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Anti-inflammatory Triterpenes and Glyceryl Glycosides from *Kandelia candel* (L.) Druce

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Abstract – Phytochemical investigation of *Kandelia candel* resulted in the isolation of six triterpenes (**1** - **5**) and two glyceryl glycosides (**6** and **7**) and their structures were determined by comparing the spectroscopic data with those of reported values. In present study, we described the inhibitory effects of fractions and isolated compounds from *K. candel* on pro-inflammatory cytokines (IL-12 p40, IL-6, and TNF-α) production in lipopolysaccharide (LPS) stimulated bone marrow-derived dendritic cells (BMDCs). Results indicated that compounds **3**, **6**, and **7** showed potent inhibition on IL-6 production (IC_{50} values at less than 0.5 μM, respectively). Meanwhile, compounds **6** and **7** exhibited strong inhibitory effects on the production of TNF-α (IC_{50} values of 1.7 ± 0.1 and 5.5 ± 0.2 μM). Compounds **1** and **3** were also showed the inhibitory effects on IL-12 p40 production (IC_{50} values of 8.9 ± 0.4 and 3.3 ± 0.1 μM, respectively).
Keywords – *Kandelia candel*, Rhizophoraceae, IL-12 p40, IL-6, TNF-α, Anti-inflammation

Introduction

Inflammation is defined as part of a complex biological response of vascular tissue toward exogenous harmful stimuli.[1] It is mediated by a variety of soluble factors especially a group of secreted polypeptides known as cytokines which played a key role in the modulation of immune responses.[2] Cytokines such as IL-6, IL-12 p40, and TNF-α, are produced by many different cell types and often show overlapping activities regulating proliferation or differentiation, depending on the type and developmental state of the target cells involved. Among cytokines, the bioactive form of IL-12 is a disulfide-linked heterodimer of p35 and p40 subunits in which the soluble p40 subunit of IL-12 strongly antagonizes IL-12 bioactivity.[3] IL-6 is mediated its effects on cells through a complex mechanism and known to be a multifunctional cytokine that participates in several biological events, including immune responses, hematopoiesis and acute-phase reactions.[4] TNF-α is well- characterized, affected by variety of cells and involved in induce production of cytokines, endothelial gene regulation, chemotaxis, leukocyte adherence, and the activation of fibroblast.[5] The dendritic cells (DC) maturation process is accompanied by the production of various cytokines that orchestrate the inflammatory and immune response against cancer cells or pathogen microorganisms. In particularly, DCs potential to induce T cell to secrete IL-12,[6,7] and release IL-6, and TNF-α.[8,9,10] In previous studies, BMDCs produce pro-inflammatory cytokines when stimulated with LPS.[11] The changes of cytokine production were confirmed with the transcription levels of these cytokines. DCs were capable of inflammatory cytokines. In the stimulation of LPS, DCs started to generate this cytokines (specially IL-12).

Mangroves are a diverse group of trees that grow in

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intertidal tropical and subtropical forests. Mangrove extracts have been used for diverse medicinal purposes and are known to exhibit antibacterial, antiherpetic, and antihelminthic activities.[12] *Kandelia candel* (L.) Druce (Rhizophoraceae) is widely distributed in the Asian coastline. This plant was used as a folk medicine against rheumatoid arthritis. Previous studies showed the excellent antioxidant activities of tannins from *K. candel*.[13] This study describes the isolation, structural determination of seven compounds (**1** - **7**), as well as the evaluation of inhibitory effects of fractions and isolated compounds on pro-inflammatory cytokines IL-12 p40, IL-6, and TNF-α production in LPS-stimulated BMDCs.

Experimental

General experimental procedures – Optical rotations were determined on a JASCO P-2000 polarimeter (Hachioji, Tokyo, Japan). IR spectra were obtained on a Bruker TENSOR 37 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany). The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL 400 NMR spectrometer (JOEL, MA, USA) and TMS was used as an internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70 - 230 mesh and 230 - 400 mesh, Merck, Darmstadt, Germany) and YMC RP-18 resins (30 - 50 μm, Fuji Silysia, Kasugai, Japan). Thin layer chromatography (TLC) used pre-coated silica gel 60 F_{254} (1.05554.0001, Merck, Darmstadt, Germany) and RP-18 F_{254S} plates (1.15685.0001, Merck, Darmstadt, Germany) and compounds were visualized by spraying with aqueous 10% H_2SO_4 and heating for 3 - 5 min.

