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Antioxidant and anti-inflammatory effects in lipopolysaccharide-induced THP-1 cells of coumarins from the bark of *Hesperethusa crenulata* R.

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Abstract

All parts of Thanakha (*Hesperethusa crenulata* R.) have been used as traditional skin care herbal material in Myanmar. In this study, coumarins from *H. crenulata* R. bark were isolated through solvent extraction, systematic solvent fractionation, and repeated column chromatography. Spectroscopic analyses using ESI-MS, 1D NMR (1H and 13C), 2D NMR (gHSQC and gHMBC), specific rotation, circular dichroism, and IR spectrometry revealed three coumarins 2R-7-hydroxy-8-(2,3-dihydroxy-3-methylbutyl)-coumarin (compound **1**), peucedanol (compound **2**), and methylpeucedanol (compound **3**), which were first isolated from Thanakha tree. Antioxidant capacities of three coumarins decreased as follows: compound **2** > compound **3** > compound **1**. Treatments of lipopolysaccharide-induced THP-1 human monocytic cells with compounds **2** and **3** at 378.8 μ M and 359.7 μ M inhibited tumor necrosis factor- α production by approximately 32.7% and 13.3%, respectively, compared with the negative control. In summary, these results suggest that Thanakha bark extracts can be used as a potent antioxidant and anti-inflammatory source for cosmetic ingredients.

Keywords: Antioxidant capacity, *Hesperethusa crenulata* R., Methylpeucedanol, Peucedanol, Tumor necrosis factor- α

Introduction

Hesperethusa crenulata R. (Thanakha) is distributed in Southeast and South Asia, Myanmar, India, Malaysia, and Sri Lanka [1]. Thanakha paste made from the pulp of *H. crenulata* has been used for cosmetic purposes in Myanmar for over 2000 years. The traditional cream is applied to the face and arms for cooling and protection from sunburn [2]. For that reason, several cosmetic companies located at Myanmar have used Thanakha as an ingredient of cosmetic products [1, 3, 4].

Characteristic features of *H. crenulata* physiology are aromatic volatile oil and a variety of secondary metabolites including coumarins, steroids, indoles, benzoquinones, and flavonoids [1, 5, 6]. Also, several studies have reported that *H. crenulata* extract exhibits various biological activities such as antioxidant, anti-inflammatory, and anti-coagulant effects [1, 5, 7, 8]. A monophenol peucedanol was previously reported to show free-radical-scavenging activity and inhibition of lipid peroxidation [9–11]. Peucedanol inhibited the activities of cholinesterases such as acetylcholinesterase and butyrylcholinesterase [11]. Lipopolysaccharides (LPS) is a component of Gram-negative bacteria that induces an inflammatory response to ultimately lead to production of cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-1. Coumarins isolated

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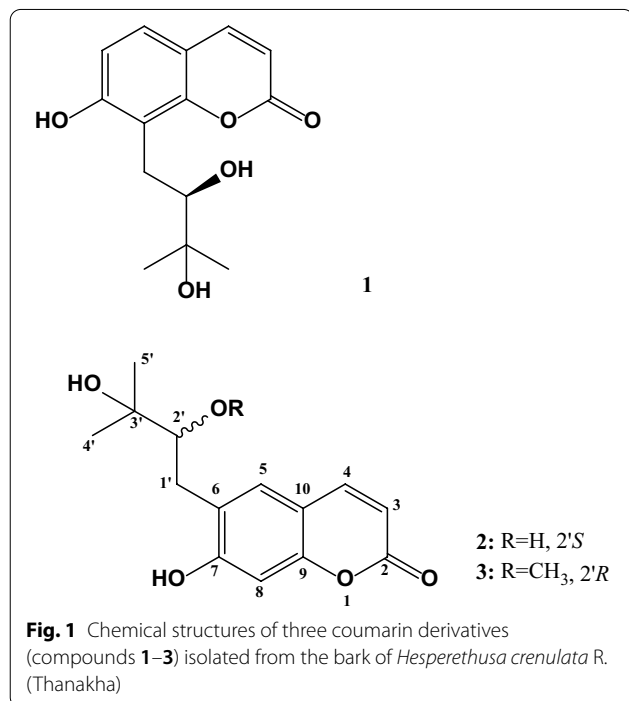
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from the bark of *H. crenulata* R. have been reported to inhibit TNF- α production by blocking activation of nuclear factor kappa B and mitogen-activated protein kinases in LPS-activated THP-1 cells [12, 13].

