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α-Ecdysone suppresses inflammatory responses via the Nrf2 pathway in lipopolysaccharide-stimulated RAW 264.7 cells

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ABSTRACT

The search for new biologically active compounds is a topic of current research because of their ubiquitous availability and low toxicity. Plants of the *Silene* genus contain secondary metabolites known as phytoecdysteroids that reportedly have various biological activities. α-Ecdysone is a phytoecdysteroid with biological activity that has not been thoroughly investigated to date. Therefore, we investigated the immunomodulatory and anti-inflammatory effects of α-ecdysone on LPS-treated RAW264.7macrophage cells and in a zebrafish model. To explore these activities, RAW264.7 cells were pretreated with α-ecdysone (0.1–10 μM) for 24 h and then with LPS to induce inflammation. We assayed membrane fluidity, lysosomal enzyme activity, and superoxide generation to determine the immunomodulatory activity. Using ELISA, we examined the levels of the pro-inflammatory cytokines prostaglandin (PGE_2) and interleukin-1β (IL-1β), as well as the protein expression of cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α) and heme-oxygenase-1 (HO-1) by immunoblotting. We also investigated the subcellular localization of the nuclear transcription factor (NF-κB) subunits and expression of the mitogen-activated protein kinase (MAPK) pathway. We found that α-ecdysone is a potent immunostimulator that enhances membrane fluidity and lysosomal enzyme activity and generates superoxide anions. Simultaneously, α-ecdysone inhibited nitric oxide levels and suppressed the levels of pro-inflammatory mediators and cytokines. Furthermore, α-ecdysone increased HO-1 and nuclear factor erythroid 2-related factor (Nrf2) production, mitigated NF-κB subunit proteins in the nucleus and decreased MAPKs and Akt activation. These results suggest that α-ecdysone is a good immunostimulator with anti-inflammatory effects that occur via inhibition of pro-inflammatory mediators and cytokines through stimulation of HO-1 and Nrf-2 production.

1. Introduction

Inflammation is an intricate immune response of the host defense against diverse detrimental physiological stimuli that involves activation of various immune cells and tissues that act together for the restoration of normal cell structure and function [1]. Inflammation is an essential component of the immune system that has also been implicated in pathogenesis of many diseases ranging from rheumatoid arthritis to complications of sepsis. Macrophages play a crucial role in inflammation, as they are recruited to inflamed tissues with concomitant and excessive production of pro-inflammatory cytokines and mediators [2]. Production of NO and PGE_2 is governed by iNOS, eNOS and COX-2 expression and considered to be responsible for inflammatory progression [3].

NF-κB is a ubiquitous transcription factor that plays a pivotal role in regulating inflammatory response. During inflammatory conditions, NF-κB that is localized in the cytoplasm as a homodimer or heterodimer phosphorylates and is translocated into the nucleus. Following translocation, it binds to the promoter of those genes that express pro-inflammatory cytokines and mediators [4]. Lines of evidence indicated that MAPKs and PI3K/Akt pathways also regulate the expression and activation of NF-κB. Phosphorylation of MAPKs such as ERK, p38, and JNK play a critical role in NF-κB activation and production of proinflammatory cytokines [5]. Additionally, the PI3K/Akt signaling pathway acts as a negative regulator of LPS-induced acute inflammatory response factors that induce the expression of iNOS and

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COX-2 in RAW 264.7 cells [6].

Oxidative stress is associated with the increased production of ROS, which causes various forms of protein oxidation that drive the inflammatory cascade [7]. Different enzymatic anti-oxidants and anti-inflammatory systems including phase II detoxifying enzymes and stress proteins such as HO-1 help to combat inflammatory stresses. HO-1 belongs to the heat shock protein family, which contributes to diverse immune responses via attenuation of complement-dependent inflammation. Previous studies have suggested that HO-1 knock out mouse models are prone to chronic sepsis disease induced by proinflammatory endotoxin [8]. Expression of HO-1 is regulated by the redox-sensitive transcription factor Nrf2, which plays a protective role by inducing detoxifying genes through AREs [9]. An increasing number of reports have also suggested that HO-1 has the most AREs on its promoter, making it a highly effective therapeutic target for protection against inflammatory diseases [10].

Plants of higher kingdoms produce diverse physiologically active compounds that have been used in treatment of various human ailments for several decades. Ecdysteroids are natural anabolic agents that are currently being marketed with dietary supplements for modulation of a broad range of biological processes [11]. Plants of the *Silene* genus (Caryophyllaceae) comprise one of the best known groups of ecdysone-containing plants. Ecdysteroids are found in high levels in > 100 species of plant representatives of the *Silene* genus [12]. In the past decade, *Silene linicola* has been identified and studied as a new source of ecdysteroids. *S. linicola* is known to contain 12 phytoecdysteroids, including seven ecdysteroids that have been isolated from the aerial organs of *S. linicola*. One of these ecdysteroids was identified as α-ecdysone [13].

However, the precise mechanisms by which suppression of the LPS-stimulated inflammatory response mediated by α-ecdysone remain unclear. The present study investigated the anti-inflammatory effects of α-ecdysone exerted via Nrf2-dependent HO-1 induction in LPS-induced RAW 264.7 cells. Additionally, this suppression has been implemented through inhibition of NO production via down-regulation of iNOS and eNOS along with PGE_2 and COX-2 levels by inactivation of the NF-κB pathway. To the best of our knowledge, this is the first study of the anti-inflammatory activity of α-ecdysone.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise noted, all chemicals were of pure grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS (*Escherichia coli* 055: B5), Griess reagent, neutral red and other reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA. Primary antibodies for β-actin, c-JUN, c-fos, HO-1, NF- κB p50, NF- κB p65, COX-2, Lamin-B, Akt, p-Akt, JNK, p-JNK, p38 and p-p38 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). iNOS, eNOS and TNF-α were obtained from Abcam (Cambridge, MA, USA) and Nrf-2 was acquired from Enzo Life Sciences Co., USA.

