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Cardiospermum halicacabum Extract Inhibits the Production of Nitric Oxide and Reactive Oxygen Species in LPS-Stimulated RAW 264.7 Cells through Erk/p38 Signalling Pathways

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Abstract

The medicinal plant *Cardiospermum halicacabum* has been extensively studied for its potential in various therapeutic activities, including anti-inflammation, antioxidant, antibacterial, antifungal and antiparasitic effects. However, the specific molecular mechanisms responsible for its anti-inflammatory properties remain largely unknown. In order to shed light on this aspect, the current investigation was aimed at elucidating the underlying mechanisms through which *C. halicacabum* exerts its anti-inflammatory response. The different concentrations of *C. halicacabum* extract were tested for cytotoxicity on RAW264.6 murine macrophage cells by MTT assay. The inhibition of nitric oxide (NO) in LPS-induced cell pre-treated with the extract was evaluated by Griess assay using Griess reagent. Flow cytometry and fluorescent microscopy were used to examine the reactive oxygen species (ROS) in the treated cells. The expression of iNOS and the phosphorylation status of Erk1/2, p38 and NF-κB were determined by western blotting. The *C. halicacabum* extract at concentrations up to 125 μg/mL did not cause the cytotoxicity to the RAW264.6 murine macrophage cells. Incubation of LPS-induced cells with the extract significantly minimized the production of NO as compared with the LPS-stimulated cells. Further, the extract significantly reduced ROS generation in LPS-induced cells. In the present of *C. halicacabum*, LPS-treated cells had the reduced NOS expression as well as had low phosphorylation on Erk1/2, p38 and NF-κB. *C. halicacabum* attenuates NO and ROS production under the regulation of Erk1/2 and p38 pathway as well as NF-κB.

Keywords: Anti-inflammation, *Cardiospermum halicacabum*, Nitric oxide, Reactive oxygen species, Erk1/2, NF-κB

Introduction

Cardiospermum halicacabum (L.) or balloon vine is commonly distributed in Asian and African countries [1]. In folk medicine, *C. halicacabum* has been used to treat cough, hyperthermia, nervous illnesses, amenorrhea, rheumatism, chronic bronchitis, stiffness of limbs and snakebite [2,3]. The plant's roots, leaves, and seeds yield a variety of bioactive compounds, including flavonoids, triterpenoids, glycosides, tannins, proteins, saponin, fatty acids, carbohydrates and volatile esters [4,5].

C. halicacabum exhibits a range of beneficial effects, such as anti-inflammatory, antioxidant, antibacterial, antifungal and antiparasitic activities [4,5]. Recently, the presence of rutin in *C. halicacabum* leaves has been identified as the active molecule with anti-inflammatory activity [6,7]. Rutin is a natural flavonoid glycoside, which showed the anti-inflammatory effect by reducing the production of NO in RAW 264.7 cell line and in carrageenan-induced paw edema [8,9]. In addition, the flavonoids found in the chloroform extract of *C. halicacabum* exhibited anti-cancer activities [10].

Activation of macrophage by lipopolysaccharid (LPS), a component of cell wall of the gram-negative bacteria, induces many enzymes within mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinase (Erk1/2), c-JUN NH_2-terminal protein kinase (JNK), and p38 mitogen-activated protein kinase (p38) [11]. These MAPK enzymes contribute to mediate the phosphorylation of various downstream signalling proteins required for the inflammatory gene transcriptions [12]. In addition, LPS induces macrophages to secrete pro-inflammatory mediators such as NO. NO plays an essential role to eradicate the phagocytosed pathogen and the circumstance extracellular pathogens [13]. Nevertheless, an overabundance of NO can have a detrimental impact as it has the potential to harm tissues, thereby exacerbating acute and chronic inflammations [14]. The synthesis of NO is facilitated by 3 distinct isoforms of NO synthase enzymes, namely endothelium NO synthase (eNOS), neural NO synthase (nNOS) and inducible NO synthase (iNOS) [15]. Besides NO, ROS are generated from macrophage in response to LPS induction. The term “ROS” contains many molecules including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen [16]. ROS also serves as the mediators for host defense against the pathogens through the oxidative burst [16]. However, the excessive production of ROS is associated with the inflammatory progression and cell death [17]. It has been shown that suppression of ROS and NO production can limit inflammatory-related disorders [18,19]. Thus, inhibition of NO and ROS has been used as the criteria to evaluate anti-inflammatory activity of the medicinal plants in various publications [8,18,20].

Nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) is a transcription factor that plays a key role to regulate the transcription of diverse proinflammatory genes including cytokines, chemokines and adhesion molecules [21]. In resting cells, NF-κB protein is in the inactive state in the cytoplasm by associating with its inhibitory, inhibitor of nuclear factor kappa B (IκB) [22]. Signals from surface receptors including cytokine receptor, toll-like receptor and T and B cell receptor trigger the phosphorylation of IκBs by IκB kinase (IKK) [22]. On the other hand, it has been reported that IKK can be activated by ROS [23]. Phosphorylated IκBs result in IκBs degradation by proteasome and then allows the migration of NF-κB into the nucleus, where it binds DNA element of target genes for transcription [22,24]. Activation of NF-κB is required for the expression of diverse inflammatory cytokines, chemokines, as well as the inducible nitric oxide synthase (iNOS) for NO production [25].

C. halicacabum has been extensively studied for its anti-inflammatory properties; however, the specific mechanisms responsible for this activity remain to be fully elucidated. Thus, the current research endeavors to investigate the molecular pathways involved in the anti-inflammatory effects of *C. halicacabum* extract. Our findings demonstrate that *C. halicacabum* effectively suppresses the production of NO and ROS induced by LPS, concomitant with the downregulation of Erk1/2, p38 and NF-κB phosphorylation.

Materials and methods

Preparation of the *C. halicacabum* crude extract

Leaves of *C. halicacabum* were supplied from Charoensuk Pharma Supply Co., Ltd. in Thailand. The leaves of *C. halicacabum* were subjected to a cleaning process and subsequently dried in an oven (Memmert, Germany) at a temperature of 60 °C. After drying, the leaves were finely ground using a mortar. A total of 100 g of the ground dried *C. halicacabum* was then mixed with 100 mL of 80 % ethanol and left to soak for a duration of 48 h at room temperature. The resulting extract suspension was filtered through Whatman No. 1 filter paper and subsequently concentrated by evaporating the solvent using a rotatory evaporator (Büchi, Konstanz, Germany) at a temperature of 40 °C under reduced pressure. The concentrated extract was then transformed into a paste-like consistency and dried at room temperature. Finally, the dried extract was stored in airtight containers and kept at a temperature of –20 °C until it was ready for assay.

Cell viability assay

The effect of the *C. halicacabum* on cell viability was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as the method previously described with minor modification [26]. Murine macrophage, RAW264.7 cells (EP-CL-0190, Elabscience, USA) at a density of 1×10^4 cells/well were seeded into 96-well plates for overnight to allow cell attachment. Then, cells were treated with different concentrations of the *C. halicacabum* extract ranged 0 - 125 µg/mL and further incubation for 24 h. Cells incubated with DMEM (Dulbecco's Modified Eagle Medium) containing the 0.2 % vehicle (dimethyl sulfoxide, DMSO) were used as a control. In addition, the effect of LPS on cell viability was also tested by incubating the cells with the extract in the presence of 1 µg/mL for 24 h. After 24 h, 20 µL of 5 mg/mL MTT dissolved in PBS (phosphate buffer saline) was added to each well with additional incubation for 2 h. The supernatant was gradually removed before adding 100 µL of DMSO into each well to dissolve the formazan crystals. The optical density was measured at 540 nm using a microplate reader (Synergy H1, BioTek, USA) to quantify the number of viable cells, which was proportional to the optical density. Percentage of cell viability of *C. halicacabum*-treated cells was calculated in comparison with the control group. Data were expressed as mean ± SD from 3 different occasions with triplicate wells for each experiment.

Determination of NO production

The NO production from RAW 264.7 cells after treatment was quantified by Griess reagent as previously described [26]. RAW264.7 macrophages were seeded in a 96-well culture plate (Nunc™, Roskilde, Denmark) at a cell density of 5×10^4 cells/well and allowed to adhere to the plate overnight. Various concentrations ranging from 0 - 125 µg/mL of the extract were applied into each well and left incubated for 30 min. After incubation, 1 µg/mL LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was then added into each well and further incubated for 24 h. Indomethacin (50 µM; Sigma, St. Louis, MO, USA) was used as a positive control. The culture supernatant containing NO was collected and the nitrite (a stable breakdown product of NO) was analyzed by the Griess reagent kit (Invitrogen, Oregon, USA) according to the manufacturer's instruction. Cell supernatant (150 µL) was mixed in a 96-well plate with 20 µL of Griess reagent and 130 µL deionized water. The samples were additionally incubated for 30 min at room temperature before measuring the absorbance at 570 nm using a microplate reader (Synergy H1, BioTek, USA). A nitrile solution was utilized to generate a standard curve as described earlier. The resulting standard curve displayed the relationship between nitrile concentration (x-axis) and absorbance at 570 nm (y-axis), enabling the calculation of NO concentration. The data presented represents the mean ± SD from 3 separate instances, with triplicate wells for each experiment. To determine the percentage of NO inhibition in each condition, the cells treated with LPS without samples were employed as a reference control.

$$\%Inhibition = \frac{A-B}{A} \times 100 \qquad (1)$$

where A = concentration of nitrile production in LPS-treated cells without the extract and B = concentration of nitrile production of LPS-stimulated cultures in the present of plant extracts.

