin and eosin staining, tumor necrosis factor-α, interleukin (IL)-1 β, IL-6 and macrophage inflammatory protein-2 pro-inflammatory mediators and myeloperoxidase are measured by enzyme-linked immunosorbent assay. Molecular mechanism of Bilobalide in lung inflammation whereby cyclooxygenase-2 (COX-2), induced nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1), and the activation of the nuclear factor-kappa B (kB) (p65)/IkB are determined by immunoblotting technique. Moreover, pretreatment of Bilobalide significantly downregulated the expression of COX-2, iNOS, and phosphorylation of p65 and induced the IkB activation in the lung. Further, Bilobalide prevented the oxidative stress by upregulating the expression of HO-1 in lung tissues, and gene expression of sepsis-induced anti-oxidative enzymes (catalase, MnSOD, CuZnSOD, and GPx-1) in the Bilobalide-treated mice were induced dose-dependently and it determined by a quantitative-real time-polymerase chain reaction. **Conclusion:** Hence, we suggested Bilobalide has the ability to act as a possible therapeutic candidate against sepsis caused ALI.

Key words: Acute lung injury, antioxidant, bilobalide, inflammation, nuclear factor-kappa B, sepsis

SUMMARY

- Bilobalide significantly ameliorates histopathological alterations by reducing alveolar damage and inflammatory responses
- Bilobalide inhibits the sepsis-induced pro-inflammatory mediator responses by blocking nuclear factor-kappa B translocation and its activation in murine and macrophage models.



Abbreviations used: ALI: Acute lung injury, COX-2: Cyclooxygenase-2, NMBA: Neuromuscular blocking agent, NF-kB: Nuclear factor-kappa B, TNF-α: Tumor necrosis factor, IL-1 β: Interleukin1 β.

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DOI: 10.4103/pm.pm_448_20

Access this article online
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INTRODUCTION

Sepsis is one of the life-threatening medical disorder, characterized by the immodest inflammatory response, coagulation, and multiple organ injuries stimulated by microbial infection.[1] The lung, one of the important susceptible organs face approximately 40% acute lung injury (ALI) prevalent by sepsis.[2,3] Till now, there is no specific medication available to treat the sepsis-induced ALI. General supportive care for the sepsis-induced ALI patients involves, short-term use of the neuromuscular blocking agent in early-stage and anti-inflammatory drugs in the effective treatment.[4,5] The role of corticosteroid and Statins are reported to reduce inflammation in sepsis-induced ALI. However, recent multicenter trials of the above-mentioned cares are disclosed there is no mortality benefit in sepsis-induced ALI.[6,7]

The pathophysiological alteration of sepsis prompted ALI, uncontrolled accumulation of the acute inflammation by stimulating the pro-inflammatory mediators in the host cells through activating several pathways. Recent shreds of evidence indicate nuclear factor-kappa B (NF-κB) encourages the pro-inflammatory gene expression in

Cite this article as: Wang F, Huang J, Li J, Chen K, Zhang X, Zhang Y, *et al*. Bilobalide, A bioactive compound on sepsis-induced acute lung injury through its anti-inflammatory and antioxidative activity. Phcog Mag 2021;17:163-9.

the lung. NF-kB leads to excessive accumulation of tumor necrosis factor (TNF)-α, interleukin (IL)-1 β, IL-6, (macrophage inhibitory protein [MIP])-2, and nitrite oxide (NO). Especially, inducible nitric oxide synthase (iNOS) is involved in the production of NO in the inflammatory pathogenesis. While the over-expression of NO is believed to induce central inflammatory mediator secretion and it dangerous to humans. Besides, uninhibited inflammatory cell migration into the lungs also can lead to inflating the myeloperoxidase (MPO), reactive oxygen species (ROS) production that induces the uncontrolled inflammatory mediator production, lung injury, and/or death. Another important target is the catalytic activity of enzymatic antioxidants in neutralizing specific free radicals for instance catalase, superoxide dismutase (SOD) and glutathione increase the cellular ability against ROS/oxidative free radicals. Hydrogen peroxide (H_2O_2) is the product of superoxide catalyzed by SOD dismutase and serially reduction of H_2O_2 to water carried out by catalase and glutathione peroxidase (GPx).[8] Therefore, inhibition of inflammation and oxidative activity is a promising therapeutic approach to sepsis-induced ALI.[9-11]

Bilobalide is a terpenoid trilactone present in the Chinese traditional medicinal plant *Ginkgo biloba* L., has shown a wide variety of therapeutic applications against clinical disorders like neurodegenerative disorder,[12] vascular dementia,[13] and liver damage.[14] Also, several favorable biological effects of *G. biloba* extract anticipated the protection of nerve cell death,[15] free radical scavenging, anti-inflammation, antitumor, anti-aging,[16,17] cardioprotective property,[18] and necrosis to the immortalized skin cells. Even so, potent anti-inflammatory property and molecular mechanism of Bilobalide against ALI have not yet been revealed. Therefore, this study evidenced that the anti-inflammatory and antioxidant role of Bilobalide and its fundamental mechanism of sepsis-mediated activation of NF-κB/IKK and HO-1 pathway in male C57BL/6J murine model.