2.2. Compound isolation

α-Ecdysone was isolated from *S. linicola* using ethanol distillation followed by $CHCl_3$ treatment and final extraction with ethylacetate [13]. The purity of α-ecdysone was determined by high-performance liquid chromatography (HPLC) analysis. Analysis of α-ecdysone (1 mg/ml in methanol) was conducted using an Agilent HPLC 1100 series (Santa Clara, CA, USA) equipped with a Zorbax-Eclipse XDB–C18 (3.0 mm × 150 mm, 3.5 μm) column that was maintained at a constant temperature of 25 °C. The sample was filtered (Millipore, 0.22 μm) and aliquots of 20 μl were injected into the column. The mobile phase, which consisted of A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid), was applied at a constant flow rate of 0.5 ml/min. The gradient started with 5% B and increased to 15% B (20 min), then 100% B (30 min), which was held for 5 min, after which it was decreased to 15% over 40 min. Detection was conducted at 247 nm for 45 min.

2.3. Cell culture and compound treatment

RAW 264.7, a murine macrophage cell line (ATCC, Rockville, MD), was maintained in DMEM (Sigma-Aldrich; St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Gaithersburg, MD) and 1% penicillin-streptomycin cocktail at 37 °C in a 5% CO_2 incubator. α-Ecdysone was dissolved in 5% DMSO and cells were treated with several concentrations of α-ecdysone (0.1, 1, 5, and 10 μM) for 24 h for experimental analysis.

2.4. MTT assay for cell survival

Cell survival was evaluated based on reduction of the water soluble yellow dye MTT into an insoluble blue formazan product. After treatment with α-ecdysone for 24 h, 10 μl of MTT working solution (5 mg/ml in PBS) was added to each well for 4 h. The generated formazan crystals were then solubilized by adding 50 μl of DMSO and quantified spectrophotometrically at 540 nm using an ELISA reader (Bio-Tek Instrument Co., WA, USA). The percentage cell proliferation was calculated as follows: (absorbance of treated cells / absorbance of control cells) × 100.

2.5. Neutral red assay

Effects of α-ecdysone on membrane fluidity of macrophages was determined by the neutral red uptake capacity of cells as previously described [14], with some minor modifications. The optical density was determined at 490 nm and phagocytosis was expressed as OD values.

2.6. Determination of cellular lysosomal activity

The cellular lysosomal enzyme was evaluated by measuring the acid phosphatase activity in macrophages as previously described [15]. Briefly, 100 μl of 10 mM p-NPP solution and 50 μl of 0.1 M citrate buffer (pH 5.0) were added. After 30 min of incubation, 150 μl of 0.2 M borate buffer (pH 9.8) was added and the absorbance at 405 nm was measured. The percentage activity was then calculated as follows:

Lysosomal enzyme activity

= OD sample − OD negative control/OD negative control × 100

2.7. NBT dye reduction assay

A NBT dye reduction assay was conducted as previously described, with some minor modifications [14]. Following incubation, 20 μl of phorbol myristate acetate (10 ng/ml) and 20 μl of NBT solution in PBS (1.5 mg/ml) were added, the mixture was incubated and the absorbance was measured at 570 nm. The percentage NBT reduction was calculated as follows:

NBT reduction%

= OD sample − OD negative control/OD negative control × 100

2.8. NO measurement

The nitrite concentration in the medium was measured according to the Griess reaction [15]. Macrophages were incubated with PBS as a blank control and LPS (100 ng/ml; 12 h) in the presence of α-ecdysone (0.1, 1, 5 and 10 μM) and 20 μM Indo as a positive control. The absorbance of the mixture at 540 nm was determined and the calculated

concentration was taken as an indicator of NO production. Sodium nitrite was used to generate a standard curve.

2.9. ELISA for cytokine determination

IL-1β and prostaglandin levels in cell supernatants were determined by sandwich ELISA (Invitrogen, Frederick, MD, USA) and a PGE_2 kit (Enzo Life Sciences Co., USA), respectively. After incubation, cell supernatants were collected and assays were performed according to the manufacturer's instructions.

2.10. Immunoblotting

After incubation, cells were harvested and lysed with RIPA buffer for whole cell protein isolation. For cytoplasmic and nuclear protein isolation, we used a NE-PER nuclear protein extraction kit (Thermo Scientific) according to the manufacturer's instructions. Protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Sigma-Aldrich, St. Louis, MO, USA). For western blot analysis, 50 μg of protein in each lane was run on SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics, Indianapolis, IN, USA) by electroblotting. The blots were then incubated with the appropriate primary antibodies followed by HRP-conjugated secondary antibody and visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia, Piscataway, New Jersey).

2.11. Thiobarbituric acid-reactive substances (TBARS) assay

The lipid peroxide content of RAW 264.7 cells was determined using the modified TBARS assay method as previously described [16]. For analysis, the mixture was heated at 100 °C for 10 min, after which the absorbance was read at 532 nm (Bio-Tek Instrument Co., WA, USA). MDA standard was prepared from 1,1,3,3-tetraethoxypropane.

2.12. Zebrafish model to detect morphological alterations

Detection of morphological alterations by LPS in zebrafish eggs was conducted as previously described by our group [18]. Briefly, LPS treatment was administered to the larvae of zebrafish (2 dpf) in 24 well plates, after which samples were incubated in E3 medium for 24 h. Upon completion of the incubation, larvae were treated with α-ecdysone (0.1, 1, 5 and 10 μM), after which they were incubated for an additional 24 h and morphological images were captured.

2.13. Statistical analysis

Each experimental condition was performed in triplicate ($n = 3$), and all data were expressed as the means ± standard deviation (SD). Statistical analyses were conducted using a student's *t*-test, and differences were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Effects of α-ecdysone on viability of RAW264.7 macrophages

The peak corresponding to α-ecdysone was identified at a retention time of 22.676 min with a purity of 93.14% by HPLC analysis (Fig. 1A). To determine the cytotoxic effects of α-ecdysone in RAW264.7 cells, an MTT assay was conducted to measure the cell viability. The results showed that exposure of α-ecdysone to different concentrations for 24 h did not significantly influence the viability of macrophages (Fig. 1B). Taken together, these results indicate that α-ecdysone was safe to use for murine macrophage cells at the tested concentrations; therefore, the same range of concentrations was used for all subsequent experiments.

