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Protective effect of phlorotannins from *Eisenia bicyclis* against lipopolysaccharide-stimulated inflammation in HepG2 cells

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ABSTRACT

In this study, four bioactive phloroglucinol derivates including phloroglucinol (**1**), eckol (**2**), dioxinodehydroeckol (**3**), and dieckol (**4**) were isolated from *Eisenia bicyclis* and characterized by nuclear magnetic resonance (NMR) spectroscopic methods. Moreover, the anti-inflammatory activity of these compounds was investigated on human hepatoma cell line HepG2 cells stimulated by lipopolysaccharide (LPS). It was demonstrated that LPS can induce the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) as well as the expression of inflammatory mediators as cyclooxygenase-2 (COX-2), and inducible nitric oxide synthases (iNOS) from HepG2 cells. Among isolated compounds, compound (**1**) exhibited significant inhibition on LPS-stimulated inflammatory responses in HepG2 cells without any cytotoxicity. Herein, compound (**1**) suppresses the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α and the expression of COX-2 and iNOS. Thus, these results indicated that phlorotannins isolated from *E. bicyclis*, especially compound (**1**), can be used as a beneficial source for preventing and treating inflammation response.

1. Introduction

Inflammation is the normal physiological and immune response to tissue injury. The inflammatory process is a protective response that occurs in response to trauma, infection, tissue injury and noxious stimuli (Choy et al., 2008). The systemic inflammatory reaction (acute phase response) is induced by many noxious stimuli but in all cases the inflammatory cytokines (Koj and Jura, 2003). These metabolic alterations can be induced by an endotoxin treatment such as lipopolysaccharide (LPS). The effects of LPS are in turnmediated by cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and it has been shown that many of the metabolic effects of infection, inflammation and trauma can be induced by these cytokines (Hardardóttir et al., 1994).

A variety of natural resources have traditionally been used in oriental folk medicine to treat inflammatory disease. Extracts of seaweed also exhibit significant anti-inflammatory activity (Kang et al., 2012; Yoon et al., 2011a). *Eisenia bicyclis* is common perennial brown alga belonging to the family Laminariaceae that inhabits the middle Pacific coast around Korea and Japan. Previously, *E. bicyclis* was reported that has

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various bioactive derivatives, such as phlorotannins, polysaccharides, pyropheophytin, peptides and oxylipin, and it has been studied for many beneficial bioactivities including anti-inflammatory, anti-allergic, and anti-tumors activities (Okada et al., 2004; Yoon et al., 2011b; Whitaker and Carlson, 2006). The major bioactive phenolic compounds in *E. bicyclis* were reported to be phlorotanins include eckol, phlorofucofroeckol A, dieckol, and 8,8′-bieckol (Eom et al., 2012). In recent years, these compounds have been investigated for their anti-oxidative and anti-inflammatory effects in macrophages (Yoon et al., 2011b; Kim et al., 2011). However, the anti-inflammatory activity of these phlorotannins in hepatocytes remains to be evaluated. Hepatocellular carcinoma (HepG2) cell stimulated with LPS could result in the release of inflammatory mediators that contribute to the pathogenesis of liver failure/injury.

Therefore, this study was designed to estimate the anti-inflammatory effects of isolated compounds constituents from *E. bicyclis* by measuring the production of cytokines as well as expression of inducible nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2) in LPS-stimulated hepatocellular carcinoma cell lines.

2. Materials and methods

2.1. Extraction and fractionation

The edible brown alga, *E. bicyclis* was collected on Ulleung Island, Korea, in September 2010. As previously reported (Eom et al., 2011), the sample was rinsed with fresh water to eliminate forging materials such as sand and shells. Next, the sample was desalinated by soaking three times in two volumes of water for 1 h. The desalinized sample was cut into an efficient size for extraction using a grinding mill. The dried *E. bicyclis* powder (500 g) was extracted by stirring extraction unit (Dongwon Scientific Co., Korea) with methanol (MeOH, 3 times × 10 L) at 67 °C. The MeOH extract (137 g) was suspended in water and partitioned with *n*-hexane (Hex, 1.0 L × 3 times), dichloromethane (CH_2Cl_2, 1.0 L × 3 times), ethyl acetate (EtOAc, 1.0 L × 3 times), and *n*-butanol (*n*-BuOH, 1.0 L × 3 times) in turn. In this solvent fractionation method, dried Hex- (21.2 g), CH_2Cl_2 (1.3 g), EtOAc (16.0 g), *n*-BuOH (23.6 g) and H_2O soluble (74.9 g) extract were obtained.