MATERIALS AND METHODS

Chemicals

Bilobalide, phosphate-buffered saline (PBS), sodium dodecyl sulfate (SDS), 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) was procured from the Sigma chemicals, USA. All the enzyme-linked immunosorbent assay (ELISA) assay kits and polymerase chain reaction (PCR) kits were attained from Cayman Chemicals, USA, and Applied Biosystems, CA, USA, respectively.

Mice and grouping

Approximately 24–30 g (8–10 weeks aged) weighed, male C57BL/6J were kept in a specific-pathogen-free conditioned room at the temperature 25°C and relative humidity was 50 ± 10% under dark and light cycle at 12 h, water and food are delivered *ad libitum* and all the protocols are given by Committee for control and supervision on experiments on animals.

Mice are divided into five groups: (i) Sham or control group (Same amount of sterile saline [0.9%] is injected to the sham and cecal ligation and puncture (CLP) groups through intraperitoneal way), (ii) CLP group, (iii) CLP + Bilobalide (20 mg/kg body weight [b. w] of mice) group, (iv) CLP + Bilobalide (40 mg/kg) group, (v) CLP + Bilobalide (80 mg/kg) group.

Mice acute lung injury induction and treatment

To the mice, sepsis induction was done by CLP technique[19] and Bilobalide treated with respective concentrations for 48 h before CLP induction through intraperitoneally. After 48 h of Bilobalide treatment, mice are anesthetized via 75 mg/kg of ketamine/15 mg/kg of xylazine mixture solution through the intraperitoneal way. Following hair removal abdomen is disinfected with isopropanol (75%). The incision (0.5–1 cm) was made in the lower abdomen. Then, the cecum was introduced below the ileal papilla and the incision was closed. The cecum is introduced to the sham mice group with the absence of ligation or puncture. Finally, the mice are refused by sterile saline and subjected to food and water. After 24 h of CLP induction, all the mice groups are sacrificed and collected the lungs for further analysis.

Assessment of lung injury

The lungs are collected and the wet weight is measured immediately and placed in a hot air oven at 75°C–80°C on 48 h for measuring the dry weight of the lung. The ratio of lung weight in the wet and dry conditions is directly proportional to the edema infiltration. The damage of the lung (Alveolar permeability) was also evaluated by determining the total protein concentration present in BAL fluid using the Bradford assay method.

Assessment of inflammation and Myeloperoxidase activity

The right lobes of the lungs are lavaged three to four times using 1.0 ml ×1 PBS which contains 1M ethylenediaminetetraacetic acid solution, then the BAL fluid is centrifuged and stored the collected supernatant in a deep freezer (−80°C) for further analysis. Also, the pelleted cells are collected and erythroid cells are lysed using ACk lysis buffered solution by centrifuging at 1500 rpm for 10 min. Then the pelleted cells are resuspended with 200 µl of sterile ×1 PBS and the neutrophil is counted by the Wright-Giemsa staining method.

Further, MPO activity is measured in the lung tissue homogenate. Tissue homogenate is prepared by homogenizing the lung tissue using Radioimmunoprecipitation Assay buffer. Then, it centrifuged at 1500 rpm for 15 min at 4°C and the supernatant is collected. From the collected supernatant, the presence of MPO is assessed by the ELISA method as per the protocol given by the manufacturer.

Histological analysis

Following 24 h of sepsis instillation, the left lobe of the lung is isolated and fixed with 4% paraformaldehyde for 48 h. Then it was dehydrated, embedded in paraffin, cut into 5 µm thickness, and pigmented using hematoxylin and eosin (H and E). Then, injury of the lung was scored by ImageJ software.

Quantification of pro-inflammatory mediator secretion

Following 24 h of post-CLP exposure, pro-inflammatory cytokine production (TNF-α, IL-1 β, IL-6, and MIP-2 in BAL fluid and prostaglandin-E2 tissue homogenate of lunging each experimental groups are evaluated by ELISA kits and conformed the protocols of the manufacturer.