3.2. α-Ecdysone causes enhanced membrane fluidity in macrophages

Rapid functioning of phagocytic macrophages was assessed by neutral red uptake assay to determine the membrane fluidity of macrophages, which is essential to particle uptake. As shown in Fig. 2, phagocytosis was found to increase with increasing concentrations of α-ecdysone, which differed significantly from the control group. Although all concentrations were found to be effective, the highest concentration (10 μM) of α-ecdysone was shown to have best activity, indicating enhanced phagocytic potential of macrophages. LPS was included as a positive control for comparison of the phagocytotic activity of α-ecdysone.

3.3. Enhanced lysosome membrane permeabilization and increased respiratory burst was induced in RAW 264.7 by α-ecdysone

The acid phosphatase activity of macrophages was determined based on the ability of phosphatases to catalyze the hydrolysis of p-NPP to p-nitrophenol and the measured absorbance was shown to be co-related to lysosomal activity. Secretion of lysosomal enzyme is pre-requisite to antigen degradation by phago-lysosome fusion. Production of superoxide anions is an eventual process during particle phagocytosis by macrophages that is generally accompanied by ROS generation. This oxidative burst can be detected through NBT reduction assay, which produces a purple blue color from original yellow by absorbing the superoxide ions that are generated during phagocytosis. In our study (Fig. 3A–B), we found that α-ecdysone significantly enhanced lysosomal activity and NBT reduction in a concentration dependent fashion, with 5 and 10 μM being highly effective. To compare our results, we have included LPS as a positive control for lysosomal secretion and PMA as a positive control for superoxide generation.

3.4. α-Ecdysone suppresses NO production via induction of HO-1 expression

Apart from normal defense mechanisms, NO represents toxic and proinflammatory mediators in acute and chronic inflammatory diseases. Analysis of the potential anti-inflammatory properties of α-ecdysone revealed that macrophages treated with medium alone did not release NO, whereas LPS treatment caused higher NO production. When α-ecdysone (0.1–10 μM) was added to the medium, NO production was reduced in a dose-dependent manner (Fig. 4A). Furthermore, α-ecdysone was found to decrease the MDA levels in a concentration-dependent manner following LPS stimulation (Fig. 4B), confirming the reduction of proinflammatory mediators.

Because the NO production levels were suppressed by α-ecdysone in LPS-stimulated RAW 264.7 cells, we next examined the effects of this combination on the protein levels of two isoforms of NO synthase; namely, inducible (iNOS) and endothelial (eNOS) NO synthase [17]. Using the nontoxic dose ranges established, we determined the protein levels of iNOS and eNOS by immunoblotting. LPS treatment increased the expression levels of both iNOS and eNOS, whereas marked dose-dependent decreases in protein levels were observed in response to α-ecdysone treatment (Fig. 4C). It was previously suggested that antioxidant enzyme HO-1 played a crucial role in regulation of inflammatory response, which prompted us to examine the protein expression levels of HO-1 in LPS-activated macrophage cells. Western blot analysis demonstrated that 5 and 10 μM of α-ecdysone caused marked changes in HO-1 expression, but no considerable changes in protein expression levels were observed in response to treatment with 0.1 and 1 μM of α-ecdysone (Fig. 4C). These results suggest that α-ecdysone suppresses NO levels by upregulating HO-1 expression.

3.5. α-Ecdysone inhibits the production of pro-inflammatory cytokines

Pro-inflammatory cytokines such as PGE_2 and IL-1β are secreted early during inflammation, and their elevated levels play a crucial role

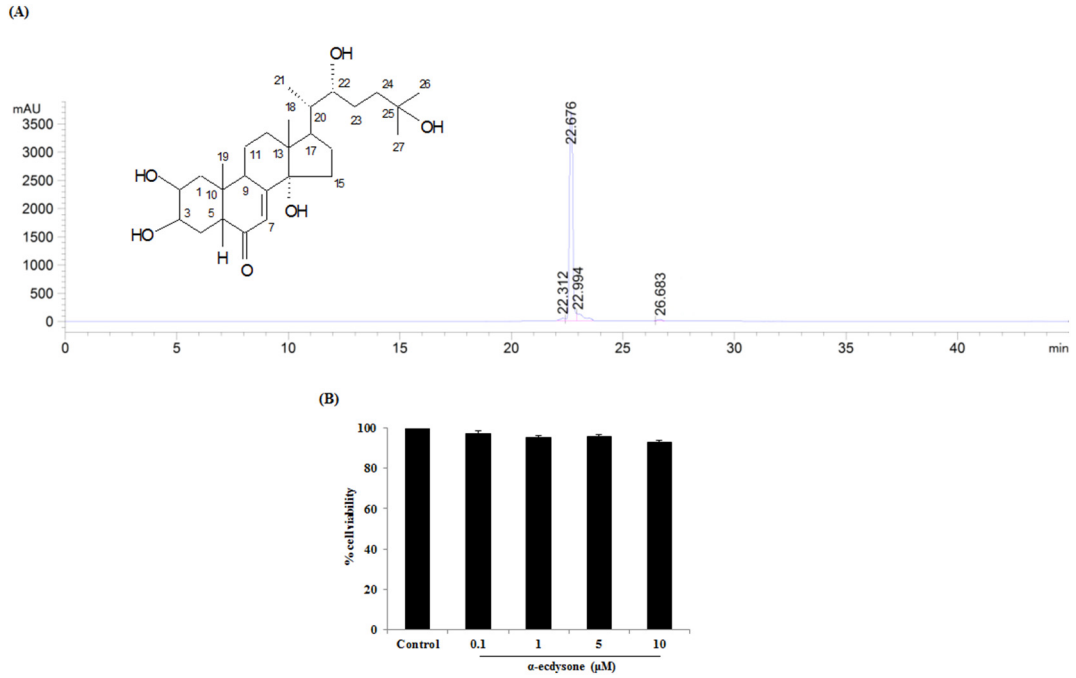

Fig. 1. Cytotoxicity profile of α-ecdysone. (A) Chemical structure of α-ecdysone. Numbers 1–27 represents carbon atoms. HPLC chromatogram of the α-ecdysone recorded at 247 nm. (B) After 24 h of incubation with α-ecdysone, macrophage cells were subjected to MTT assay for cell viability. The control group was set as a reference with a value of 100%. The data represent the mean ± SD of three independent experiments, $n = 3$.

