

Research Article

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5 β -Hydroxypalisadin B isolated from red alga *Laurencia snackeyi* attenuates inflammatory response in lipopolysaccharide-stimulated RAW 264.7 macrophages

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In this study, four compounds isolated from the red alga *Laurencia snackeyi* were evaluated for their potential anti-inflammatory effect in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. These compounds were tested for their inhibitory effects on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. Since 5 β -hydroxypalisadin B showed the best activity it was further tested for the production of prostaglandin-E₂ (PGE₂), expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), the release of pro-inflammatory cytokines tumor necrotic factor-alpha (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6). 5 β -Hydroxypalisadin B significantly reduced the PGE₂ release and suppressed the iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. It also significantly reduced the release of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6. These findings provide the first evidence of anti-inflammatory potential of 5 β -hydroxypalisadin B isolated from the red alga *L. snackeyi* and hence, it could be exploited as an active ingredient in pharmaceutical, nutraceutical and functional food applications.

Key Words: anti-inflammation; bioactive; functional ingredient; *Laurencia snackeyi*; macrophage; secondary metabolite

INTRODUCTION

Since the dawn of 21st century, there has been an increase in the search for natural bioactive compounds as lead metabolites for pharmaceutical, cosmeceutical, and functional food industry (Heo et al. 2010, Lee et al. 2013b). This is partly due to the increase in health awareness and stringent regulations pertaining to the addition of synthetic chemicals in food and pharmaceuticals. This was further accelerated with the findings of undesirable

side effects related to the use of synthetic additives in food and nutraceuticals. Hence, there has been a steady increase in scientific findings to demonstrate possible applications of plant based active components as functional ingredients in various industrial applications (Tripathi et al. 2013). One of the focus areas of scientific research is the discovery of lead pharmaceutical with anti-inflammation properties.



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Tissue inflammation is a complex biological response to harmful stimuli. It is also a protective attempt to remove the injurious stimuli and initiate the healing process of the tissue. In these cases, macrophages play a key role in inflammation (Kim et al. 2006). Anti-inflammatory agents can be used to treat inflammatory reactions and most of these agents act by preventing the release of inflammatory mediators or inhibiting the action of released mediators on their target cells. Today, there is an urgent need to explore anti-inflammatory chemicals with less toxicity. In sight of this, intensive research is being carried out to explore and discover non-toxic anti-inflammatory agents. Researchers are venturing into marine resources to discover anti-inflammatory bioactive agents (Mohsin and Kurup 2011).

Marine organisms are known to produce a wide diversity of unprecedented bioactive secondary metabolites. One of the unique metabolites produced by the marine organisms is halogenated secondary metabolites. Red algae genus *Laurencia* is known to incorporate halogen atoms like bromine and chlorine, which are not readily available to terrestrial species (Suzuki et al. 2002). Members of the algal genus *Laurencia* produce a rich and diverse range of secondary metabolites including sesquiterpenes, diterpenes, and triterpenes (Suzuki et al. 2002, Tan et al. 2011). These halogenated secondary metabolites are well known to exhibit biological properties such as antibacterial and anti-biofouling (Vairappan 2003, Suzuki and Vairappan 2005, Vairappan et al. 2008).

In our previous work, we reported the isolation and characterization of sesquiterpenes from *L. snackeyi* as chemotaxonomical markers (Tan et al. 2011). However, the biological properties of these secondary metabolites have not been reported in details. Therefore, as part of an effort to search for bioactive natural products, we examined the anti-inflammatory properties of the isolated compounds in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Hence, based on this investigation we introduce 5 β -hydroxypalisadin B as a potential anti-inflammatory agent. Findings from this investigation could suggest the possibility of using natural chemicals such as 5 β -hydroxypalisadin B as functional ingredients.

