Anti-inflammatory effects of alkaloid enriched extract from roots of *Eurycoma longifolia* Jack

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ABSTRACT

**Objective:** To examine the *in vitro* and *in vivo* anti-inflammatory effects of the alkaloid enriched extract (ELA) from the roots of *Eurycoma longifolia*. **Methods:** The *in vitro* anti-inflammatory effects of ELA were evaluated by examining its inhibitory activities against nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expressions in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The level of NO produced in the culture media was determined by Griess method. The iNOS and COX-2 protein expressions were analyzed by Western blot. The *in vivo* effect of ELA was evaluated on LPS-induced septic shock in mice model. Mice mortality was monitored for 5 days after injection of LPS. The chemical contents of the ELA were determined by using various chromatographic and spectroscopic techniques. **Results:** The ELA was found to exhibit a significant anti-inflammatory effect in both *in vitro* and *in vivo* models. The results demonstrated that ELA dose-dependently inhibited LPS-induced NO production as well as the protein iNOS and COX-2 expressions. In the septic shock model, ELA dose-dependently protected mice from LPS-induced mortality. Further study on the isolated components of ELA indicated that 9,10-dimethoxycanthin-6-one may contribute significantly to the anti-inflammatory effects of the extract. **Conclusions:** These results suggest that ELA exhibits the anti-inflammatory activity via suppression of pro-inflammatory mediators such as NO, iNOS, and COX-2 and protects mice from LPS-induced mortality in septic shock model.

1. Introduction

Inflammation is a normal immune system response of the body to cell damage and stimuli. Cytokine networks and pro-inflammatory genes regulate the pathogenesis of inflammation[1]. Overproduction of these inflammatory mediators such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2) may lead to many diseases such as rheumatoid arthritis,
asthma, multiple sclerosis, systemic lupus erythematosus, and atherosclerosis[2-4]. Therefore, control of these inflammatory mediators may be important means for the prevention of multiple inflammatory diseases[5].

Eurycoma longifolia (E. longifolia) Jack has been used in Southeast Asian countries as traditional remedies for sexual dysfunction, constipation, exercise recovery, fever, loss of libido, aging, stress, malaria, osteoporosis, diabetes, high blood pressure, cancer, leukemia, and glandular swelling[6]. Chemical studies revealed that this plant is a rich source of biologically active compounds including phenolics, quassinoids, canthin-6-one alkaloids, β-carboline alkaloids, triterpenoids, lactones, neolignans and steroids[7]. Alkaloid isolated from E. longifolia exhibited various biological effects including antimalarial, cytotoxic, and anti-ulcer activities[7]. Recently, a β-carboline alkaloid isolated from the hairy root cultures of E. longifolia showed potent anti-inflammatory activity through the activation of Nrf2/heme oxygenase-1 pathway[8]. The anti-inflammatory effect of the methanol extract of this plant was also reported[9].

In this study, we evaluated the anti-inflammatory activity of the alkaloid enriched fraction from the roots of E. longifolia. The in vitro molecular study on macrophage cells and in vivo anti septic shock were also carried out to demonstrate its potential application in anti-inflammation.

2. Materials and methods

2.1. Plant materials

The plant E. longifolia was collected in March 2017 at Nghe An province, Vietnam and was identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). The voucher specimen (BB-01) was deposited at the Institute of Marine Biochemistry (VAST).

2.2. Preparation of alkaloid enriched fraction

The roots of E. longifolia (5 kg) was air dried, ground to powder and extracted with methanol (1/5 v/v) at room temperature (3 times × 24 h each). The organic layer was filtered and concentrated under vacuum to obtain the crude extract of methanol (200 g). This crude extract was suspended in 1 mol/L HCl (1/15 w/v) and partitioned with ethyl acetate (3 times × 1.5 L each) to yield ethyl acetate and water-soluble layer. This layer was adjusted to pH 10 by NaOH and filtered to obtain the alkaloid solution. The solution was then partitioned with ethyl acetate (3 times × 1.5 L each), the organic layer was collected and concentrated under vacuum to obtain the alkaloid enriched fraction (ELA, 10 g).

