Chemical constituents from Vietnamese mangrove *Calophyllum inophyllum* and their anti-inflammatory effects

Nguyen Van Thanh b,1, Hyun-Jae Jang c,1, Le Ba Vinh a,b,1, Kieu Thi Phuong Linh b, Phan Thi Thanh Huong b, Nguyen Xuan Cuong b, Nguyen Hoai Nam b, Chau Van Minh b, Young Ho Kim a,⁎, Seo Young Yang a,⁎

a College of Pharmacy, Chungnam National University, Daejeon 34134, Republic of Korea
b Institute of Marine Biochemistry (IMBC), Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam
c Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, 30 Cheongiu, Chungbuk 28116, Republic of Korea

ARTICLE INFO

Keywords: Anti-inflammatory
*Calophyllum inophyllum*
NF-κB
Triterpenoid

ABSTRACT

In a search for anti-inflammatory activity in resources from Vietnamese mangroves, we found that a methanolic extract from the leaves of *Calophyllum inophyllum* (CIL) showed significant anti-inflammatory effects in vitro. Using various chromatographic techniques, we subsequently isolated 12 compounds (1–12) from a methanolic extract of CIL, including two novel compounds (1–2). The inhibitory effects of these compounds on lipopolysaccharide-induced nitric oxide (NO) production in RAW264.7 cells were also evaluated. Compound 1 significantly suppressed NO production (IC50 = 2.44 ± 0.88 μM), the secretion of pro-inflammatory cytokines (including interleukin-1 beta and tumor necrosis factor alpha), and the expression of inducible nitric oxide synthase through downregulation of nuclear factor-kappa-B signaling cascades. These results suggest that *C. inophyllum* leaves might be a useful resource for the development of drugs for the treatment of inflammation.

1. Introduction

Inflammation is one of a series of complex processes that induce pathogenesis of inflammatory diseases and disorders, including autoimmune diseases, cancer, metabolic syndromes, and cardiovascular diseases [1]. During the process of inflammation, diverse pro-inflammatory cytokines and mediators, such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6, prostaglandins (PGEs), and nitric oxide (NO), are released, mainly by activated macrophages [2]. Activated macrophages emerge in response to exogenous stress or endogenous signals, such as pathogens and/or damage-associated molecular patterns [3,4]. These are key mediators that induce inflammatory disorders and may have the potential as therapeutic targets [5].

Nuclear factor-kappa-B (NF-κB) signaling pathways, which activate transcription factors in the nucleus (e.g., RelA/p50 complex), are implicated in controlling pro-inflammatory genes [6,7]. Because abnormal overproduction and accumulation of cytokines/mediators contribute to inflammatory pathogenesis [8,9], it is important to ensure normal regulation of NF-κB cascades for improving the prognosis of inflammatory diseases.

The mangrove *Calophyllum inophyllum* Linn. (Guttiferae) is an evergreen shrub found in tropical regions, such as Southeast Asia, India, and East Africa [10]. This species is used in traditional medicine as a sedative and an herbal remedy for the treatment of several ailments, such as wound care, sore throat, eye diseases, burns, pain, and inflammation [10,11]. Coumarins, xanthones, flavonoids, and triterpenoids are major chemical constituents of *C. inophyllum* extracts [12–14], which have previously been reported to possess diverse pharmacological effects, including anti-microbial [15], anti-inflammatory [15], anti-osteoporosis [16], anti-cancer [17], wound healing [18], anti-arthritic [10], anti HIV [19], and antioxidant activities [20].

As part of our ongoing investigations of the metabolites and biological activities of Vietnamese mangroves [21], herein, we isolated and elucidated the structures of two new triterpenoids (1 and 2) obtained from *C. inophyllum* (Fig. 1), together with ten known compounds, and evaluated their anti-inflammatory activity.
2. Experimental

2.1. General experimental procedures

Optical rotation were recorded by a JASCO P-2000 digital polarimeter (Tokyo, Japan). High resolution quadrupole time-of-flight mass spectra were recorded on an Agilent 6530 Accurate-Mass spectrometer (CA, USA). The 1D and 2D NMR spectra were recorded on a Bruker AVANCE III HD 500 FT-NMR spectrometers (MA, USA) with TMS used as an internal standard. Thin layer chromatography (TLC), Silica gel, YMC RP-18, and Sephadex LH-20, were provided by Merck (Darmstadt, Germany). Compounds were detected with UV radiation (254 and 365 nm) and spraying the plates with 10% H2SO4 followed by heating. Chemical reagents together standard compounds were obtained from Sigma-Aldrich. HPLC purification was carried out on an Agilent 1200 series preparative system equipped with an Agilent 1200 diode array detector.