Immunoblot assay

An equal amount of protein samples (30 µg per sample) is subjected to the wells of 10% concentrated SDS-polyacrylamide gel electrophoresis. After separation, the membrane blotted onto polyvinylidene difluoride membrane to transferring the protein samples. Then, the membrane blocked using 5% fat-free skim milk at room temperature for 60 min. Then, the membrane keeps into the relevant primary antibody against iNOS, COX-2, HO-1, p-NF-kB, IkB-a, and β-actin for overnight incubation at 4°C. Following incubation, the membrane is washed and incubated with the HRP-labeled secondary antibody at room temperature

for 60 min. Then, protein bands are visualized and photographed using an enhanced chemiluminescence detection kit and band intensity is calculated by ImageJ software.

Griess assay

NO_2^- accumulation is the notable identifier of nitrous oxide (NO) production in the lung tissue homogenate. An equal amount of Griess reagent which consists of sulfanilamide (1%), naphthyl ethylene diamine dihydrochloride (0.1%), and phosphoric acid (2%) was added to the protein lysate of the lung and it kept at room temperature for 10 min. A standard curve was made by using sodium nitrate ($NaNO_2$) and the amount of nitrite is quantified at 550 nm in a spectrophotometer.

Gene expression analysis

Total RNA is isolated in the left lobes of the lung tissues using TRIzol reagent (Invitrogen, CA, USA). Then, 1 μg of total RNA is reverse transcribed into complementary DNA (cDNA) by reverse transcription kits (Applied Biosystems, CA). Then, the quantitative-real time-(qRT)-PCR reaction is executed by SYBR Green PCR Master Mix (Applied Biosystems, CA) using Applied Biosystems 7300 qRT-PCR machine, and cycle threshold values (computed tomography) are automatically calculated in an average of triplicates and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences of the sense and antisense gene are following catalase (forward-AATCAGAAGGCAGTCCTCCC-3′ and reverse 5′-TCGGGGAGCACAGAGTGTAC-3′), MnSOD (for ward-5′-ATGTCTGTGGGAGTCCAA-3′ and reverse 5′-TGAAGGTAGTAAGCGTGCTC-3′, CuZnSOD (forward-5′-GACAAACCTGAGCCCTAAG-3′ and reverse-5′-CGACCTTG CTCCTTATTG-3′, GPx-1 forward-5′-GACTACACCGAGATG AACGAT-3′ and reverse-5′-CACTTCGCACTTCTCAAACA-3′ and GAPDH (forward-5’-CAATGACCCCTTCATTGACC-3’ reverse-5’-GACAAGCTTCC CGTTCTCAG-3’).

Cell culture maintenance

RAW264.7 cells are seeded in Dulbecco's modified Eagle medium which contains fetal bovine serum (10%) and 0.1% of antibiotic/antimycotic solution under the humidified condition at 37°C with 5% CO_2.

Cell viability assay

MTT reagent is used to assess the cell viability. Briefly, 1.0×10^4 of RAW264.7 cells are cultured per well of 96-well culture plate and keep it in a CO_2 incubator on 24 h. Then, the cells are introduced by 10, 50, or 100 μg/ml of Bilobalide for 4 h before 1 μg/ml of LPS instillation. 20 μl of MTT (5 mg/ml) reagent was added to all the wells and kept in a dark condition for 4 h at 37°C. After the incubation, media was discarded and crystal formazan was liquefied with DMSO (150 μl) solution. Then, the absorbance was recorded at 490 nm using a microtiter plate reader. Control group value is considered as 100% of viability.

Nuclear factor-kappa B p65 DNA binding activity assay

To examine the NF-κB DNA-binding level, the cellular nuclear proteins were isolated from both control and bilobalide treated RAW264.7 cells with the aid of an extraction kit (Cayman Chemicals, USA). Then the NF-κB p65 subunit DNA-binding level was investigated in the extracted nuclear proteins with the help of assay kits as per the manufacturer guidelines (Cayman Chemicals, USA). A specific DNA, which contains an NF-κB response element sequence, is immobilized on the 96-well plate bottom. NF-κB p65 subunit is identified via the addition of a specific primary antibody against NF-κB p65. A secondary antibody conjugated to HRP is mixed to provide absorbance at 450 nm.

Statistical analysis

Values were shown such as mean ± standard deviation (SD). Statistical data were accomplished non-parametrical manner by Kruskal–Wallis test to determine the significant difference between different experimental groups. Statistical significant is established as $P \leq 0.05$.