Detection of ROS by fluorescent microscope

The potential of the *C. halicacabum* extract on the inhibition of the intracellular ROS production was monitored using a ROS indicator, chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein (CMH_2DCFDA). Raw 264.7 cells were seeded into ibidi µ-slide 8 well™ slides (Ibidi, Munich, Germany). Cells were pre-treated with the extract at 5, 50 and 125 µg/mL for 30 min before treating with 1 µg/mL LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 60 min. The cells treated with 50 µM Indomethacin (Sigma, St. Louis, MO, USA) were used as the positive control, while the cells cultivated in the 0.2 % vehicle (dimethyl sulfoxide, DMSO) served as the negative control. Following treatment, the cells were exposed to CMH_2DCFDA (ThermoFisher Scientific, Milano, Italy) for 20 min according to the manufacturer's instructions. Subsequently, the green fluorescence-labeled cells were observed under a fluorescence microscope (Nikon Eclipse Ti, Nikon®, Melville, NY, USA). The Mean Correlated Cell Fluorescence (CTCF) was determined using the following equation.

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean Fluorescence of background readings}) \qquad (2)$$

Detection of ROS by flow cytometry

Intracellular ROS production after treating the cells with *C. halicacabum* was also confirmed by flow cytometry. Cells were treated as mentioned above. After incubation for 24 h, cells were stained with CMH_2DCFDA for 30 min in a CO_2 incubator (Panasonic®, Kadoma, Japan). Cells were then centrifuged (TOMY Digital Biology Co., Ltd., Tokyo, Japan) and resuspended with complete medium followed by analyzing using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), and CMH_2DCFDA-stained cells were analyzed with CellQuestPro software.

Western blotting

The RAW 264.7 macrophage cells were plated at a cell density of 1×10^6 cells in 6-well plate for overnight. On the following day, cells were pre-treated with the extract at 5, 50 and 125 µg/mL for 30 min before stimulating with 1 µg/mL LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for additional 24 h. Indomethacin (50 µM; Sigma, St. Louis, MO, USA) was also used as a positive control, whereas cell cultivation in the 0.2 % vehicle (dimethyl sulfoxide, DMSO) was used as a negative control. Upon treatment, cells were collected and lysed in Ripa buffer plus Phosphatase and Protease inhibitor (PI) (Thermo Fisher Scientific). Total protein lysates were then subjected to western blot analysis. Specific antibodies against iNOS, COX-2, Erk1/2, phosphorylated-Erk1/2, p38, phosphorylated-p38, NF-κB, β-actin and GAPDH (Cell signalling Technology), were used. Protein bands were observed using a CCD camera (Imagequant LAS 4000, GE Healthcare, Chicago, IL, USA). The intensity of the protein bands was measured using ImageJ software. The densitometric ratio of the target proteins and either β-actin or GAPDH was determined and then normalized to the untreated cells' value.

Statistical analysis

ANOVA coupled with Tukey's posthoc analysis through GraphPad Prism software was employed to detect significant variations among the experimental groups. The results were displayed as mean ± SD from 3 independent experiments, each with at least triplicate samples. A *p*-value less than 0.05 was considered indicative of a significant difference.

Results and discussion

Results

C. halicacabum did not affect on cell viability

We initially explored the cytotoxic effects of *C. halicacabum* on RAW264.7 murine macrophage by quantifying cell viability using the MTT assay. Different concentrations of the extract ranging from 0 - 125 µg/mL were cultured with murine macrophage in the present or absence of the inflammation inducer, 1 µg/mL LPS for 24 h. The results showed that treatment of RAW264.7 cells with all tested concentrations exerted no effect on cell viability as compared to the control, cells with 0.2 % v/v DMSO (**Figure 1**). The cell viability levels from all tested concentrations were more than 90 %. Likewise, cell viability did not change when the LPS was added together with the extract to RAW264.7 cells. Altogether, the *C. halicacabum* extract with a concentration range of 0 - 125 µg/mL showed no toxicity to RAW264.7 macrophages. Therefore, the *C. halicacabum* extract at a concentration of 0 - 125 µg/mL was used for further investigation on the anti-inflammatory study.

