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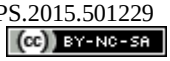
ISSN 2231-3354 

Anti-inflammatory Constituents from Branches of *Corylus hallaisanensis* Nakai

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ABSTRACT

Development of bioactive ingredients from natural sources has long been the research project of our laboratory. In this study, the extract from *Corylus hallaisanensis* Nakai branches was investigated and their anti-inflammatory constituents were identified. The prepared ethanol extract was successively partitioned into n-hexane, ethyl acetate, n-butanol and aqueous layers. Upon anti-inflammatory screenings, ethyl acetate fraction exhibited good nitric oxide production inhibitory activity in lipopolysaccharide-induced RAW 264.7 cells. Further phytochemical studies for the ethyl acetate fractions led to isolation of four constituents such as β-sitosterol (**1**), 3,3',4'-tri-O-methylellagic acid (**2**), carpinontriol A (**3**) and carpinontriol B (**4**). All of the compounds were isolated for the first time from this plant. The isolates **2**, **3** and **4** showed considerable inhibition on the production of nitric oxide in the RAW 264.7 cell without causing cell toxicities. And compounds **3** and **4** reduced the production of interleukin-6, an inflammatory cytokine, in dose-dependent manner in RAW 264.7 cells. Based on these results, *C. hallaisanensis* extracts could be potentially applicable as anti-inflammatory agents in pharmaceutical or cosmetic industries.

INTRODUCTION

Inflammation is described as cellular events in response to infection and tissue injury. Inflammatory responses include enhanced vascular permeability and blood supply as well as increased migration of immune cells at damaged site (Yoon *et al*., 2009).

Also it is characterized by the abundant production of nitric oxide (NO), prostaglandin (PG) E_2, and cytokines such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6). These inflammatory mediators are involved in the pathogenesis of a vast number of human diseases. Therefore, modulation of pro-inflammatory mediators is important measure for the search of anti-inflammatory agents (Deng *et al*., 2011; Hadad and Levy, 2012). The foundation of modern pharmacologic medicine is based in ethnobotanical traditions utilizing indigenous flora. Anti-inflammatory agents from plant sources has been extensively studied by using cell-based (Kim *et al*., 2011; Ko *et al*., 2013) or animal model (Jeong *et al*., 2013) assay protocols. *Corylus hallaisanensis* Nakai is a deciduous woody plant belonging to the family Betulaceae.

This flora is endemic to Korea, and inhabits a specific area in the Jeju Island. It has fruit as an edible nut, and has been used as folk medicine. Some Butulaceae species have been reported to contain large quantities of tannins such as pedunculagin, casuarinin and stachyurin (Jin *et al*., 1998). As far as we know, however, there is no report on the biological properties either on the chemical constituents for the extracts of *C. hallaisanensis*.

In this study, ethanol extracts from the branches of *C. hallaisanensis* were investigated on anti-inflammation effect by using lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells. In addition, the isolation and identification of the bioactive substances in the extracts were also described.

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MATERIALS AND METHODS

Plant material

The branches of *C. hallaisanensis* Nakai were collected in June of 2011 from Halla Botanical Garden in Jeju Island, Korea. Voucher specimen (sample number 322) was deposited at the herbarium of Department of Chemistry and Cosmetics, Jeju National University.

Extraction and isolation

The procedure for the isolation of the compounds **1**-**4** is summarized in Figure 1. The shade dried branches of *C. hallaisanensis* (802 g) were cut into small pieces and extracted three times with 70% aq. ethanol using a mechanical stirrer at room temperature for 24 hr. The resulting ethanol solutions were combined and filtered. The filtrate was concentrated using a rotary evaporator at a temperature 37℃. Part of the ethanol extract (30.0 g) was suspended in water (1 L) and fractionated into *n*-hexane (2.2 g), ethyl acetate (4.7 g), *n*-butanol (7.1 g) and water (15.6 g) portions. The ethyl acetate (EtOAc) layer was subjected to vacuum liquid chromatography (VLC) on silica gel using step-gradients (*n*-hexane/EtOAc to EtOAc/MeOH, 300 mL each) to provide 30 fraction (V1-V30). The compounds **1** (4.2 mg) and **2** (24.4 mg) were obtained from fractions V5 and V10 respectively by recrystallization over methanol. The fraction V19 was purified by silica gel column chromatography (CC) with chloroform-methanol (8:1) to give compound **3** (8.0 mg). The fraction V16 was subjected to Sephadex LH-20 CC with chloroform-EtOAc-methanol (5:15:1) to afford the compound **4** (10.2 mg)

