Asian Pacific Journal of Tropical Biomedicine

Journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.250265

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

Dao Thi Thanh Hien[1†], Tran Phi Long[2†], Tran Phuong Thao[2], Jeong-Hyung Lee[2], Duong Thu Trang[3], Nguyen Thi Thu Minh[3], Pham Van Cuong[3], Do Thi Ngoc Lan[4], Nguyen Hai Dang[3], Nguyen Tien Dat[5✉]

[1]*Deparment of Traditional Pharmacy, Hanoi University of Pharmacy, 13-Le Thanh Tong, Hoan Kiem, Hanoi, Vietnam*

[2]*Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon, Gangwon-Do 200-701, Republic of Korea*

[3]*Advanced Center for Bio-organic Chemistry, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*

[4]*National Institute of Drug Quality Control, 48-Hai Ba Trung, Hoan Kiem, Hanoi, Vietnam*

[5]*Center for Research and Technology Transfers, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*

ARTICLE INFO

Article history:
Received 23 October 2018
Revision 25 November 2018
Accepted 5 January 2019
Available online 21 January 2019

Keywords:
Eurycoma longifolia
Anti-inflammation
Alkaloid
9,10-dimethoxycanthin-6-one

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,

✉Corresponding author: Nguyen Tien Dat, Center for Research and Technology Transfers, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.
Tel: +84-24-37568422
Fax: +84-24-37568422
E-mail: ngtiend@imbc.vast.vn

Foundation project: This research is funded by Vietnam Academy of Science and Technology under grant number VAST04.03/17-18.

†These authors make equal contributions to this work.

How to cite this article: Hien DTT, Long TP, Thao TP, Lee JH, Trang DT, Minh NTT, et al. Anti-inflammatory effects of alkaloid enriched extract from roots of *Eurycoma longifolia* Jack. Asian Pac J Trop Biomed 2019; 9(1): 18-23.

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, triterpenoid, 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 w/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 CO_2 incubator in humidified atmosphere with 5% CO_2 at 37 ℃.

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 $\times 10^5$ 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 IC_{50} 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 ℃ 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) ℃ 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 (*Escherichia coli* 0111:B4; Sigma-Aldrich, 20 mg/kg body weight) 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.

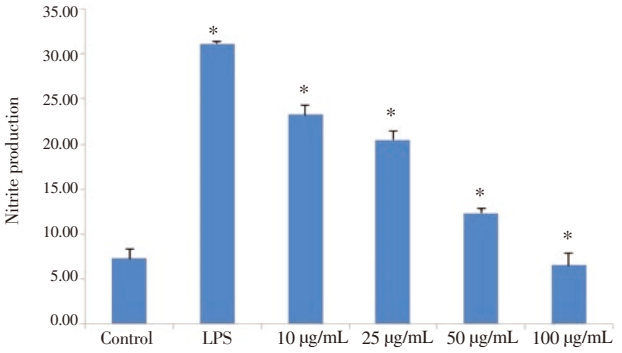


Figure 1. Inhibitory effects of ELA on LPS-induced NO production in RAW264.7 cells.

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-methoxycanthin-6-one (2), 9,10-dimethoxycanthin-6-one (3) and 5-methoxycanthin-6-one (4) (Figure 2).

9-Hydroxycanthin-6-one (1): yellow powder. ESI-MS (positive): *m/z* 237 $[M+H]^+$. ^{1}H 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). ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm): 115.8 (C-1), 145.8 (C-2), 139.9 (C-4), 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).

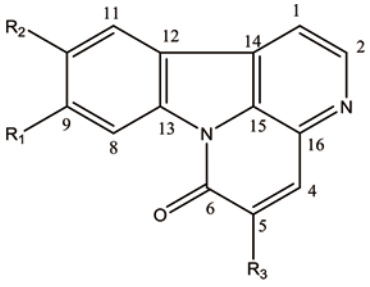
9-Methoxycanthin-6-one (2): orange powder. ESI-MS (positive): *m/z* 251 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 3.91 (3H, s, O-CH_3), 6.93 (1H, d, *J* = 10.0 Hz, H-5), 7.13 (1H, dd, *J* = 2.5, 10.0 Hz, H-10), 7.95 (1H, d, *J* = 2.0 Hz, H-8), 8.08 (1H, d, *J* = 10.0 Hz, H-11), 8.12 (1H, d, *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). ^{13}C 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), 129.3 (C-14), 131.5 (C-15), 135.0 (C-16), 55.7 (9-OCH_3).