RESULTS

Bilobalide reduces cecal ligation and puncture-induced inflammatory response in septic mice

Histopathological alterations in lungs of sepsis-induced ALI in mice with or without Bilobalide pretreatment are investigated by H and E staining and the changes are assessed by light microscopy [Figure 1]. In the sham group, normal alveolar walls are typically normal and no inflammatory cell infiltration. In the sepsis-induced mice, excessive infiltration of inflammatory cells, alveolar damages, inter-alveolar wall thickening, hemorrhages, and edema are shown in the lungs. However, Bilobalide pre-treatment repressed the inflammatory cell permeation and histopathological alterations in the lungs by a dose-dependent manner. Based on these results, lung injury score was calculated and it confirms the same result.

Bilobalide ameliorates cecal ligation and puncture-induced lung injury in acute lung injury mice

The protective role of Bilobalide against CLP-induced lung injury is estimated by measuring the ratio of wet-to-dry of the lung and total protein concentration in BAL fluid. When compared to the untreated group, CLP significantly increased the wet-to-dry ratio of lung and total protein concentration in BAL fluid of ALI mice. Nevertheless, pretreatment with Bilobalide expressively ameliorated the CLP increased wet-to-dry ratio of lung and total protein concentration in BAL fluid in a dose-dependent manner [Table 1]. These findings assured that Bilobalide dose-dependently reduces the sepsis-induced inflammatory response and injury in the lungs.

Bilobalide attenuates myeloperoxidase activity and neutrophil permeation

Then, the effect of Bilobalide on MPO activity and neutrophil permeation were examined in the homogenate of lung tissue and BAL fluid respectively. These are also the histological markers of the

Table 1: Effect of bilobalide on the cecal ligation and puncture-induced lung injury in acute lung injury mice

Experimental groups	Wet/dry weight ratio	Protein in BALF
Group I	1.65±0.36	0.28±0.95
Group II	3.69±0.18*	3016.49±0.53*
Group III	3.71±0.49	3025.62±0.19
Group IV	3.14±0.37#	2507.93±0.48#
Group V	2.65±0.94#	1536.47±0.62#

The pre-treatment with bilobalide expressively ameliorated the CLP increased wet-to-dry ratio of lung and total protein concentration in BAL fluid. Values were depicted as a mean±SD of triplicates (n=6). The significance level was determined by using Kruskal–Wallis test. *P<0.05 when compared with control; #P<0.05 when compared with the sepsis-induced group. SD: Standard deviation; CLP: Cecal ligation and puncture; ALI: Acute lung injury

neutrophil cell invasion and lung burden. After 24 h of CLP induction, MPO activity and neutrophil infiltration were significantly increased compared to the sham mice. Moreover, Bilobalide dose-dependently reduced MPO activity in lung tissue lysate and neutrophil infiltration in BAL fluid [Figure 2]. These results were further supported that Bilobalide significantly ameliorates inflammatory cell infiltration.

Bilobalide inhibits cecal ligation and puncture-induced inflammatory mediator secretion in BAL fluid

To study the inhibitory property of Bilobalide on inflammatory mediators production by CLP method, the amount of TNF-a, IL-1b, IL-6, and MIP-2 in the BAL fluid was quantified by ELISA technique. Table 2 presented sepsis significantly increased TNF-α, IL-1 β, IL-6, and MIP-2 presence in the BAL fluid of CLP-mice, compared to non-septic mice. Pretreatment with Bilobalide significantly inhibits the above pro-inflammatory mediators compared to the Septic mice group.

Role of Bilobalide on cecal ligation and puncture-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in sepsis mice

The possible underlying mechanism of Bilobalide in CLP-induced mice, the influence of Bilobalide on COX-2 and iNOS expression in all mice groups are examined. As shown in Figure 3, sepsis induction was notably increased COX-2 and iNOS expression in lung tissue homogenates and was dose dependently repressed by pre-treatment of Bilobalide. As well as, Bilobalide treatment dose-dependently down-regulates the NO and PGE2 presence in the lung tissue lysate of septic mice. The above result reliable with the result of iNOS and COX-2 expression in the Bilobalide treated mice group.