β-Sitosterol (1)

^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.36(1H, d, *J* = 5.3 Hz, H-6), 3.55(1H, m, H-3), 1.01 (3H, s, H-19), 0.93 (3H, d, *J* = 6.6 Hz, H-21), 0.87 (3H, t, *J* = 7.8 Hz, H-29), 0.85 (3H, d, *J* = 7.1, H-26), 0.82 (3H, d, *J* = 6.9 Hz, H-27), 0.69 (3H, s, H-18); ^{13}C NMR($CDCl_3$, 100 MHz) δ 37.4 (C-1), 29.9 (C-2), 72.0 (C-3), 42.5 (C-4), 141.0 (C-5), 121.6 (C-6), 32.1 (C-7), 31.9 (C-8), 50.3 (C-9), 36.7 (C-10), 21.3 (C-11), 40.0 (C-12), 42.5 (C-13), 57.0 (C-14), 24.5 (C-15), 28.4 (C-16), 56.2 (C-17), 12.0 (C-18), 20.0 (C-19), 36.4 (C-20), 19.0 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.3 (C-25), 19.6 (C-26), 19.2 (C-27), 23.3 (C-28), 12.2 (C-29).

3,3',4'-tri-O-Metylellagic acid (2)

^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.57 (1H, s, H-5), 7.49 (1H, s, H-5'), 4.04(3H, s, OCH_3-3), 4.03 (3H, s, OCH_3-3'), 3.98 (3H, s, OCH_3-4'); ^{13}C NMR(DMSO-d_6, 100 MHz) δ 113.3 (C-1), 141.6 (C-2), 140.8 (C-3), 153.8 (C-4), 111.8 (C-5), 111.6 (C-6), 158.5 (C-7), 112.4 (C-1'), 140.9 (C-2'), 140.1 (C-3'), 152.7 (C-4'), 107.4 (C-5'), 111.1 (C-6'), 158.3 (C-7'), 61.3 (OCH_3-3), 61.0 (OCH_3-3'), 56.7 (OCH_3-4').

Carpinontriol A (3)

^{1}H NMR (CD_3OD, 400 MHz) δ 7.05 (1H, dd, *J* = 8.0, 2.4 Hz, H-5), 7.00 (1H, dd, *J* = 8.0, 2.4 Hz, H-15), 6.79 (1H, d, *J* = 8.0 Hz, H-4), 6.77 (1H, d, *J* = 8.0 Hz, H-16), 6.65 (1H, d, *J* = 2.0 Hz, H-19), 6.56 (1H, d, *J* = 2.0 Hz, H-18), 4.44 (1H, dd, *J* = 6.4, 2.2 Hz, H-12), 4.07~4.00 (2H, m, H-8, H-9), 3.74 (1H, d, *J* = 19.6 Hz, H-10a), 3.53 (1H, d, *J* = 15.6 Hz, H-13a), 2.98 (1H, dd, *J* = 16.0, 3.0 Hz, H-7a), 2.88 (1H, dd, *J* = 15.9, 9.2 Hz, H-7b), 2.87 (1H, dd, *J* = 15.6, 6.6 Hz, H-13b), 2.75 (1H, dd, *J* =18.8, 8.6 Hz, H-10b); ^{13}C NMR (CD_3OD, 100 MHz) δ 128.2 (C-1), 127.5 (C-2), 153.5 (C-3), 117.4 (C-4), 130.9 (C-5), 130.3 (C-6), 39.6 (C-7), 69.3 (C-8), 73.8 (C-9), 47.0 (C-10), 218.8 (C-11), 78.2 (C-12), 39.5 (C-13), 129.7 (C-14), 131.6 (C-15), 117.1 (C-16), 153.7 (C-17), 135.1 (C-18), 135.6 (C-19).