9,10-Dimethoxycanthin-6-one (3): light yellow powder. ESI-MS (positive): *m/z* 281 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 3.94 (3H, s, 10-OCH_3), 3.96 (3H, s, 9-OCH_3), 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). ^{13}C 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-OCH_3), 56.0 (10-OCH_3).

5-Methoxycanthin-6-one (4): yellow powder. ESI-MS (positive): *m/z* 251 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$), δ (ppm): 4.06 (3H, s, O-CH_3), 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). ^{13}C NMR (125 MHz, $CDCl_3$), 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-OCH_3).

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 IC_{50} value of 33.11 μM but less potent compared to cardamonin (IC_{50} 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).



1: R_1=OH; R_2=R_3=H
2: R_1=OMe; R_2=R_3=H
3: R_1=R_2=OMe; R_3=H
4: R_1=R_2=H; R_3=OMe

Figure 2. Structures of compounds 1-4.

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).

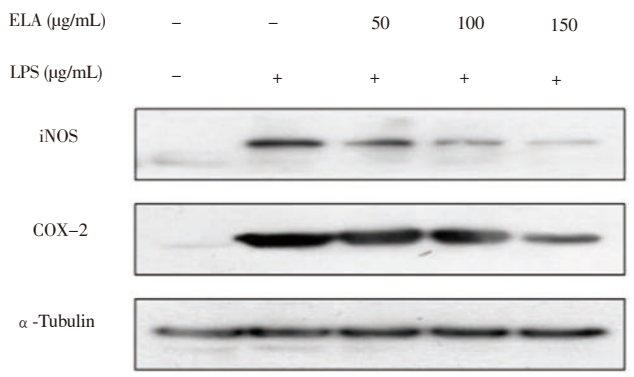


Figure 3. Inhibitory effect of ELA on LPS-induced iNOS and COX-2 expressions in RAW264.7 murine macrophage cells.

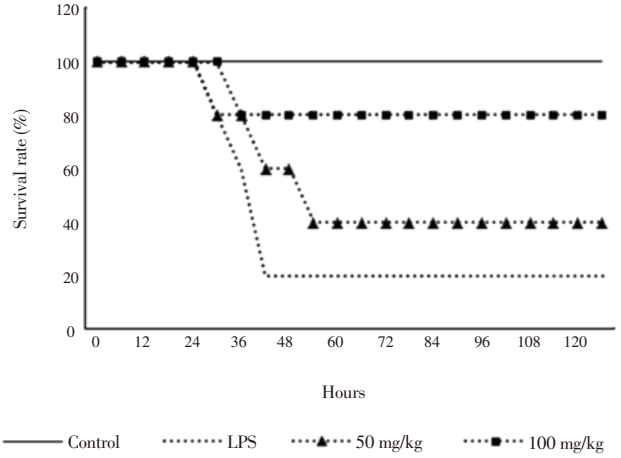


Figure 4. Effect of ELA on survival rate of mice treated with LPS. Each point represents the survival rate (%) of mice at the indicated time after LPS treatment. The level of significance was evaluated by log-rank test.

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 E_2. 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 E_2[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 E_2. The effect of NO on cytokines has been well reported. Accordingly, NO suppression can decrease LPS-induced TNF- α and IL-1 β, 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[30,31]. Consistently, 9,10-dimethoxycanthin-6-one (3) possessing two methoxy groups showed stronger anti-inflammatory effects than other compounds with or only one methoxy group. There have been several studies on the anti-inflammatory activities 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- κ B inhibitory activity in TNF- α -stimulated HEK-293/NF- κ B-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 IC_{50} 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 IC_{50} value of 33.11 μM. Canthin-6-one and its derivative 5-(1-hydroxyethyl)-canthin-6-one, isolated from the stem barks of *Ailanthus altissima*, showed potent NO production inhibitory activity with the IC_{50} 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- κ B 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. Noteworthily, 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.