2.2. Isolation and structural analysis

The EtOAc fraction (16.0 g), which exhibited a most potent protective effect, was subjected to Sephadex LH-20 (Merck, Darmstadt, Germany; 100% MeOH) and was further purified by LiChroprep RP-18 column (Merck, Darmstadt, Germany; 20% MeOH to 100% MeOH, gradient) only to afford the phlorotannins. Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany; 0.25 mm). The spots were detected by UV irradiation (254, 365 nm) and by spraying with vanillin-H_2SO_4 reagent. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a Varian VNS600 instrument (Varian, Palo Alto, USA), using DMSO-d_6 solvent peak (2.49 ppm in ^{1}H and 39.7 ppm in ^{13}C NMR) as an internal reference standard. The standard pulse sequences programmed into the instruments were used for each 2D measurement fast atom bombardment mass spectrometry (FAB-MS) spectra were obtained on a Micromass Autospec OA-TOF spectrometer (Micromass, Manchester, UK). All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

2.3. Cell culture

HepG2 (hepatocellular carcinoma cell) cells obtained from the American Type Culture Collection (ATCC; Manassas, USA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco BRL, NY, USA), penicillin (100 U/mL; Gibco BRL) and streptomycin (100 μg/mL; Gibco BRL) under a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Dimethyl sulfoxide (DMSO) was obtained from Junsei Chemical Co. (Tokyo, Japan). LPS was purchased from Sigma–Aldrich (5×10^6 units/mL; MO, USA).

2.4. Cell viability test

Cell viability was assayed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). This assay is based on the ability of a mitochondrial succinate dehydrogenase in viable cells, which catalyzes the conversion of the pale yellow MTT to a dark blue formazan crystal that accumulates within the cells. Briefly, the cells were typically pretreated with LPS (1 μg/mL) for 24 h and then treated with *E. bicyclis* MeOH extract, its solvent extracts, and isolated phlorotannins for 24 h. The treated cells were washed and incubated with MTT solution (1 mg/mL, final concentration) for 3 h. Then the media were removed and the formazan was dissolved in DMSO. The solution was transferred to microtiter plates and the color was quantified by the absorbance at 570 nm (Vo et al., 2011).

2.5. Western blot analysis

The cells were seeded in 6-well culture plates at a density of 1×10^6 cells/well and grown in 2 mL of growth media for 24 h to reach 60–70% confluences. The cells were typically pretreated with LPS (1 μg/mL) for 24 h and then treated with various concentrations of compound (**1**) for 24 h. Treated cells were homogenized in a buffer [1% Triton X-100, 100 mM sodium pyrophosphate, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM phenylmethylsulfonyl fluoride, pH 7.4]. The homogenates were centrifuged at 12,000 × *g* for 20 min at 4 °C and the supernatant was used to determined protein concentration. Protein extracts were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated in a phosphate buffered saline containing 5% skim milk and 0.1% Tween-20 at 4 °C overnight to reduce non-specific binding and then blotted with a iNOS, COX-2, IL-1β, IL-6, and TNF-α antibodies (1:1000; Santa Cruz Biotechnology, CA, USA) according to the manufacturer's instructions. Proteins were visualized using a peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology) and enhanced chemiluminescence (ECL).

Comparing the intensity of bands was measured by densitometric analysis and normalized by staining housekeeping protein, β-actin (Santa Cruz Biotechnology).

2.6. Statistical analysis

Statistical analysis was performed by Student's *t*-test. A value of $P < 0.05$ based on at least three or more independent experiments was considered to be statistically significant.

