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ORIGINAL ARTICLE

Anti-Inflammatory Activity of *Corylopsis coreana* Uyeki Extracts and Isolated Compounds by Inhibiting Nitric Oxide and Cytokine Production

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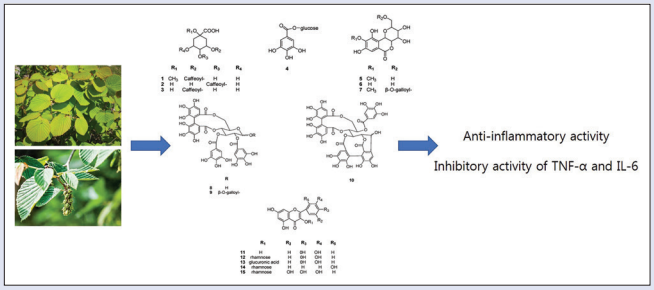
ABSTRACT

Context: *Corylopsis coreana* Uyeki (Korean winter hazel) belongs to the *Hamamelidaceae* family. It is cultivated as an ornamental plant in South Korea. In traditional folk medicine, *C. coreana* has been used to treat irritated skin and inflammatory diseases. **Aims:** To study the anti-inflammatory effects of *C. coreana* Uyeki leaf and bark extracts as well as their major active compounds, we evaluated *in vitro* levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and nitric oxide (NO) using RAW 264.7 macrophages. **Materials and Methods:** Production of TNF-α and IL-6 cytokines was measured by ELISA assay, and NO inhibitory activity was tested by Griess assay. **Results:** Extracts of leaves and barks, as well as isolated compounds from these extracts, showed remarkable anti-inflammatory activities by inhibiting NO, TNF-α, and IL-6 cytokine production. In particular, the leaf extract showed more potent inhibitory activities than that of barks. **Conclusions:** Ellagitannins and flavonoids were probably related to the anti-inflammatory activity of CL (leaves) extract, providing evidence of the anti-inflammatory property of CL.

Key words: Anti-inflammatory ability, *Corylopsis coreana uyeki,* cytokine production inhibition, interleukin-6, nitric oxide production inhibition, tumor necrosis factor-α

SUMMARY

- Fifteen phenolic compounds isolated from Corylopsis coreana Uyeki,including tannins and flavonoids.
- Tannins and flavonoids showed potent anti-inflammatory activities.
- *C. coreana* Uyeki leaves extract showed more stronger inhibitory activity on cytokine production than barks.



Abbreviations used: TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; CL: *Corylopsis coreana*; NO: Nitric oxide; LPS: Lipopolysaccharide; Tel 1: Tellimagrandin I; Tel 2: Tellimagrandin II; Cas: Casuarinin; Quer: Quercetin; EGCG: Epigallocatechin gallate.

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INTRODUCTION

Inflammatory diseases such as rheumatoid arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and psoriasis are common diseases worldwide.[1] Inflammation is the primary defense reaction of the host. It is considered to be a beneficial and necessary attempt by the host to eliminate an aggressive agent. However, serious diseases may develop if chronic inflammation occurs.[2] Anti-inflammatory drugs are used to minimize such damage and improve patient's quality of life. However, currently marketed anti-inflammatory drugs cause a range of adverse effects.[3] Therefore, herbal medicines have been developed as alternatives.

Nitric oxide (NO) is a free radical generated through the conversion of L-arginine to citrulline. The reaction is catalyzed by NO synthase (NOS). High levels of NO have been closely correlated with the pathophysiology of various inflammatory diseases. Therefore, suppression of excessive release of ROS and NO could protect against oxidative stress and the development of inflammation-related diseases.[4] Macrophages play important roles in inflammatory response. When activated, macrophages will release NO; pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α; and lipid mediators including prostaglandin (PG) E2 to promote inflammation by stimulating cellular migration to the target site.[5] Lipopolysaccharide (LPS) is known to activate various mammalian cell types and induce the expression of various pro-inflammatory mediators.[6]

Corylopsis coreana Uyeki (Korean winter hazel; CL) belongs to the *Hamamelidaceae* family (witch hazel family).[7] CL is cultivated as an ornamental plant in South Korea. There are a few research reports about species in the genus *Corylopsis* as well as CL. *Hamamelis virginiana* (witch hazel) has been used as a folk medicine for the treatment of irritated skin and inflammatory diseases.[8] Witch hazel bark is widely used in skin care products for sunburn and atopic eczema.[9] Hamamelitannin and simple phenolics have been isolated from the bark of witch hazel.[10] Their