This study was performed to discover principal components to play a role as functional cosmetic materials. The reason for focusing on coumarin compounds is that they have excellent anti-inflammatory effect reported as above. So skin improvement effect was also expected. And structural feature is as a benzopyrone derivative, coumarin has a heterocyclic C6-C3 structure of the coumarin nucleus (Fig. 1). It was two known coumarins, 2*R*-7-hydroxy-8-(2,3-dihydroxy-3-methylbutyl) coumarin (compound 1) and peucedanol (compound 2), along with a new coumarin methylpeucedanol (compound 3), were isolated from the bark of *H. crenulata* through solvent extraction, systematic solvent fractionation, and repeated column chromatography and identified using NMR spectroscopic analyses (¹H-NMR, ¹³C-NMR, Q-TOF LC/MS, and IR). All three coumarins were first isolated from the bark of Thanakha plant in this study.

Therefore, we aimed to evaluate antioxidant capacities of these three coumarins (compounds 1–3) isolated from the Thanakha bark in this study and their inhibitory effects on TNF- α production in LPS-induced THP-1 cells.



Materials and methods

Reagents

2,2'-zino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), vitamin C, sodium acetate, 2,4,6,-tripyridyl-*s*-triazine (TPTZ), HCl, FeCl₃·6H₂O, LPS from *Escherichia coli* O55:B5 were purchased from Sigma Chemical Co., LLC (St Louis, MO, USA). The three coumarins (compounds 1–3; purity > 95%) used in this study were isolated from the ethyl acetate fraction of a 70% (v/v) aqueous methanol extracts of *H. crenulata* R. barks using column chromatography. Roswell Park Memorial Institute (RPMI)-1640 medium, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Welgene Inc. (Gyeongsan, Republic of Korea). All other reagents used were of analytical or HPLC grade.

Plant materials

The barks of *H. crenulata* R. were purchased from the market located at Taungbi Village, Old Bargan, Myanmar in June, 2018, and identified by Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. A voucher specimen (KHU0180607) was deposited at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Republic of Korea.

General experimental procedures

Silica gel (230–400 mesh; Merck, Darmstadt, Germany) and Sephadex LH-20 (Merck) were used for column chromatography (c.c.). Preparative-liquid chromatography (prep-LC) (LC-Forte/R; YMC, Kyoto, Japan) was carried out using cartridge YMC-DispoPack (ODS-25, 80 g and 120 g; YMC). Thin-layer chromatography (TLC) analysis was performed using on a Kieselgel 60F₂₅₄ plate (Merck) and a RP-18 F_{254s} plate (Merck), and detection was performed by a UV lamp at 254 and 365 nm and 10% H₂SO₄ solution by spraying and heating. ¹H and ¹³C NMR along with 2D NMR data were obtained on a Bruker AVANCE II 400 (¹H NMR at 400 MHz and ¹³C NMR at 100 MHz) spectrometer (Bruker, Rheinstetten, Germany) in CDCl₃ with TMS as internal standard. ESI/MS was obtained on a 6530 Accurate-Mass Q-TOF LC/MS (Agilent Technologies GmbH, Waldbronn, Germany). IR spectra were obtained from a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). Curves of CD was recorded on a Jasco spectropolarimeter type J-715 at 21 C (scan range, 200–400 nm; and cell length, 0.1 cm).