Carpinontriol B (4)

^{1}H NMR (CD_3OD, 400 MHz) δ 7.05 (1H, dd, *J* = 8.2, 2.5 Hz, H-15), 6.99 (1H, dd, *J* = 8.1, 2.2 Hz, H-5), 6.79 (1H, d, *J* = 8.2 Hz, H-16), 6.76 (1H, d, *J* = 8.2 Hz, H-4), 6.66 (1H, s, H-19), 6.36 (1H, s, H-18), 4.73 (1H, dd, *J* = 11.8, 4.5 Hz, H-8), 4.23 (1H, d, *J* = 10.1 Hz, H-10), 3.91 (1H, d, *J* = 10.1 Hz, H-9), 3.54 (1H, ddd, *J* = 20.0, 12.7, 2.1 Hz, H-12a), 3.14 (1H, m, H-13a), 3.05 (1H, dd, *J* = 15.9, 4.5 Hz, H-7a), δ 2.93 (1H, m, H-12b), δ 2.85 (1H, m, H-7b), δ 2.82 (1H, m, H-13b); ^{13}C NMR (CD_3OD, 100 MHz) δ 127.0 (C-1), 127.9 (C-2), 153.6 (C-3), 117.3 (C-4), 130.6 (C-5), 130.3 (C-6), 37.1 (C-7), 68.7 (C-8), 69.9 (C-9), 78.8 (C-10), 215.7 (C-11), 37.7 (C-12), 25.3 (C-13), 130.9 (C-14), 129.4 (C-15), 117.2 (C-16), 152.8 (C-17), 135.0 (C-18), 134.8 (C-19).

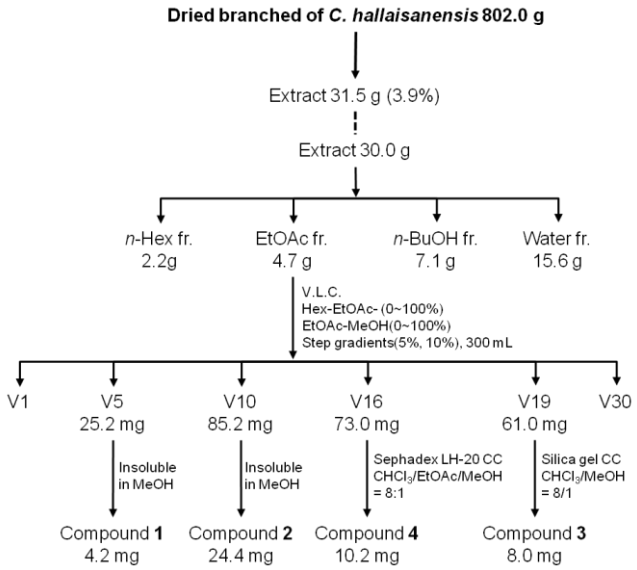

Fig. 1: Isolation scheme of the compounds 1 - 4 from the extract of *C. hallaisanensis.*

Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's media (DMEM; GIBCO Inc., NY, USA) supplemented with penicillin (100 Units/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C.

Measurement of nitric oxide (NO) concentration

Nitric oxide production was assayed by measuring the nitrite in the supernatants of cultured RAW 264.7 cells. The cells were seeded at densities of 2 x 10^5 cells/mL in 24 well culture plates and were cultured for 18 hr. The cells were stimulated with lipopolysaccharide (LPS, 1 μg/mL) and treated with various concentrations of samples (ethanol extracts, solvent fractions, compounds **2-4**) for 24 hr. The supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and was incubated at room temperature for 10 min. The concentrations of nitrite were then determined at an optical density of 540 nm. Nitrite concentration was determined by comparing the optical density with the standard curve obtained with sodium nitrite. All experiments were performed in triplicate.