Table 2: Effect of bilobalide on the levels of inflammatory mediators in the BAL fluid

Experimental groups	TNF-α	IL-1β	IL-6	MIP-2
Group I	47.17	23.8	0.69	0.28
Group II	156.54*	110.01*	254.93*	13.87*
Group III	153.95	109.65	258.9	12.13
Group IV	129.19#	68.08#	124.69#	5.34#
Group V	77.53#	38.23#	57.41#	2.59#

Bilobalide treatment significantly decreased the TNF-α, IL-1β, IL-6, and MIP-2 levels in the BAL fluid of CLP mice. Values were depicted as a mean±SD of triplicates (n=6). The significance level was determined by using Kruskal–Wallis test. *P<0.05 when compared with control; #P<0.05 when compared with the sepsis-induced group. CLP: Cecal ligation and puncture; TNF: Tumor necrosis factor; IL: Interleukin; SD: Standard deviation; MIP: Macrophage inflammatory protein

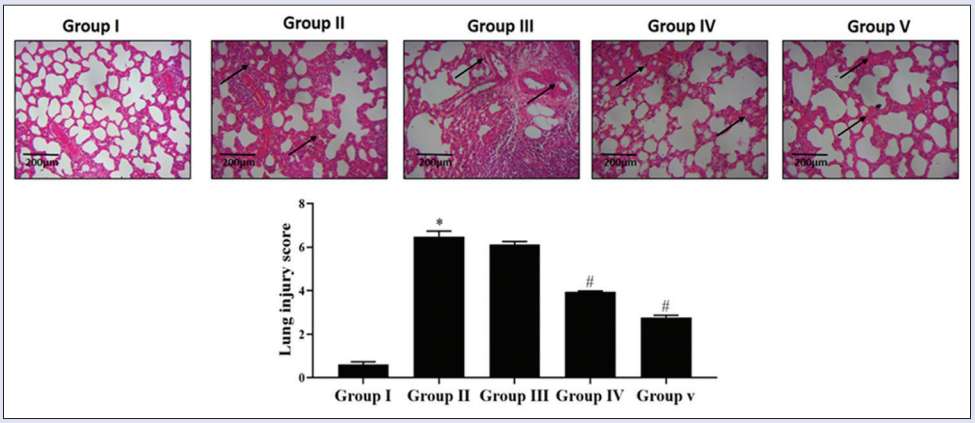

Figure 1: Effect of Bilobalide on reduces cecal ligation and puncture-induced inflammatory response in septic mice. The normal alveolar tissue architecture and no inflammatory conditions were noted in control (Group I). The excessive inflammatory cells infiltration, alveolar damages, and edema were noted in sepsis-induced mice (Group II). Bilobalide (20, 40, and 80 mg/kg) pretreatment repressed the inflammatory cell permeation and histopathological alterations in the lungs (Group III-V). Values were depicted as a mean ± standard deviation of triplicates (n = 6). The significance level was determined by using Kruskal–Wallis test. Note: "*" indicates P < 0.05 when compared with control; "#" indicates P < 0.05 when compared with the sepsis-induced group. Histological alterations were pointed out by using arrow marks

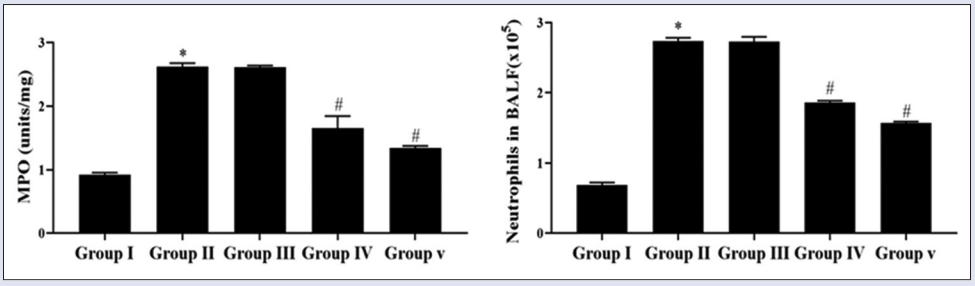

Figure 2: Effect of Bilobalide on the MPO activity and neutrophil permeation. The Bilobalide treatment dose-dependently reduced the myeloperoxidase activity in the lung tissues and neutrophil infiltration in BAL fluid of sepsis-induced mice. Values were depicted as a mean ± standard deviation of triplicates (n = 6). The significance level was determined by using Kruskal–Wallis test. Note: "*" indicates P < 0.05 when compared with control; "#" indicates P < 0.05 when compared with the sepsis-induced group.

Anti-oxidative properties of Bilobalide in septic mice

The role of the antioxidative property of Bilobalide was determined by analyzing the gene expression of catalase, MnSOD, CuZnSOD, and GPx-1 in septic mice. Sepsis induction notably reduced the catalase, MnSOD, CuZnSOD, and GPx-1 expression in gene level compared to the untreated [Figure 4a]. Whereas, treated with Bilobalide significantly increased the expression of the above AOEs in lung tissues. Also, sepsis induction repressed the HO-1 expression compared to the sham group mice. Bilobalide dose-dependently augmented the HO-1 expression [Figure 4b]. Altogether, these results are indicated that Bilobalide can reduce the CLP-induced oxidative damage through increasing AOEs and HO-1 expression.