Plant material – The plant *K. candel* was collected at Xuan Thuy National Park, Nam Dinh province, Vietnam in July 2013 and identied by Dr. Nguyen The Cuong (Institute of Ecology and Biological Resources, VAST). A voucher specimen (XT_CB01C) was deposited at the Herbarium of the Institute of Marine Biochemistry, VAST, Vietnam.

Extraction and isolation – The fresh plant *K.candel* branches and leaves was dried under shiny for dried sample. The dried sample (3 kg) were well grinded and extracted three times with MeOH at room temperature for 6 h and filtered each to give a MeOH residue (500 g) after removal of the solvent under reduced pressure. This residue was supended in water (2 L) and partitioned in turn with CH_2Cl_2 (3 × 2 L) and EtOAc (3 × 2 L) to furnish corresponding fractions: CH_2Cl_2 (B, 112 g) and EtOAc (C, 180 g) and water layer (D, 2 L). The water layer was added to Diaion HP-20 CC eluted with methanol-water (0% to 100% of methanol) to get five fractions (D1 - D5). Compound **1** (6.0 mg) was obtained in fraction D2 after subjecting it to YMC RP-18 CC with acetone-water (1-2, v-v) followed by silica gel CC with *n*-hexane-EtOAc (5-1, v-v). The EtOAc fraction (C, 180 g) was crudely separated by silica gel CC using gradient concentration of methanol in dichloromethane (from 50% to 100%). Fractions were pooled after TLC analysis to give four fractions (C1 to C4). Fraction C4 (0.2 g) was separated into seven subfractions (C4.1 - C4.7) by silica gel CC elutied with dichloromethane-methanol-water (15-1-0.01, v-v-v) and further purified by YMC RP-18 CC with solvent system acetone-methanol-water (1-1-1, v-v-v) to afford compounds **6** (7.0 mg) and **7** (6.5 mg). The dichloromethane fraction (B, 112 g) was separated by silica gel CC with gradient solvent system of *n*-hexane-acetone (from 100% *n*-hexane to 100% acetone) to give five fractions (B1-B5). Fraction B2 was divided into six subfractions (B2.1-B2.6) by silica gel CC using solvent system *n*-hexane-dichloromethane-ethylacetate (20-0.8-1, v-v-v). Compounds **2** (8.0 mg) and **4** (6.0 mg) were obtained from subfraction B2.4 after purified by silica gel CC with *n*-hexane-dichloromethane-methanol (2-2-0.05, v-v-v). Subfraction B2.2 was separeated by silica gel CC with *n*-hexane-dichloromethane-acetone (1-8-0.1, v-v-v) and followed by silica gel CC with dichloromethane-ethylacetae (30-1, v-v) afford **5** (7.5 mg). Finally, compound **3** (5.5 mg) was purified from fraction B4 by silica gel CC with dichloromethane-acetone (50-1, v-v) and followed by silica gel CC with *n*-hexane-acetone (3-1, v-v), YMC RP-18 CC with acetone-water (5-1, v-v).

Oleanolic acid (**1**) – White needles; $[\alpha]_D^{17}$: +34.15 (*c* 0.13, MeOH); IR (KBr) ν_{max} 3435, 2934, 1689, 1460, 1365, and 1036 cm^{-1}; spectral data were consitent with the literature.[15]

Ursolic aldehyde (2) – Colourless needles; $[\alpha]_D^{17}$: +41.00 (*c* 0.10, CH_3Cl); IR (KBr) ν_{max} 3435, 2946, 1725,1456, 1029, and 752 cm^{-1}; spectral data were consitent with the literature.[16]

***cis*-3-*O*-*p*-Hydroxycinnamoyl ursolic acid (3)** – Colourless needles; $[\alpha]_D^{17}$: +40.16 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3367, 2944, 1694, 1605, 1513, 1454, 1388, 1167, 1020, and 850 cm^{-1}; spectral data were consitent with the literature.[17]

***β*-Sitosterol (4)** – White amorphous powder; $[\alpha]_D^{17}$: −139.60 (*c* 0.10, CH_3Cl); IR (KBr) ν_{max} 3383, 2935, 1463, 1381, and 1061 cm^{-1}; spectral data were consitent with the literature.[18]

Betulin (5) – White powder; $[\alpha]_D^{17}$: +75.20 (*c* 0.12, CH_3Cl); IR (KBr) ν_{max} 2933, 1686, 1454, 1214, 1028, and 880 cm^{-1}; spectral data were consitent with the literature.[19]