Extraction and isolation of coumarins from the bark of *H. crenulata*

Dried bark powder (5 kg) was extracted overnight in 70% aqueous methanol (MeOH; 45 L) at room temperature. The concentrated extract was poured into water (3 L) and extracted with ethyl acetate (EtOAc; 3 L × 3) and then *n*-butanol (3 L × 3) to give the EtOAc extract (140 g; HCE), *n*-butanol extract (45 g; HCB), and water extract (395 g; HCW). The EtOAc extract was applied to silica gel c.c. (5 × 22 cm) and eluted with *n*-hexane–EtOAc (10:1 → 5:1 → 3:1 → 1:1; 2 L of each) → CHCl₃–MeOH (10:1 → 7:1 → 5:1 → 3:1 → 1:1; 2 L of each) while monitoring with TLC to produce 20 fractions (HCE-1 to HCE-20). Fraction HCE-9 (940 mg) was submitted to SiO₂ c.c. (4 cm × 20 cm) and eluted with *n*-hexane–EtOAc (4:1 → 3:1; 6 L of both), yielding 11 fractions (HCE-9-1 to HCE-9-11) and a purified compound **2** (HCE-9-4, 31.5 mg, elution volume/total volume (Ve/Vt) 0.27–0.36, TLC (Kieselgel 60F₂₅₄) R_f 0.51, *n*-hexane–EtOAc = 1:1). Fraction HCE-10 (4.8 g) was subjected to SiO₂ c.c. (4 cm × 20 cm) and eluted with *n*-hexane–EtOAc (4:1; 6 L), yielding 12 fractions (HCE-10-1 to HCE-10-12) along with a purified compound **1** (HCE-10-9, 137.6 mg, Ve/Vt 0.67–0.75, TLC (Kieselgel 60F₂₅₄) R_f 0.26, *n*-hexane–EtOAc = 1:1). Fraction HCE-12 (327 mg) was applied to a preparative-LC cartridge: YMC-DispoPack AT (ODS-25, 80 g; and particle size, 25 μm) and eluted in 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile (7:3) at a flow rate of 10 mL/min using a UV detector at 254 and 356 nm. Repeated collection of a peak at 29.5 min led to isolation of a purified compound **3** (49 mg). Chemical structures of three coumarin derivatives (compounds **1**–**3**) from the Thanakha bark extracts are shown in Fig. 1.

(2′R)-7-hydroxy-8-(2,3-dihydroxy-3-methylbutyl)-coumarin (compound 1)

White amorphous powder (CH₃OH); [α]_D²⁵ = +45° (*c* = 0.5, MeOH); IR (KBr) ν_{max} 3477, 2950, 1702, 1629 cm⁻¹; positive ESI–MS *m/z* 247 [M–OH+H]⁺; ¹H-NMR (400 MHz, CDCl₃, δ_H) 7.64 (1H, d, *J* = 9.1 Hz, H-4), 7.28 (1H, d, *J* = 8.1 Hz, H-5), 6.76 (1H, d, *J* = 8.1 Hz, H-6), 6.21 (1H, d, *J* = 9.1 Hz, H-3), 4.80 (1H, m, H-2′), 3.32 (2H, m, H-1′), 1.38, 1.26 (both 3H, both s, H-4′, H-5′); ¹³C-NMR (100 MHz, CDCl₃, δ_C) 163.7 (C-2), 160.9 (C-7), 151.3 (C-9), 143.8 (C-4), 128.7 (C-5), 114.0 (C-8), 113.1 (C-6), 112.6 (C-10), 106.6 (C-3), 91.3 (C-2′), 71.7 (C-3′), 27.5 (C-1′), 25.9, 24.0 (C-4′, C-5′).

Peucedanol (compound 2)

Yellow amorphous powder (CH₃OH); [α]_D²⁵ = –58.5 (*c* = 0.07, MeOH); IR (KBr) ν_{max} 3495, 3320, 1715, 1690 cm⁻¹; positive ESI–MS *m/z* 247 [M–OH+H]⁺; ¹H-NMR

Table 1 ¹H- and ¹³C-NMR spectral data of compounds **2** and **3** in CDCl₃

No.	¹ H-NMR		¹³ C-NMR	
	2	3	2	3
2			163.8	160.7
3	6.19 (1H, d, <i>J</i> = 9.1)	6.26 (1H, d, <i>J</i> = 9.1)	114.2	113.2
4	7.84 (1H, d, <i>J</i> = 9.1)	7.64 (1H, d, <i>J</i> = 9.1)	146.3	143.3
5	7.39 (1H, s)	7.33 (1H, s)	125.1	125.0
6			127.4	129.5
7			165.4	161.1
8	6.71 (1H, s)	6.81 (1H, s)	98.3	98.9
9			157.0	154.9
10			112.3	112.1
1′a	3.25 (2H, m, H-1′)	3.02 (1H, dd, <i>J</i> = 2.2, 14.0)	30.4	32.5
1′b	3.25 (2H, m, H-1′)	2.56 (1H, dd, <i>J</i> = 10.5, 14.0)		
2′	4.76 (1H, m, H-2′)	3.65 (1H, br.d, <i>J</i> = 10.5)	92.6	77.6
3′			72.4	72.8
4′-CH₃	1.30 (3H, s)	1.38 (3H, s)	25.5	26.3
5′-CH₃	1.24 (3H, s)	1.27 (3H, s)	25.4	23.6
OCH₃		3.92 (3H, s)		56.0

δ in ppm from TMS, *J* value in Hz

(400 MHz, CDCl₃, δ_H) and ¹³C-NMR (100 MHz, CDCl₃, δ_C) see Table 1.

