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Anti-inflammatory, Anti-oxidative and Anti-bacterial Activities of the Constituents Extracted from Leaves of *Taliparititi hamabo*

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ABSTRACT. *Taliparititi hamabo* is a plant growing around salt marshes in the Lava Coast region of Jeju Island, Korea. In this study, the extract of *T. hamabo* leaves was investigated for the anti-inflammatory, anti-oxidative and anti-bacterial activities and their active constituents were identified. In the anti-inflammatory tests using lipopolysaccharide-stimulated RAW264.7 cells, the ethyl acetate (EtOAc) fraction inhibited the nitric oxide production without causing cell toxicity. Moreover, the EtOAc fraction reduced pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-6) as well as prostaglandin E_2. In the anti-oxidative studies with DPPH and $ABTS^+$ radicals, potent scavenging activities were observed in the EtOAc and *n*-butanol fractions. Upon the anti-bacterial tests using *Staphylococcus epidermidis*, EtOAc and *n*-butanol fractions exhibited good activities. Through the phytochemical studies on EtOAc fraction, three components were isolated by repeated column chromatography; oleic acid (**1**), *p*-hydroxyphenethyl-*trans*-ferulate (**2**), nicotiflorine (**3**). Based on these results, the extract of *T. hamabo* leaves can be developed as natural resources for cosmetic applications.

Key words: *Taliparititi hamabo*, Anti-inflammation, Anti-oxidation, Anti-bacterial, Isolation

INTRODUCTION

Many of skin troubles are reported to be caused by over-production of free radicals and inflammation.[1] Our body continuously produces various reactive oxygen species (ROS) such as $\cdot O_2^-$, $\cdot OH$ and H_2O_2 in the metabolic process of routine daily activities. Under normal physiological conditions, the production and removal of free radicals in the body are in a state of dynamic balance. However, when the body is under stressful situations, the body comes to produce too much ROS, or in some cases the ROS scavenging system is reduced. If the balance between production and scavenging of ROS is broken, inflammation occurs.[2,3]

Inflammation is a common underlying pathological process. When the body is affected by harmful stimuli such as trauma and infection, it can cause systemic inflammatory response. Among them, macrophages involved in the inflammatory process produce large amounts of nitric oxide (NO) and pro-inflammatory cytokines.[4]

Recently the fields of dermatology and cosmetics have begun to focus on the human skin microbiome, as the skin microbiota is involved in the health of the skin ecosystem. Among skin microbes, *Staphylococcus epidermidis* is a commensal bacterium that acts as a skin microbiota sentinel. These sentinels are playing a key role in the skin ecosystem. However, studies have pointed out that when the skin is stimulated by inflammation, *S. epidermidis* will increase the release of pro-inflammatory cytokines such as TNF-α and IL-6 thereby inducing an intensified inflammatory response.[5]

Taliparititi hamabo is a plant of Malvaceae family, mainly distributed in China and Korea. This plant is a deciduous shrub growing along the coast. In Korea, it grows around salt marshes in the Lava Coast region of Jeju Island.[6] Literature survey indicated that the root extract of *T. hamabo* has insecticidal, detoxifying, antipyretic and diuretic effects.[7] However, there are no research reports on the biological activities and chemical constituents for the plant leaves. Therefore, in this study, the anti-inflammatory, anti-oxidative and anti-bacterial activities of the ethanol extract and solvent fractions were evaluated from the leaves of *T. hamabo*. In addition, in order to identify the active phytochemicals, the extract was subjected to repeated column chromatography, and the chemical structures of the isolated compounds were determined based on the spectroscopic studies.

EXPERIMENTAL

Plant Material

The leaves of *T. hamabo* were collected from Hallim-

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eup of Jeju, Korea in September 2020. The dried samples were prepared and deposited in the Natural Products Chemistry Laboratory, Department of Chemistry and Cosmetics, Jeju National University (No. 504).