2.2. Plant material

The leaves of *Calophyllum inophyllum* were acquired at Ca Mau, Vietnam, in July 2017, and taxonomically identified by Dr. Nguyen The Cuong. The vouchers specimen in this study (DTCB.CM 05) is deposited at the herbarium of IMBC, VAST, Vietnam and College of Pharmacy, Chungnam National University.

2.3. Extraction and isolation

The dried whole plant of *Calophyllum inophyllum* (2.5 kg) were extracted with methanol (10L × 3 times) under reflux condition. Evaporation of the solvent under reduced pressure gave MeOH extract (250 g). The MeOH extract was suspended in H2O and successively separated with n-hexane, CH2Cl2, and EtOAc to yield n-hexane (30.9 g), CH2Cl2 (76.0 g), EtOAc (11.0 g), and water layer, respectively.

The water layer was fractionated on a Diaion HP-20 CC eluting with gradients solvent systems of MeOH/H2O (0–100% MeOH, step-wise) to give three fraction W1–W3. The W1 fraction (2.7 g) was subjected on silica gel CC eluted with n-hexane/acetone (100:1, 100: 2, 100: 4) to give compounds 3 (6.0 mg) and 4 (4.0 mg). Similarly, fraction W2 (2.9 g) was isolated using Sephadex LH-20 (MeOH/H2O 1:2) to give compound 5 (5.0 mg), 6 (4.0 mg), 8 (7.0 mg), and 11 (10.9 mg).

The CH2Cl2 fraction (76 g) was subjected to silica gel CC and eluted with n-hexane/acetone (100:1, 50:1, 25:1, 10:1, 5:1 and 2:1) to afford seven fractions (D1 – D7). Compounds 7 and 12 was obtained from fraction D2 after subjecting it on silica gel CC and eluted with n-hexane/EtOAc (15:1, v/v) to give. Fraction D6 was isolated by YMC CC and eluted with MeOH/H2O (1:1, v/v) to afford seven subfractions (D6A–D6G), respectively. Further purification of subfraction D6E (1.92 g) by silica gel CC eluted with CH2Cl2/EtOAc (15:1), followed by Sephadex LH-20 CC with MeOH-H2O (2:1), provided compounds 9 (2.5 mg) and 10 (1.4 mg). Finally, compounds 1 (1.4 mg) and 2 (1.8 mg) were purified by semi-preparative RP HPLC (ACN/ H2O 75/25) from the subfraction D6G (822.9 mg).

2.3.1. 27-{(E)-p-coumaroyloxy}canophyllic acid (1)

White amorphous powder; C39H56O6; [α]D21: +71.5 (c 0.1, MeOH); UV (MeOH) νmax (log ε) nm: 200 (2.13); IR (KBr): νmax 3296, 2928, 2856, 1680, 1436, 1386, 1076 cm−1; 1H and 13C NMR data, see Table 1; HR-QTOF-MS: m/z 619.4010 [M−H]− (calcd for [M−H]− 619.4004).

2.3.2. 27-{(Z)-p-coumaroyloxy}canophyllic acid (2)

White amorphous powder; C39H56O6; [α]D21: +30.0 (c 0.1, MeOH); UV (MeOH) νmax (log ε) nm: 200 (2.13); IR (KBr): νmax 3295, 2926, 2856, 1680, 1436, 1386, 1076 cm−1; 1H and 13C NMR data, see Table 1; HR-QTOF-MS: m/z 619.4005 [M−H]− (calcd for [M−H]− 619.4004).

2.4. Nitric oxide (NO) assay for assessment of anti-inflammatory activity

The murine macrophage cell line, RAW 264.7 (TIB-71, ATCC, Manassas, VA, USA), was obtained from American Type Culture Collection (ATCC). The NO assay was performed by previously described method [22]. Briefly, RAW 264.7 cells (1 × 105 cells/well) were cultured into 96-well plates for 24 h. Cells were pre-treated with samples for 1 h before treatment with LPS (0.1 µg/mL) for 24 h. The cell culture supernatants (100 µL) was mixed with an equal volume of Griess reagent. Cell cytotoxicity was determined by the MTT assay after 24 h incubation with samples [22].