Cell viability assay

The cell viability was determined by the MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Briefly, cells were seeded into 24 well culture plates. After 18 hr, the cells were stimulated with LPS (1.0 μg/mL) and treated with samples for 24 hr at 37℃ under 5% CO_2 condition. MTT reagent (500 μg/mL) was added to the medium and it was allowed to stand for 4 hr. Finally the medium was removed, and the formazan crystals were dissolved in DMSO. The cell viability was evaluated as the relative absorbance of the control group.

Detection of pro-inflammatory cytokine interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α)

The pre-incubated RAW 264.7 cells were treated with LPS (1 μg/mL) and the samples (EtOAc layer, compound **4**) for 24 hr. Then, the inhibition effect of samples on TNF-α and IL-6 production in LPS-treated RAW 264.7 cells was determined by an enzyme-linked immunosorbent assay (ELISA) kit, as described in the manufacturer's instructions.

Statistical analysis

All data were obtained in triplicate and are represented as means ± standard error (SE). Significant differences between treatments were determined by the Student's *t* test in one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Nitric oxide (NO) is a signaling molecule that plays critical role in the pathogenesis of inflammation in living organism. The overproduction of NO under pathological conditions can cause a toxic effects leading to inflammatory disease. Therefore, down-regulation of NO synthesis in the presence of inflammatory stimuli is relevant to the development of anti-inflammatory agents (Hyun *et al*., 2015).

In order to evaluate anti-inflammatory activity of *C. hallaisanensis* extracts, we examined its extract and fractions (*n*-hexane, EtOAc, *n*-butanol and water layers) on NO production using RAW 264.7 macrophage cells. The amount of NO product was highly increased when stimulated by lipolpolysaccharude (LPS), and the decrease of its production was compared when treated with *C. hallaisanensis* extract samples. Among the five samples, the EtOAc layer (100 μg/mL concentration) remarkably inhibited NO production by 99.5% (Fig. 2). The *n*-hexane and *n*-butanol fractions exhibited much lower activities.

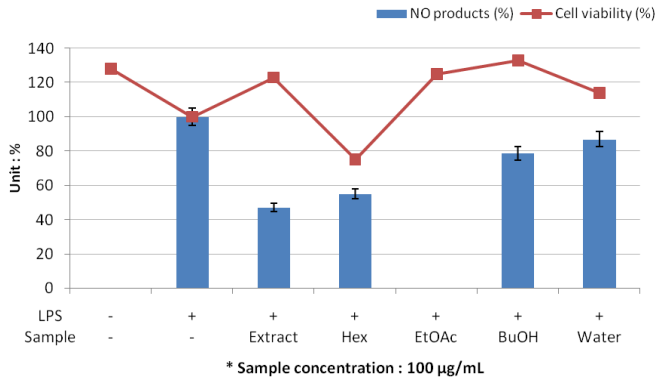

Fig. 2: Effect of extract and solvent fractions from *C. hallaisanensis* on NO production and cell toxicities in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg/mL of LPS only, or with LPS plus *C. hallaisanensis* extract (100 μg/mL) for 24 h. Nitric oxide production was determined by using the Griess reagent. Cell viability was determined after 24 h culture of cells stimulated with LPS (1 μg/mL) in the presence of *C. hallaisanensis*. The data represent the mean±SD of triplicate experiments.