Extraction and Isolation

The dried and crushed leaves (1.0 kg) of *T. hamabo* were stirred with 70% aqueous ethanol at room temperature for 24 h. The resulting ethanol solution was filtered, and the filtrate was concentrated at 40 ℃ using a rotary evaporator and freeze-dried to afford powder extract (233.2 g). A portion of extract (150.0 g) was suspended in water and partitioned to give *n*-hexane (*n*-Hex, 14.5 g), EtOAc (6.7 g) and *n*-butanol (*n*-BuOH, 20.3 g) soluble fractions. The EtOAc fraction (5 g) was subjected to vacuum liquid chromatography (VLC) on silica gel using a step gradient (*n*-Hex/EtOAc to EtOAc /methanol, 300 mL each) to provide 32 fractions (Fr. V1-V32). The Fr. V9 was identified as compound **1** (82 mg). Compound **2** (63.4 mg) was isolated from combined Fr. V20-21 through Sephadex LH-20 column chromatography (CC) with eluent chloroform-methanol (5:1). Compounds **3** (111 mg) was purified from Fr. V26 by using Sephadex LH-20 CC with chloroform–methanol (3:2).

Anti-inflammatory Activities

Cell Culture

The murine macrophage cell line RAW264.7 was purchased from American Type Cell Culture (ATCC, USA). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Inc., USA) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal bovine serum (FBS, GIBCO Inc., USA). The cells were incubated in an atmosphere of 5% CO_2 at 37 ℃.

Measurement of Nitric Oxide (NO) Production

Nitric oxide production was determined by measuring nitrite in the supernatant of cultured RAW264.7 cells. The cells were seeded in 24-well culture plates at a density of 2×10^5 cells/well for 18 h. The cells were then stimulated with LPS (100 ng/mL) and various concentrations of samples for 24 h. The supernatant was mixed with an equal volume of Griess reagent and was incubated at room temperature for 10 min. Absorbance was measured at 540 nm. The nitrite amounts in the test samples were calculated from $NaNO_2$ standard curve.

Cell Viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. RAW264.7 cells (2×10^5 cells/well) were incubated in 24-well plates for 18 h. The cells were then stimulated with LPS (100 ng/mL) in the presence of various concentrations of samples and cultured for 24 h. MTT (500 μg/mL) reagent was added to the medium and left to stand for 4 h. After removing the supernatant, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm.

Detection of TNF-α, IL-6 and Prostaglandin (PG) E_2

Enzyme-linked immunosorbent assay (ELISA) was used to determine the production of TNF-α, IL-6 and PGE_2. RAW264.7 cells (2×10^5 cells/well) were dispensed in 24-well plates and cultured for 18 h. The cells were then stimulated with LPS (1 μg/mL) and various concentrations of samples for 24 h. After, the supernatant was harvested and were then assayed according to the ELISA kit (TNF-α; BD Biosciences, IL-6; Invitrogen, PGE_2; R&D Systems).

Anti-oxidative Activities

DPPH Radical Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity experiment was applied to Blois's method. The DPPH reagent was diluted to 0.2 mM with EtOH and then used. 20 μL of each sample and 180 μL of DPPH solution were placed in a 96-well plate, and reacted at room temperature for 15 min. After the reaction, absorbance was measured at 515 nm. The concentration was expressed as SC_{50} when the free radicals in the samples were decreased to 50%. The experiment used BHT as a positive control.

$ABTS^+$ Radical Scavenging Assay

ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity was tested by applying Re's method. A 7.4 mM ABTS solution and 2.5 mM potassium persulfate were mixed at a ratio of 1:1 and reacted at room temperature in the dark for 16 h to form $ABTS^+$ radical. The $ABTS^+$ solution was diluted with ethanol so that the absorbance at 700 nm was 0.78 ± 0.02 and used in the experiment. Then, 20 μL of each sample was added in a 96-well plate, and 180 μL of diluted $ABTS^+$ solution was added and reacted at room temperature for 15 min. After the reaction, absorbance was measured at 700 nm. The concentration was expressed as SC_{50} when the free radicals in the samples were decreased to 50%. The experiment used BHT as a positive control.

Anti-bacterial Activities

Bacteria Culture

S. epidermidis was purchased from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Korea). Subculture of *S. epidermidis* was conducted every 24 h under aerobic conditions in a 37 ℃ incubator using Tryptic Soy Broth (TSB) as the medium.

Paper Disc Diffusion Method

To measure the anti-bacterial activity of the samples, the clear zone of each strain was confirmed using the paper disc diffusion method. 5 mL of the bacterial suspension (1.5×10^6 CFU/mL, 0.8% agar) was inoculated on semi-solidified TSB medium (1.5% agar) in the petri dish. Paper discs about 8 mm diameter size were impregnated with 40 μL of the test samples, then put on the medium surface and incubated at 37 ℃ for 24 h. The zone of inhibition around each disc was observed and measured diameter size of clear zone. Erythromycin was used as a positive control.

