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Original Research Article

Skin care benefits of bioactive compounds isolated from *Zanthoxylum piperitum* DC. (Rutaceae)

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Abstract

Purpose: *To investigate skin care efficacies of Zanthoxylum pipetitum extract and isolated compounds.*
Methods: *Ethanol extracts of leaves, branches and fruits of what were partitioned into n-hexane, chloroform, ethyl acetate, n-butanol and aqueous layers and some fractions were further analyzed to isolate five compounds. The isolated compounds were identified based on the proton and carbon nuclear magnetic resonance (NMR) spectra. Cosmetic efficacy tests of the extracts and isolated compounds were evaluated by in vitro tests.*
Results: *Phytochemical studies of the chloroform and ethyl acetate layers led to the isolation of five compounds; quercitrin (1), afzelin (2), hydroxy-α-sanshool (3), α-sanshool (4) and hyperoside (5). In activity tests, the extracts showed inhibitory activity against inflammation response and melanin synthesis, and induction of procollagen type I C-peptide (PIP). Among the isolated compounds, hydroxy-α-sanshool (3) and α-sanshool (4) displayed significant anti-inflammatory activity.*
Conclusion: *The results demonstrate that Z. piperitum extract and its active compounds possess a significant potential as a cosmeeutical agent for enhancing skin quality.*

Keywords: *Zanthoxylum piperitum, Quercitrin, Afzelin, Hydroxy-α-sanshool, α-Sanshool, Hyperoside, Anti-inflammatory, Anti-aging*

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INTRODUCTION

The dream of fending off aging signs on human face may be as long as human civilization. In consideration of the global aging population, it is more important than ever to develop interventions that keep health in old age and delay the occurrence of age-related diseases. [1]. Aging is a complex and multifactorial process accompanied by several functional and aesthetic changes on the skin, one of which is linked to the reduction of collagen. Reduced synthesis of collagen types I is characteristic of chronologically aged skin. In a study of collagen production in chronologically aged skin, the content of type I collagen, the major collagen in the skin and a marker of collagen synthesis, is decreased by 68% in old skin compared to young skin, and cultured young fibroblasts generate more type I collagen than old cells [2]. Collagen impart strength and elasticity to skin, and its degeneration with aging causes skin to become

fragile, with easy bruise and loss of youthful appearance [3]. So, the stimulation of collagen synthesis is an important criterion to develop potential skin care ingredients, especially anti-aging products.

Melanin plays an important role in preventing skin cancer caused by UV rays and it is a pigment that is biosynthesized from L-tyrosine in melanocytes. Melanogenesis is the process, mediated by several enzymes such as Tyrosinase, TRP-1 and TRP-2, producing melanin in the skin and within the hair follicles [4]. Decline of melanogenesis results in mottling, gray hair formation and sunburn. On the other hand, excessive production of melanin leads to the dark spots on the skin: a prominent aging sign. Therefore, controlling melanogenesis is critical for anti-aging cosmeceutical products [5].

Molecular inflammation is a major biological mechanism that correlates to the aging process and age-related diseases. The major factors of inflammatory reactions (i.e., IL-1b, IL-6, TNF-a, COX-2, and iNOS) have all been found to be relevant during the aging process [6]. In another study, it was demonstrated that the inflammatory process is intricately involved in the aging process [7]. Nitric oxide (NO) is a signaling molecule synthesized by a family of nitric oxide synthase (NOS) isoforms found in most tissues [8]. Nitric oxide (NO) is an important intracellular molecule that regulates various biological functions, including neurotransmission, macrophage-mediated cytotoxicity, and smooth muscle relaxation. On the other hand, excess production of NO has been related to oxidative stress and several diseases such as chronic inflammation, stroke, arthritis, diabetes, autoimmune disease, and septic shock [9]. Therefore, NO inhibitors are regarded as valuable in the development of treatment or drugs to control the inflammation related to human diseases and aging.

Zanthoxylum piperitum is an aromatic medicinal plant belonging to the Rutaceae family and distributed in Northeast Asia; Korea, Japan and China. It has characteristic citrus-like flavor and is also used as traditional spices and medicines in Korea [10]. It has been used for the treatment of stomachache, vomiting, diarrhea and moist dermal ulcer. Components such as sanshool, sanshoamide, methyl 2, 4-dimethoxy-5-hydroxycinnamate, xanthoxylol and flavonoids have been isolated from *Z. piperitum* leaves [11].