Funding

This research is funded by Vietnam Academy of Science and Technology under grant number VAST04.03/17-18.

References

[1] Sharma B, Dabur R. Role of pro-inflammatory cytokines in regulation of skeletal muscle metabolism: A systematic review. *Curr Med Chem* 2018 (in press). Doi: 10.2174/0929867326666181129095309.

[2] Lind M, Hayes A, Caprnda M, Petrovic D, Rodrigo L, Kruzliak P, et al. Inducible nitric oxide synthase: Good or bad? *Biomed Pharmacother* 2017; **93**: 370-375.

[3] Rankin JA. Biological mediators of acute inflammation. *AACN Clin Issues* 2004; **15**(1): 3-17.

[4] Isomaki P, Punnonen J. Pro- and anti-inflammatory cytokines in rheumatoid arthritis. *Ann Med* 1997; **29**(6): 499-507.

[5] Mitchell JA, Larkin S, Williams TJ. Cyclooxygenase-2: Regulation and

relevance in inflammation. *Biochem Pharmacol* 1995; **50**(10): 1535-1542.

[6] Rehman SU, Choe K, Yoo HH. Review on a traditional herbal medicine, *Eurycoma longifolia* Jack (Tongkat Ali): Its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules* 2016; **21**(3): 331.

[7] Mutschlechner B, Schwaiger S, Tran TVA, Stuppner H. Development of a selective HPLC-DAD/ELSD method for the qualitative and quantitative assessment of commercially available *Eurycoma longifolia* products and plant extracts. *Fitoterapia* 2018; **124**: 188-192.

[8] Nguyen HD, Choo YY, Nguyen TD, Nguyen HN, Chau VM, Lee JH. 7-Methoxy-(9H-beta-carbolin-1-il)-(E)-1-propenoic acid, a beta-carboline alkaloid from *Eurycoma longifolia*, exhibits anti-inflammatory effects by activating the Nrf2/Heme Oxygenase-1 pathway. *J Cell Biochem* 2016; **117**(3): 659-670.

[9] Han YM, Woo SU, Choi MS, Park YN, Kim SH, Yim H, et al. Antiinflammatory and analgesic effects of *Eurycoma longifolia* extracts. *Arch Pharm Res* 2016; **39**(3): 421-428.

[10]Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**(1-2): 55-63.

[11]Hatziieremia S, Gray AI, Ferro VA, Paul A, Plevin R. The effects of cardamonin on lipopolysaccharide-induced inflammatory protein production and MAP kinase and NFkappaB signalling pathways in monocytes/macrophages. *Br J Pharmacol* 2006; **149**(2): 188-198.

[12]Moreira GL, Maria VI, António RJ. An overview on cardamonin. *J Med Food* 2014; **17**(6): 633-640.

[13]Kardono LB, Angerhofer CK, Tsauri S, Padmawinata K, Pezzuto JM, Kinghorn AD. Cytotoxic and antimalarial constituents of the roots of *Eurycoma longifolia*. *J Nat Prod* 1991; **54**(5): 1360-1367.

[14]Miyake K, Tezuka Y, Awale S, Li F, Kadota S. Canthin-6-one alkaloids and a tirucallanoid from *Eurycoma longifolia* and their cytotoxic activity against a human HT-1080 fibrosarcoma cell line. *Nat Prod Commun* 2010; **5**(1): 17-22.

[15]Readel KE, Seigler DS, Young DA. 5-Methoxycanthin-6-one from *Leitneria floridana* (Simaroubaceae). *Biochem Syst Ecol* 2003; **31**(2): 167-170.

[16]Lu Z, Huang W, Wang L, Xu N, Ding Q, Cao C. Exhaled nitric oxide in patients with chronic obstructive pulmonary disease: A systematic review and meta-analysis. *Int J Chron Obstruct Pulmon Dis* 2018; **13**: 2695-2705.

[17]Stuehr DJ, Haque MM. Nitric oxide synthase enzymology in the twenty years after the Nobel prize. *Br J Pharmacol* 2018 (in press).

[18]Garcia-Ortiz A, Serrador JM. Nitric oxide signaling in T cell-mediated immunity. *Trends Mol Med* 2018; **24**(4): 412-427.