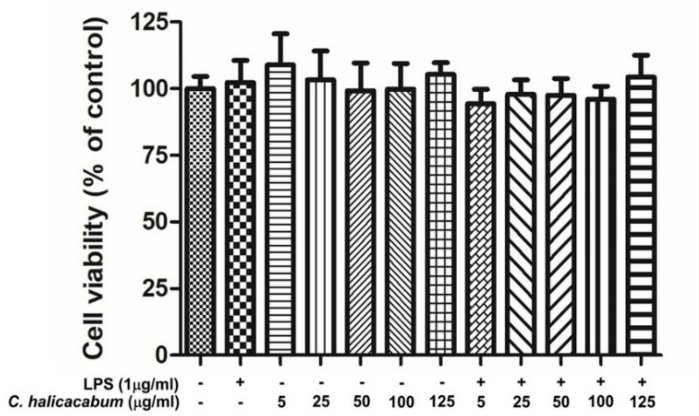

Figure 1 Effect of the different concentration of *C. halicacabum* on cell viability of RAW 264.7 macrophages. Raw 264.7 macrophages were treated with the extract of *C. halicacabum* at concentration ranged from 0 - 125 µg/mL for 24 h in the present or absent of 1 µg/mL LPS. The control cells were treated with medium containing vehicles (0.2 % v/v DMSO) without the extract and LPS. Cell viability was assessed by MTT assay, and the percentage of cell viability was calculated as compared with 100 % of the control. Data from at least 3 independent experiments performed in at least triplicates are expressed as mean ± SD, *n* = 3.

C. halicacabum inhibits NO production in LPS-stimulated RAW 264.7 cells

The anti-inflammatory potential of *C. halicacabum* extract was evaluated by quantifying the production of NO in RAW264.7 cells stimulated with LPS, using the Griess assay. Notably, a significant augmentation in the production of NO was detected in the LPS-stimulated cells. Treatment of the LPS-stimulated cells with the *C. halicacabum* extract at the concentration of 0 - 125 µg/mL significantly caused the reduction of NO production in a dose-dependent manner (**Figure 2(A)**). The highest inhibitory was found at 82.41 ± 11.41 % followed by 78.45 ± 9.09 % after treatment the cells with the extract's concentration of

125 and 100 µg/mL, respectively (**Figure 2(B)**). Interestingly, the LPS-stimulated cells treated with 50 µg/mL of the extract achieved the inhibitory effect on NO production comparable to the standard drug, indomethacin. Cells cultured in medium alone or with 125 µg/mL of the extract did not induce NO production. Thus, *C. halicacabum* exerts the inhibitory effect on NO production from the LPS-activated RAW264.7 macrophages. *C. halicacabum* at 5, 50 and 125 µg/mL corresponding to low, intermediate, and high concentration was used for further experiments.

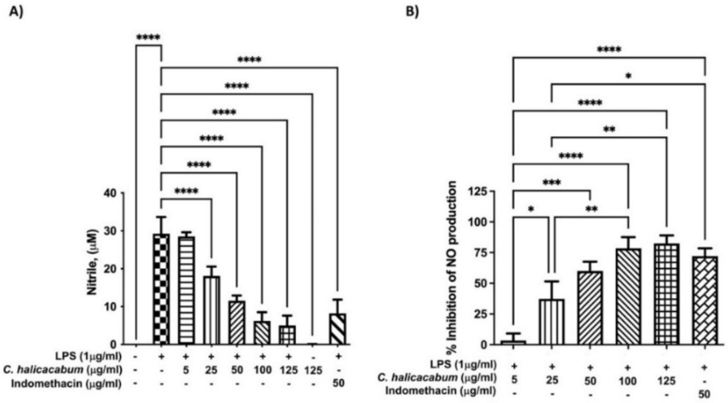

Figure 2 *C. halicacabum* inhibited the NO production in LPS-stimulated RAW264.7 cells: (A) RAW264.7 cells were treated with indicated concentrations of *C. halicacabum* extract for 30 min; followed by adding LPS (1 µg/mL) to the cells and additional incubated for 24 h. Indomethacin was used as a positive control. Griess reagent was used to quantify nitrite levels in the culture media. Data expressed as mean ± SD from 3 independent experiments. ***$p < 0.001$ vs cells treated with LPS only. (B) The percentage of inhibition on NO production treated with of *C. halicacabum* was calculated using LPS-induced cells as the reference condition and presented as mean ± SD from 3 independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