Thus, the purpose of this study was to investigate the leaves, branches and fruits of *Z. piperitum* for for enhanced skin quality, and also to identify the plant's bioactive compounds.

EXPERIMENTAL

Plant materials

Z. piperitum specimens were collected in Jeju, the island in the south of Republic of Korea, in August, 2017.

Extraction and fractionation

Ethanol (EtOH) and 1, 3-butylene glycol (BG) were used as solvents for general extractions in this study. 1, 3-butylene glycol (BG) is one of the most useful solvents in the cosmetic formula and known to extract similar compounds with alcohols in our previous trials. To identify active compounds, the dried leaves of *Z. piperitum* (150 g) were extracted with 50 % EtOH at room temperature for 24 h. The extract was filtered and concentrated using a rotary evaporator at 40℃. A part of the concentrate (21.0 g) was suspended in water and successively fractionated into *n*-hexane (Hex), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol and water portions. The EtOAc layer was subjected to normal silica gel column chromatography (CC), and 8 fractions (Fr.1 to 8) were eluted with $CHCl_3$ and methanol (MeOH). Then, the Fr. 8 gave compound **1** (358 mg) and the Fr. 6 was further separated using Sephadex LH-20 with MeOH to isolate compound **2** (16.0 mg). Finally, Compound **3** (16.0 mg) was separated from the $CHCl_3$ layer (200 mg) using Sephadex LH-20 with $CHCl_3$/MeOH (20:1).

The dried branches of *Z. piperitum* (400 g) were extracted and fractionated as above. The Hex layer was subjected to normal silica gel and Sephadex LH-20 CCs successively, and the fractions with $CHCl_3$ and MeOH gave compound **4** (1.0 mg). The dried fruits of *Z. piperitum* (400 g) were extracted and fractionated as above. The EtOAc layer was subjected to normal silica gel CC, and 15 fractions (fr. 1 to 15) were eluted with $CHCl_3$ and MeOH. The Fr. 4 was further fractionated by Sephadex LH-20 with MeOH to purify compound **1** (49.5 mg) again. Compound **5** (85.1 mg) was obtained from a precipitate in the EtOAc layer, and finally compound **3** (158.8 mg) was separated from the Hex layer (494 mg) using Sephadex LH-20 with $CHCl_3$/MeOH (15:1).

Anti-inflammation assay

The production of nitric oxide was evaluated by measuring the nitrite in the cultured Murine macrophage cell line RAW 264.7. The cells were

cultured in plates containing Dulbecco's Modified Eagle Medium (DMEM, 200 µL/well) supplemented with penicillin (100 units/ml), streptomycin sulfate (100 µg/mL) and 10 % fetal bovine serum (FBS) in a humidified atmosphere of 5 % CO_2. The cells (2×10^5/well) were treated with 1 µg/mL lipopolysaccharide (LPS) and various concentrations of samples for 24 h. Then, 100 µL of cell culture media was added to 100 µl Griess reagent (50 µl of 0.1 % napthyl ethylenediamine-HCl and 50 µL of 1 % sulphanilamide in 5 % phosphoric acid), and incubated at room temperature for 10 min. The generated nitrite was measured using an ELISA reader at a wavelength of 570 nm (SpectraMax M5, Molecular Devices, California, USA).

The concentration of nitrite was measured in comparison to standard curve of the sodium nitrite, as previously reported [12]. Cytotoxicity assay was performed alongside using MTT (3-(4, 5-dimethylthiaol-2-yl)-2-5-diphenyltetrazolium bromide) reagent in accordance the protocol of Mosmann *et al* [13].

The expression of TNF-α was determined in the culture supernatant of RAW 264.7 cells. Cell culture supernatant samples were centrifuged at 3000 g for 20 min and the supernatants were transferred to new tubes and frozen immediately at –80 °C. The assay was performed following the protocol of ELISA kits (R&D Systems, MTA00B) for TNF-α.