MATERIALS AND METHODS

Halogenated secondary metabolites

Red alga, *L. snackeyi* (Weber-van Bosse) Masuda was collected at the depth of 5 meters by scuba divers at Pu-

lauSulug Island, Kota Kinabalu, Sabah, Malaysia. The voucher specimen (BORH2010012) was deposited in the BORNEENSIS Collection of Institute for Tropical Biology and Conservation, University Malaysia Sabah. Collected specimens were cleaned off epiphytes, sand and organic debris, brought to the laboratory under 4°C in a chiller. In the laboratory the algae were rinsed in three exchanges of double distilled water and subjected to air-drying under 24°C away from direct sunlight. Partially dried algal thallus (220 g) was extracted with MeOH for 5 days. The MeOH solution was concentrated in vacuo and partitioned between Et₂O and H₂O. The Et₂O solution was washed with water, dried over anhydrous Na₂SO₄, and evaporated to leave dark green oil (1.9 g). Chemical profiling of the crude extract was done by spotting crude extract on SiO₂ gel F₂₅₄ thin layer chromatography (TLC) and developed in toluene (100%) and hexane : EtOAc (3 : 1) solvent systems, visualized by UV light (254 nm) and molybdophosphoric acid and heated. The crude extract (1.0 g) was then fractionated by Si gel column chromatography with a step gradient (hexane / EtOAc in the ratio of 9 : 1, 8 : 2, 7 : 3, 5 : 5 and 100% EtOAc). Fraction eluted with hexane-EtOAc (9 : 1) was subjected to preparative TLC with toluene to give LS-F (8.0 mg, 0.8%). Fraction eluted with hexane-EtOAc (8 : 2) was subjected to preparative TLC with toluene to give 3 (18.7 mg, 1.9%), 2 (33.7 mg, 3.4%), and 4 (18.2 mg, 1.8%). Fraction eluted with hexane-EtOAc (5 : 5) was subjected to preparative TLC with CHCl₃ to give 1 (10.3 mg, 1.0%). Yields of the respective compounds were calculated based on the weight of the crude extracts. Compounds 1 to 4 were subjected to ¹H-NMR, ¹³C-NMR, and 2D NMR measurements such as ¹H-¹H COSY, HSQC, HMBC, and NOESY. ¹H-NMR (600 MHz) and ¹³C-NMR (150 MHz) spectra were recorded with a JEOL ECA600 instrument (JEOL, Tokyo, Japan), with tetramethyl silane as internal standard. High-resolution mass spectroscopy data was measured using Shimadzu LCMS-IT-TOF (Shimadzu, Tokyo, Japan). Optical rotations were measured on an AUTOPOL IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). Other physical characteristics were obtained as described by Kamada and Vairappan (2012).

Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The RAW 264.7 cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 100 U mL⁻¹ of penicillin, 100 μ g mL⁻¹ of streptomycin and 10% fetal

bovine serum. The cells were incubated and maintained in an atmosphere of 5% CO₂ at 37°C.

Determination of nitric oxide (NO) production

RAW 264.7 cells (1×10^5 cells mL⁻¹) were plated in a 24-well plate and after 16 h the cells were pre-incubated with the purified compounds at 37°C for 1 h. Then further incubated for another 24 h with LPS (1 µg mL⁻¹) at the same temperature. Then, quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production (Lee et al. 2007). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the optical density at 540 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader. The fresh culture medium was used as a blank in every experiment.

Lactate dehydrogenase (LDH) cytotoxicity assay

RAW 264.7 cells (1.5×10^5 cells mL⁻¹) were plated in 96-well plate and after 16 h the cells were pre-incubated with the purified compounds for 1 h at 37°C. Then the cells were further incubated for another 24 h with LPS (1 µg mL⁻¹) at the same temperature. After the incubation, LDH level in the culture medium was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Determination of prostaglandin E₂ (PGE₂) production

RAW 264.7 cells (1×10^5 cells well⁻¹) were pretreated with 5β-hydroxypalisadin B for 2 h and then treated with LPS (1 µg mL⁻¹) to allow cytokine production for 24 h. The PGE₂ levels in the culture medium were quantified using a competitive enzyme immunoassay kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The release of PGE₂ was measured relative to that of the control value.

Western blot analysis

RAW 264.7 cells (1.0×10^6 cells mL⁻¹) were pre-incubated for 16 h and then treated with LPS (1 µg mL⁻¹) in the presence or absence of 5β-hydroxypalisadin B. After in-