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antimutagenic and anticancer activities against various forms of cancer have been reported.[11] Recently, chalcone compound isosalipurposide has been successfully isolated from CL.[12] Its cytoprotective effect against oxidative damage is mediated through Nrf2 activation and induction of its target gene expression in hepatocytes.[12] Biological evaluation of CL flos has shown that 100% ethanolic extract of CL flos has the highest antimicrobial activity against methicillin-resistant *Staphylococcus aureus* strain.[13] In addition, the flower of CL flos may be a useful source to identify compounds to cure and prevent septic arthritis based on high-performance liquid chromatography analysis and optimization of extraction conditions.[13] Previously, we had reported the isolation of 15 compounds from leaves of CL and their antioxidative and antiproliferative properties.[14] The objective of the present study was to investigate the anti-inflammatory activities of CL bark and leaf extracts and their isolated compounds.

MATERIALS AND METHODS

Extraction, isolation, and identification

Dry leaves and barks of CL (1.8 kg each) were extracted for 72 h at room temperature with 80% aqueous MeOH (8.0 L). Concentrated leaf extract was then used to isolate compounds that were identified as described in our previous article.[14]

Materials for bioassays

RAW 264.7 macrophage cell line was purchased from Korean Cell Line Bank. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, lipopolysaccharide (LPS) from *Escherichia coli*, dimethylsulfoxide, phosphate-buffered saline, human IL-6, human TNF-α, L-N^G-monomethyl arginine citrate (L-NMMA), and Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Mouse monocyte-macrophage RAW 264.7 cells were maintained in DMEM media supplemented with penicillin (100 U/mL) and 10% FBS at 37°C in a humidified incubator with 5% CO_2 and 95% air. The medium was changed every 3 days.

RAW 264.7 macrophage cell culture for nitric oxide production measurement

RAW 264.7 macrophage cell culture was done by seeding at a density of 1×10^5 cells per well at a total volume of 200 µL in a 96-well plate. These cells were incubated at 37°C in 5% CO_2 for 3 h to ensure adherence. Afterward, the medium was removed, and the cells were treated with CL extracts (100, 50, 25, 12.5, and 6.125 µg/mL, DMEM medium) or compounds (100, 50, 25, 12.5, and 6.125 µM, DMEM medium) for 1 h followed by stimulation with 10 µg/ml LPS for 24 h. Finally, the spent media were collected and analyzed for NO production with Griess reagent by mixing 100 µL of cell culture supernatant with the same volume of Griess reagent. NO production inhibitory activity (I) was calculated as inhibition rate (%) which was calculated using the following formula:

$$I = \frac{1 - (\text{A sample} - \text{A blank})}{\text{A control} - \text{A blank})} \times 100$$

where

A sample: Absorbance of compound-treated cell sample

A control: Absorbance of only LPS-treated cell sample

A blank: Absorbance of nontreated cell sample.

RAW 264.7 macrophage cell culture for cytokine production measurement

RAW 264.7 macrophage cell culture was done by seeding at a density of 4.5×10^5 per well at a total volume of 1 mL in a 24-well plate. These cells were incubated at 37°C in 5% CO_2 for 3 h to ensure adherence. Afterward, the medium was removed, and the cells were treated with CL extracts, positive control (L-NMMA), or compounds (100, 50, 25, 12.5, and 6.125 µg/ml, DMEM medium) and incubated for 1 h followed by stimulation with 50 µg/mL LPS for 6 h. Finally, the spent media were collected and analyzed for TNF-α and IL-6 production using ELISA assay kits according to the manufacturer's recommendations.

Statistical analysis

All data were expressed as mean ± standard deviation. One-way analysis of variance followed by *post hoc* Tukey's test was used to determine statistical significance in differences. All analyses were performed for data from at least three independent experiments. Each experiment was performed in triplicates. In all experiments, untreated cells were considered as blank (B) and treated cells with only LPS were considered as control (C). Statistical significance was considered at $P < 0.05$.