3. Results and discussion

3.1. Cytotoxicity of methanol extract and its fraction from *E. bicyclis* on LPS-stimulated HepG2 cells

In our search for natural products that possess anti-inflammatory activity, we prepared MeOH crude extracts of *E. bicyclis*. The evaporated MeOH extract was partitioned with Hex, CH_2Cl_2, EtOAc, *n*-BuOH. As shown in Fig. 1, the treatment of *E. bicyclis* MeOH extract and its solvent fractions except *n*-BuOH fraction resulted in significant enhancement in cell viability compared to the cell viability of control (58.6 ± 2.3%). Among the solvent fractions, EtOAc soluble fraction exhibited the lowest cytotoxicity effect (99.4 ± 0.7%) at a concentration of 100 μg/mL. This result indicates that *E. bicyclis* extracts exert considerable hepatoprotective effect in HepG2 cells. According to Yoon et al. (2011b), the EtOAc fraction of *E. bicyclis* exhibited the highest radical scavenging activity and was reported to contain various antioxidant compounds as marine-derived polyphenols, phlorotannins, which are only known in brown algae. The hepatoprotective activities of EtOAc fraction from *E. bicyclis* were evaluated and five phlorotannins were isolated and identified from EtOAc fraction of *E. bicyclis* (Kim et al., 2011) (Fig. 2).

3.2. Isolation of active compounds exhibiting hepatoprotective effects against inflammation

In order to elucidate the anti-inflammation agents from *E. bicyclis* with low cytotoxicity, we isolated active compounds from the EtOAc fraction using the cell viability assay as described in Section 2. The structural analysis using NMR and FAB-MS revealed the compounds were to be phloroglucinol (**1**), eckol (**2**), dioxinodehydroecko (**3**), and dieckol (**4**).

Compound **1** (phloroglucinol): the compound was identified by TLC with an authentic sample.

Compound **2** (eckol): light brown powder, FAB-MS *m/z* 373 $[M+H]^+$. ^1H NMR (DMSO-d_6, 600 MHz): δ 9.46 (1H, s, OH-9), 9.41 (1H, s, OH-4), 9.14 (2H, s, OH-2, 7), 9.11 (2H, s, OH-3′, 5′), 6.14 (1H, s, H-3), 5.96 (1H, d, *J* = 2.4 Hz, H-8), 5.80 (1H, d, *J* = 1.8 Hz, H-6), 5.79 (1H, d, *J* = 3.0 Hz, H-4′), 5.72 (2H, d, *J* = 1.8 Hz, H-2′, 6′). 13C-NMR (DMSO-d_6, 150 MHz): δ 160.6 (C-1′), 159.0 (C-3′, 5′), 153.2 (C-7), 146.3 (C-9), 146.1 (C-2), 142.8 (C-5a), 142.1 (C-4), 137.4 (C-10a), 123.4 (C-1), 122.9 (C-9a), 122.5 (C-4a), 98.7 (C-8), 98.4 (C-3), 96.4 (C-4′), 93.9 (C-2′), 93.8 (C-6), 93.7 (C-6′).

Compound **3** (dioxinodehydroeckol): pale brown powder, FAB-MS *m/z* 371 $[M+H]^+$. ^1H NMR (DMSO-d_6, 600 MHz): δ 9.73 (1H, s, OH-1), 9.59 (1H, s, OH-9), 9.56 (1H, s, OH-6), 9.24 (1H, s, OH-3), 9.23 (1H, s, OH-11), 6.10 (1H, s, H-7), 6.04 (1H, d, *J* = 2.7 Hz, H-2), 6.01 (1H, d, *J* = 2.7 Hz, H-10), 5.84 (1H, d, *J* = 2.7 Hz, H-4), 5.82 (1H, d, *J* = 2.7 Hz, H-12). ^{13}C NMR (DMSO-d_6, 150 MHz): δ 153.3 (C-3), 153.0 (C-11), 146.3 (C-1), 146.1 (C-9), 142.1 (C-4a), 141.7 (C-12a), 140.1 (C-6), 137.2 (C-7a), 131.6 (C-13b), 125.9 (C-5a), 122.6 (C-8a), 122.4 (C-13a), 122.2 (C-14a), 98.8 (C-2, 10), 97.5 (C-7), 93.9 (C-4, 12).