cubation for 24 h, the cells were harvested, washed twice with ice-cold phosphate-buffered saline, and the cell lysates were prepared with lysis buffer (50 mM L⁻¹ Tris-HCl [pH 7.4], 150 mM L⁻¹ NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], and 1 mM L⁻¹ EDTA) for 20 min on ice. Cell lysates were centrifuged at 14,000 ×g for 20 min at 4°C. Then protein contents in the supernatant were measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Cell lysates (30-50 µg) were subjected to electrophoresis in SDS-polyacrylamide gels (8-12%), and the separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was pre-incubated with blocking solution (5% skim milk in Tris buffered saline containing Tween-20) for 90 min at room temperature. Then the membrane incubated with anti-mouse inducible nitric oxide synthase (iNOS; 1 : 1,000; Calbiochem, La Jolla, CA, USA) and anti-mouse cyclooxygenase-2 (COX-2; 1 : 1,000; BD Biosciences Pharmingen, San Jose, CA, USA) for overnight at room temperature. After washing, the blots were incubated with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (1 : 5,000; Amersham Pharmacia Biotech, Little Chalfont, UK) for 90 min at room temperature. The bands were visualized on X-ray film using ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

Determination of pro-inflammatory cytokines (tumor necrotic factor-alpha [TNF-α], interleukin [IL]-1β, and IL-6) production

The inhibitory effect of the sample on the production of pro-inflammatory cytokines from LPS stimulated RAW 264.7 cells was determined according to a previously described method (Cho et al. 2000). Briefly, RAW 264.7 cells (1×10^5 cells mL⁻¹) were pretreated with 5β-hydroxypalisadin B for 2 h and then treated with LPS (1 µg mL⁻¹) to allow production of pro-inflammatory cytokines for 24 h. Supernatants were used for the assay using an ELISA kit (R & D Systems) according to the manufacturer's instructions.

Statistical analysis

All the data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Duncan's multiple range test. p-values of less than 0.05 (p < 0.05) were considered as significant.

RESULTS AND DISCUSSION

Red algae belonging to the genus *Laurencia* (family Ceramiales) are a rich source of halogenated compounds of sesquiterpene, diterpene, C₁₅-acetogenin and bromoalene skeletons. To date, diverse variety of structurally unusual secondary metabolites have been isolated from red algal genus *Laurencia*. These metabolites are mostly halogenated with bromine (Br) and chlorine (Cl) atoms (Mao and Guo 2010). Halogenated compounds have been reported to possess multiple biological properties such as antioxidant and antibacterial activities (Vairappan et al. 2008). The novelty of halogenated metabolite structures and the potential biological significance of these chemicals have provoked the present investigation. In this investigation, we evaluated the possible anti-inflammatory potentials of four secondary metabolites isolated from the red alga *L. snackeyi*. Isolated compounds could be categorized into three halogenated snydererans (1, 2, 3) and one non halogenated sesquiterpene (4).

Identification of halogenated secondary metabolites

A total of 220 g of partially air-dried *L. snackeyi* gave 0.9% of dark green oily lipid crude extract. Crude extract was subjected to SiO₂ gel column chromatography, preparative thin layer chromatography and high performance liquid chromatography (HPLC) to obtain four pure halogenated metabolites. These were further subjected to ¹H-NMR, ¹³C-NMR, ¹H-¹H-COSY, HSQC, HMBC, NOESY, and high resolution mass spectroscopy (LCIT-TOFMS), spectroscopy experiments. Their spectroscopic data are given below to supplement assignments in the original spectroscopic data published:

5β-Hydroxypalisadin B (1): C₁₅H₂₄O₂Br₂, colourless oil, [α]_D²⁸-12.0° (CHCl₃; 1.0), ¹H-NMR (CDCl₃, 600 MHz) δ: 1.14 (3H, s, H₃-15), 1.20 (3H, s, H₃-14), 1.52 (1H, ddd, *J* = 3.6, 3.6, 12.6 Hz, H-8), 1.60 (1H, s, H-6), 1.67 (3H, s, H₃-13), 1.78 (3H, s, H₃-12), 1.84 (1H, ddd, *J* = 4.5, 12.6, 12.6 Hz, H-8), 2.24 (1H, m, H-9), 2.28 (1H, m, H-9), 3.43 (1H, dd, *J* = 7.8, 10.5 Hz, H-1), 3.68 (1H, dd, *J* = 3.0, 10.5 Hz, H-1), 3.84 (1H, dd, *J* = 5.1, 12.0 Hz, H-10), 4.46 (1H, br s, H-2), 4.46 (1H, br s, H-5), 5.91 (1H, d, *J* = 6.3 Hz, H-4). ¹³C-NMR (CDCl₃, 150 MHz) δ: 35.6 (t, C-1), 68.4 (d, C-2), 140.8 (s, C-3), 132.9 (d, C-4), 70.8 (d, C-5), 56.0 (d, C-6), 78.2 (s, C-7), 38.4 (t, C-8), 33.0 (t, C-9), 66.2 (d, C-10), 41.4 (s, C-11), 21.0 (q, C-12), 25.7 (q, C-13), 18.9 (q, C-14), 30.7 (q, C-15).