Bilobalide inhibits the activation of nuclear factor-kappa B (p65) in septic mice

NF-kB (p65) signaling cascade phosphorylation plays a main starring role in inflammatory mediator's production. Therefore, we determined the sepsis-induced activation of NF-kB (p-p65) and Bilobalide treatment mice groups. As shown in Figure 5, sepsis exaggerated the activation of NF-kB (p-p65) was in the lungs. On the other hand, pre-treatment with Bilobalide downregulated the phosphorylation of NF-kB (p-p65) in septic mice lungs. Also, Bilobalide pre-treatment inhibited sepsis-induced down-regulation of IkB-a. These findings suggest that downregulation of the NF-kB signaling activation could be liable for the anti-inflammatory effect of Bilobalide in a murine model.

Inhibitory property of Bilobalide on the binding activity of nuclear factor-kappa B and DNA in RAW 264.7 cells

Then, we analyzed the role of Bilobalide on the binding activity of NF-kB and DNA in RAW264.7 cells. Before this study, an MTT assay was used for the determination of cell viability. From this analysis, we found Bilobalide is non-toxic up to 100 μg/ml [Figure 6a]. Further, pretreatment with Bilobalide significantly repressed the LPS-stimulated binding activity of NF-kB and DNA [Figure 6b]. This result suggests that Bilobalide could be repressed LPS-induced inflammatory damage by inhibiting the binding activity of NF-kB and DNA.

DISCUSSION

Sepsis, one of the inflammatory syndrome triggered by the infection. It is also influencing aspect for the development of Sepsis-associated ALI and there are no effective treatments to cure.[20] Currently, the septic disorder might resemble by murine CLP model.[21] Sepsis-induced excessive inflammatory responses cause hypoxemia in order to make the alveolar–capillary membrane damage in ALI. Neutrophils are infiltrated and obstruct the alveolar–capillary barrier permeability. So targeting the inflammatory cell infiltration using naturally available products provide positive responses for the prevention of ALI.[22-24] However, the current study explored the protecting role of Bilobalide against septic ALI developed murine model. Our findings revealed the preventive role of Bilobalide against septic ALI was mostly refereed by inhibiting the inflammatory and inducing antioxidative properties. The underlying mechanism of these activities is reducing the iNOS, COX-2, increasing the AOEs expression, and inactivating the NF-kB signaling pathway.

The augmented alveolar permeability enriches the protein-rich fluid penetration into the alveolar and interstitial region that leads to an increase the lung edema and which may encourage the pathological alteration to ALI triggered mice.[23,25] In the development of ALI, Neutrophil invasion into the alveolar and interstitial regions has a vital role. One of the important histological markers for the inflammatory responses and neutrophil infiltration in injured lung tissue is MPO.[26-28] Our experiment shows pretreatment with bilobalide significantly alleviated the sepsis-induced ALI by reducing histological alteration, inflammatory cell infiltration, thickening of the alveolar wall, and capillary permeability. It was well confirmed