RESULTS AND DISCUSSION

The RAW264.7 cell is a murine macrophage widely being used for model of *in vitro* inflammatory events. When the macrophage cells are stimulated with lipopolysaccharides (LPS), the levels of nitrite, a stable oxidized product of nitric oxide (NO) are increased in the culture medium. Accordingly, LPS-induced RAW 264.7 cells were employed to monitor the change of NO production with the extract and solvent fractions from *T. hamabo* leaves.

The *T. hamabo* leaves were extracted with 70% ethanol, and the obtained extract was sequentially separated into *n*-Hex, EtOAc, *n*-BuOH and H_2O soluble fractions according to the order of polarity. First, we used a concentration of 100 μg/mL of the extracts and fractions in this experiment. As a result, *n*-Hex and EtOAc fractions significantly reduced the NO production, but cytotoxicity was observed in the case of *n*-Hex fraction (*Fig.* 1). Therefore, we selected the EtOAc fraction for further test within the range of appropriate concentrations, where no cytotoxicity was detected. As shown in *Fig.* 2, the EtOAc fraction exhibited no toxicity with a concentration of up to 200 μg/mL inhibiting NO production by 85.8% with a manner of dose-dependence.

When RAW264.7 cells are stimulated with LPS, they secrete several pro-inflammatory cytokines including TNF-α and IL-6. PGE_2 is an important inflammatory mediator produced by cyclooxygenase (COX)-2, which could be also quantified in the supernatants of LPS-treated macrophages. In order to evaluate further anti-inflammatory mechanism by the EtOAc fraction, we measured expression of pro-inflammatory cytokines and PGE_2 using ELISA

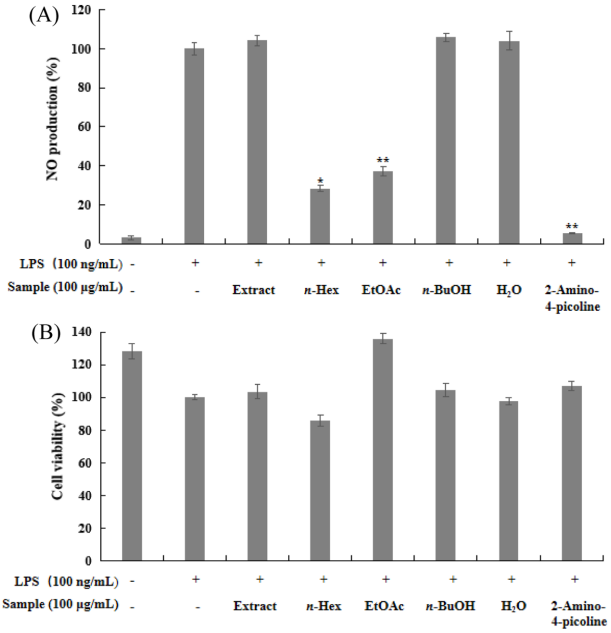

***Figure* 1.** Effects of extract and solvent fractions from *T. hamabo* leaves on NO production (A) and cell viability (B) in LPS-induced RAW264.7 cells. The data represent the mean±SD of triplicate experiments. *$p < 0.05$; **$p < 0.01$.

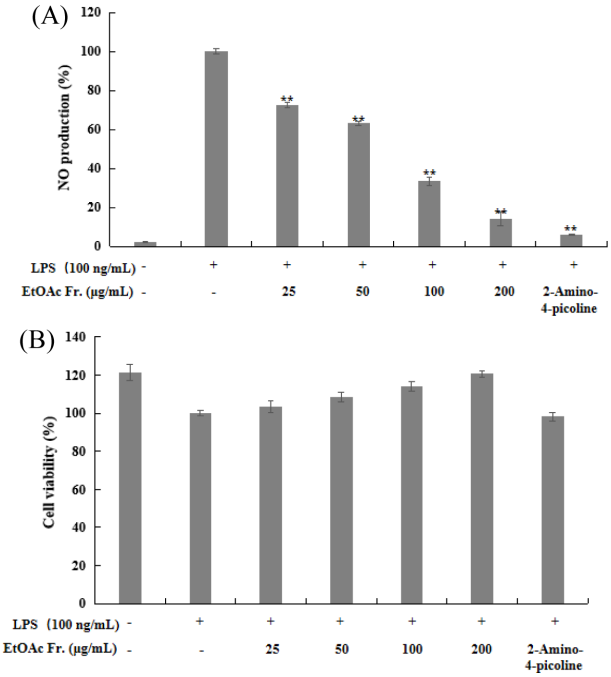

***Figure* 2.** Effects of EtOAc fraction from *T. hamabo* leaves on NO production (A) and cell viability (B) in LPS-induced RAW264.7 cells. The data represent the mean±SD of triplicate experiments. *$p < 0.05$; **$p < 0.01$.