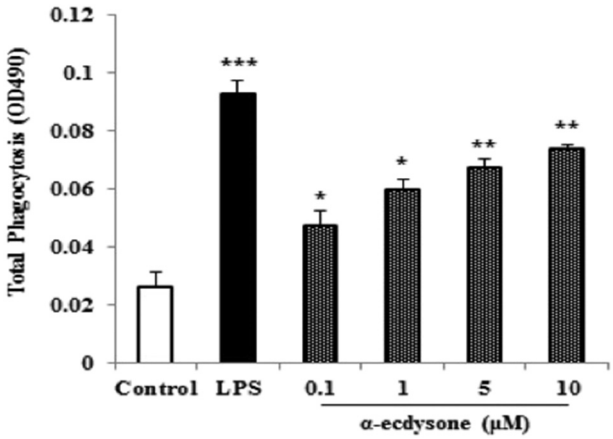

Fig. 2. Effect of α-ecdysone on macrophage phagocytosis. A neutral red uptake assay was conducted to determine the membrane fluidity of macrophages, with LPS taken as a positive control. The data represent the means ± SD of three independent experiments, n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

in the development of various acute and chronic inflammatory diseases. Therefore, we determined whether α-ecdysone has the potential to inhibit the release of these cytokines. Significant increases in the levels of PGE_2 (Fig. 5A) and IL-1β (Fig. 5B) were observed in LPS stimulated macrophage cells relative to control cells. Pro-inflammatory cytokine secretion in LPS-stimulated macrophages was inhibited by α-ecdysone exposure in a dose-dependent manner (Fig. 5A–B). Because COX-2 is the primary enzyme responsible for PGE_2 synthesis, the expression levels of COX-2 were determined by western blotting. The COX-2 protein levels were undetectable in the control, whereas stimulation by LPS alone markedly increased COX-2 expression. Reduced expression of COX-2 protein was observed in the presence of α-ecdysone along with LPS treatment, and these changes occurred in a dose-dependent manner (Fig. 5C). Similarly, the reduced expression of TNF-α was found to be in accordance with the expression of COX-2 (Fig. 5C). These results indicate that α-ecdysone inhibits the initial phase of the LPS-stimulated inflammatory response.

3.6. α-Ecdysone suppresses the TLR-4 pathway to protect cells from inflammation

The toll like receptor-4 pathway is the major molecular mechanism of LPS induced inflammation. Because we found that α-ecdysone exerted anti-inflammatory activity, we investigated its effects on the TLR signaling pathway by western blot analysis. As shown in Fig. 6, α-ecdysone significantly downregulated the key proteins of the TLR-pathways such as TLR-4, MYD88, IKK-α and IKK-ϒ, and these changes occurred in a concentration-dependent manner. These findings suggested that α-ecdysone played a role in downregulation of the TLR-4 pathway.

3.7. α-Ecdysone prevents NF-κB translocation driven by Nrf-2 activation and MAPKs inhibition

NF-κB activation is the eventual event in TLR-4 activation as TLR-4 marker proteins were found to be inhibited by α-ecdysone, we further investigated its effects on NF-κB inhibition. The translocation of NF-κB subunits was measured by cytosolic and nuclear extraction followed by western blot analyses. LPS stimulation caused the translocation of p50 and p65 protein from cytoplasm (Fig. 7A) to the nucleus (Fig. 7B). However, the protein expression in the nucleus was markedly reduced by pretreatment with α-ecdysone in LPS-stimulated cells. Because α-ecdysone inhibited pro-inflammatory mediators production and induced HO-1 expression, we found that nuclear fractions of α-ecdysone treated macrophage cells showed a gradual increase in Nrf-2 levels relative to cytosolic fractions, indicating involvement of HO-1 in the LPS-induced inflammatory pathway (Fig. 7A–B).

To further investigate whether α-ecdysone regulates MAPK proteins responsible for NF-κB activation, we examined the phosphorylation levels of JNK, p38 and ERK in LPS-stimulated macrophages. Pre-treatment with α-ecdysone strongly inhibited the phosphorylation of p38 MAPK, JNK and ERK in LPS-stimulated cells (Fig. 7C). Suppression of JNK and p38 was associated with concomitant down-regulation of c-fos

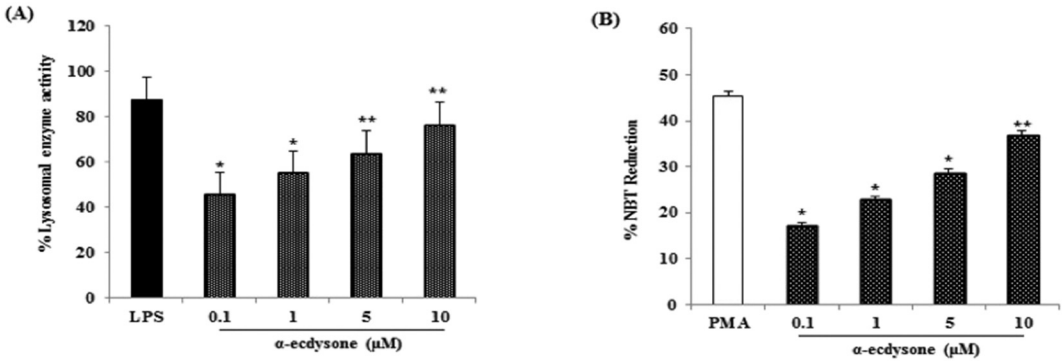

Fig. 3. α-Ecdysone induces membrane permeabilization and superoxide anion production. In vitro phagocytosis responses of macrophages were assayed by (A) % lysosomal enzyme activity and (B) % NBT reduction, where LPS and PMA were used as positive controls, respectively. The data represents the means ± SD of three independent experiments, $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

and c-JUN expression (Fig. 7B). Another signaling molecule, Akt, promotes NF-κB activation, which stimulates COX-2 and iNOS expression. α-Ecdysone markedly suppressed phosphorylation of Akt in LPS-stimulated cells (Fig. 7C). These results were further confirmed in the presence of ERK inhibitor (PD98059), p38 inhibitor (SB203580), Akt inhibitor (Wortmannin) and JNK inhibitor (SP600125). No significant differences were observed in cell viability and NO secretion was found to be markedly suppressed in the presence of α-ecdysone as well as specific kinase inhibitors in LPS-stimulated RAW 264.7 cells (Fig. S1). These data collectively suggest additional characteristics of α-ecdysone regulatory effects on the NF-κB pathway are mediated by the cooperative involvement of Nrf-2 activation and inhibition of MAPKs phosphorylation.