Palisadin B (2): C₁₅H₂₄OBr₂, colourless oil, [α]_D²⁸ + 7.8°

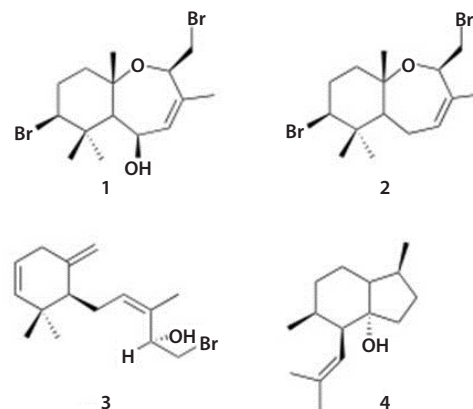


Fig. 1. Chemical structures of the isolated compounds from *Laurencia snackeyi*. 5β-Hydroxypalisadin B (1), palisadin B (2), palisol (3), and pacifigorgiol (4).

(CHCl₃; 2.0), ¹H-NMR (CDCl₃, 600 MHz) δ: 0.95 (3H, s, H₃-14), 1.15 (3H, s, H₃-15), 1.36 (3H, s, H₃-13), 1.69 (3H, s, H₃-12), 1.77 (2H, m, H-8), 1.77 (1H, m, H-6), 2.05 (2H, m, H-5), 2.25 (2H, m, H-9), 3.41 (1H, dd, *J* = 11.0, 7.0 Hz, H-1β), 3.73 (1H, dd, *J* = 11.0, 3.0 Hz, H-1α), 3.95 (1H, dd, *J* = 12.0, 5.0 Hz, H-10), 4.54 (1H, br s, H-2), 5.63 (1H, d, *J* = 8.0 Hz, H-4). ¹³C-NMR (CDCl₃, 150 MHz) δ: 36.2 (t, C-1), 70.7 (d, C-2), 136.1 (s, C-3), 129.4 (d, C-4), 25.9 (t, C-5), 52.8 (d, C-6), 77.5 (s, C-7), 36.7 (t, C-8), 32.9 (t, C-9), 66.3 (d, C-10), 40.8 (s, C-11), 21.0 (q, C-12), 22.0 (q, C-13), 17.9 (q, C-14), 30.7 (q, C-15).

Palisol (3): C₁₅H₂₃OBr, colourless oil, [α]_D²⁸ + 4.6° (CHCl₃; 0.5), ¹H-NMR (CDCl₃, 600 MHz) δ: 0.94 (3H, s, H₃-14), 1.01 (3H, s, H₃-15), 1.70 (3H, s, H₃-12), 2.00 (1H, m, H-6), 2.00 (1H, m, H-5), 2.31 (1H, m, H-5), 2.66 (2H, br s, H-8), 3.38 (1H, dd, *J* = 9.0, 5.0 Hz, H-1), 3.49 (1H, dd, *J* = 9.0, 9.0 Hz, H-1), 4.69 (1H, s, H-13), 4.73 (1H, ddd, *J* = 9.0, 5.0, 1.0 Hz, H-2), 4.90 (1H, s, H-13), 5.33 (1H, m, H-4), 5.38 (1H, ddd, *J* = 10.0, 1.0, 1.0 Hz, H-10), 5.51 (1H, ddd, *J* = 10.0, 4.0, 4.0 Hz, H-9). ¹³C-NMR (CDCl₃, 150 MHz) δ: 38.4 (t, C-1), 71.0 (d, C-2), 133.8 (s, C-3), 130.5 (d, C-4), 25.6 (t, C-5), 53.5 (d, C-6), 146.0 (s, C-7), 33.1 (t, C-8), 123.7 (d, C-9), 137.5 (d, C-10), 37.8 (s, C-11), 18.2 (q, C-12), 110.7 (t, C-13), 25.9 (q, C-14), 31.0 (q, C-15).