Compound **4** (dieckol): pale brown powder, FAB-MS *m/z* 743 $[M+H]^+$. ^1H NMR (DMSO-d_6, 600 MHz): δ 9.65 (1H, s, OH-9), 9.55 (1H, s, OH-9″), 9.45 (1H, s, OH-4″), 9.40 (1H, s, OH-4), 9.31 (2H, s, OH -3‴, 5‴), 9.23 (1H, s, OH-2″), 9.18 (1H, s, OH-2), 9.17 (1H, s, OH-7″), 9.10 (2H, s, OH-3′, 5′), 6.16 (1H, s, H-3″), 6.14 (1H, s, H-3),

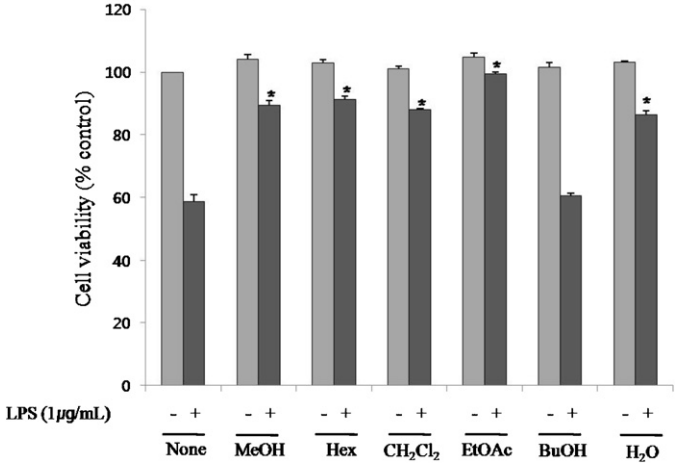

Fig. 1 – Protective effect of *Eisenia bicyclis* methanol extracts and its fractions against lipopolysaccharide (LPS)-stimulated inflammation in HepG2 cells. Cells were stimulated with LPS (1 μg/mL) for 24 h and then treated with *E. bicyclis* methanol extracts and its fractions (100 μg/mL) for 24 h. The data represent the mean ± SD of triplicate experiments.

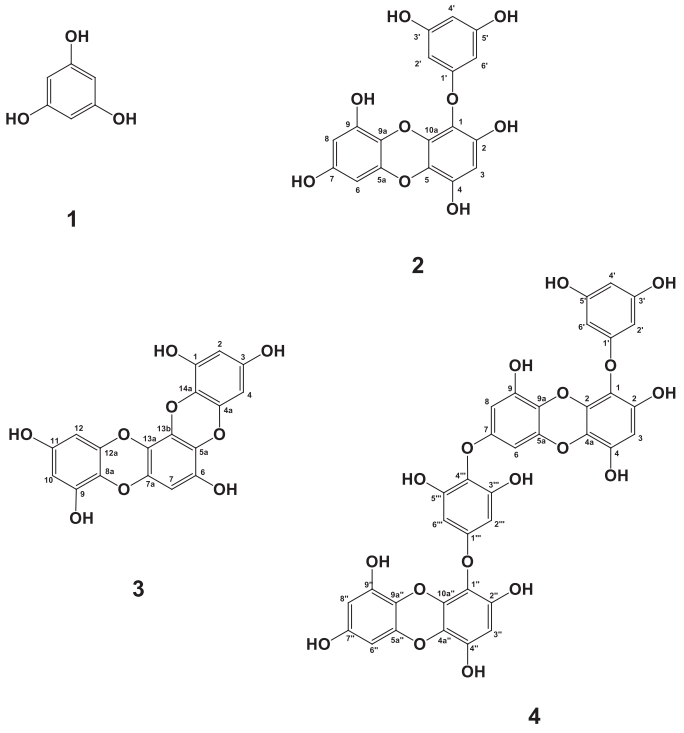

Fig. 2 – Phlorotannins (1–4) isolated from *Eisenia bicyclis*.