RESULTS

Isolation and identification

Fifteen compounds were isolated from CL leaf extract. They were identified as 3-caffeoylquinic acid methyl ester (1), 4-caffeoylquinic acid (2), 3-caffeoylquinic acid (3), 3-O-galloyl-β-D-glucopyranoside (4), bergenin (5), nor-bergenin (6), 11-galloylbergenin (7), tellimagrandin I (8), tellimagrandin II (9), casuarinin (10), quercetin (11), quercitrin (12), quercetin 3-O-β-D-glucuronide (13), datiscetin 3-O-β-D-rhamnopyranoside (14), and myricetin 3-O-β-D-rhamnopyranoside[15] by comparing their spectral (MS, NMR) data with values reported in the literature. Amounts of these compounds and their spectrum data have been described in detail in our previous article [Figure 1].[14]

Inhibitory activity on nitric oxide production

At an interface between innate and adaptive immune systems, the production of NO by NOS is a crucial step. Therefore, we initially evaluated the ability of bark and leaf extracts and all the isolated compounds to inhibit NO production from LPS-treated RAW 246.7 cells. In murine macrophage RAW cells, LPS stimulation alone can induce iNOS transcription and protein synthesis as well as subsequent NO production. Therefore, RAW cells have been used as an excellent model for screening potential inhibitors.[15] Results of IC_{50} values of extracts and isolated compounds on NO production in LPS-stimulated RAW cells are summarized in Table 1.

Both leaf and bark extracts significantly decreased NO accumulation in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner [Table 1]. The leaf extract had a slightly better inhibitory effect than that of the bark extract (IC_{50} values of 37.84 and 39.64 µg/mL, respectively). For comparison, the positive control L-NMMA had stronger inhibitory activity (IC_{50} value of 7.12 µg/mL) than that of leaf and bark extracts of CL. At high concentrations of 100 µg/mL, leaf and bark extracts showed higher NO production inhibitory activity than that of the positive control (93%, 95%, and 88%, respectively. Data not shown).

In addition, inhibitory activities of compounds isolated from leaf extract on NO production were investigated. Compounds 2, 4, 5, 6, 12, and 15 exhibited IC_{50} values of more than 100 µM, whereas compounds 8, 9, 10, and 11 showed the most potential NO inhibitory activity, with IC_{50} values of 44.14 ± 11.60, 24.10 ± 4.15, 28.52 ± 6.16, and