Pacifigorgiol (4): C₁₅H₂₆O, colourless oil, [α]_D²⁸ + 41.0° (CHCl₃; 0.5), ¹H-NMR (CDCl₃, 600 MHz) δ: 0.77 (3H, d, *J* = 7.0 Hz, H₃-14), 0.97 (3H, d, *J* = 6.0 Hz, H₃-15), 1.07 (1H, m, H-8β), 1.07 (1H, m, H-4α), 1.22 (1H, m, H-4β), 1.33 (1H, m, H-5β), 1.43 (1H, m, H-6), 1.61 (1H, m, H-5α), 1.60 (1H, m, H-9β), 1.60 (3H, s, H₃-13), 1.74 (3H, s, H₃-12), 1.97 (1H, m, H-7), 1.97 (1H, m, H-9α), 2.06 (1H, m, H-3), 2.06 (1H, m, H-8α), 2.54 (1H, dd, *J* = 10.5, 4.0 Hz, H-2), 5.07 (1H, d, *J* =

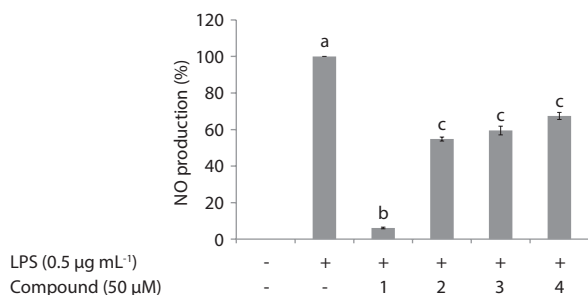


Fig. 2. Inhibitory effect of the compounds on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages. Incubation of the compounds with cells in response to LPS for 24 h; the NO levels in the medium were measured. Values are presented as mean \pm standard deviation of three determinations. Values are compared with the control (no compound) and the different alphabets (a-c) on the values are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test (Duncan's multiple range test). 5 β -Hydroxypalisadin B (1), palisadin B (2), palisol (3), and pacifigorgiol (4).

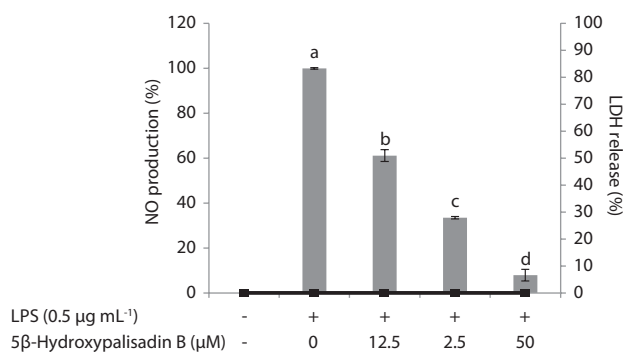


Fig. 3. Effect of 5 β -hydroxypalisadin B on lipopolysaccharide (LPS)-induced nitric oxide (NO) production and lactate dehydrogenase (LDH) release in RAW 264.7 macrophages. Incubation of 5 β -hydroxypalisadin B with cells in response to LPS for 24 h; the NO levels in the medium were measured. Values are presented as mean \pm standard deviation of three determinations. Values are compared with the control (without the sample) and the different alphabets (a-d) on the values are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test.

10.5 Hz, H-10). ¹³C-NMR (CDCl₃, 150 MHz) δ : 83.9 (s, C-1), 47.8 (d, C-2), 29.6 (d, C-3), 34.9 (t, C-4), 24.0 (t, C-5), 49.4 (d, C-6), 30.3 (d, C-7), 34.9 (t, C-8), 30.3 (t, C-9), 121.2 (d, C-10), 134.4 (s, C-11), 26.3 (q, C-12), 18.4 (q, C-13), 19.4 (q, C-14 or C-15), 19.0 (q, C-15 or C-14).

Independent structural elucidation and comparison of spectroscopic data with publish data, lead to the identity of the isolated metabolites as 5 β -hydroxypalisadin B (1), palisadin B (2), palisol (3), and pacifigorgiol (4) (Paul and Fenical 1980, Kuniyoshi et al. 2001, Suzuki and Vairappan

2005). The final confirmations of the compounds were made based on their HRMS and optical rotation values and were similar to the ones in the published documents. In addition, the purities of the compounds were determined via HPLC and ¹H-NMR to be >99%. The chemical structures of the isolated compounds are shown in Fig. 1.