6.02 (1H, d, J = 3.0 Hz, H-8), 5.99 (1H, d, J = 3.0 Hz, H-8″), 5.95 (2H, s, H-2‴, 6‴), 5.82 (1H, d, J = 3.0 Hz, H-6), 5.81 (1H, d, J = 3.0 Hz, H-6″), 5.80 (1H, d, J = 1.8 Hz, H-4′), 5.72 (2H, d, J = 1.8 Hz, H-2′, 6′). ^{13}C NMR (DMSO-d_6, 150 MHz): δ 160.2 (C-1′), 158.7 (C-3′) 158.6 (C-5′), 155.8 (C-1‴), 154.2 (C-7), 153.0 (C-7″), 151.1 (C-3‴, 5‴), 146.0 (C-2, 9″), 145.8 (C-2″, 9), 142.5 (C-5a″), 142.3 (C-5a), 141.9 (C-4″), 141.8 (C-4), 137.2 (C-10a), 137.0 (C-10a″), 124.2 (C-4‴), 124.0 (C-9a), 123.2 (C-4a), 123.1 (C-4a″), 122.6 (C-9a″), 122.2 (C-1, 1″), 98.3 (C-3), 98.2 (C-3″), 98.0 (C-8, 8″), 96.1 (C-4′), 94.4 (C-2‴, 6‴), 93.8 (C-6″), 93.6 (C-2′, 6′), 93.5 (C-6).

3.3. *Cytotoxicity effect of phloroglucinol (1) from E. bicyclis on LPS-stimulated HepG2 cells*

All compounds had different cytotoxicity in HepG2 cells within the test concentration. Among isolated compounds from *E. bicyclis*, compound (**1**) was found to be the most active with a lowest cytotoxicity (99.7 ± 0.7%) at a concentration of 100 μg/mL (Fig. 3). The protective activity was in dose dependent manner and no significant difference on cell viability was observed over at a concentration of 100 μg/mL (data not

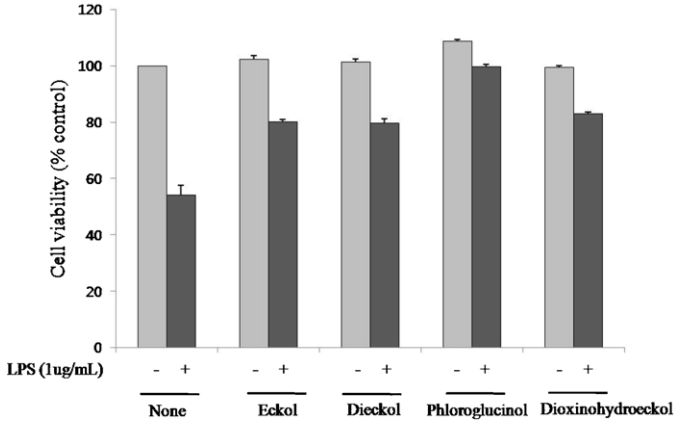

Fig. 3 – Protective effect of phlorotannins isolated from ethyl acetate soluble fraction of *Eisenia bicyclis* methanol extracts against lipopolysaccharide (LPS)-stimulated inflammation in HepG2 cells. The cells were stimulated with LPS (1 μg/mL) for 24 h and then treated with isolated compounds from *E. bicyclis* (100 μg/mL) for 24 h. The data represent the mean ± SD of triplicate experiments.

shown). Considering the results in this study, we suggested that compound (1) from *E. bicyclis* can be a potential marine-derived polyphenol for liver disease prevention.

3.4. *Effect of phloroglucinol isolated from E. bicyclis on the production of cytokines such as IL-1β, IL-6, TNF-α and expression of iNOS and COX-2 in LPS-stimulated HepG2 cells*

Next, we evaluated the inhibitory effect of phlorotannins on the production of cytokines such as IL-1β, IL-6, TNF-α and expression of iNOS and COX-2. It was found that compound (1) exhibited the highest hepatoprotective activity against LPS-stimulated inflammation in HepG2 cell. As shown in Fig. 4, the production of IL-1β, IL-6, and TNF-α were significantly elevated in HepG2 cell by the treatment of LPS (1 μg/mL) compared to unstimulated cells. However, the production of these cytokines decreased by the treatment of compound (1) in dose dependent manner. Moreover, compound (1) was found to be effective against the expression COX-2 and iNOS in LPS-stimulated HepG2 cells. The suppressive effect of compounds on iNOS and COX-2 expression may contribute to the reduction of NO and PGE_2 production in the stimulated cells.