2.3. Isolation of compounds from ELA

The ELA extract (10 g) was chromatographed on a silica gel column eluted with increasing the volume of methanol in dichloromethane (0% → 100%) to obtain five fractions ELA1→ELA5. The sub-fraction ELA2 (2.1 g) was chromatographed on a silica gel column and eluted with solvent system of dichloromethane:acetone (10/1, v/v) to yield compound 1 (17 mg). The fraction ELA3 (3.1 g) was chromatographed on a silica gel column and eluted with hexane/ethyl acetate (1/1 v/v) to obtain compound 2 (12 mg) and the sub-fraction ELA3.4. Compounds 3 (15 mg) and 4 (4 mg) were obtained from the sub-fraction ELA3.4 on a silica gel column eluting with dichloromethane/methanol (20/1 v/v).

2.4. Cell culture

Murine macrophage RAW264.7 cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and streptomycin-penicillin (Invitrogen, Carlsbad, CA, USA). Cultures were maintained in a CO2 incubator in humidified atmosphere with 5% CO2 at 37 °C.

2.5. Assay for inhibition of NO production

The effects of samples on the NO production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells were examined as described previously[8]. The cells were seeded in 96-well plate at 2 ×103 cells/well and incubated for 18 h. The plates were pretreated with ELA (from 10 µg/mL to 100 µg/mL) or compounds (from 1 µM to 100 µM) for 30 min and then incubated for another 24 h with or without 1 µg/mL LPS (Escherichia coli 0111:B4; Sigma Aldrich, USA). A total of 100 µL of the culture supernatant were transferred to other 96-well plate and 100 µL of Griess reagent were added. The absorbance of the reaction solution was read at 570 nm with a XMark microplate reader (BioRad, USA). The remaining cell solutions in cultured 96-well plate were used to evaluate cell viability by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay[10]. Cardamonin, a known NO production inhibitor[11,12], was used as a positive control. Its IC50 value for inhibition of NO production was calculated as 0.64 µg/mL (or 2.37 µM) from the similar assay.

2.6. Western blot analysis

Cells were harvested and lysed in a lysis buffer [150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1% NP-40, 5 mM sodium orthovanadate, and protease inhibitors cocktail (BD Biosciences)] and then centrifuged at 4 °C and 15 000 rpm for 10 min. An equal
amount of protein was separated onto sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked in 5% skim milk for 1 h at room temperature, probed with the appropriate primary antibodies, washed, and then incubated with the corresponding secondary antibody. The signal was detected using the enhanced chemiluminescence system (Intron, Seongnam, Korea). α-Tubulin was detected for protein loading control.

2.7. In vivo effects of ELA on LPS–induced mortality in IRC mice

Male IRC mice (20-22 g) were housed in plastic cages and maintained at (22 ± 2) °C and 50%-60% relative humidity with 12 h light-dark cycles throughout the experiment and the animals were maintained in these facilities for at least 1 week before the experiment. Animal experiments were approved by the Animal Research Committee of Kangwon National University (protocol number KW-180119-3, issued date: 05. Feb., 2018). The effect of ELA on LPS-induced septic mortality was determined as previously described[8]. In brief, four IRC mice groups (5 mice per group) were administered orally with ELA at concentrations of 50 mg/kg and 100 mg/kg dissolved in dimethyl sulfoxide: corn oil (1:9 by volume) or control vehicle 1 h before LPS injection. Mice survival was monitored for 5 d after injecting LPS. The log-rank test was used to compare the survival significance.

2.8. Statistical analysis

Data was expressed as the mean ± standard deviations (SD). Statistical significance was assessed by the two-tailed unpaired Student’s t-test and P value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of ELA on NO production in LPS–stimulated RAW264.7 cells

Our results revealed that ELA significantly inhibited the NO production in LPS-stimulated RAW264.7 murine macrophages in a dose-dependent manner (Figure 1). Its IC_{50} value was determined as (28.18 ± 0.11) µg/mL compared to the positive control, cardamonin (IC_{50} value of 0.64 µg/mL). Similar to cardamonin, ELA did not affect the cell viability at tested concentrations in MTT assay (data not shown). It was indicated that the inhibitory effects of NO production of ELA was not a result of cytotoxicity.