C. halicacabum inhibited ROS production in LPS-stimulated RAW 264.7 cells

ROS are the key messenger molecules that are involved in inflammation progression. ROS is produced mainly by innate cells such as neutrophils and macrophages in a host-defense response to invading pathogens [27]. Thus, to confirm the potential of *C. halicacabum* in inhibiting ROS production, we next investigate the effect of *C. halicacabum* on ROS generation in LPS-treated cells. Detection of ROS can be done by using ROS-tracking molecules, chloromethyl derivative of 2',7'-dichlorodihydrofluorescein (CMH$_2$DCFDA). This nonfluorescent CMH$_2$DCFDA is passively diffused across the cell membrane, where it is subsequently deacetylated by intracellular esterase [28]. Upon reaction with ROS, the H$_2$DCFDA is transformed to fluorescent 2',7'-dichlorofluorescein (DCF) [29], which can be subsequently monitored by fluorescent microscope and flow cytometry. Augment of intracellular ROS was observed in cells treated with LPS for 24 h (**Figures 3(A)** and **3(B)**). Interestingly, the excessive ROS generation was dramatically dropped when cells were pretreated with the extract at 50 and 125 µg/mL ($p < 0.05$, **Figure 3(B)**) before stimulating with LPS. The ROS level in cells pretreated at 125 µg/mL of the extract was comparable to the control cells (**Figure 3(B)**). We further confirmed the effect of *C. halicacabum* extract on ROS inhibition by flow cytometric analysis. This CMH$_2$DCFDA was also used to probe intracellular ROS for flow cytometry analysis. The number of ROS-positive cells was about 1 % in untreated cells. LPS-treated cells showed a dramatic increase in ROS-positive cells (12.12 ± 2.75 %) (**Figures 3(C)** and **3(D)**). A gradual decrease of ROS-positive cells was observed when cells were incubated with the *C. halicacabum* extract at 5 and 50 µg/mL (9.80 ± 0.59 and 9.32 ± 0.36, respectively). A sharp decrease of cells with ROS was found in cells treated with *C. halicacabum* extract of 125 µg/mL ($p < 0.05$, **Figure 3(B)**). The findings from flow cytometry analysis were closely correlated with a fluorescent microscope in which *C. halicacabum* extract inhibited ROS production of LPS-stimulated cells. Thus, these findings reveal that *C. halicacabum* exerts the potent anti-inflammatory effects.

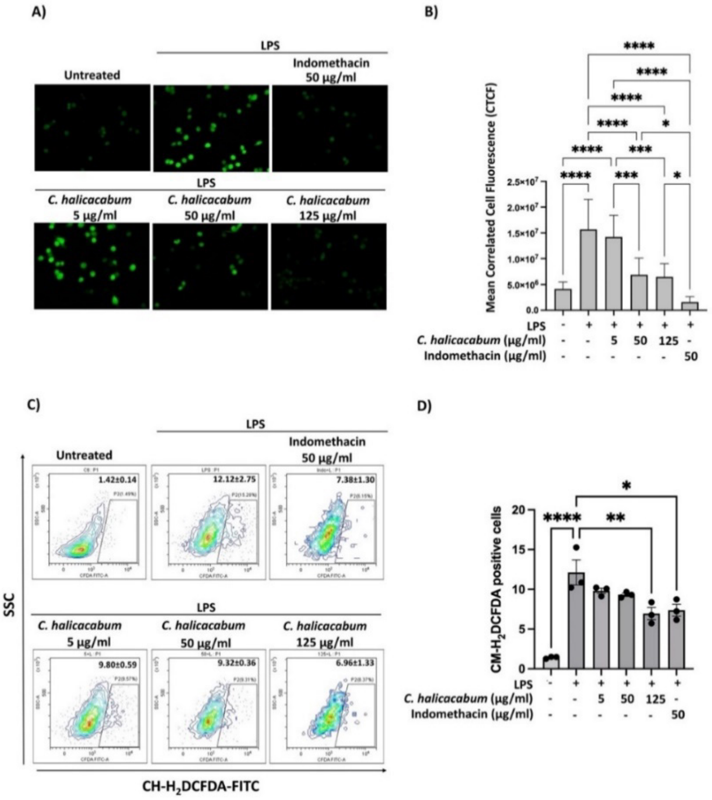

Figure 3 ROS was inhibited in LPS-stimulated RAW264.7 treated with *C. halicacabum* extract: (A) RAW264.7 cells were treated with indicated concentrations of *C. halicacabum* extract for 30 min; followed by adding LPS (1 µg/mL) to the cells and additional incubated for 60 min. Indomethacin was used as a positive control. Chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein (CMH_2DCFDA) was used to monitor the production of ROS under a fluorescent microscope. The representative pictures were from 1 of 3 independent experiments. (B) CTCF was calculated and expressed as mean ± SD from 3 independent experiments. (C) RAW264.7 cells were treated as above. The chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein (CMH_2DCFDA) was used to probe the intracellular ROS and analyzed by flow cytometry. The representative pictures from 1 of 3 independent experiments were shown. (D) CMH_2DCFDA-positive cells in percentage were depicted as mean ± SD from 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Downregulation of iNOS expression in C. halicacabum treated LPS-stimulated RAW264.7 cells

The expression of iNOS protein, which is the upstream regulator of NO generation was then examined. As shown in **Figure 4**, expression of iNOS protein was increased when RAW264.7 macrophages were induced by LPS. Treatment of the cells with the *C. halicacabum* before stimulating with LPS caused the reduction of iNOS protein level in a dose-dependent manner. Additionally, the expression of COX-2 protein, necessary for the synthesis of the pro-inflammatory mediator PGE2, was analyzed. Consistent with the iNOS results, a reduction in COX-2 expression was observed following pre-treatment with *C. halicacabum* extract before LPS induction (**Figure 4**). Overall, these findings suggest that the *C. halicacabum* extract inhibited NO production by suppressing iNOS expression in activated RAW264.7 cells.