2.5. ELISA assay

For the quantitative determination of pro-inflammatory cytokines or mediators (IL-1β, TNF-α, IL-6, and PGE2), RAW 264.7 cells was plated into 96-well plates (2 × 103 cells/well) and were cultured for 24 h. Cells were stimulated by LPS (0.1 μg/mL) for 24 h except for control group, after pre-treating with or without samples at 5 and 10 μM concentration.
κ analysis was performed to detect iNOS, COX-2, p65, p-IκB, Lamin B, and β-actin protein expression in the RAW264.7 cells, as described in previous report [23].

Results are expressed as the means ± standard error of the mean (SEM), and the statistical analyses were performed using Student’s t-test in Prism 5 software (GraphPad software, San Diego, CA, USA). A probability value of 0.05 (P < 0.05) was considered a significant difference between the only LPS-treated group and experimental group.

3. Results and discussion

3.1. Isolation of compounds 1–12

The methanolic extract of dry C. inophyllum leaves was fractioned with n-hexane, dichloromethane, ethyl acetate, and water. Using several chromatographic techniques (i.e., silica gel, RP-18, Sephadex LH-20, and semipreparative RP-HPLC), compounds 1–12 were isolated. On the basis of comprehensive analysis of spectroscopic data and comparison with previous data, the chemical structures of the known compounds (3–12) were elucidated as methyl shikimate (3), (3S,5S,6R,7E,9R)-3,5,6-trihydroxy-β-1-phenyl-3-O-β-D-glucopyranoside (4), benzyl-O-α-D-glucopyranosyl (1→6)-β-D-glucopyranoside (5), hexyl rutinoside (6), canepholl (7), kaempferol-3-O-α-D-rhamnoside (8), 27-[(Z)-p-coumaroyloxy]friedelin-28-carboxylic acid (9), (22E,24R)-24-methyl-Sa-cholesta-7,22-diene-3β,5β-triol (10), ammonioflavone (11), and 3-oxo-friedel-28-oxic acid (12).

3.2. Elucidation of the coumaroyl ester triterpenoid structure

Compound 2 was isolated as a white powder. Its molecular formula was determined to be C_{39}H_{56}O_{6} (with 12 degrees of unsaturation) based on 1H and 13C NMR data (Table 1), and the HR-QTOF-MS (observed [M−H]− ion at m/z 619.4005, calculated for [M−H]− 619.4004). The 13C NMR spectrum of 2 displayed 39 carbon signals, which, with the assistance of HSQC, were assigned as four sp² and six sp³ quaternary carbons, six sp³ methines, twelve sp³ methylenes, and six methyls. Among them, one carboxylic acid carbon (δC 182.8), and two oxygen-bearing carbons (δC 65.0 and 72.7) were identified. Specific signals, including one ester carbonyl (δC 166.8), four aromatic protons of a para-substituted benzene ring (δH 6.82 and 7.20 (each 2H, d, J = 8.5 Hz)), and two mutually cis-coupled olefinic protons (δH 5.85 and 6.86 (each 1H, d, J = 12.5 Hz)), were characteristic of a (Z)-p-coumaroyl moiety. The 1H NMR spectrum of 2 further showed signals for an oxymethine (δH 3.72 (1H, d, J = 2.0 Hz)), an oxymethylene (δH 4.42/4.52 (each 1H, d, J = 12.0 Hz)), five singlet methyls (δH 0.96, 0.87, 0.89, 0.96, and 1.02), and a doublet methyl (δH 0.92 (J = 7.0 Hz)). The above-mentioned NMR data, in combination with the molecular formula, suggested that 2 was a pentacyclic triterpene acid containing a (Z)-p-coumaroyl group.

Comparison of the 1D NMR data of 2 (Table 1) with those of 27-hydroxyacetate canepholl acid previously isolated from the same plant revealed that 2 had the same 3-hydroxy-27-oxygenated-friedelan-28-oxic acid triterpenoid nucleus [24]. The HMBC spectrum indicated that the (Z)-p-coumaroyl moiety was linked to the nucleus through C-27 by correlation from H-27α (δH 4.42) to C-9′ (δC 166.7). The planar structure of 2 was also confirmed with the aid of COSY and HMBC cross-peaks (Fig. 2).