Effect of the compounds on LPS-induced NO production

NO is usually generated by macrophages as part of the human immune responses. The chronic expression of NO could also be associated with various carcinomas and inflammatory conditions. In addition, NO production could also be increased by the production of iNOS under pathological conditions (Heo et al. 2010). Therefore, it is clear that inhibition of NO production may have therapeutic value over inflammatory diseases.

Initially, anti-inflammatory properties of the compounds were assessed via inhibitory effect of NO production in RAW 264.7 macrophages. All the four compounds significantly inhibited NO production in the tested cell line. In contrast, stimulation of the cells with LPS resulted in an enhancement of NO concentration in the medium. However, pretreatment of RAW 264.7 cells with the compounds decreased the NO production in different magnitudes. Fig. 2 illustrates the relative effects of the compounds on NO production.

In comparison, 5 β -hydroxypalisadin B (1) exhibited the strongest inhibitory effect on NO production among the tested compounds. Furthermore, LPS-induced NO production was dose-dependently decreased by 5 β -hydroxypalisadin B (1) with a maximum of 90% inhibition observed at the concentration of 50 μ M (Fig. 3). Moreover, the IC₅₀ value was recorded as 17.56 μ M. In addition, LDH assay confirmed that 5 β -hydroxypalisadin B (1) did not affect cell viability at the tested concentrations. Therefore, the inhibitory effect of 5 β -hydroxypalisadin B (1) on NO production does not appear to be due to a cytotoxic effect on RAW 264.7 cells.

Since 5 β -hydroxypalisadin B exhibited the best activity as compared to the other three compounds, further experiments were conducted to determine its potential as an anti-inflammatory agent. It is also noteworthy to state that both compounds 1 and 2 have the same basic structure. The difference is just in the presence of hydroxyl moiety in compound 1. Significant increase in the anti-inflammation activity could be attributed to the presence of hydroxyl functionality.

Effect of 5β-hydroxypalisadin B on LPS-induced PGE₂ production

PGE₂ is the most abundant prostanoid in humans and involved in regulating many different fundamental biological functions (Nakagawa 2011). In addition to their important mediator role in the inflammatory process, prostaglandins play a pivotal role in maintaining the ho-

meostasis of various tissues (Kang et al. 2011, Wijesinghe et al. 2013). Induction of COX-2 activity and subsequent generation of PGE₂ are closely related to the NO production (Chang et al. 2006). Therefore, with the profound inhibitory effect on NO production exhibited by 5β-hydroxypalisadin B was further evaluated for its ability to inhibit the LPS-induced PGE₂ production in RAW 264.7 macrophages.

Inhibition of PGE₂ production in LPS-stimulated RAW 264.7 cells was assessed by measuring PGE₂ in culture medium harvested from the cells treated with or without 5β-hydroxypalisadin B (1) and LPS. LPS markedly increased PGE₂ production, compared with control cells. However, cells pretreated with 5β-hydroxypalisadin B (1) slightly inhibited LPS-induced PGE₂ production in a dose-dependent manner (Fig. 4). 5β-Hydroxypalisadin B (1) showed no inhibition at the concentration of 12.5 μM. However, 5β-hydroxypalisadin B (1) inhibited the LPS-induced PGE₂ release by 37.4% at 50 μM. Nevertheless, the inhibitory effect of 5β-hydroxypalisadin B (1) on PGE₂ release was not as potent as the inhibitory effect on NO production.

Our results indicated that 5β-hydroxypalisadin B could induce the anti-inflammatory activity by inhibiting the PGE₂ production in RAW 264.7 macrophages. As previously reported fucoxanthin and a polysaccharide isolated from brown seaweed *Ecklonia cava* significantly suppressed the release of PGE₂ in RAW 264.7 cells (Heo et al. 2010, Kang et al. 2011).

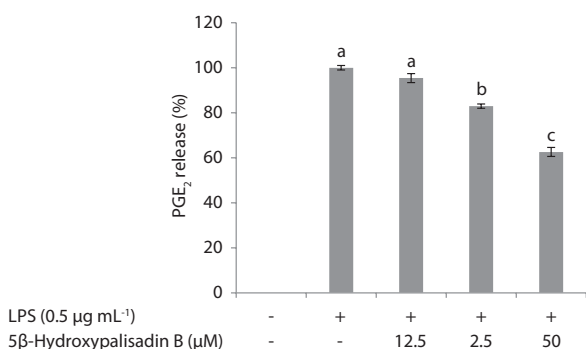


Fig. 4. Effect of 5β-hydroxypalisadin B on lipopolysaccharide (LPS)-induced prostaglandin-E₂ (PGE₂) production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of 5β-hydroxypalisadin B, the concentrations of PGE₂ in the medium were measured. Values are presented as mean ± standard deviation of three determinations. Values are compared with the control (without the sample) and the different alphabets (a-c) on the values are significantly different at p < 0.05 as analyzed by Duncan's multiple range test.