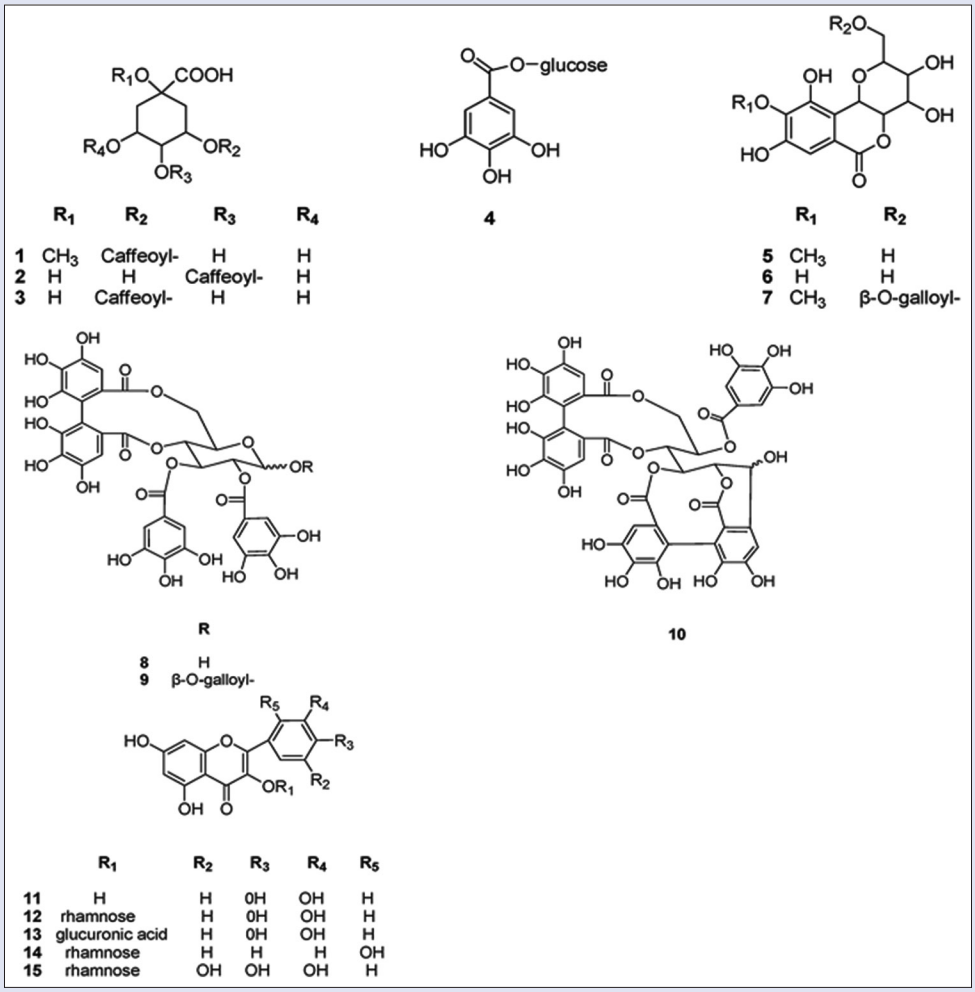

Figure 1: Structures of compounds 1–15 isolated from leaves of *Corylopsis coreana*

Table 1: Inhibitory effect of extracts and compounds on nitric oxide production in RAW macrophages

Sample	IC_{50} (μg/mL)	Compound	IC_{50} (μM)
Leaf extract	37.84±6.23[b]	1	46.63±13.36[a,b,c,d]
Bark extract	39.64±1.16[b]	2	>100[d]
L-NMMA	7.12±0.28[a]	3	71.37±14.55[a,b,c,d]
		4	>100[d]
		5	>100[c,d]
		6	>100[e]
		7	49.06±5.10[a,b,c,d]
		8	44.14±11.60[a,b,c,d]
		9	24.10±4.15[a]
		10	28.52±6.16[a,b]
		11	37.96±17.48[a,b,c]
		12	>100[c,d]
		13	45.21±5.22[a,b,c,d]
		14	88.74±3.81[b,c,d]
		15	>100[e]
		L-NMMA	14.61±2.65[a]

Data are presented as mean±SD of three experiments. Values with different letters (a, b, c, d, e) are statistically significantly different at $P<0.05$). SD: Standard deviation; L-NMMA: L-N^G-monomethyl arginine citrate

37.96 ± 17.48 μg/mL, respectively. Therefore, compounds 8, 9, 10, and 11 were selected to evaluate their inhibitory activities against cytokine production [Table 1].

Inhibitory activity against cytokine production

Due to their NO production inhibitory activity, compounds 8, 9, 10, and 11 were assessed for their anti-inflammatory activity on LPS-induced pro-inflammatory cytokines IL-6 and TNF-α which are known to play a crucial role in host defense and inflammation. RAW cells were pretreated with isolated compounds for 1 h at different concentrations (100, 50, and 25 μM) followed by stimulation with LPS for 6 h. Epigallocatechin-3-gallate (EGCG), a bioactive component of green tea, is known to exert anti-inflammatory effects on immune cells.[16] It was used as a positive control in this study.

Inhibitory activity on tumor necrosis factor-α production

For CL extracts, during the incubation time of 6 h, blank RAW cells produced 7.21 ng/mL of TNF-α. After stimulation with LPS, TNF-α production was increased about 37 folds (up to 248.09 ng/mL). The concentration of TNF-α was reduced by treatment with CL extract in a dose-dependent manner. TNF-α production in cell treatment with leaf and bark extracts at 100 μg/mL was inhibited by 97% and 99%, respectively, compared to that in LPS-stimulated untreated RAW cells. At a concentration of 50 μg/mL, leaf extract again showed high inhibitory activity on TNF-α production, causing 78% decrease in TNF-α level, better than bark extract and EGCG (43% and 52% decrease, respectively). At a concentration of 25 μg/mL, leaf extract, bark extract, and EGCG inhibited

the production of TNF-α by 41%, 25%, and 47%, respectively [Figure 2].

At compound level as well as at extract level, after 6 h of incubation, 0.74 ng/mL TNF-α was produced by blank RAW cells. The concentration of TNF-α in LPS-induced cells was 5.47 ng/mL, an increase of about 8 folds. The concentration of TNF-α was decreased more by treatment with selected compounds at higher concentration except 11. TNF-α production after treatment with 25 μg/mL of 11 was lower than that after treatment with 11 at higher concentrations (50 and 100 μg/mL). At the same concentration, most compounds resulted in lower concentration of TNF-α than that of treatment with EGCG, the positive control [Figure 3].