![Figure 1. Inhibitory effects of ELA on LPS-induced NO production in RAW264.7 cells.](image)

Each value indicates the mean ± SD from three independent experiments. Control value was obtained in the absence of LPS and ELA. Asterisks indicate statistical significance (P < 0.05) compared with the control.

3.2. Effect of compounds isolated from ELA on NO production in LPS–stimulated RAW264.7 cells

Compounds 1-4 were isolated from ELA by using various chromatographic techniques. Their structures were determined by using spectroscopic data and by comparison with the reported data[13-15]. They were elucidated as 9-hydroxycanthin-6-one (1), 9-methoxy-9H-canthin-6-one (2), 9,10-dimethoxy-9H-canthin-6-one (3) and 5-methoxy-9H-canthin-6-one (4) (Figure 2).

9-Hydroxycanthin-6-one (1): yellow powder. ESI-MS (positive): m/z 237 [M+H]+. 1H NMR (500 MHz, DMSO-d_{6}), δ (ppm): 6.93 (1H, d, J = 10.0 Hz, H-5), 6.98 (1H, dd, J = 2.0, 8.5 Hz, H-10), 7.95 (1H, d, J = 2.0 Hz, H-8), 8.06 (1H, d, J = 3.5 Hz, H-4), 8.08 (1H, d, J = 5.0 Hz, H-1), 8.11 (1H, d, J = 8.5 Hz, H-11), 8.72 (1H, d, J = 5.0 Hz, H-2), 13C NMR (125 MHz, DMSO-d_{6}), δ (ppm): 115.8 (C-1), 145.8 (C-2), 139.9 (C-3), 128.0 (C-5), 158.9 (C-6), 102.9 (C-8), 160.4 (C-9), 114.0 (C-10), 124.5 (C-11), 115.5 (C-12), 140.5 (C-13), 128.9 (C-14), 131.6 (C-15), 135.0 (C-16).

9-Methoxy-9H-canthin-6-one (2): orange powder. ESI-MS (positive): m/z 251 [M+H]+. 1H NMR (500 MHz, DMSO-d_{6}), δ (ppm): 3.91 (3H, s, O-CH₃), 6.93 (1H, d, J = 10.0 Hz, H-5), 7.13 (1H, dd, J = 2.5, 10.0 Hz, H-10), 7.95 (1H, d, J = 2.0 Hz, H-8), 8.08 (1H, d, J = 10.0 Hz, H-11), 8.12 (1H, d, J = 5.0 Hz, H-1), 8.18 (1H, d, J = 8.5 Hz, H-4), 8.74 (1H, d, J = 5.0 Hz, H-2), 13C NMR (125 MHz, DMSO-d_{6}), δ (ppm): 116.1 (C-1), 145.8 (C-2), 139.8 (C-4), 128.0 (C-5), 158.8 (C-6), 100.6 (C-8), 161.1 (C-9), 113.0 (C-10), 124.1 (C-11), 116.8 (C-12), 140.1 (C-13), 128.3 (C-14), 131.5 (C-15), 135.0 (C-16), 55.7 (O-CH₃).

9,10-Dimethoxy-9H-canthin-6-one (3): light yellow powder. ESI-MS (positive): m/z 281 [M+H]+. 1H NMR (500 MHz, DMSO-d_{6}), δ (ppm): 3.94 (3H, s, O-CH₃), 3.96 (3H, s, O-CH₃), 6.94 (1H, d, J = 9.5 Hz, H-5), 7.92 (1H, s, H-11), 8.06 (1H, d, J = 10.0 Hz, H-4), 8.09 (1H, s, H-8), 8.15 (1H, d, J = 5.0 Hz, H-1), 8.77 (1H, d, J = 5.0 Hz, H-2), 13C NMR (125 MHz, DMSO-d_{6}), δ (ppm): 115.9 (C-1), 145.3 (C-2), 139.3 (C-4), 127.8 (C-5), 158.3 (C-6), 106.0 (C-8),...
151.7 (C-9), 147.7 (C-10), 99.7 (C-11), 116.0 (C-12), 133.4 (C-13), 129.5 (C-14), 131.1 (C-15), 134.9 (C-16), 55.8 (9-OCH3), 56.0 (10-OCH3).