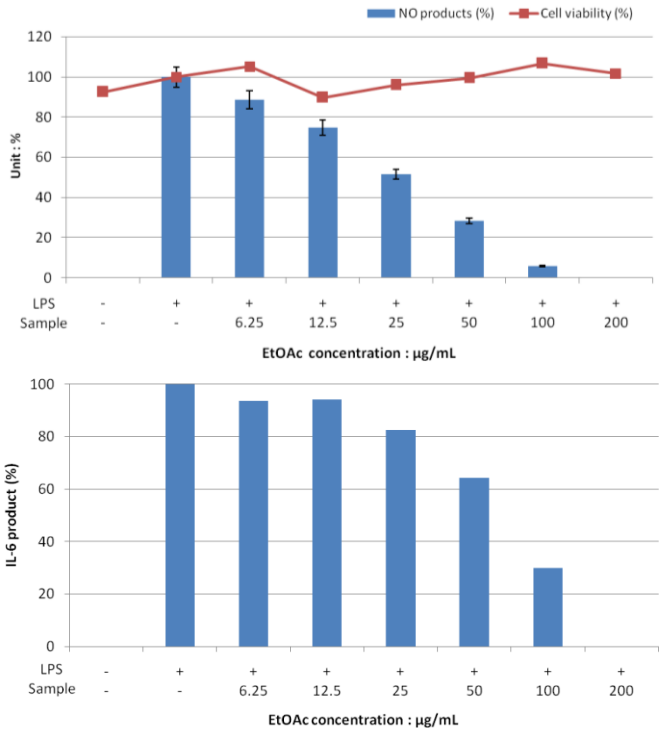

Fig. 3: Effect of ethyl acetate fractions from *C. hallaisanensis* on NO production and cell toxicities (a), IL-6 (b) in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg/mL of LPS only, or LPS plus various concentrations (6.25-200 μg/mL) of EtOAc fraction for 24 h. Nitric oxide production was determined by using the Griess reagent. Cell viability was determined after 24 h culture of cells stimulated with LPS (1 μg/mL) in the presence of EtOAc fraction. IL-6 produced and released into culture medium was assayed using the ELISA method. The data represent the mean±SD of triplicate experiments.

As EtOAc fraction was further studied under various concentrations (6.25 to 200 µg/mL), it showed NO inhibition activity in a concentration-dependent manner with IC_{50} of 24.9 µg/mL (Fig. 3a). Under the employed concentrations, the EtOAc fraction did not cause any cell toxicities as determined by the appearance of formazan crystal in MTT assay. In addition, the EtOAc fraction showed significant inhibition effect on the production of IL-6, an inflammatory cytokine, in dose-dependently with IC_{50} 71.4 µg/mL (Fig 3b) based on ELISA analysis.

As the EtOAc fraction showed potent anti-inflammatory activities, it was chosen for phytochemical studies to determine the active constituents. Through chromatographic purification procedures, four known compounds **1-4** were isolated (Fig. 4). Their chemical structures were identified primarily by analyzing the nuclear magnetic resonance (NMR) spectroscopy data.

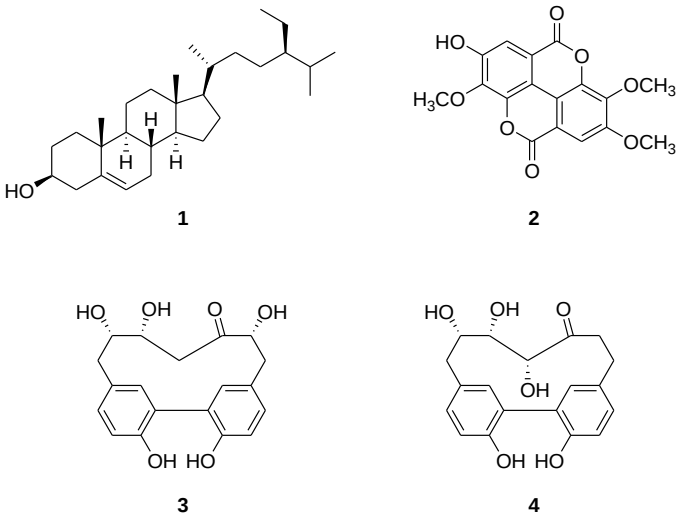

Fig. 4: Chemical structures of compounds **1 - 4** isolated from *C. hallaisanensis*.