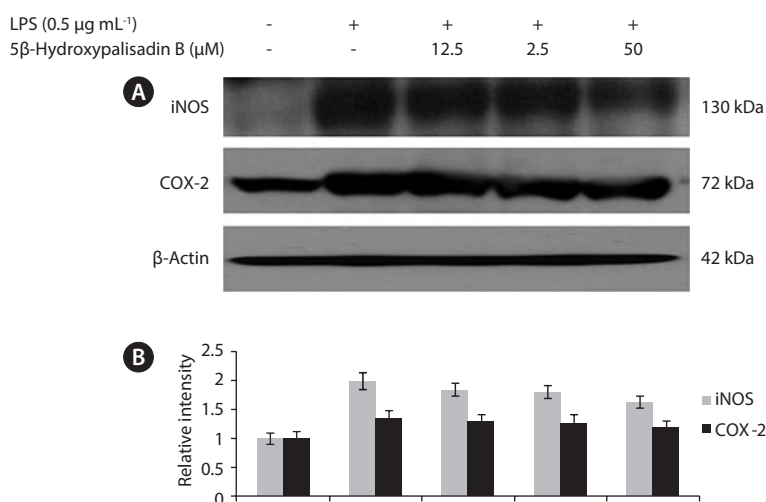


Fig. 5. (A) Effect of 5β-hydroxypalisadin B on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression in RAW 264.7 macrophages. The cells were incubated with LPS for 24 h in the presence or absence of 5β-hydroxypalisadin B. Cell lysates were electrophoresed and the expression levels of iNOS and COX-2 were detected with specific antibodies. (B) Signal intensities of the target proteins were determined using densitometric analysis. Values are presented as mean ± standard deviation of three determinations.

Effect of 5 β -hydroxypalisadin B on LPS-induced iNOS and COX-2 protein expression

In order to determine the effect of 5 β -hydroxypalisadin B (1) on the expression of iNOS and COX-2, the inhibitory effects were investigated using Western blot analysis. Fig. 5 showed the influence of 5 β -hydroxypalisadin B (1) on iNOS and COX-2 protein expression in RAW 264.7 macrophages. The iNOS and COX-2 protein expressions were markedly increased when the macrophages were treated only with LPS compared to the control. However, 5 β -hydroxypalisadin B (1) dose-dependently suppressed iNOS and COX-2 protein expressions as indicated in the figure, though it was not prominent in COX-2.

Inhibition of iNOS, the enzyme mediating macrophage NO production has been shown to block prostaglandin release in RAW 264.7 macrophages (Ahmad et al. 2002). In addition, COX-2 enzymatic activity catalyzes the first committed step in prostaglandin synthesis (Savonenko et al. 2009). Some of the previous reports demonstrated that certain active compounds might have the potential to affect NO production and iNOS enzyme activity (Chang et al. 2006). Our results demonstrated that the iNOS and COX-2 protein expressions were markedly decreased when the macrophages was treated with 5 β -hydroxypalisadin B. Therefore, it could be suggested that 5 β -hydroxypalisadin B inhibited the NO production by decreasing both iNOS and COX-2 protein expression in RAW 264.7 cells.

Effect of 5 β -hydroxypalisadin B on production of LPS-induced pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6)

Since 5 β -hydroxypalisadin B (1) exhibited inhibition against pro-inflammatory mediators, such as NO, PGE₂, and iNOS, its effects on pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 were further investigated. Pretreatment of macrophages with 5 β -hydroxypalisadin B (1) considerably inhibited the production of cytokines TNF- α , IL-1 β , and IL-6 in a similar pattern (Fig. 6A-C). Release of the cytokines was significantly influenced by 5 β -hydroxypalisadin B (1) and suppression of the release of cytokines by 5 β -hydroxypalisadin B (1) showed a concentration dependent profile. Possibly 5 β -hydroxypalisadin B (1) exerts anti-inflammatory effects like decreasing NO and / or PGE₂ productions by down-regulating the expression level of pro-inflammatory mediators such as iNOS and / or COX-2 or pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 in LPS stimulated macrophages.