The relative stereochemistry of 2 was deduced by analysis of its J_{1,2} coupling constants and NOESY experiments. The small vicinal coupling constants (near 0 Hz) of H-2β (δH 1.86, br d, J_{gem} = 10.5 Hz) and H-12α (δH 1.95, br d, J_{gem} = 14.5 Hz) with their neighboring protons were revealed to be in an equatorial configuration. The NOESY correlations of H-12βaxial/H-18 and H-25, H-18/H-26 and H-30, H-25/H-24, H-2αaxial/H-4 and H-10, H-10/H-8, H-8/H-27β, and H27α/H-19α, confirmed the friedelane skeleton of 2 (Fig. 3). H-3 was assigned to be in an equatorial orientation based on its small coupling constant (δH 3.72, br d, J = 2.0 Hz) as well as NOE cross-peaks from H-3 to H-2α, H-2β, H-4,
and H₃-23. Consequently, compound 2 was elucidated as 27-{(Z)-p-coumaroyloxy}canophyllic acid.

Compound 1 was obtained as a white powder. The molecular formula was established as C₃₉H₅₆O₆ by HR-QTOF-MS data (m/z 619.4010 [M–H]–) which indicated that 1 was an isomer of 2. The ¹H and ¹³C NMR spectra of 1 were almost identical to those of 2, with only slight changes in the chemical shifts and J values of H-7′ (δH 7.61, 1H, d, J = 16.0 Hz from δH 6.86, 1H, d, J = 12.5 Hz) and H-8′ (δH 6.31, 1H, d, J = 16.0 Hz from δH 5.85, 1H, d, J = 12.5 Hz), which revealed that 1 possessed an (E)-p-coumaroyl unit. This assumption was also verified by COSY and HMBC correlations (Fig. 2). By analysis of proton coupling constants and the NOESY spectrum, the relative configuration of 1 was found to be the same as that of 2. Thus, 1 was determined as 27-{(E)-p-coumaroyloxy}canophyllic acid.

3.3. Effects of compounds 1–12 on NO production in activated macrophages

NO is one of the key mediators for inflammatory responses and pathogenesis. All extracted C. inophyllum isolates were tested for inhibitory effects on LPS-stimulated NO production in RAW264.7 cells. Studies have demonstrated that extracts of CIL have an anti-inflammatory effect through suppression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expression [15]. Thus, we hypothesized that compounds 1–12 isolated from CIL may also have anti-inflammatory activity. Without cytotoxic effects, compounds 1, 2, 9, and 12 exhibited significant inhibition of NO production in macrophages activated by LPS, with IC₅₀ values of 2.44 ± 0.88, 7.00 ± 1.13, 15.46 ± 0.18, and 22.47 ± 1.39 µM, respectively (Table 2). Among the coumaroyl ester triterpenoids showing potent
3.4. Effects of compounds 1 and 2 on pro-inflammatory cytokine expression

IL-1β, TNF-α, and IL-6 are well known as representative pro-inflammatory cytokines that are produced mainly by activated macrophages, and are implicated in inflammation responses [25]. The effects of compounds 1 and 2 on LPS-stimulated inflammatory cytokine production were assessed using ELISA. Treatment with compounds 1 and 2 resulted in diminished concentrations of IL-1β and TNF-α, but IL-6 and PGE2 were unaffected at the tested concentrations, 5 and 10μM (Fig. 4). Dysregulation of IL-1β and TNF-α is associated with systemic inflammatory disorder, sepsis, and autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease [5]. Therefore, compounds 1 and 2 could represent effective therapies to ameliorate the impacts of abnormal inflammation responses.

### Table 2
Inhibitory effects of compounds 1–12 on LPS-induced NO production.a

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.44 ± 0.88</td>
</tr>
<tr>
<td>2</td>
<td>7.00 ± 1.13</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>5</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>6</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>7</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>8</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>9</td>
<td>15.46 ± 0.18</td>
</tr>
<tr>
<td>10</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>11</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>12</td>
<td>22.47 ± 1.39</td>
</tr>
<tr>
<td>Dexamethasoneb</td>
<td>0.012 ± 0.007</td>
</tr>
</tbody>
</table>

Data are shown as the IC50 values (means ± SEM) of three independent experiments (n = 3).

a Cell viability was more than 70% at the IC50 concentration.

b Dexamethasone was used as the positive control.