Methylpeucedanol (compound 3)

Brown amorphous powder (CH₃OH); [α]_D²⁵ = +81.0 (*c* = 0.01, MeOH); IR (KBr) ν_{max} 3488, 1708, 1627 cm⁻¹; ECD (MeOH) 333 (Δε = 4.1); positive HRFABMS *m/z* 279.1223 [M+H]⁺ (calcd for C₁₅H₁₉O₅, 279.1228); ¹H-NMR (400 MHz, CDCl₃, δ_H) and ¹³C-NMR (100 MHz, CDCl₃, δ_C) see Table 1.

Measurements of antioxidant capacities

The antioxidant capacity of compounds **1**–**3** was assessed using the ABTS and FRAP (ferric reducing antioxidant power) assay and expressed in mg vitamin C equivalents (VCE)/g of compound. The ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.). The reaction between ABTS radicals and the appropriately diluted samples was allowed to proceed at 37 °C for 10 min, and then the decrease in absorbance of the resulting solution was measured at 734 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.).

The antioxidant capacity using FRAP assay, FRAP reagent solution was prepared by mixing 0.3 M sodium acetate buffer solution (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl₃·6H₂O at a ratio of 10:1:1. The reactions between the 950 μL of FRAP reagent solution

and 50 μL of diluted samples were reacted for 30 min at room temperature, and then the absorbance at 593 nm was immediately measured by a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.).

Cell culture

THP-1 cells, a human monocyte-like cell line, were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 $\mu\text{g}/\text{ml}$ penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37 $^{\circ}\text{C}$ in a humidified 5% CO_2 incubator. The cells were sub-cultured every 3–4 days and seeded onto 96-well plates. After incubation for 24 h, cells were stimulated with *Lactobacillus plantarum* K8 lysate and/or LPS.

Enzyme-linked immunosorbent assay (ELISA)

After cells were stimulated with compounds 1–3 and/or LPS, and culture supernatants were collected and used for the TNF- α ELISA assay. Human TNF- α capture antibody (R&D #MAB610) and human TNF- α biotinylated antibody (R&D #BAF210) were used according to the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times. Data are presented as the mean \pm standard deviation. Statistical analysis was performed using the Prism 5.0 software (GraphPad Software, USA). The significance of differences was assessed using an unpaired one- or two-tailed Student's *t* test. Differences were considered statistically significant when the *p* value was < 0.05 .

Results and discussion

Identification of coumarins from the bark of *H. crenulata* R.

The presence of coumarins in the EtOAc layer obtained from the 70% (v/v) aqueous MeOH extract of *H. crenulata* bark was confirmed by TLC, in which the spots showed UV absorption at 254 and 330 nm and an orange color under 10% (v/v) sulfuric acid and heating. Repeated SiO_2 and ODS column chromatography for EtOAc fraction led to isolation of a new (compound 3, yield: approximately 0.001%) and two known (compounds 1, yield: approximately 0.003% and 2, yield: approximately 0.001%) coumarin derivatives were first isolated from the Thanakha bark.

The known coumarins were respectively identified as 2*R*-7-hydroxy-8-(2,3-dihydroxy-3-methylbutyl) coumarin (compound 1) and peucedanol (compound 2) based on spectroscopic data including NMR, MS, and specific rotation values and by comparing their spectroscopic data with those reported in the literature [11].

Compound 3 was obtained as a brown amorphous powder and was separated on TLC, sprayed with 10%