5-Methoxycanthin-6-one (4): yellow powder. ESI-MS (positive): m/z 251 [M+H]+. 1H NMR (500 MHz, CDCl3), δ (ppm): 4.06 (3H, s, O-CH3), 7.19 (1H, s, H-4), 7.51 (1H, ddd, J = 1.0, 1.0, 1.0 Hz, H-10), 7.67 (1H, ddd, J = 1.0, 1.0, 1.0 Hz, H-9), 7.81 (1H, d, J = 5.5 Hz, H-8), 8.07 (1H, d, J = 8.0 Hz, H-11), 8.68 (1H, d, J = 5.5 Hz, H-1), 8.75 (1H, d, J = 5.5 Hz, H-2). 13C NMR (125 MHz, CDCl3), δ (ppm): 117.4 (C-1), 145.8 (C-2), 109.8 (C-4), 154.4 (C-5), 155.2 (C-6), 113.7 (C-8), 130.4 (C-9), 125.7 (C-10), 122.4 (C-11), 125.1 (C-12), 129.2 (C-13), 127.4 (C-14), 138.9 (C-15), 136.8 (C-14), 56.8 (5-OCH3).

The in vitro anti-inflammatory effects of compounds 1-4 were evaluated on the NO production in LPS-stimulated RAW264.7 cells. According to the results, only compound 3 exhibited significant inhibitory activity against NO production with the IC50 value of 33.11 µM but less potent compared to cardamonin (IC50 value of 2.37 µM). The other compounds either showed no activity or induced cytotoxicity to the tested cells up to 50 µM (data not shown).

3.3. Effect of ELA on expressions of iNOS and COX-2 in RAW264.7 cells

The inhibitory effect of ELA on the expressions of iNOS and COX-2 in LPS-stimulated RAW264.7 cells was also investigated. According to the results, ELA effectively suppressed the expressions of iNOS and COX-2 proteins in a dose-dependent manner (Figure 3). The amount of internal control, α-tubulin, remained unchanged.

3.4. Effect of ELA on the LPS-induced mortality rate in IRC mice

The effect of ELA on LPS-induced mortality in mice for 5 d was evaluated. After LPS administration, LPS-treated mice decreased in the survival rate at 30 h, and caused 80% mortality after 42 h. At 48 h, the survival rate of mice administered LPS alone or pretreated with ELA at 50 and 100 mg/kg was 20% or 60% and 80%, respectively (Figure 4).

4. Discussion

NO plays as important signaling molecules in various physiological and pathophysiological responses[16]. Therefore, a strategy for the development of anti-inflammatory agents is to inhibit the NO production by immune cells, particularly macrophages. Following the stimuli such as LPS, macrophages induce the expression of several inflammatory cytokines like tumor necrosis factor-α (TNF-α), inflammatory mediators, such as NO and prostaglandin E2. Therefore, drugs that inhibit the overproduction of NO may have potential therapeutic effects in treatment of anti-inflammatory diseases. Our data demonstrated that ELA dose-dependently inhibited the NO production in the LPS-stimulated macrophages.

NO is synthesized by nitric oxide synthases (NOS). There are three different isoforms of NOS including constitutive neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) nitric oxides synthases[17,18], among which, iNOS is expressed in response to inflammatory stimuli such as LPS, pro-inflammatory cytokines and interferon gamma (IFN-γ)[16,19]. iNOS catalyzes the formation and release of a large amount of NO[20,21]. Cyclooxygenase (COX) is
the enzyme involved in the conversion of arachidonic acid to a huge number of biologically active mediators including prostaglandins. COX exists in two isoforms including COX-1 and COX-2. COX-2, induced in response to various stimuli, is activated at the site of the inflammation[21,22]. It has been known that COX-2 is a key mediator of inflammatory pathways. COX-2 appears to be the dominant source of prostaglandin formation in inflammation, including prostaglandin E₂[23,24]. In this study, our results suggested that the extract ELA inhibited COX-2 in a concentration dependent manner, so it could also suppress the expression of prostaglandin E₂. The effect of NO on cytokines has been well reported. Accordingly, NO suppression can decrease LPS-induced TNF-α and IL-1β, the pro-inflammatory cytokines, but enhance IL-10, the anti-inflammatory cytokine, in macrophages. Endogenous NO was also reported to upregulate TNF-α production in transfected phorbol myristate acetate-differentiated U937 cells[25,26]. Our results indicated that the ELA effectively suppressed the expression of iNOS and COX-2 in RAW264.7 cells, suggesting that ELA decreased NO production levels by inhibition of the expressions of both proteins.