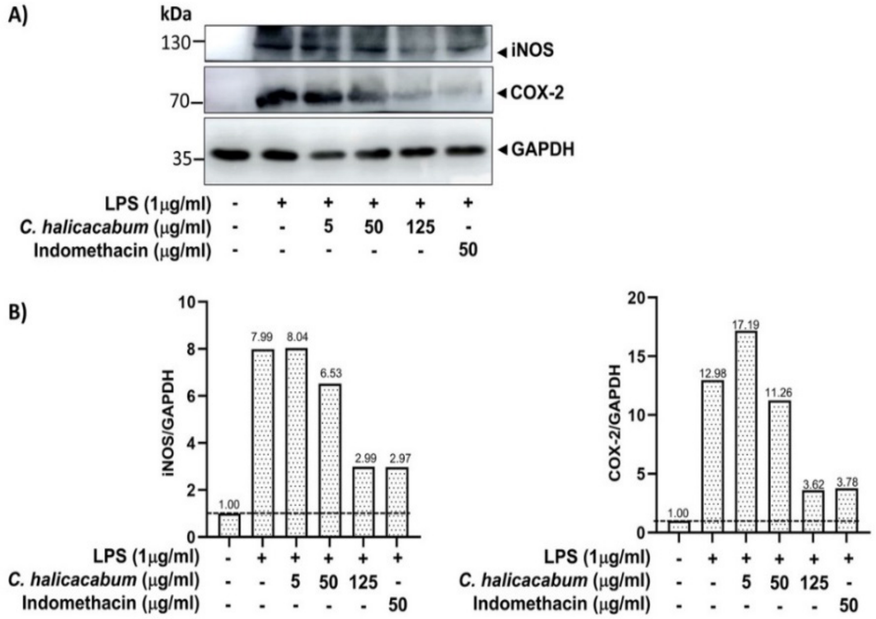

Figure 4 Downregulation of iNOS protein in LPS-stimulated RAW264.7 treated with *C. halicacabum* extract: (A) After exposing RAW264.7 cells with *C. halicacabum* extract for 30 min, cells were then activated with 1 µg/mL LPS (lipopolysaccharide) for an additional 24 h. The cell lysates were prepared before subjecting to western blot analysis and the PVDF (polyvinylidene difluoride) membrane containing protein is probed with a specific antibody against mouse iNOS protein. Antibody against *β*-actin is used as a loading control. One representative experiment from 3 independent experiments was shown. (B) The band intensity of each protein was quantified. The protein of interest was normalized with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and relative to the control (untreated cells) as indicated in a number on the graph.

C. halicacabum attenuates LPS-induced Erk1/2 and p38 phosphorylation

It has been reported that the expression of iNOS and COX-2 is regulated through MAPK pathway [30]. Here, we also examined the effect of *C. halicacabum* on the activation of 2 kinases in MAPK pathway, Erk1/2 and p38. Cells treated with LPS caused the augment of Erk1/2 and p38 phosphorylation. The phosphorylation of Erk1/2 and p38 was reduced when the cells were pretreated with the extract at concentrations of 5 and 50 µg/mL before the addition of LPS (**Figures 5(A)** and **5(B)**). Interestingly, a remarkable decrease in the phosphorylation of Erk1/2 and p38 by 58.99 and 55.82 %, respectively, was observed when the cells were pretreated with the extract at a higher concentration of 125 µg/mL, compared to the group treated with LPS alone (**Figures 5(A)** and **5(B)**). These findings suggest that *C. halicacabum* may exert its effects on iNOS and COX-2 expression, at least partially, through the regulation of Erk1/2 and p38.