3.8. α-Ecdysone prevents LPS induced morphological deformities in zebrafish larvae

The combination of in vitro (macrophage) and in vivo (zebrafish) analyses has gained momentum in recent years and become an excellent strategy for investigations of inflammation and identification of novel compounds. The key characteristics and advantages of *Danio rerio* are: (i) high fecundity and small size, (ii) rapid *ex utero* development, (iii) optical transparency of the embryos and larvae, (iv) rapid assessment of pharmacological activity by absorption of compounds from surrounding medium through the gastrointestinal tract or across the skin, (v) maintenance of cellular components of the innate immune system, (vi) accessibility of transgenic fluorescent reporter lines, (vii) and the ability to conduct immunological studies on the behavior of

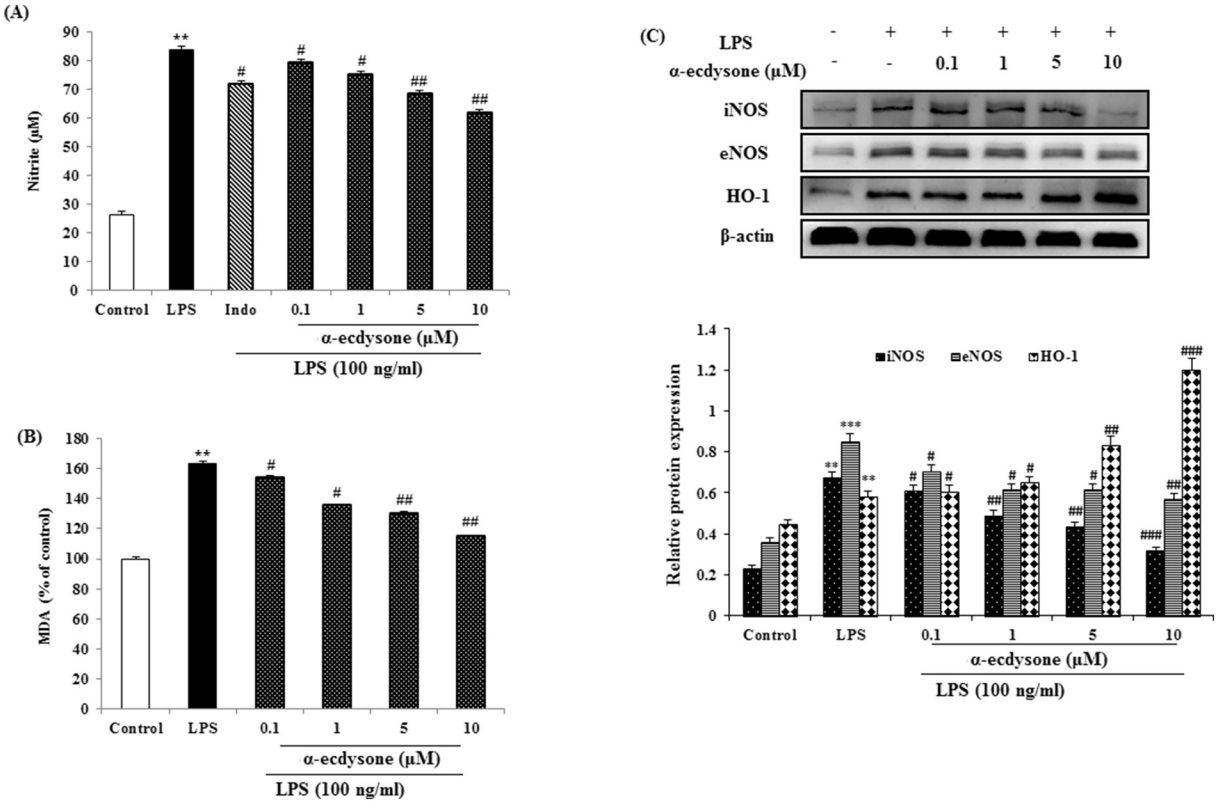

Fig. 4. α-Ecdysone suppresses oxidative stress. (A) Nitrite and (B) MDA production was determined in LPS-stimulated macrophage cells in the presence or absence of α-ecdysone (0.1, 1, 5 and 10 μM) for 24 h. Indomethacin (Indo; 20 μM) was used as a positive control. (C) Expression of iNOS, eNOS and HO-1 was assayed by immunoblotting and quantified by ImageJ. The data represent the means ± SD of three independent experiments, n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs LPS.