(2*S*)-3-*O*-Octadeca-9*Z*,12*Z*,15*Z*-trienoylglyceryl-6'-*O*-(*α*-D-galactopyranosyl)-*β*-D-galactopyranoside (6) – White amorphous powder; $[\alpha]_D^{17}$: +14.00 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3338, 2931, 1730,1604, 1452, 1381, 1257, 1168, 1071, and 949 cm^{-1}; spectral data were consitent with the literature.[20]

(2*S*)-3-*O*-Octadeca-9*Z*,12*Z*,15*Z*-trienoylglyceryl-*O*-*β*-D-galactopyranoside (7) – White amorphous powder; $[\alpha]_D^{17}$: +3.73 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3332, 2927, 1734, and 1069 cm^{-1}; spectral data were consitent with the literature.[20]

Anti-inflammatory assay

Cell culture and viability – Bone marrow-derived dendritic cells were grown from wild-type C57BL/6 mice (Orient Bio Inc., Seoul, Korea).[21] The mouse tibia and femur was obtained by flushing with Dulbecco's modified Eagle's medium to yield bone marrow cells. The cells were cultured in Rosell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 50 μM *β*-mercaptoethanol, and 2 mM glutamine supplemented with 3% J558L hybridoma cell culture supernatant containing granulocyte-macrophage colony-stimulating factor (GM-CSF). The culture medium was replaced with fresh medium every other day. At day six of culture, non-adherent cells and loosely adherent DC aggregates were harvested, washed, and resuspended in RPMI 1640 supplemented with 5% FBS.

Cell viability was evaluated by the MTT methods. Briefly, MTT was added to the cell culture medium for 4 h. The supernatant was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 540 nm. The percentage of dead cells was determined relative to the control group.

Inhibitory effect of sample stimulated on anti-inflammatory cytokine productions – We used LPS-stimulated BMDCs as a model for testing the inhibitory effects of extracts and isolated compounds on the secretion of pro-inflammatory cytokines IL-12 p40, IL-6, and TNF-α. The BMDCs were incubated in 48-well plates containing 1×10^5 cells per well, and then treated with the extracts and isolated compounds at different concentrations for 1 h before stimulation with 10 ng/mL LPS from *Salmonella minnesota* (Alexis, Famingdale, NY, USA). Supernatants were harvested 18 h after stimulation. Concentrations of murine TNF-α, IL-6, and IL-12 p40 in the culture supernatant were determined by ELISA (BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. SB203580, an inhibitor of cytokine suppressive binding protein/p38 kinase, was used as a positive control.

Statistical analysis – All data were expressed as mean ± S.D. of at least three independent experiments performed in triplicates. Statistical significance is indicated by one-way ANOVA followed by Dunnetts multiple comparison test using GraphPad Prism 6 program (GraphPad Software Inc., San Diego, CA, USA), $P < 0.05$.