(v/v) sulfuric acid, and heated to show an orange color. It showed absorbance bands due to hydroxy (3475 cm^{-1}), conjugated ketone (1635 cm^{-1}), double bond (1615 , 1605 cm^{-1}), and aromatic (1515 , 1480 cm^{-1}) groups in the IR spectrum. The molecular weight was 278 from the molecular ion peak at m/z 279 $[\text{M}+\text{H}]^+$ in positive ESI/MS. The ^1H -NMR spectrum showed olefin methine proton signals at δ_{H} 7.64 (1H, d, $J=9.1$ Hz, H-4) and δ_{H} 6.26 (1H, d, $J=9.1$ Hz, H-3), which were attributed to one α -pyrone, and olefin methine proton signals at δ_{H} 7.33 (1H, s, H-5) and δ_{H} 6.81 (1H, s, H-8) due to a 6,7-disubstituted coumarin moiety. One oxygenated methine proton signal at δ_{H} 3.65 (1H, br.d, $J=10.5$, H-2'), one germinal methylene proton signal at δ_{H} 3.02 (1H, dd, $J=2.2$, 14.0, H-1a') and δ_{H} 2.56 (1H, dd, $J=2.2$, 14.0, H-1b'), one methoxy signal at δ_{H} 3.92 (3H, s, H-OCH₃), and two methyl proton signals at δ_{H} 1.38 (3H, s, 4'-CH₃) and δ_{H} 1.27 (3H, s, 5'-CH₃) were due to one oxygenated-isopentenyl unit. The ^{13}C -NMR spectrum exhibited 15 carbon resonances including one methoxy δ_{C} 56.0 (OCH₃) signal indicating compound 3 to be composed of one coumarin and one isoprene moiety. The multiplicity of each carbon was determined by a DEPT spectrum. The chemical shift of the signals in the ^{13}C -NMR spectrum of compound 3 was similar to that of compound 2, peucedanol (see Table 1), with an additional methoxy moiety. The carbon signals due to one carbonyl at δ_{C} 160.7 (C-9); two oxygenated olefin quaternary carbons at δ_{C} 161.1 (C-7) and δ_{C} 154.9 (C-9); two olefin quaternary carbons at δ_{C} 126.5 (C-6) and δ_{C} 112.1 (C-10); and four olefin methines at δ_{C} 143.3 (C-4), δ_{C} 125.0 (C-5), δ_{C} 113.2 (C-3), and δ_{C} 98.9 (C-8) corresponded to the carbons of a coumarin moiety with one oxygenated and one carbonated substituent. In addition, carbon signals for one oxygenated quaternary methine at δ_{C} 72.8 (C-3'), one oxygenated methine at δ_{C} 77.6 (C-2'), one methylene at δ_{C} 32.5 (C-1'), and two methyl at δ_{C} 26.3 (C-CH₃) and δ_{C} 23.6 (C-CH₃) suggested the presence of one isopentenyl unit with two hydroxyl groups. Attachment of the methoxy moiety at C-2' was identified by the cross-peak between the oxygenated methine proton signal at δ_{H} 3.65 (H-2') and the methoxy carbon signal at δ_{C} 56.0 in the HMBC spectrum. This was confirmed by HMBC correlations at δ_{H} 3.65 (H-2')/ δ_{C} 126.5 (C-6), δ_{C} 77.6 (C-2'), and δ_{C} 72.8 (C-3'); δ_{H} 3.01 (H-1a') and at δ_{H} 2.56 (H-1b')/ δ_{C} 77.6 (C-2') and δ_{C} 72.8 (C-3'). The absolute configuration of C-2' was determined to be *R* from the positive cotton effect at 333 nm. Its similar coumarin, (+)-peucedanol methyl ether, 6-[(2*R*)-2,3-dihydroxy-3-methylbutyl]-7-methoxychromen-2-one, showed the positive cotton effect at 333 nm. Therefore, the chemical structure of compound 3 was determined to be 6-[(2*R*)-3-hydroxy-2-methyl-3-methylbutyl]-7-hydroxychromen-2-one,

Table 2 Total phenolic and flavonoid content and antioxidant capacity of compounds 1–3

Compound	Antioxidant capacity (mg vitamin C equiv./g DW ^a)	
	ABTS ^b	FRAP ^c
1	4.58 ± 0.19	n.d. ^d
2	54.12 ± 5.93	5.52 ± 0.91
3	40.15 ± 0.36	9.75 ± 1.47

^a Dry weight^b 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay^c Ferric reducing antioxidant power assay^d Not detected

and was revealed to be a new coumarin, named methylpeucedanol.