Melanogenesis inhibition assay

B16 melanoma cells were cultured into 6-well plates at 1×10^5 cells/well for 24-h, followed by treatment with various concentrations of extracts or fractions. After 3 days incubation, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 1 M NaOH containing 10% DMSO, at 60℃ for 1 h. Melanin contents were determined using a standard curve of synthetic melanin (Sigma-Aldrich, USA), and absorbance was measured at 405 nm. The values were expressed by the total protein contents.

Collagen synthesis activity in normal Human Dermal Fibroblast (HDFn) cell

Human Dermal Fibroblast (HDFn) cells were cultured a microplate at 5×10^4 cells/well with DMEM (containing 10 % FBS and 100 unit/mL penicillin-streptomycin) and incubated at 37 ℃ in a humidified atmosphere of 5 % CO_2 overnight. After incubation, extracts or fractions were added to the plate, diluted in serum-free media, and cells were cultured for another 24 h. The supernatants of culture were collected after centrifugation at 15,000 rpm in 4 ℃ for 30 sec, and PIP tests was performed according to the supplier's protocol (Takara, MK101; 15).

Nuclear magnetic resonance spectroscopy

The chemical structure was determined by the interpretation of the ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra on a JEOL (JNM-ECX 400) instrument. Deuteriomethanol (CD_3OD), deuteriochloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-D6) were used to dissolve the isolated compounds.

Statistical analysis

Results were presented as Means ± standard deviation (SD), and they were analyzed by Student's t-test using Microsoft Excel 2013 (Microsoft Corporation, USA) for independent samples. The level of significance was set at $p < 0.05$.

RESULTS

Chemical structure of five compounds isolated from *Z. piperitum* was investigated using NMR spectroscopy. Compounds **1**, **2** and **5** showed typical flavonoid signals in the ^1H-NMR and ^{13}C-NMR spectra. The ^1H-NMR signals (δ 6.9 ~ δ 7.3) of the compound **1** showed proton positions of the B-ring. The existence of H-6 and H-8 were confirmed by two broad doublet signals (δ 6.2 and 6.4). The ^1H-NMR spectrum indicated a rhamnoside with distinguishing peaks (δ 0.9 and 5.3). By comparing the obtained data to known values, compound **1** was confirmed to be quercitrin [14]. By the same process, compounds **2** and **5** were confirmed to be afzelin [15] and hyperoside [16], respectively. Compounds **3** and **4** showed 15 signals in ^1H and ^{13}C NMR spectra, where oxygen-bearing or nitrogen-bearing sp^3 carbons were identified. The presence of eight sp^2 carbons and one carbonyl carbon was verified. These results led to the identification of compounds **3** and **4** as hydroxy-*α*-sanshool and *α*-sanshool [17].

The anti-inflammatory activity were assessed for extracts of *Z. piperitum* and isolated compounds by measuring the level of NO and TNF-α in LPS-stimulated macrophage cells. As shown in Table. 1, BG extracts of leaves, branches and fruits inhibited NO production up to 43%, 50% and 66%, respectively. Among the isolated compounds, hydroxy-*α*-sanshool (**3**) and *α*-sanshool (**4**) displayed significant reduction of NO production (Figure 2).

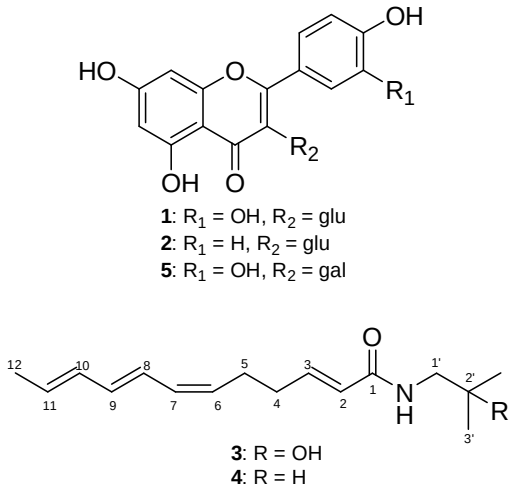

Figure 1: Chemical structure of compounds **1**~**5** isolated from *Z. piperitum.*

Quercitrin (1)