Compound **1** showed 29 signals in ^{13}C NMR spectrum, where one oxygen-bearing sp^3 carbon and two sp^2 carbons were identified. The presence of six methyl groups were also indicated based on ^1H and DEPT NMR signals. By the inspection of the other spectroscopic data, compound **1** was identified as *β*-sitosterol (Sohn *et al*., 2009). Compound **2** showed five singlet peaks at δ 7.57 (1H, s), 7.49 (1H, s), 4.04 (3H, s), 4.03 (3H, s) and 3.98 (3H, s) in ^1H NMR spectrum. The unusual up-filed shifted carbonyl signals at δ 158.5 and 158.3 were noted in ^{13}C NMR spectrum, which suggested a structure of an ellagic acid derivative. ^{13}C NMR spectrum also indicated the presence of three methoxy carbons with signals at δ 61.3, 61.0 and 56.7 ppm. By comparison of the obtained data to literature values, compound **2** was confirmed as 3,3',4'-tri-*O*-methylellagic acid (Fan et al., 2005). In ^{13}C NMR spectra, compounds **3** and **4** showed 19 carbon peaks respectively with twelve sp^2 carbons corresponding to two aromatics and seven sp^3 aliphatic carbons, which are characteristic indications for the presence of a diarylheptanoid skeleton. The carbon signals at δ 218.8 and 215.7 indicated the presence of carbonyl group for the compounds **3** and **4** respectively. The signals at δ 69.3, 73.8 and 78.2 in ^{13}C NMR spectrum suggested three oxygen-bearing sp^3 carbons in the compound **3**. Also the corresponding carbons peaks at δ 68.7, 69.9 and 78.8 were observed in the compound **4**, which indicated that the compounds **3** and **4** possess similar structures. By the further analysis of 2D NMR data, the compounds **3** and **4** were identified as diarylheptanoid derivatives, carpinontriol A and carpinontriol B, respectively (Lee et al., 2002). All of the compound **1-4** were isolated for the first time from *C. hallaisanensis*.

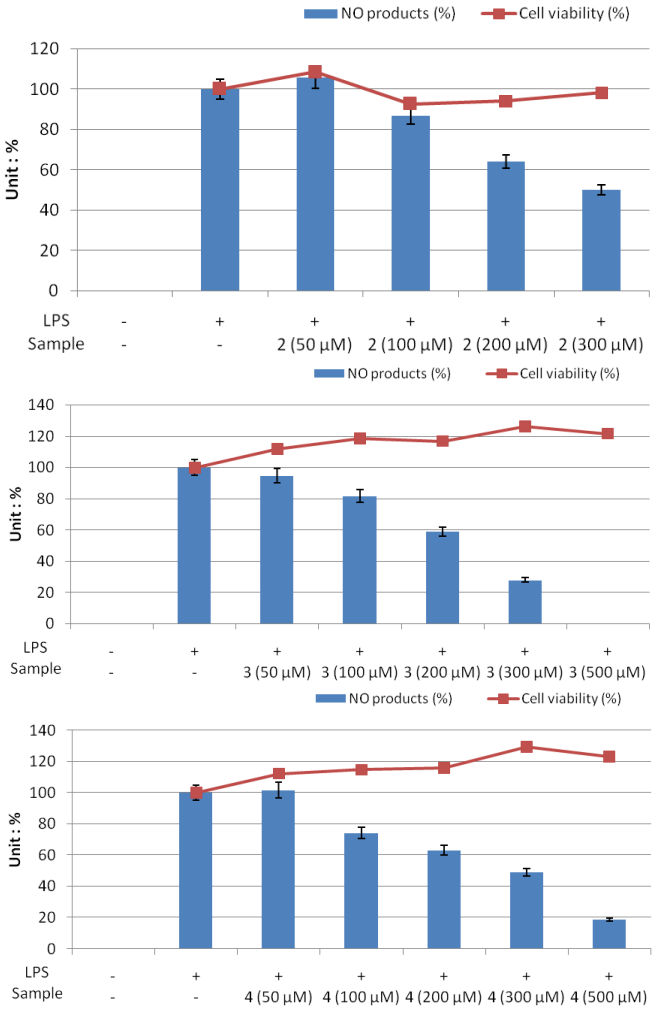

Fig. 5: Effect of isolated compounds **2** (Fig. 5a), **3** (Fig. 5b) and **4** (Fig. 5c) on NO production and cell toxicities in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 µg/mL of LPS only, or LPS plus various concentrations of isolated compounds for 24 h. Nitric oxide production was determined by using the Griess reagent. Cell viability was determined after 24 h culture of cells stimulated with LPS (1 µg/mL) in the presence of isolated compounds. The data represent the mean±SD of triplicate experiments.