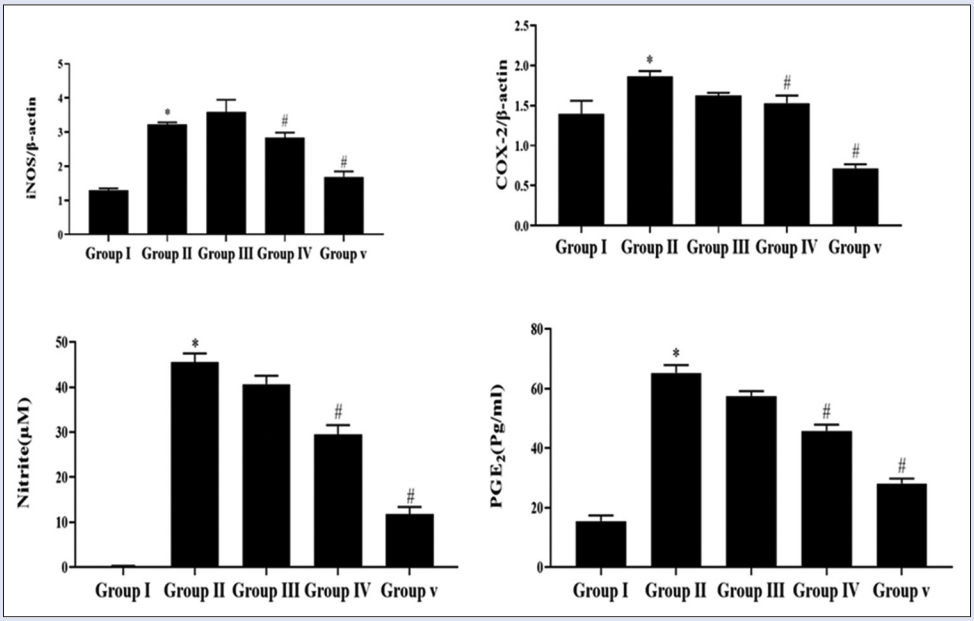

Figure 3: Effect of Bilobalide on the cyclooxygenase-2 and induced nitric oxide synthase expression in sepsis mice. Bilobalide treatment effectively downregulates the induced nitric oxide synthase and cyclooxygenase-2 expression and also NO and PGE2 levels in the lung tissues of septic mice. Values were depicted as a mean ± standard deviation of triplicates ($n = 6$). The significance level was determined by using Kruskal–Wallis test. Note: "*" indicates $P < 0.05$ when compared with control; "#" indicates $P < 0.05$ when compared with the sepsis-induced group

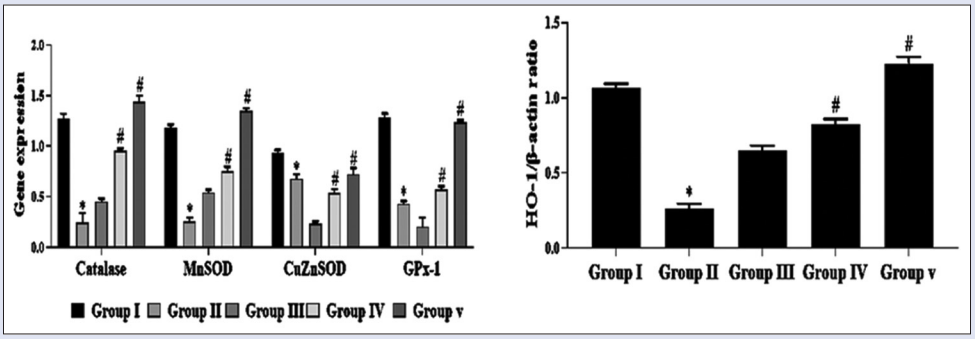

Figure 4: Effect of Bilobalide on the expression of antioxidants in the septic mice. The Bilobalide treatment notably augmented the catalase, MnSOD, CuZnSOD, and GPx-1 expressions in the septic mice. Bilobalide also dose-dependently augmented the HO-1 expression. Values were depicted as a mean ± standard deviation of triplicates ($n = 6$). The significance level was determined by using Kruskal–Wallis test. Note: "*" indicates $P < 0.05$ when compared with control; "#" indicates $P < 0.05$ when compared with the sepsis-induced group

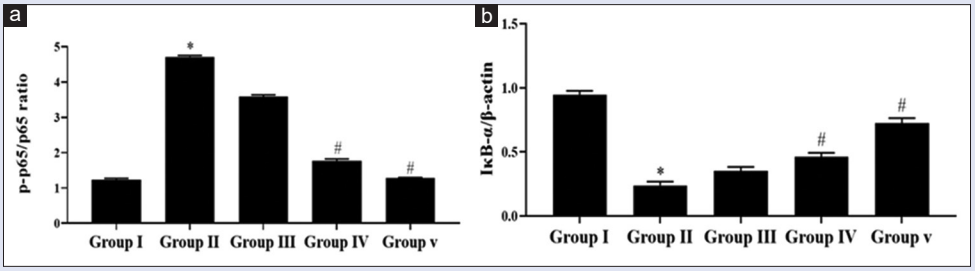

Figure 5: Effect of Bilobalide on the phosphorylation of nuclear factor-kappa B (p65) in the septic mice. The pre-treatment with Bilobalide was down-regulated the phosphorylation of nuclear factor-kappa B (p-p65) in the lung tissue of septic mice. Bilobalide pre-treatment was also up-regulated the IkB-a in the septic mice. Values were depicted as a mean ± standard deviation of triplicates ($n = 6$). The significance level was determined by using Kruskal–Wallis test. Note: "*" indicates $P < 0.05$ when compared with control; "#" indicates $P < 0.05$ when compared with the sepsis-induced group