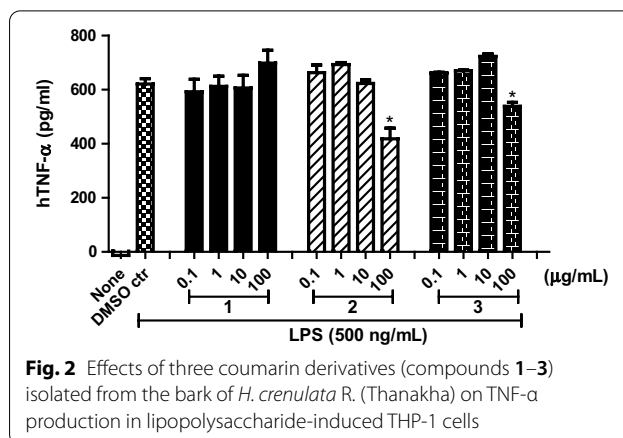
Antioxidant capacity of three coumarins (compounds 1–3)

The antioxidant capacities of compounds 1–3 are presented in Table 2. Antioxidant capacities measured by the ABTS radicals increased as follows: compound 1 < compound 3 < compound 2, whereas those measured by the FRAP assay increased as follows: compound 1 < compound 2 < compound 3.

The extract of *H. crenulata* R. has been reported to have antioxidant capacity due to the presence of phenolic compounds such as coumarins and flavones. Coumarins such as peucedanol and its glycoside in *Peucedanum japonicum* Thunb leaf were previously reported to have DPPH-radical-scavenging activity and AAPH-induced lipid peroxidation [9, 14]. Like those previous results, three coumarins tested in this study acted as antioxidants. These three coumarins, a group of monophenol isolated from Thanakha barks, have free hydroxyl (–OH) group in the coumarin backbone, that can donate hydrogen or electron for the reduction of free radicals and transition metals.

Anti-inflammatory effects of three coumarins isolated from the *H. crenulata* R. bark

To test the anti-inflammatory effects of the coumarins isolated from the bark from *H. crenulata* R., TNF- α production was measured in LPS-stimulated THP-1 cells in this study. THP-1 cells were pre-treated with various coumarins for 18 h and re-treated with LPS (500 ng/ml) for 4 h. The change in TNF- α production was not significant in the treatment with compound 1 compared with the DMSO control (Fig. 2). As shown in Fig. 2, compounds 2 and 3 at 378.8 μ M and 359.7 μ M inhibited the production of TNF- α in the culture media by 32.66 ± 11.09%

**Fig. 2** Effects of three coumarin derivatives (compounds 1–3) isolated from the bark of *H. crenulata* R. (Thanakha) on TNF- α production in lipopolysaccharide-induced THP-1 cells

and 13.33 ± 4.21%, respectively, suggesting that peucedanol and methylpeucedanol have the anti-inflammatory effects through reduced production of TNF- α , one of pro-inflammatory cytokines.

The stem bark extracts from *H. crenulata* R. has been reported to show potent anti-inflammatory effects in murine macrophage [1]. Many coumarins are previously reported to possess anti-inflammatory property. The two coumarins (compounds 2 and 3) showed anti-inflammatory effects via the inhibited production of TNF- α in human monocyte-like THP-1 cells. Peucedanol showed anti-inflammatory activity through the nitric oxide production inhibition in macrophage-like RAW 264.7 cells [10]. As compounds 2 and 3 had higher antioxidant capacity than compound 1 (Table 2), they were higher anti-inflammatory effects than compound 1 (Fig. 2), suggesting that antioxidant coumarin plays a role as an anti-inflammatory agent.

In conclusion, we isolated and identified three coumarin derivatives (compound 1, 2*R*-7-hydroxy-8-(2,3-dihydroxy-3-methylbutyl)-coumarin; compound 2, peucedanol; and compound 3, methylpeucedanol) from the bark of *H. crenulata* R. (Thanakha) in this study. These three compounds showed antioxidant capacities due to the presence of a free hydroxyl group on the coumarin backbone. Peucedanol and methylpeucedanol, a newly reported compound, had inhibitory effects on production of TNF- α in LPS-induced THP-1 cells. All parts of Thanakha (*Hesperethusa crenulata* R.) have been used as traditional skin care herbal material in Myanmar. The results of this study suggest that Thanakha bark extract is used as potent anti-inflammatory ingredients of functional foods and cosmetics. In addition to molecular biological mechanisms for the new substance (compound 3), in vivo anti-inflammatory animal studies will be needed in the future.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-021-00665-8>.

Additional file 1. Raw data of NMR spectra for compound (1–3).

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Authors' contributions

S-SL, D-OK, and N-IB designed this study. S-SL, H-GK, E-HP, KJK, M-HB, GK, H-JJ, C-GL, and M-CS performed experiments and data curation. S-SL wrote original draft. D-OK supervised the project. D-OK and N-IB reviewed and edited the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The data and materials used in this study are available under permission from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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