The anti-inflammatory activities were assessed for the isolated compounds **1-4** by measuring the level of nitric oxide in LPS-treated macrophage cells. As shown in Fig. 5, the compounds **2**, **3** and **4** reduced the NO synthesis dose-dependently with IC_{50} of 310.6, 223.1 and 289.6 µg/mL, respectively. Diarylheptanoids **3** and **4** showed slightly higher activities compared to ellagic acid derivative **2**. These compounds were further investigated for their cytotoxic effects using MTT assay. As shown in Figure 5, they did

not damage the cell viability at the employed concentrations (50, 100, 200, 300, 500 μM) in macrophage cells. Therefore, it is assumed that down-regulation of NO production in the isolates **2-4** is not caused by cell toxicities but mediated by some cell signal pathways.

As shown in Figure 3b, the EtOAc fraction decreased the levels of interleukin 6 (IL-6) in LPS-treated RAW 264.7 macrophages. Therefore, the isolates **1-4** were also examined on their effect on the production of inflammatory mediator IL-6. As a result, the carpinontriols **3** and **4** showed the inhibition of IL-6 synthesis in a concentration-dependent manner with IC_{50} values ranging from 200 to 500 μM (Fig. 6).

The compound **2** showed very low activities in this test. Therefore, it is assumed that the IL-6 inhibition activities shown in EtOAc fraction is derived from those in compounds **3** and **4**. Tumor necrosis factor α (TNF-α) is another cytokine playing important role in inflammatory disease. The inhibition effect of the compounds **1-4** on TNF-α secretion was determined by an enzyme-linked immunosorbent assay (ELISA) kit. However, all of the compounds **1-4** did not affect the production of TNF-α in LPS-treated RAW 264.7 cells.

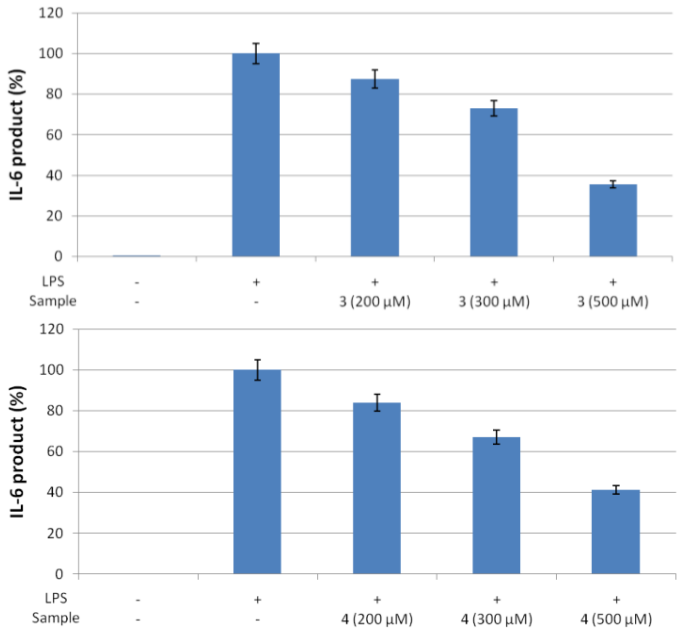

Fig. 6: Effect of isolated compounds **3** (Fig. 6a) and **4** (Fig. 6b) on IL-6 production in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg/mL of LPS only, or LPS plus various concentrations (200-500 μM) of compound **3** and **4** for 24 h. IL-6 produced and released into the culture medium was assayed using the ELISA method. The data represent the mean±SD of triplicate experiments.

In summary, the present study indicated that the ethyl acetate fraction of C. *hallisanensis* branches possessed strong inhibition activities on nitric oxide synthesis in LPS-stimulated RAW 264.7 murine macrophages.

The phytochemical study of the ethyl acetate fraction led to the isolation of four known compounds, *β*-sitosterol (**1**), 3,3',4'-tri-*O*-methylellagic acid (**2**), carpinontriol A (**3**) and carpinontriol B (**4**). The isolated compounds **2-4** exhibited the down-regulation of the inflammatory mediators such as NO or IL-6 in LPS-induced macrophages. Thus, these results suggested that the extract of *C. hallisanensis* branches could have a potential for use as anti-inflammatory agents.

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