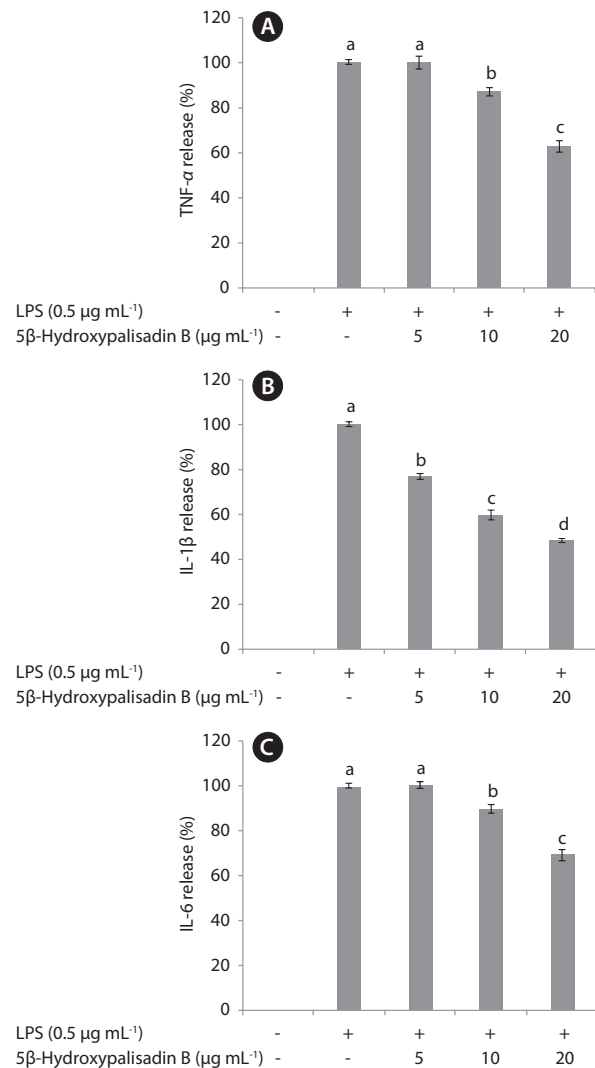


Fig. 6. Effect of 5 β -hydroxypalisadin B on lipopolysaccharide (LPS)-induced tumor necrotic factor-alpha (TNF- α) (A), interleukin (IL)-1 β (B), and IL-6 (C) production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of 5 β -hydroxypalisadin B, the concentrations of the pro-inflammatory cytokines in the medium were measured. Values are presented as mean \pm standard deviation of three determinations. Values are compared with the control (without the sample) and the different alphabets (a-d) on the values are significantly different at p < 0.05 as analyzed by Duncan's multiple range test.

Pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α , which are mainly produced by activated monocytes or macrophages, stimulate bone resorption and also enhance the production of PGE₂ in several types of cells (Hernández-Ledesma et al. 2009). It is well known that a high concentration of pro-inflammatory cytokines plays a critical role in the induction of iNOS through activation of nuclear factor- κ B. The endogenous production of TNF- α contributes to the induction of iNOS in response

to inflammatory stimuli (Lee et al. 2013a). Therefore, identification of the inhibitors of iNOS as therapeutically important agents is essential (Toma et al. 2011). In this study, our results showed that 5 β -hydroxypalisadin B inhibited the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α up to a significant extent, suggesting that the suppression of iNOS by 5 β -hydroxypalisadin B might be associated with the attenuation of the cytokine release. Since the inflammatory stimulators such as LPS induce cytokines in the process of macrophage activation, the inhibition of cytokine production or function is a key mechanism in the control of inflammation (Kang et al. 2011, Lee et al. 2011).

CONCLUSION

In this study, we reported the first results regarding the anti-inflammatory activity of 5 β -hydroxypalisadin B, a brominated secondary metabolite isolated from the red algae *L. snackeyi*. 5 β -Hydroxypalisadin B suppressed the NO production, iNOS and COX-2 expression and cytokine release in RAW 264.7 macrophages. Thus, the findings of this study may facilitate awareness about anti-inflammatory properties of *L. snackeyi* and help future developments in pharmaceuticals, nutraceuticals and functional foods from this relatively underutilized resource.

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