^{1}H NMR (400 MHz, CD_3OD): 7.33 (1H, d, *J*=2.3 Hz, H-2'), 7.28 (1H, dd, *J*=2.3, 8.7 Hz, H-6'), 6.91 (1H, d, *J*=8.7 Hz, H-5'), 6.33 (1H, d, *J*=1.8 Hz, H-8), 6.16 (1H, d, *J*=2.3, H-6), 5.35 (1H, d, *J*=1.3, H-1''), 3.33 ~ 4.25 (4H, m, H-2'', H-3'', H-4'', H-5''), 0.95 (3H, d, *J*=5.9, H-6'')
^{13}C NMR (100 MHz, CD_3OD): δ 179.5 (C-4), 165.7 (C-7), 163.1 (C-5), 159.3 (C-9), 158.4 (C-2), 149.7 (C-4'), 146.3 (C-3'), 136.2 (C-3), 123.0 (C-1', C-6'), 117.0 (C-5'), 116.4 (C-2'), 105.9 (C-10), 103.5 (C-1''), 99.9 (C-6), 94.8 (C-8), 73.8 (C-4''), 72.1 (C-3''), 72.0 (C-2''), 71.9 (C-5''), 17.7 (C-6'').

Afzelin (2)

^{1}H NMR (400 MHz, CD_3OD): 7.75 (2H, d, *J*=8.7 Hz, H-2', 6'), 6.92 (2H, dd, *J*=8.7 Hz, H-3', 5'), 6.35 (1H, d, *J*=1.8 Hz, H-8), 6.18 (1H, d, *J*=2.3, H-6), 5.37 (1H, d, *J*=1.3, H-1''), 3.32 ~ 4.22 (4H, m, H-2'', H-3'', H-4'', H-5''), 0.92 (3H, d, *J*=5.9, H-6'')
^{13}C NMR (100 MHz, CD_3OD): 179.7(C-4), 166.1(C-7), 163.3(C-5), 161.6(C-4'), 159.3(C-9), 158.6(C-2), 136.3(C-3), 132.0(C-2', 5'), 116.6(C-3', 5'), 122.7(C-1'), 106.0(C-10), 103.6(C-1''), 100.0(C-6), 94.9(C-8), 73.3(C-4''), 72.2(C-3''), 72.1(C-2''), 72.0(C-5''), 17.7(C-6'').

Hydroxy-*α*-sanshool (3)

^{1}H NMR (400 MHz, CD_3OD): δ 6.79 (1H, m, H-3), δ 5.99 ~ 6.19 (4H, m, H-7, 8 , 9, 10), δ 5.79 (1H, m, H-2), δ 5.73 (1H, m, H-11), δ 5.37 (1H, m, H-6), δ 3.24 (2H, m, H-1'), δ 2.34 (2H, m, H-5), δ 2.29 (2H, m, H-4), δ 1.78 (3H, m, H-12), δ 1.23 (6H, s, H-3', 4')
^{13}C NMR (100 MHz, CD_3OD): 169.1(C-1), 145.3(C-3), 134.8(C-9), 133.2(C-10), 131.0(C-11), 130.7(C-7), 130.6(C-6), 126.6(C-8), 125.2(C-2), 71.7(C-2'), 51.2(C-1'), 33.3(C-4'), 27.5(C-5), 27.3(C-3', 4')

α-Sanshool (4)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.82 (1H, m, H-3), δ 6.00 ~ 6.37 (4H, m, H-7, 8 , 9, 10), δ 5.78 (1H, m, H-2), δ 5.72 (1H, m, H-11), δ 5.39 (1H, m, H-6), δ 3.15 (2H, dd, *J*=6.4, 6.8 Hz H-1'), δ 2.26 (2H, m, H-4), 2.26 (2H, m, H-5), δ 1.79 (3H, m, H-12), δ 1.79 (2H, m, H-2')
^{13}C NMR (100 MHz, $CDCl_3$): 166.1 (C-1), 143.6 (C-3), 133.6 (C-9), 131.9 (C-10), 130.3 (C-11), 129.7 (C-7), 129.8 (C-6), 125.4 (C-8), 124.4 (C-2), 28.7 (C-2'), 47.0 (C-1'), 32.2 (C-4'), 29.8 (C-5), 20.3 (C-3', 4')