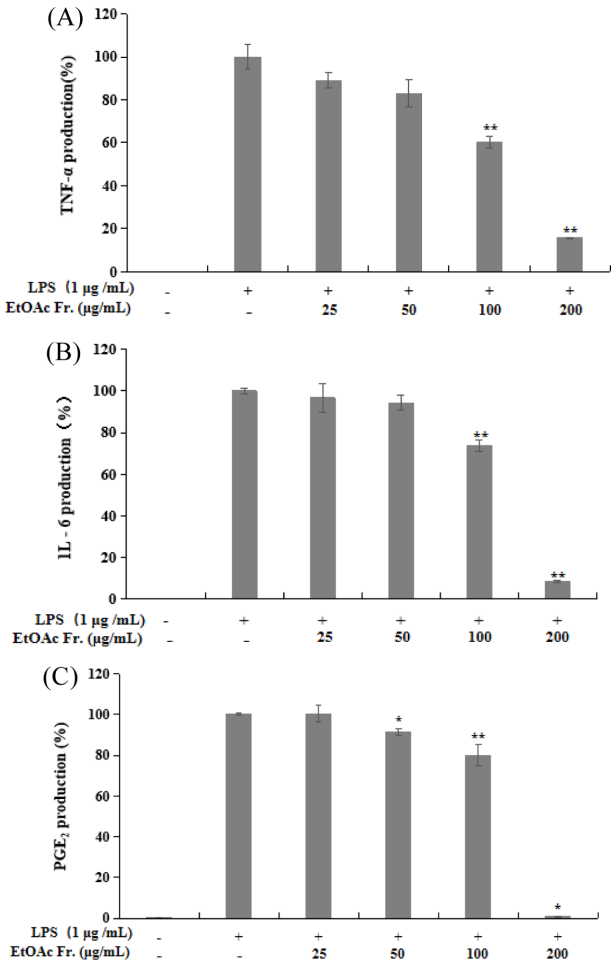

***Figure* 3.** Effects of EtOAc fraction from *T. hamabo* leaves on production of TNF-α (A), IL-6 (B) and PGE_2 (C) and cell viability in LPS-indued RAW264.7 cells. The data represent the mean ±SD of triplicate experiments. *$p < 0.05$; **$p < 0.01$.

kit. As a result, as shown in *Fig.* 3, it was observed that release of TNF-α, IL-6 and PGE_2 was reduced in a concentration-dependent manner respectively.

DPPH is a stable free radical and, when contacted with an antioxidant, is converted into colorless hydrazine. The degree of discoloration reflects the scavenging ability of antioxidant compounds.[8] ABTS can be oxidized by $K_2S_2O_8$ to generate $\cdot ABTS^+$, blue-green radical species. In the presence of antioxidants, $\cdot ABTS^+$ is readily turned into colorless ABTS. To identify the anti-oxidative activities of the fraction samples from *T. hamabo* leaves, DPPH and $ABTS^+$ radical scavenging activities were evaluated. In the DPPH radical scavenging studies, the EtOAc and *n*-BuOH fractions showed higher activities with SC_{50} values of 73.9 and 44.3 μg/mL respectively, which indicated their better activities than that of the positive control BHT. Also, $ABTS^+$ radical scavenging activity was observed in the EtOAc and *n*-BuOH fractions showing SC_{50} values of 44.1 and 88.4 μg/mL respectively (*Table* 1).

***Table* 1.** SC_{50} Values of Extract and Solvent Fractions from *T. hamabo* Leaves on DPPH and $ABTS^+$ Radical Scavenging

SC_{50}[1)] (μg/mL)	DPPH radical	$ABTS^+$ radical
Extract	148.1	>100
n-Hex	138.0	>100
EtOAc	73.9	44.1
n-BuOH	44.3	82.4
H_2O	260.1	>100
BHT	76.6	18.8