[19]Yun HY, Dawson VL, Dawson TM. Neurobiology of nitric oxide. *Critical Rev Neurobiol* 1996; **10**(3-4): 291-316.

[20]Robinson MA, Baumgardner JE, Otto CM. Oxygen-dependent regulation of nitric oxide production by inducible nitric oxide synthase. *Free Rad Biol Med* 2011; **51**(11): 1952-1965.

[21]Pautz A, Art J, Hahn S, Nowag S, Voss C, Kleinert H. Regulation of the expression of inducible nitric oxide synthase. *Nitric Oxide* 2010; **23**(2): 75-93.

[22]Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* 1996; **271**(52): 33157-33160.

[23]Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 2011; **31**(5): 986-1000.

[24]Lee JH, Jung NH, Lee BH, Kim SH, Jun JH. Suppression of heme oxygenase-1 by prostaglandin E2-protein kinase A-A-kinase anchoring protein signaling is central for augmented Cyclooxygenase-2 expression in lipopolysaccharide-atimulated RAW 264.7 macrophages. *Allergy Asthma Immunol Res* 2013; **5**(5): 329-336.

[25]Yan L, Wang S, Rafferty SP, Wesley RA, Danner RL. Endogenously produced nitric oxide increases tumor necrosis factor- α production in transfected human U937 cells. *Blood* 1997; **90**(3): 1160-1167.

[26]Wu CH, Chen TL, Chen TG, Ho WP, Chiu WT, Chen RM. Nitric oxide modulates pro- and anti-inflammatory cytokines in lipopolysaccharide-activated macrophages. *J Trauma* 2003; **55**(3): 540-545.

[27]Jung WK, Choi I, Lee DY, Yea SS, Choi YH, Kim MM, et al. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages *via* the p38/ERK and NF-kappaB pathways. *Int J Biochem Cell Bio* 2008; **40**(11): 2572-2582.

[28]Guo LY, Hung TM, Bae KH, Shin EM, Zhou HY, Hong YN, et al. Anti-inflammatory effects of schisandrin isolated from the fruit of *Schisandra chinensis* Baill. *Eur J Pharmacol* 2008; **591**(1-3):293-299.

[29]Saba E, Shafeeq T, Irfan M, Lee YY, Kwon HW, Seo MG, et al. Anti-inflammatory activity of crude venom isolated from parasitoid wasp, *Bracon hebetor* Say. *Mediators Inflamm* 2017; **2017**: 6978194.

[30]Okunrobo LO, Usifoh CO, Uwaya JO. Anti-inflammatory and gastroprotective properties of some chalcones. *Acta Pol Pharm* 2006; **63**(3): 195-199.

[31]Yang H, Du Z, Wang W, Song M, Sanidad K, Sukamtoh E, et al. Structure-activity relationship of curcumin: Role of the methoxy group in anti-inflammatory and anticolitis effects of curcumin. *J Agric Food Chem* 2017; **65**(22): 4509-4515.

[32]Ngoc PB, Pham TB, Nguyen HD, Tran TT, Chu HH, Chau VM, et al. A new anti-inflammatory beta-carboline alkaloid from the hairy-root cultures of *Eurycoma longifolia*. *Nat Prod Res* 2016; **30**(12): 1360-1365.

[33]Tran TV, Malainer C, Schwaiger S, Atanasov AG, Heiss EH, Dirsch VM, et al. NF-kappaB inhibitors from *Eurycoma longifolia*. *J Nat Prod* 2014; **77**(3): 483-488.

[34]Kim HM, Lee JS, Sezirahiga J, Kwon J, Jeong M, Lee D, et al. A new canthinone-type alkaloid isolated from *Ailanthus altissima* Swingle. *Molecules* 2016; **21**(5): E642. Doi: 10.3390/molecules21050642..

[35]Cho SK, Jeong M, Jang DS, Choi JH. Anti-inflammatory effects of canthin-6-one alkaloids from *Ailanthus altissima*. *Planta Med* 2018; **84**(8): 527-535.

[36]Rehman SU, Choe K, Yoo HH. Review on a traditional herbal medicine, *Eurycoma longifolia* Jack (Tongkat Ali): Its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules* 2016; **21**(3): 331.