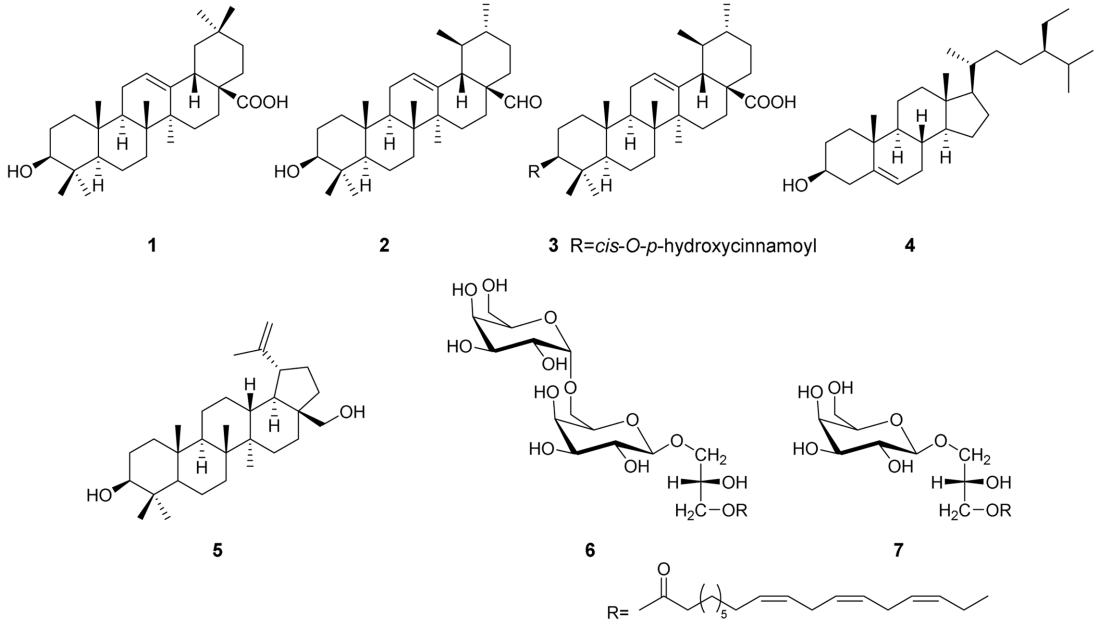

Fig. 1. The structures of isolated compounds (**1** - **7**) from *K. candel.*

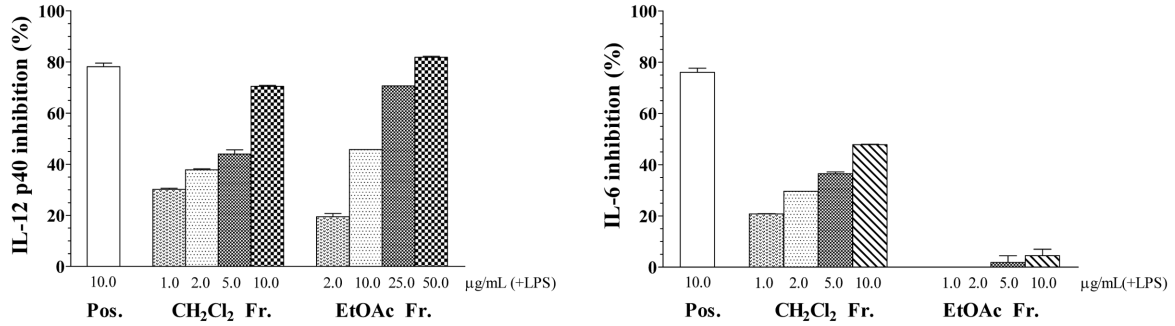

Fig. 2. Effect of CH_2Cl_2 and EtOAc fractions of *K. candel* on IL-12 p40 and IL-6 production in LPS-stimulated BMDCs. Data were presented as inhibition rate (%) compared to the value of vehicle-treated DCs. SB203580 was used as positive control (**Pos.**). $P < 0.05$.