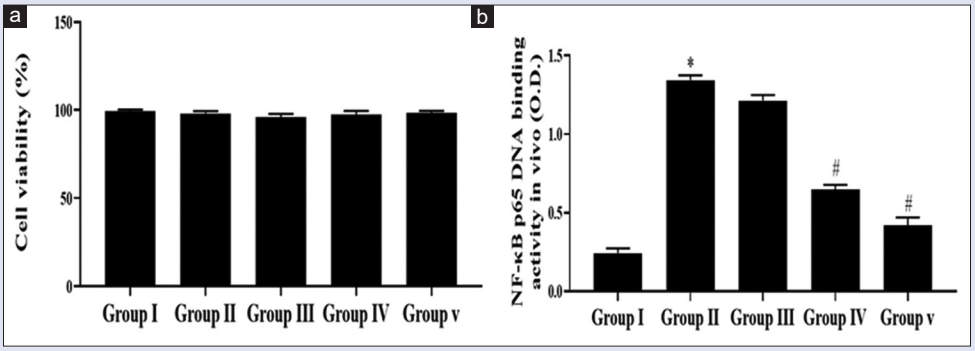

Figure 6: Inhibitory property of Bilobalide on cell viability and binding activity of nuclear factor-kappa B and DNA in RAW 264.7 cells. The Bilobalide treatment showed the non-toxicity up to 100 μg/ml. Further, pretreatment with Bilobalide significantly repressed the LPS-stimulated binding activity of nuclear factor-kappa B, and Values were depicted as a mean ± standard deviation of triplicates ($n = 6$). The significance level was determined by using Kruskal–Wallis test. Note: "*" indicates $P < 0.05$ when compared with control; "#" indicates $P < 0.05$ when compared with the sepsis-induced group

by measuring other factors such as, wet-to-dry weight ratio, amount of total protein in BAL fluid, and pro-inflammatory mediators secretion are increased septic mice, simultaneously these factors are significantly reduced Bilobalide [Table 1]. In addition, the concentration of MPO in the lung tissue lysate and the presence of neutrophil cells presence in BAL fluid were increased significantly after CLP administration. MPO activity and neutrophil cell presence were significantly repressed by Bilobalide treatment. Overall these findings strongly showed that Bilobalide might be a potential candidate to prevent the sepsis-prompted ALI.

Excessive oxidative stress plays a critical role in ALI development. Septic condition induces COX-2 and iNOS to intermediate the inflammatory processes through the release of the prostaglandin and NO respectively.[29,30] In our study, COX-2 and iNOS expression in the lung tissues and its products like PGE and NO in the BAL fluid was markedly increased after CLP introduction, besides these were significantly decreased by pretreatment with Bilobalide [Figure 3]. Furthermore, reported that catalase, SOD, and GPx are the major anti-oxidative enzymes involved to reduce the oxidative-induced injury in the lungs. From our study, Bilobalide increased the gene expression

AOEs catalase, MnSOD, CuZnSOD, and GPx-1 and HO-1 expression in the ALI mice [Figure 4]. Based on this activity we confirmed that Bilobalide diminishes the severe condition of septic-ALI by increasing the AOEs activity.

Signaling cascade illustrated that NF-kB signaling phosphorylation/activation is the major factor, interacting with the promoters which complexes to the pro-inflammatory mediator secretion, during sepsis. Phosphorylated NF-kB (p-p65) separates from IkBa and translocates into the nucleus and provokes the target genes such as TNF-α, IL-1 β, and IL-6 transcription.[31] Hence, NF-kB is found as a vital target for curing the inflammation disorders. In this present study, Bilobalide inhibits the sepsis-induced pro-inflammatory mediator responses by blocking NF-kB translocation and its activation in murine and macrophage models [Figure 6]. As well as, the survival rate of the sepsis-induced mice was notably improved by Bilobalide.

CONCLUSION

This work concludes Bilobalide has a preventive against sepsis-induced inflammation and associated injury to the lung by its anti-inflammatory and antioxidative properties. Bilobalide significantly ameliorates histopathological alterations by reducing alveolar damage and inflammatory responses. Further, NF-kB and HO-1 signaling cascades consider as main targets for Bilobalide. These results suggested Bilobalide has the ability to act as a new therapeutic candidate to prevent the lung inflammation and oxidative-associated injury during sepsis.

Financial support and sponsorship

The project was supported by for Hospital Infection Management Office, The Hospital of Xinjiang Production and Construction Corps, Urumqi, Xinjiang, 830002, China.

Conflicts of interest

There are no conflicts of interest.

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