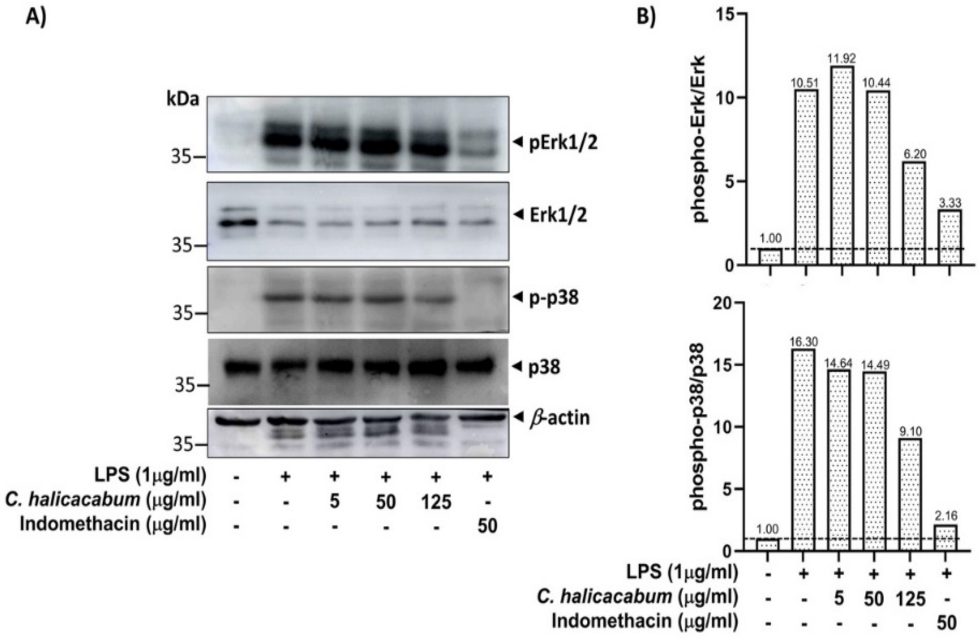

Figure 5 *C. halicacabum* extract suppressed the phosphorylation of Erk1/2 and p38 MAPK proteins in LPS-stimulated RAW264.7 cells: (A) After exposing RAW264.7 cells with *C. halicacabum* extract for 30 min, cells were then activated with 1 µg/mL LPS (lipopolysaccharide) for an additional 24 h. Western blot analysis was performed using indicated specific antibodies. Antibody against *β*-actin is used as a loading control. One representative experiment from 3 independent experiments was shown. (B) The band intensity value of each protein was quantified. The band intensity value of the interested protein was normalized with *β*-actin and relative to the control (untreated cells) as indicated in a number on the graph. Erk = extracellular signal-regulated kinase and MAPK = mitogen-activated protein kinase.

C. halicacabum attenuates LPS-induced NF-κB activation

NF-κB is a transcription factor that predominantly regulates various inflammatory mediators such as NO [25]. Signals from the receptor induces the phosphorylation of NF-κB, which then translocate to the nucleus for interacting with DNA element of genes related to inflammatory process and mediating gene transcription [22,24]. Western blot analysis demonstrated that LPS treatment resulted in NF-κB phosphorylation in murine macrophages (**Figures 5(C)** and **5(D)**). As expected, *C. halicacabum* significantly suppressed LPS-induced NF-κB phosphorylation in a dose-dependent manner. *C. halicacabum* at a concentration of 125 µg/mL inhibited NF-kB activation by 37.43 % as compared to the LPS treated group (**Figures 5(C)** and **5(D)**). Collectively, this present finding suggested that *C. halicacabum* attenuates LPS-induced NF-κB activity in murine macrophages.

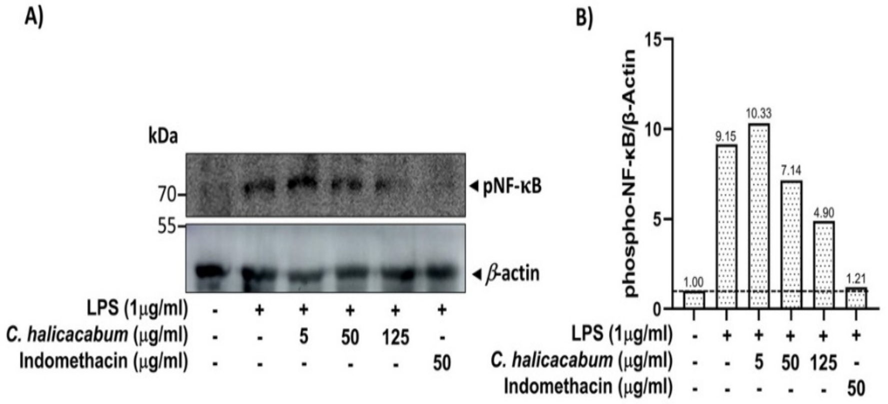

Figure 6 *C. halicacabum* extract suppressed the phosphorylation of NF-κB proteins in LPS-stimulated RAW264.7 cells: (A) After exposing RAW264.7 cells with *C. halicacabum* extract for 30 min, cells were then activated with 1 μg/mL LPS (lipopolysaccharide) for an additional 24 h. Western blot analysis was performed using phospho-NF-κB specific antibody. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. One representative experiment from 2 independent experiments was shown. (B) Band intensity of each protein was quantified. Band intensity value of phospho-NF-κB (Nuclear factor kappa-light chain enhancer of activated B cells) was normalized with GAPDH and then relative to the control (untreated cells) as indicated in a number on the graph.