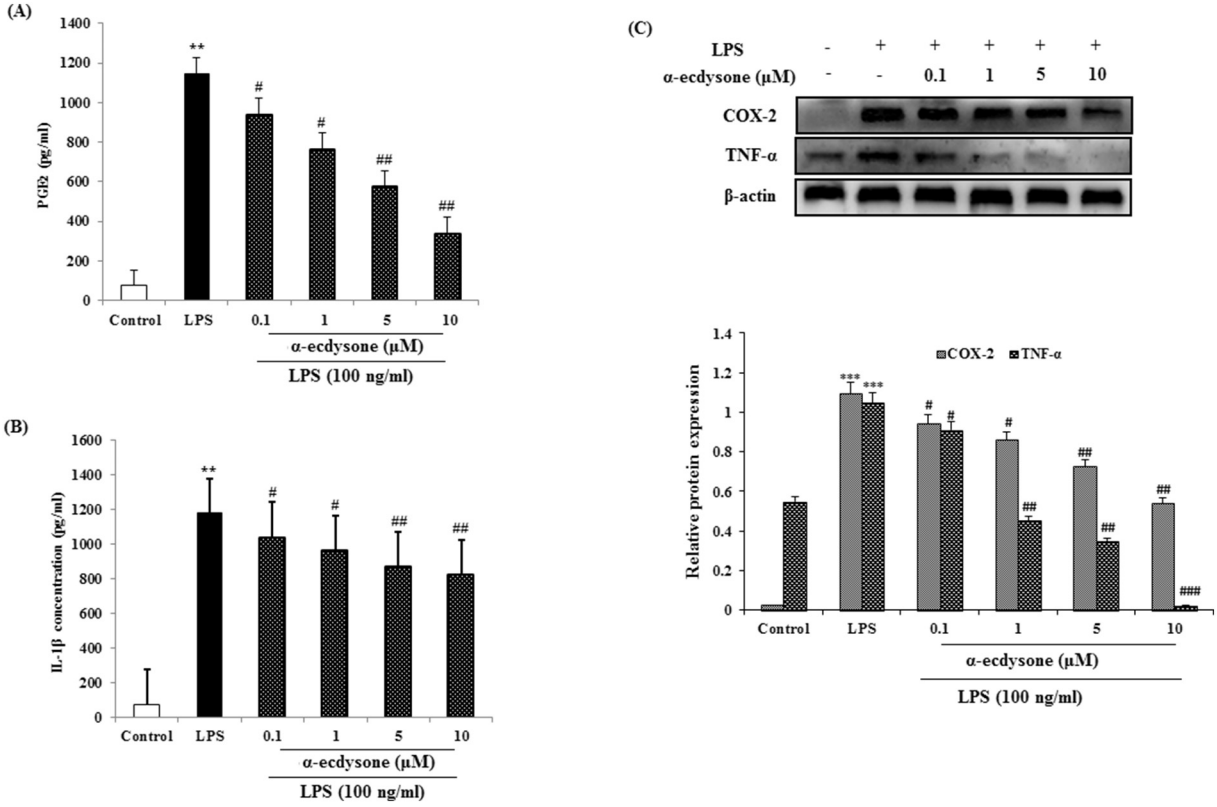

Fig. 5. Effects of α-ecdysone on inflammatory marker levels. (A) PGE_2 secretion and (B) IL-1β synthesis were determined in LPS-stimulated macrophage cells in the presence or absence of α-ecdysone (0.1, 1, 5 and 10 μM) for 24 h. (C) Expression of COX-2 and TNF-α was determined by immunoblotting and quantified by ImageJ. The data represent the means ± SD of three independent experiments, n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs LPS.

infiltrating cells in living organisms. The salient features of zebrafish serve as a discovery platform for the systemic identification of bioactive natural products [19,20].

We found that treatment with LPS altered the morphological development of larvae. As shown in Fig. 8, LPS treatment triggered spinal cord curving (SC) in zebrafish larvae, but this was recovered by treatment with α-ecdysone in a concentration-dependent manner (Fig. 8). The results of this study suggest that α-ecdysone has a protective effect against morphological deformities induced by LPS.

4. Discussion

Various studies in the past several decades have explored the biological activities of natural compounds or extracts against different ailments because of their widespread availability, higher efficacy and low toxicity toward humans. To date, numerous studies have mentioned the anti-inflammatory effects of such compounds, but few have investigated the anti-inflammatory actions at a molecular level. The present investigation provides evidence that α-ecdysone has in vitro immunomodulatory activities with low toxicity (Fig. 1) that promote

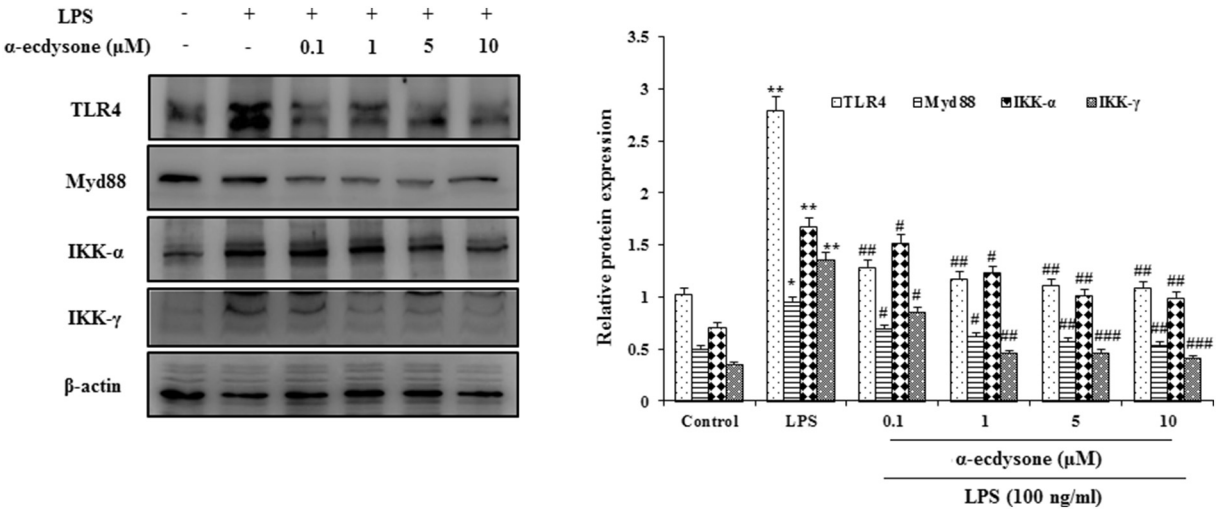

Fig. 6. Effects of α-ecdysone on LPS-induced TLR-4 pathway. Immunoblot analysis to determine the TLR-4, MYD88, IKK-α and IKK-ϒ proteins. The western blot images were quantified by ImageJ. The data represent the means ± SD of three independent experiments, n = 3. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs LPS. N.S. indicates non-significant.