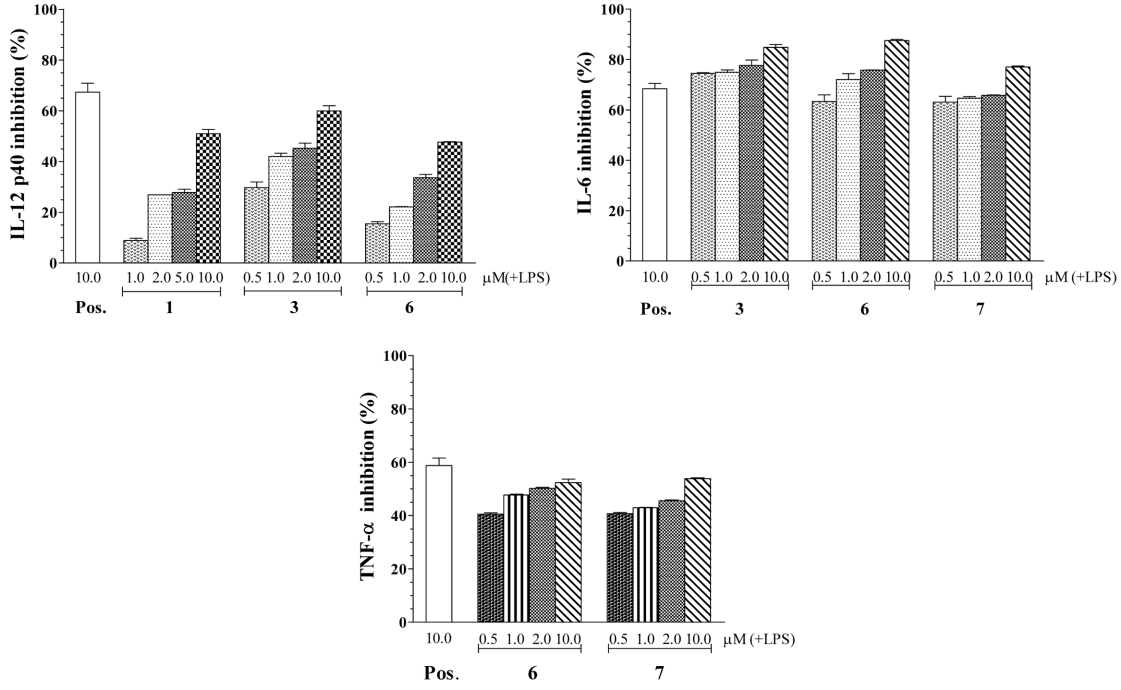

Fig. 3. Effects of selected compounds **1**, **3**, **6**, and **7** on IL-12 p40, IL-6, TNF-α productions in LPS-stimulated BMDCs. Data were presented as inhibition rate (%) compared to the value of vehicle-treated DCs. SB203580 was used as positive control (**Pos.**). $P < 0.05$.

Results and Discussion

As a part of our ongoing investigations of the anti-inflammatory effect of Vietnamese medicinal, we recently reported on the isolation, structural elucidation, and inhibitory effect of sixteen compounds from *K. candel* on pro-inflammatory cytokines production in LPS-stimulated bone marrow-derived dendritic cells (BMDCs).[14] Seven compounds (**1** - **7**) were isolated from the methanol extract of *K. candel*. Their structures were elucidated by physicochemical data in compared with those in the literatures to be oleanolic acid (**1**),[15] ursolic aldehyde (**2**),[16] *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (**3**),[17] *β*-sitosterol (**4**),[18] betulin (**5**),[19] (2*S*)-3-*O*-octadeca-9*Z*,12*Z*,15*Z*-trienoyl-glyceryl-6'-*O*-(*α*-D-galactopyranosyl)-*β*-D-galactopyranoside (**6**),[20] and (2*S*)-3-*O*-octadeca-9*Z*,12*Z*,15*Z*-trienoylglyceryl-*O*-*β*-D-galactopyranoside (**7**).[20]

The CH_2Cl_2, EtOAc fractions, and compounds (**1** - **7**) were evaluated for their inhibitory effects on pro-inflammatory cytokines (IL-12 p40, IL-6, TNF-α) production in LPS-stimulated BMDCs. These fractions from *K. candel* showed the significantly inhibited of IL-12 p40 and IL-6 production. In particularly, the dichloromethane and EtOAc fractions showed inhibition of IL-12 p40 and IL-6 production with IC_{50} values at 9.6 ± 0.4, 10.1 ± 0.4 and 13.7 ± 0.4, 19.0 ± 0.4 μg/mL, respectively (Fig. 2).

The anti-inflammatory effects of seven isolated compounds from *K. candel* were tested for inhibitory effects on the production of pro-inflammatory cytokines. SB203580 was used as a positive control. Among tested compounds, compounds **1** and **3** showed potent inhibition on IL-12 p40 production with IC_{50} values of 8.9 ± 0.4 and 3.3 ± 0.1 μM, respectively. This result is more confident for the anti-inflammatory effect of triterpenes which were described in previous study.[22] Compound **6** showed significant inhibittion of IL-12 p40 production with IC_{50} value of 11.4 ± 0.3 μM (compared to positive control, SB203580 with IC_{50} value of 5.0 ± 0.1 μM, Fig. 3). The strong inhibitory effects of three compounds **3**, **6** and **7** on IL-6 productions were observed with IC_{50} values at less than 0.5 μM, respectively. These IC_{50} values are much stronger than positive control (IC_{50}: 3.5 ± 0.1 μM, Fig. 3). Potent inhibitory effects on the production of TNF-α were also observed for compounds **6** and **7** with IC_{50} values of 1.7 ± 0.1 and 5.5 ± 0.2 μM, respectively (compared to positive control, SB203580 with IC_{50} value of 7.2 ± 0.2 μM, Fig. 3). Other tested compounds showed weak or inactive inhibitory effects ($IC_{50} > 100$ μM). The effect of galactolipids such as anti-aging, anti-viral, anti-tumor, anti-immunosuppressive, and anti-inflammatory activities were investigated in previous studies.[23] Beside potent inhibitory of inflammatory cytokines in this study, *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (**3**) has evaluated as antitumor agent.[17] This compound inhibited tumor growth in MCF-7 breast tumor cells with IC_{50} concentration at less than 20 μM. Futher studies are required to evaluate the anti-inflammatory effects of the active compounds identified in the present study.

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