Discussion

C. halicacabum has been reported as the potential medicinal herbs with versatile pharmacological activities including anti-inflammatory, anti-oxidant, anti-viral, anti-ulcer, anti-diabetic, anti-convulsant, anti-pyretic, anxiolytic, anti-cancer, anti-bacterial, anti-arthritic, anti-fungal, anti-parasitic and fertility activities [4]. In the present study, the potential of *C. halicacabum* in anti-inflammatory activity and its related biochemical mechanisms were investigated. The excess production of NO can cause the pathogenesis of inflammatory disorders such as rheumatoid arthritis (RA) [6]. Thus, NO inhibitors are the potential therapeutic options to cure inflammatory diseases. Here, we report that *C. halicacabum* exerts anti-inflammatory effects in RAW264.7 murine macrophage in response to LPS. This finding aligns with previous research [4,9,31,32]. Moreover, it has been established that rutin, found in *C. halicacabum*, is the active compound responsible for its anti-inflammatory effects [7,31]. The biosynthesis of NO depends on the activity of iNOS to convert oxygen and L-arginine to L-citrulline and NO [33]. iNOS enzyme is inducibly expressed in response to lipopolysaccharide (LPS) and/or cytokines and its expression is closely associated with the NF-κB activation [34]. Western blot analysis revealed that *C. halicacabum* markedly decrease the activation of NF-κB in LPS-activated macrophages. Our finding was consistent with the previous report in which *C. halicacabum* regulates NF-κB activation *in vitro* [32]. In resting macrophages, NF-κB is found in the cytoplasm along with its inhibitor called IκB. When LPS is detected by surface receptors, it triggers various signalling pathways that activate the IκB kinase (IKK). The activated IKK then phosphorylates IκB, which is bound to NF-κB [35]. The phosphorylated IκB is then marked for degradation and removed by proteosomes, allowing NF-κB to move into the nucleus. Once in the nucleus, NF-κB binds to the promoter region of the target gene’s DNA sequence, initiating transcription [24,35].

Likewise, ROS is also produced from macrophage in response to bacterial infection. The term “ROS” contains multiple members including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen, where H_2O_2 is studied the most [16]. The excess of ROS accumulation can cause the progression of human diseases such as atherosclerosis, cancer, and ageing [36]. Moreover, ROS works in concert with NO to enhance cell damage. Thus, controlling the level of NO and ROS is the strategy for therapeutic purposes to treat inflammation-mediated diseases. ROS is generated because of oxidative burst by NADPH oxidase activity in the mitochondria [37]. Using CMH_2DCFDA to probe intracellular ROS, our results demonstrated that *C. halicacabum* inhibited LPS-induced ROS production. It has been demonstrated previously that ROS can activate IKK [38]. This contributes to the involvement of ROS in NF-κB signalling pathway [38].

Our western blot results revealed that *C. halicacabum* inhibited LPS-induced Erk1/2 and p38 activation associated with decreased NF-κB phosphorylation. Previous findings showed that ROS was able to activate JNK1/2 and p38 proteins and then evoked the production of various inflammatory cytokines [39]. Further, the activated Erk1/2 could mediate IκBα phosphorylation directly or indirectly [40]. Additionally, NF-*κ*B translocation into the nucleus may be mediated by the activity of phosphorylated Erk1/2 and p38 MAPK [40,41]. From above data, we assumed that *C. halicacabum* inhibited LPS-induced NO production may be due to Erk1/2-mediated IκBα phosphorylation or NF-*κ*B translocation. Thus, based on above data, we could imply that ROS acts upstream of p38 and Erk1/2 activation, which then mediates the activation and nuclear translocation of NF-κB. Inhibition of NF-κB activity consequently led to decrease NO production. In addition, NADPH oxidase (NOX_2), one of enzymes that promote ROS production, is also regulated by NF-κB [42]. According to the previous literature, the reduction of LPS-induced ROS production was closely associated with the decrease of NOX_2 expression and NF-κB phosphorylation [43]. In line with our study, we found that *C. halicacabum* suppressed LPS-triggered NF-κB phosphorylation and ROS production. It has been implied that *C. halicacabum* might down-regulate NADPH oxidase expression and consequently inhibit ROS production. Recently, *C. halicacabum* has been reported to inhibit LPS-mediated ROS and NO production by interacting with A2A adenosine receptor [44], a crucial receptor found on RAW264.7 to exert anti-inflammatory effects [45]. A2A adenosine receptor also regulates the expression and activity of NOX_2 [46]. Altogether, the mode of action of *C. halicacabum* to inhibit LPS-induced ROS production might be mediated through the binding of *C. halicacabum* to A2A adenosine receptor and then inhibiting the NF-κB-driven NOX_2 expression.

Conclusions

In conclusion, the results of this study demonstrate that *C. halicacabum* exerts anti-inflammation by inhibiting the production of NO and ROS. *C. halicacabum* can suppress the expression of iNOS and COX-2 by inhibiting the phosphorylation of Erk1/2 and p38 MAPK pathway and NF-κB. Our findings confirm anti-inflammation properties of *C. halicacabum*, which may be considered as a potential agent for ameliorating the inflammatory-related diseases.

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