Septic shock is a systemic inflammatory response that develops when LPS or other microbial products stimulate the expression of various inflammatory mediators and cytokines. In the pathophysiology of septic shock, the excessive production of NO after iNOS induction has been proposed to be a major factor involved in tissue damage[27-29]. The LPS-induced mortality in mice was used to evaluate the effects of ELA on systemic inflammation. Our data indicated that oral pretreatment with ELA (50 and 100 mg/kg) dose-dependently decreased the mortality rate in the LPS-stimulated mice. Sepsis stimulated by LPS induces dysfunctional organs and promotes the production of many pro-inflammatory cytokines and mediators, such as NO, iNOS, and COX-2. Therefore, inhibiting the production of these cytokines and mediators can protect mice from mortality in LPS-stimulated sepsis model. This finding further supports that ELA has a strong anti-inflammatory activity in vivo.

The present study demonstrated that ELA from the roots of E. longifolia Jack exhibited significant anti-inflammatory effects in vitro and in vivo. In the present study, four canthin-6-one alkaloids (1-4) were isolated and tested for their inhibitory effects on the NO production. It has been known that the addition of the methoxy group has been shown to increase the anti-inflammatory activity of E. longifolia extracts[9] and isolated components including alkaloids and quassinoids[32,33]. However, mechanism of action of alkaloid enriched extract from this plant has never been reported. β-carboline and canthin-6-one alkaloids are considered as one of the bioactive components of the plant. The β-carboline alkaloid 7-MCPA isolated from the hairy root culture of E. longifolia exhibited potent anti-inflammatory activity via activation of Nrf-2/HO-1 pathway. Some canthin-6-one alkaloids from the roots of this plant also exhibited significant NF-κB inhibitory activity in TNF-α-stimulated HEK-293/ NF-κB-luc cells. In our study, we were able to show that 9,10-dimethoxycanthin-6-one (3) isolated from the ELA extract exhibited significant inhibitory effect on NO production in the LPS-stimulated RAW264.7 cells. The 7-MCPA inhibited NO production with the IC₅₀ value of (4.9 ± 0.8) µM; while in this study, compound 3 isolated from the ELA extract exhibited significant inhibitory effect on NO production with the IC₅₀ value of 33.11 µM. Canthin-6-one and its derivative 5-(1-hydroxyethyl)-canthin-6-one, isolated from the stem barks of Alstonia scholaris, showed potent NO production inhibitory activity with the IC₅₀ values of (15.09 ± 1.80) µM and (9.09 ± 0.34) µM, respectively. Further study showed that canthin-6-one down regulated the NF-κB and Akt pathway[34,35]. Notably, canthin-6-one was isolated from the E. longifolia previously[36]. Therefore, these canthin-6-one alkaloids significantly contribute to the anti-inflammatory activity of E. longifolia.

In conclusion, the ELA extract exhibited in vitro anti-inflammatory activity via inhibiting the NO production and the expressions of proteins iNOS and COX-2 in RAW264.7 cells. Further study on the bioactive components revealed that 9,10-dimethoxycanthin-6-one (3) isolated from the ELA may contribute to the anti-inflammatory activity of the extract. Noteworthy, our results demonstrated that oral administration of ELA at 100 mg/kg protected mice from LPS-induced mortality, suggesting that this enriched extract could be a potential candidate for the development of functional food for the prevention of inflammatory diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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