4. Discussion

LPS stimulation has demonstrated to induce iNOS expression in hepatocyte HepG2 cells. According to Zeng et al. (2008), LPS induced the increases of intracellular and extracellular nitric oxide (NO) levels in HepG2 cells. In addition, LPS stimulation is well known to induce inhibitor of kappaB (IκB) proteolysis and nuclear factor kappaB (NF-κB) nuclear translocation (Park et al., 2011; Zeng et al., 2008). NF-κB plays a critical

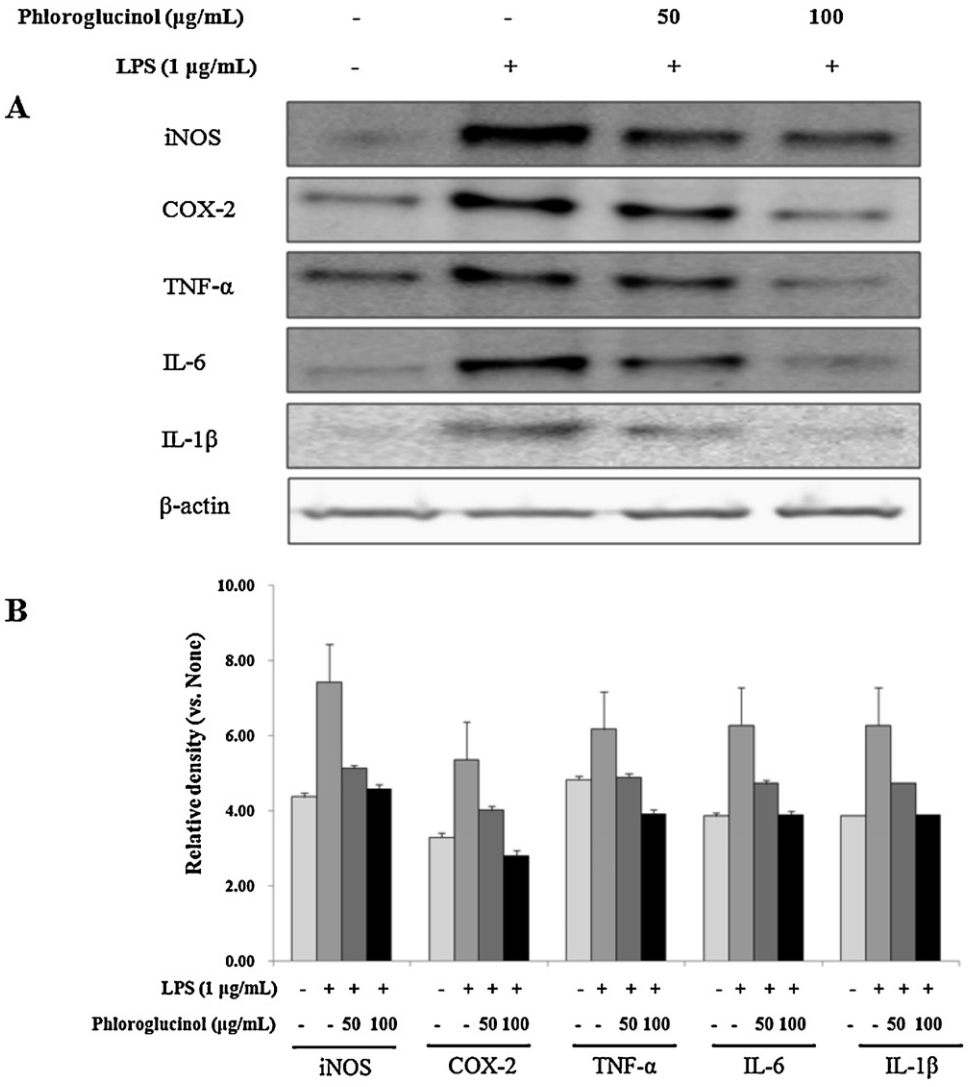

Fig. 4 – Inhibitory effect of phloroglucinol (1) on production of cytokines and expression of inflammation-mediating enzymes in lipopolysaccharide (LPS)-stimulated HepG2 cells. The cells were stimulated with LPS (1 μg/mL) for 24 h and then treated with phloroglucinol (1) (50 or 100 μg/mL) for 24 h. (A) The production of cytokines and expression of inflammation-mediating enzymes in stimulated cells. Whole cell lystates (30 μg) were prepared and the protein was subjected to 12% SDS-PAGE; expression were determined by Western blotting. (B) Values of relative density were normalized to the expression level of untreated cells.