[1)]SC_{50}: scavenging concentration for 50% of radical

***Table* 2.** Anti-bacterial Activities of *T. hamabo* Leaves

Bacterial density (1.5×10^6 CFU/mL)	Clear zone (mm)[1)]		
	S. epidermidis		
	CCARM 3709	CCARM 3710	CCARM 3711
Extract	N.A.[3)]	N.A.	N.A.
n-Hex	N.A.	N.A.	N.A.
EtOAc	11.5	N.A.	9
n-BuOH	12	9	11
H_2O	N.A.	N.A.	N.A.
Erythromycin[2)]	33.5	N.A.	30

[1)]Concentration of sample: 4 mg
[2)]Concentration of positive control: 40 μg
[3)]N.A.: No activity

S. epidermidis is a skin-associated Gram-positive strain which inhabits the epidermis layers and causes a variety of skin inflammatory symptoms.[9] The anti-bacterial activity of extract samples from *T. hamabo* was conducted using paper disc diffusion method with *S. epidermidis*. As seen in large clear zones, the EtOAc and *n*-BuOH fractions were demonstrated to possess high degree of anti-bacterial activities (*Table* 2).

As the data showed potent anti-inflammatory, anti-oxidative and anti-bacterial effects, the EtOAc fraction was selected as a target research sample to discover the active phytochemicals. The EtOAc layer was subjected to repeated column chromatography over silica gel and Sephadex LH-20. From these purification procedures, the compounds **1-3** were isolated (*Fig.* 4). The structures of the isolates were confirmed by analysis of the ^1H and ^{13}C NMR spectra, and finally by comparison of the data to the literature values. The compound **1** showed 18 carbons including a carbonyl, an olefinic bond and a methyl carbon in ^{13}C NMR spectra. The rest of the signals corresponded to CH_2 carbons. Therefore, compound **1** was identified as an unsaturated

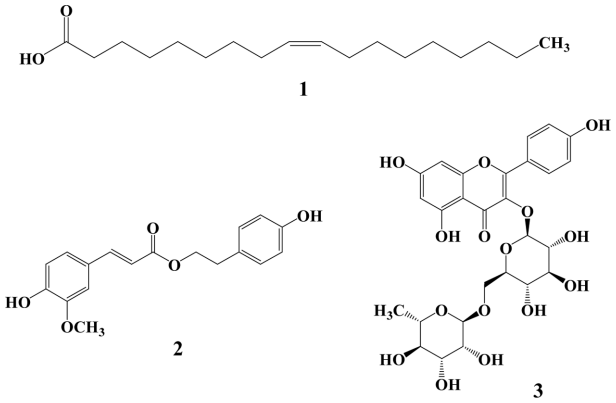

***Figure* 4.** Isolated compounds **1-3** from *T. hamabo* leaves.

fatty acid, oleic acid.[10-11] The compound **2** showed 15 carbon signals including a *trans*-olefin and two aromatic rings, which led to identify a phenylpropanoid compound, *p*-hydroxyphenethyl-*trans*-ferulate.[12] The compound **3** exhibited characteristic flavonoid skeleton to which sugar units are bound in NMR studies. The sugar unit was determined as a rutinose, therefore, the compound **3** was identified as kaempferol 3-*O*-rutinoside, nicotiflorine.[13] According to the results of literature research, compounds **1**, **2** and **3** have anti-oxidative[14-16] and anti-inflammatory[16-18] activities. Also, compounds **1** and **3** were reported to possess anti-bacterial activities.[19-20] Therefore, it is reasonable to explain that the biological activities of the *T. hamabo* leaves extracts are originated from the isolated compounds **1-3**.

CONCLUSION

In conclusion, this study demonstrates that the EtOAc fraction of *T. hamabo* leaves has a strong inhibitory effect on LPS-induced NO synthesis in RAW264.7 macrophages. In addition, the EtOAc layer reduced the production of pro-inflammatory cytokines including TNF-α, IL-6, and PGE_2 in a dose-dependent manner. In the anti-oxidative studies, the EtOAc and *n*-BuOH fractions exhibited excellent DPPH and $ABTS^+$ radical scavenging activities. Moreover, the results of anti-bacterial activity experiment using paper disc diffusion method on *S. epidermidis* indicated strong activities in both the EtOAc and *n*-BuOH fractions. Identification of active constituents on EtOAc fraction led to the isolation of three compounds; oleic acid (**1**), *p*-hydroxyphenethyl-*trans*-ferulate (**2**) and nicotiflorine (**3**). Based on these results, extract of *T. hamabo* leaves could be developed as natural resources for pharmaceutical and/or cosmetic applications.

Supporting Information. Additional supporting information (NMR data for the compounds 1-3) is available in the online version of this article.

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