Inhibitory activity on interleukin-6 production

LPS treatment resulted in a significantly increased production of TL-6 (about 6 folds) compared to blank (non-LPS treatment) (IL-6 concentration of 502 and 88 pg/mL, respectively). Treatment with leaf extract, bark extract, and isolated compounds significantly suppressed IL-6 production in a dose-dependent manner, whereas treatment with positive control did not. Specifically, treatment with leaf extract, bark extract, and isolated compounds 8, 9, 10, and 11 at their highest concentration suppressed IL-6 production to 55%, 54%, 24%, 66%, 14%, and 33%, respectively [Figures 4 and 5].

DISCUSSION

Inflammation is a complex process regulated by a cascade of cytokines, growth factors, NO, and PGs by activated macrophages. NO is a short-lived molecule that has an important role in various physiologic processes. However, NO overproduction can activate nuclear factor-κB to induce the expression of pro-inflammatory mediators. It also promotes inflammation by increasing cyclic guanosine monophosphate, vascular permeability, and transmigration of inflammatory cells to inflammation sites.[1,6] NO toxicity increases considerably if it reacts with a superoxide radical and forms highly reactive peroxynitrite anion that can lead to oxidative damage and tissue injury.[17] Chronic generation of NO radical is associated with various cancers and inflammatory conditions, including diabetes mellitus and arthritis. Pro-inflammatory cytokines such as TNF-α and IL-6 evoke elevated levels of iNOS followed by a significant increase in the production of NO.[6]

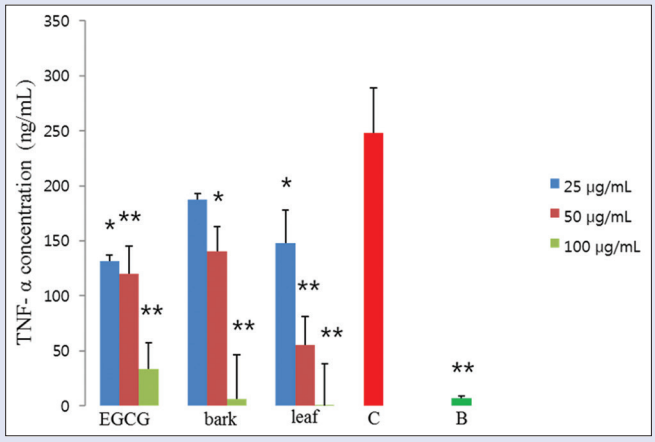

Figure 2: Inhibitory effect of extracts on tumor necrosis factor-α production in lipopolysaccharide-activated RAW macrophages. Epigallocatechin-3-gallate was used as a positive control. Data are presented as mean ± standard deviation of three experiments in triplicates. *$P < 0.05$, **$P < 0.01$, compared with the control group

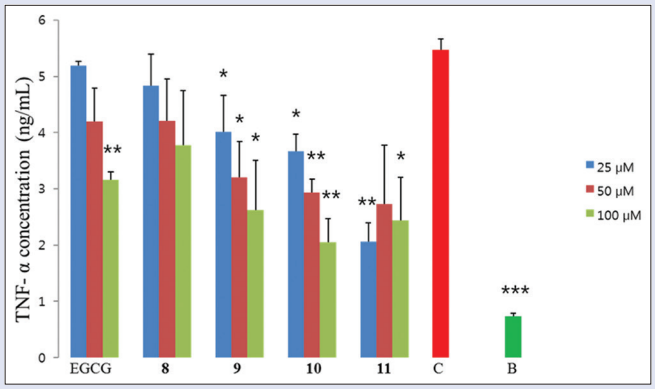

Figure 3: Inhibitory effect of selected compounds on tumor necrosis factor-α production in lipopolysaccharide-activated RAW macrophages. Epigallocatechin-3-gallate was used as a positive control. Data are presented as mean ± standard deviation of three experiments in triplicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the control group