### Fig. 4.
Effectsofcompounds 1 and 2 on LPS-induced pro-inflammatory cytokine production in the RAW264.7 cells. IL-1β, TNF-α, and IL-6 levels were determined using ELISA, with dexamethasone used as the positive control. Data are presented as the means ± SEM of duplicate experiments. *p < 0.05 compared to the group treated with LPS alone.

3.5. Effects of compounds 1 and 2 on the inflammation-related signaling pathway

Constitutive forms of nitric oxide synthases (NOS; e.g., neuronal NOS and endothelial NOS) have roles in regulating physiological functions, such as neurotransmission and pulmonary circulation.
Contrary to constitutive forms, iNOS is excessively expressed in pathological stages, including a pro-inflammatory state in which NO is maintained at high concentrations [26,27]. To investigate the correlation between their NO inhibitory effects and iNOS expression levels, we evaluated whether iNOS protein expression was suppressed by compounds 1 and 2 using Western blot analysis (Fig. 5).

Compounds 1 and 2 strongly suppressed iNOS expression in LPS-treated RAW 264.7 cells for 24 h (Fig. 5). PGE2, which is mainly produced by COX-2, is also released during the inflammatory response, and contributes to the progression of the acute inflammation processes of arthritis and cardiovascular diseases, as well as chronic inflammatory diseases [28]. Therefore, amongst the isolated phytochemicals from CIL, compounds 1 and 2 exert anti-inflammatory activity by inhibiting iNOS expression, but do not affect COX-2 in LPS-activated macrophages.

A previous study showed that the down-regulation of iNOS and COX-2 proteins by CIL was associated with blockade of the nuclear translocation of NF-κB [16]. NF-κB is a pivotal transcription factor that activates pro-inflammatory genes, such as iNOS and COX-2, highlighting the importance of NF-κB activation in inflammation [29]. The inactive form of NF-κB consists of RelA (p65)/p50 heterodimer, and inhibitors of κB (IκB) exist in the cytoplasm. Phosphorylation of IκB by IκB kinase leads to liberation of NF-κB into the nucleus, resulting in the transactivation of NF-κB to express inflammatory genes [30]. Therefore, we hypothesized that compounds 1 and 2 may influence NF-κB activity, and investigated this using Western blot analysis. We focused on whether compounds 1 and 2 affected the translocation of NF-κB to the nucleus from cytosol and nuclear extracts (Fig. 6).

Nuclear translocation of NF-κB was reduced by compound 1 when treated at 10 μM by inhibiting phosphorylation of IκB, whereas compound 2 had no significant effects at the indicated concentrations (Fig. 6). Therefore, the NF-κB inhibition activation of CIL may be attributed to the E-coumaroyl ester triterpenoid than Z-coumaroyl ester triterpenoid and corresponded with their NO inhibitory effect in Table 2.

4. Conclusions

In summary, we isolated 12 compounds, including two new triterpenoids, 1 and 2, from CIL using various chromatographic techniques and evaluated their inhibitory effects on LPS-induced NO production in RAW264.7 cells. Triterpenoids 1, 2, 9, and 12 showed anti-inflammatory activity; E-coumaroyl triterpenoid (1) exhibited the most potent inhibitory activity by inhibiting pro-inflammatory mediators such as NO, IL-1β, and TNF-α. E-coumaroyl triterpenoid also inhibited iNOS, and its anti-inflammatory activity was associated with downregulation of the NF-κB signaling pathway. These results suggest that friedelane-type triterpenoids may be active phytochemical components responsible for the anti-inflammatory effects of CIL. Our findings may provide useful therapeutic opportunities for the treatment of inflammatory diseases.

Acknowledgements

This research is funded by Vietnam Academy of Science and Technology (grant number VAST.UQBT/03/19-20), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2018R1A6A3A11047338). The authors are grateful to the Institute of Chemistry, VAST for measurement of the NMR spectra.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102921.

References

[8] C. Gabay, Interleukin-6 and chronic inflammation, Arthritis Res. Ther. 8 (2)