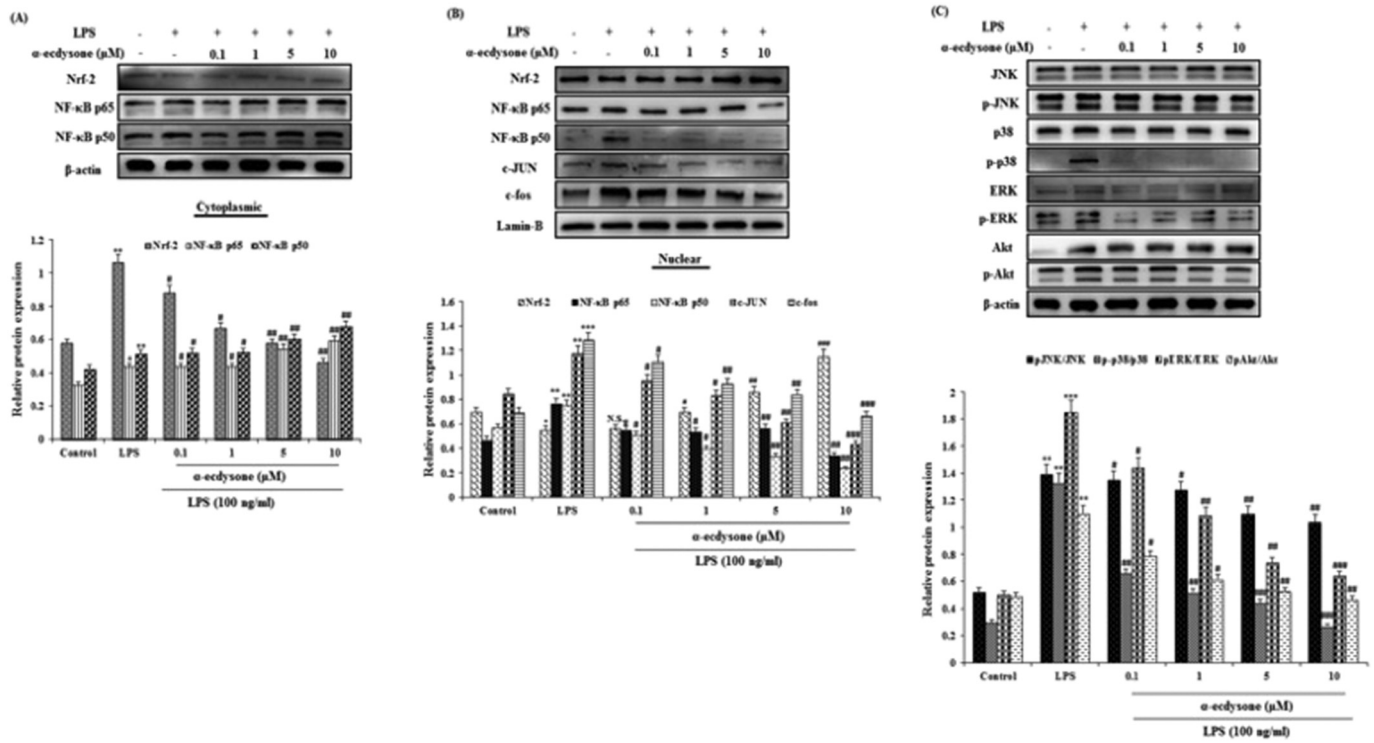

Fig. 7. Effects of α-ecdysone on LPS-induced Nrf2, NF-κB and MAPKs pathway. Immunoblot analysis to determine (A) cytosolic and (B) nuclear translocation of Nrf2 and NF-κB subunits. c-JUN and c-fos expression was checked in nuclear protein. (C) α-Ecdysone suppresses phosphorylation of MAPKs and Akt. Western blot images were quantified by ImageJ. The data represent the means ± SD of three independent experiments, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 vs control; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs LPS. N.S. indicates non-significant.

phagocytosis, suppressing cytokine production and HO-1 induction in murine macrophages.

Phagocytosis is a defense mechanism employed by macrophages in which an antigen is engulfed, and this process is accompanied by increased membrane fluidity and activation of membrane protein. The key factors involved in potentiation of phagocytosis by increasing macrophage activity are membrane fluidity, lysosomal activity and respiratory burst. In the present study, we detected a significant increase in phagocytosis in response to α-ecdysone treatment as there was a high correlation between membrane fluidity and phagocytosis, indicating enhanced capacity for engulfment (Fig. 2). Following engulfment, secretory organelles process antigens by secreting proteases, lactoferrin, and lysozymes and then deliver the antigens to the cell surface and phagosomal membranes [21]. Generation of superoxide

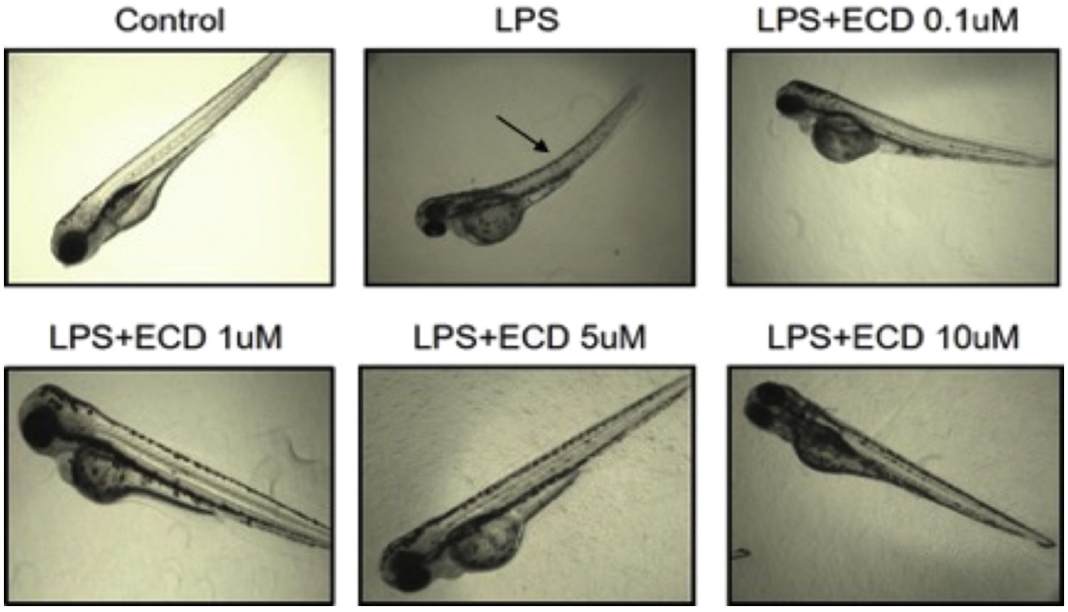

Fig. 8. Effects of α-ecdysone on LPS-induced morphological alteration. Zebrafish larvae were treated with LPS (2 dpf) in 24 well plates and were incubated in E3 medium for 24 h. Next, α-ecdysone (0.1, 1, 5 and 10 μM) was administered to the larvae. Morphological images were captured after 24 h of additional incubation. The arrow represents the spinal cord curvature (SC).