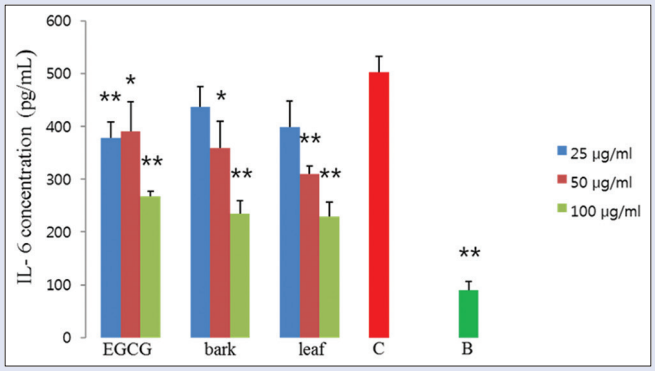

Figure 4: Inhibitory effect of extracts on interleukin-6 production in lipopolysaccharide-activated RAW macrophages. Epigallocatechin-3-gallate was used as a positive control. Data are presented as mean ± standard deviation of three experiments in triplicates. *$P < 0.05$, **$P < 0.01$, compared with the control group

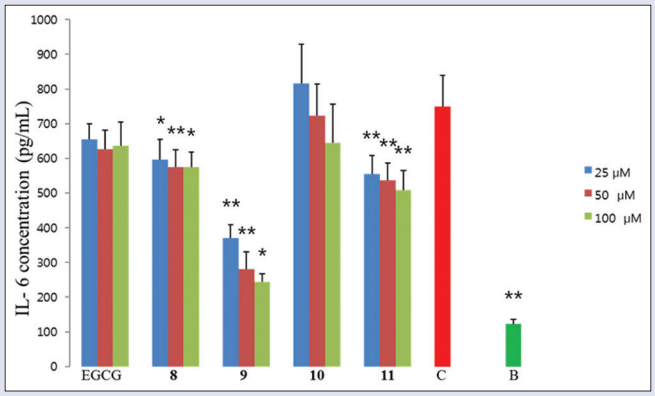

Figure 5: Inhibitory effect of selected compounds on interleukin-6 production in lipopolysaccharide-activated RAW macrophages. Epigallocatechin-3-gallate was used as a positive control. Data are presented as mean ± standard deviation of three experiments in triplicates. *$P < 0.05$, **$P < 0.01$, compared with the control group

Recently, inflammatory effects of MeOH twig extract of CL have been investigated both *in vitro* and *in vivo*. Results indicated that MeOH twig extract of CL effectively inhibited excessive NO production in transferrin-γ and LPS-stimulated mouse peritoneal macrophages without notable cytotoxicity. It also attenuated the expression of inflammatory mediators such as inducible NOS and cyclooxyganase-2 *in vivo*. The MeOH twig extract of CL also significantly suppressed paw edema induced by trypsin and carrageenan.[18] However, in our study, we focused on leaf extract of CL and isolated compounds from leaves. Isolation from bark extract was performed simultaneously with leaf extract to compare the anti-inflammatory effects of different parts of CL.

We performed studies at two levels: extract level and compound level. In terms of extract level, there were no significant differences in inhibitory activity on NO, TNF-α, or IL-6 production between leaf and bark extracts. Their cytokine production inhibitory activity was similar to that of the positive control. At high concentration, the extracts of CL had better cytokine production inhibitory activity than that of the positive control. At compound level, four compounds (8–11) significantly decreased NO production in LPS-stimulated RAW 246.7 cells. Their anti-inflammatory activity on LPS-stimulated expression of pro-inflammatory cytokines including TNF-α and IL-6 was also assessed. All compounds showed potent inhibition of TNF-α production. Among them, 11 showed the greatest inhibitory activity. For IL-6 inhibitory ability, 9 showed the greatest inhibitory activity. 10 was inactive in terms of inhibiting the production of IL-6, whereas 8 and 11 showed potent inhibitory effect on IL-6 production. Therefore, the anti-inflammatory ability presented by leaf extract of CL can be partly attributed to the presence of these compounds by decreasing the inflammatory response induced by LPS.

CONCLUSIONS

Our results indicated that ellagitannins (8–10) and flavonoids (11) were probably related to the anti-inflammatory activity of CL leaf extract, providing evidence of the anti-inflammatory property of CL. However, further researches are needed to elucidate the mechanisms involved in the anti-inflammatory activity of the isolated compounds and CL extracts.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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