role in the regulation of cell survival genes and coordinates the expressions of proinflammatory enzymes and cytokines such as iNOS, COX-2, IL-1β, IL-6, and TNF-α (Yoon et al., 2009). Therefore, the use of HepG2 cells will be a good model system for screening a candidate in developing anti-inflammatory agents. NO is a free radical with a short lifespan that plays an important role in the physiological and pathophysiological mechanisms in immunological systems. iNOS is one of the major enzymes that generates NO from L-arginine using NADPH and molecular oxygen (Cui et al., 2010; Zeng et al., 2008). In our study, the inhibitory function of compound (**1**) was confirmed by a decrease in iNOS expression level in HepG2 cells, and thus contributing to decrease the production of NO. This assumption is supported by previous reports that the inhibition of NO production is due to suppress iNOS expression (Cui et al., 2010; Heo et al., 2010).

Prostaglandin E_2 (PGE_2) is a primary product of arachidonic metabolism and is synthesized via the cyclooxygenase (COX) and prostaglandin synthase pathways. PGE_2 production is a commonly used method for the detection of COX-2 modulation and prostaglandin synthases. In addition, COX-2 is also a key enzyme in the inflammatory response of tissues to injury or infectious agents. Notably, our results showed that the expression level of COX-2 was suppressed by compound (**1**) treatment. Therefore, it could be suggested that compound (**1**) inhibited of PGE_2 production, through the inhibition of its upstream enzyme COX-2. Similarly, the previous studies have been shown that the suppression of iNOS expression cause inhibition on PGE_2 expression (Kang et al., 2011; Park et al., 2011).

On the other hand, the importance of LPS-induced proinflammatory cytokine production in liver injury is evident from numerous models of acute and chronic liver disease. Proinflammatory cytokines such as IL-1β, IL-6, and TNF-α play an essential role during the inflammatory process and liver damage. In the present study, treatment with compound (**1**) cells was effective in suppressing the production of IL-1β, IL-6, and TNF-α by LPS stimulation in HepG2 hepatocytes. These cytokines have profound effects on several cells, secrete a variety of inflammatory mediators, creating complex networks of interactions and leading to multiple inflammatory cascades (Koj and Jura, 2003). Likewise, previous studies have revealed that phloroglucinol exhibited inhibitory effects on the production of inflammatory mediators such as IL-1β, IL-6, and TNF-α in Raw264.7 cells (Kim and Kim, 2010).

The expressions of these pro-inflammatory mediators are modulated by NF-κB. Our results showed that compound (**1**) possesses the inhibitory effect on protection of cytokines and expression of inflammation-mediating enzyme. Thus, it was suggested that the inhibitory activity of compound (**1**) is due to blocking the activation of NF-κB. Many studies have determined that the inactivation of NF-κB lead to suppress the production of inflammatory mediators (Kim et al., 2010; Park et al., 2011).

Taken together, our results demonstrated that compound (**1**) inhibited the production of cytokines in LPS-activated HepG2 hepatocytes. Moreover, these anti-inflammatory profiles of compound (**1**) were mediated through the inhibition of iNOS and COX-2 expression. Accordingly, compound (**1**) seems appropriate as a potential anti-inflammatory agent via attenuation of infectious liver inflammation for developing effective treatment strategies.

5. Conclusion

In this study, phlorotannins isolated from *E. bicyclis* including phloroglucinol (**1**), eckol (**2**), dioxinodehydroeckol (**3**), and dieckol (**4**) were determined for the their protective effect against LPS-stimulated inflammation in HepG2 cells. Phloroglucinol (**1**) was found that the strongest inhibitor of inflammatory response via suppressing the production of cytokines and expression of inflammation-mediating enzymes including COX-2 and iNOS. Collectively, this study indicates the potential development of phlorotannins as beneficial hepatoprotective agents for functional food and pharmaceutical industry.

Conflict of interest statement

There is no conflict of interest among authors.

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