anions creates oxidative bursts to respond to aerobic pathogens and facilitate antigen processing. The results of p-NPP and NBT reduction assays indicate that α-ecdysone was effective at producing lysosomal enzyme and superoxide generation in a concentration dependent manner, which can enhance the capacity for intracellular antigen processing of macrophages (Fig. 3).

Macrophages are an important component of defense systems that actively participate in progression of inflammation diseases by releasing pro-inflammatory cytokines and mediators. Excessive NO production by iNOS and eNOS overexpression has been implicated in pathogenesis of septic shock, inflammation and carcinogenesis. According to our results, α-ecdysone strongly inhibits LPS-induced NO and lipid peroxide production by regulating iNOS and eNOS production (Fig. 4A–C). There is mounting evidence that plant-derived compounds such as curcumin, isoflavones, and cannabidiol [22,23] inhibit NO and MDA production and exert anti-inflammatory activities in different cells. One of the major biological effects of α-ecdysone is the induction of phase II antioxidant enzyme, which has been reported to protect against inflammatory effects by suppressing pro-inflammatory responses. We investigated the effects of α-ecdysone on protein levels of HO-1 in LPS-stimulated macrophage cells and found that it could increase HO-1 protein levels, which is crucial for inhibition of LPS-induced inflammation (Fig. 4C). HO-1 generates antioxidants that inhibit iNOS and eNOS protein expression and suppress NO production.

Previous studies have indicated an existing cross-talk between NO and prostaglandins production that contributes to inflammatory disorders. Inflammatory disorders are characterized by production of cytokines that regulate immunity such as TNF-α and IL-1β. IL-1β is an essential component for the initiation and enhancement of inflammatory responses, whereas TNF-α is an endogenous mediator of LPS-induced fever. Our data indicated that LPS caused drastic elevation of PGE_2 and IL-1β, which was inhibited by the presence of α-ecdysone (Fig. 5A–B). TNF-α is believed to mediate cytotoxicity and induce the release of cytokines. Our results demonstrated the reduction of TNF-α expression, which indicated that cytokine expression was regulated by TNF-α (Fig. 5C). Similarly, COX-2, an inducible enzyme that regulates PGE_2 production, was found to be down-regulated by α-ecdysone treatment (Fig. 5C). These findings suggest that α-ecdysone decreases NO and PGE_2 production by simultaneously attenuating iNOS, eNOS and COX-2 along with cytokine levels, and that these effects may have a potential therapeutic effect against inflammatory diseases.

HO-1 is transcriptionally activated by Nrf-2 and participates in maintenance of cellular homeostasis by attenuating inflammation in macrophages and tissues. During oxidative stress conditions such as those induced by LPS and harmful chemicals, Nrf-2 translocates to the nucleus, binds to its cis-acting response and activates antioxidant systems to mitigate inflammation. Our results showed that α-ecdysone can activate Nrf-2, resulting in induction of HO-1 activity (Fig. 6A–B). NF-κB is a transcription factor that plays a pivotal role in the regulation of gene expressions involving immune responses and inflammation. Therefore, targeting the translocation of NF-κB has the potential for treatment of inflammatory diseases. In this study, the effects of α-ecdysone on nuclear translocation of NF-κB p50 and p65 were evaluated and the results revealed it was reduced after pre-treatment of macrophages that had been induced by LPS (Fig. 6A–B). These findings indicate that α-ecdysone may interrupt the interaction of LPS with its receptors, which is in accordance with the results of previous studies [24].

NF-κB activation is regulated by either the TLR-4 pathway or cellular kinases such as MAPKs and Akt, which actively participate in inflammatory reactions. Therefore, we investigated the key proteins involved in activation of the TLR-4 pathway (TLR-4, MYD88, IKK-α and IKK-ϒ), which eventually triggers the activation of NF-κB. The results revealed the potential for α-ecdysone to downregulate the TLR-4 pathway (Fig. 6). In LPS-stimulated immune cells, MAPKs regulate pro-inflammatory signaling cascades via the activation of NF-κB. Similarly, Akt has been reported to act upstream of NF-κB activation in LPS-stimulation. The results of the present study demonstrated that α-ecdysone inhibited the phosphorylation of MAPKs and Akt, which led to the inhibition of NF-κB translocation in LPS-stimulated cells, supporting that MAPKs and Akt signaling are involved in the anti-inflammatory activity of α-ecdysone by down-regulation of the NF-κB pathway in LPS-stimulated macrophages (Figs. 7C and S1). Based on evaluation of the antioxidant properties of α-ecdysone, it is speculated that inhibition of Akt activation and the presence of phenolic rings act as electron traps that scavenge free radicals, which may contribute to α-ecdysone's anti-inflammatory activity. Moreover, the ability of α-ecdysone to protect the LPS induced morphological alteration of zebrafish larva strongly suggest that it can be used as a therapeutic drug to combat the severe effects of LPS or as a potent immunomodulator.

5. Conclusion

We demonstrated that α-ecdysone inhibits the production of pro-inflammatory mediators and cytokines known to be associated with inactivation of the NF-κB pathway via MAPK signaling, Akt phosphorylation and HO-1 activation. Elucidation of the relative mechanism of anti-inflammatory activity at the cellular and molecular levels will facilitate future application of α-ecdysone as a therapeutic agent for inflammatory diseases.

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Declaration of Competing Interest

No conflict of interest exists.

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