Hyperoside (5)

^{1}H NMR (400 MHz, DMSO-D_6): δ 7.66 (1H, dd, *J*=2.2, 8.2 Hz, H-6'), δ 7.52 (1H, dd, *J*=1.8 Hz, H-2), δ 6.81 (1H, d, *J*=8.7 Hz, H-5'), δ 6.40 (1H, d, *J*=1.8 Hz, H-8), δ 6.20 (1H, d, *J*=2.2, H-6), δ 5.37 (1H, d, *J*=7.7, H-1''), δ 3.32 ~ 4.33 (5H, m, H-2'', H-3'', H-4'', H-5'', 6'')
^{13}C NMR (100 MHz, DMSO-D_6): 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.2 (C-9), 156.2 (C-2), 148.4 (C-4'), 144.8 (C-3'), 133.4 (C-3), 121.0 (C-1'), 122.0 (C-6'), 115.9 (C-5'), 115.1 (C-2'), 103.9 (C-10), 101.7 (C-1''), 98.6 (C-6), 93.4 (C-8), 75.8 (C-5''), 73.1 (C-3''), 71.1 (C-2''), 67.9 (C-4''), 60.1 (C-6'').

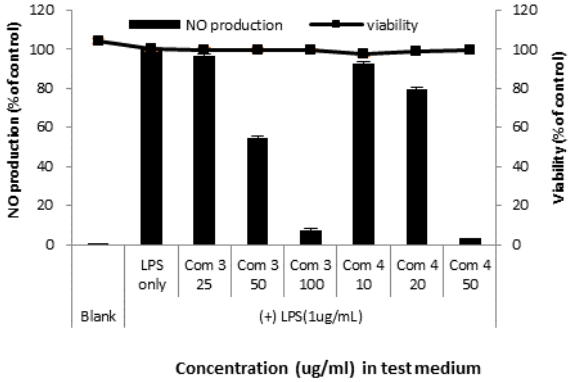

Figure 2: Effect of hydroxy-*α*-sanshool (**3**) and *α*-sanshool (**4**) on NO production by LPS-stimulated RAW 264.7 cells for 24 h

NO synthesis in macrophages can be induced by TNF-α [18], and in this assay, BG extracts of leaves, branches and fruits reduced the production of TNF-α up to 19, 23 and 17 %, (Table. 1). These results confirmed anti-inflammatory activity of *Z. piperitum* and elucidated the mechanism.

Inhibitory effect on melanin formation was measured using murine melanoma. As shown in Table 1, quantification of melanin contents indicated that all the extracts have inhibitory effects. Ethanol extracts of leaves, branches and fruits inhibited melanin synthesis up to 2715 and 25 %, respectively. Hydroxy-*α*-sanshool decreased melanin production up to 20 % at 100 µg/mL.

To examine whether *Z. piperitum* extracts increase the expression of pro-collagen type I C-peptide (PIP), human dermal fibroblast (HDFn) cells were used. Branch and fruit extracts increased the expression of PIP to 280 and 82 %, respectively (Table 1). These results confirmed that branch extract has strong activity to increase PIP expression.

Table 1: Biological activities of *Z. pipetitum* extracts

Plant part	NO Inhibition (2%, v/v)	TNF-α Inhibition (2%, v/v)	Melanin Contents Inhibition (100 µg/ml)	PIP synthesis (100 µg/ml)
Leaf	43±1.1	19±3.2	27±5.5	-
Branch	50±1.7	23±3.4	15±3.5	280±1.8
Fruit	66±3.2	17±4.4	25±15.8	82±0.7

Data are mean ± SD, and are expressed as %

DISCUSSION

Phytochemical investigation identified five constituents, quercitrin (**1**), afzelin (**2**), hydroxy-*α*-sanshool (**3**), *α*-sanshool (**4**) and hyperoside (**5**) from the extracts of *Z. piperitum* leaves, branches and fruits. Extracts of *Z. piperitum* inhibited NO production in macrophages and melanin synthesis in melanoma, both of which are important aspects of skin care agents.

These results showed good correlations to those previously reported for the same genus [19,20]. Aerial parts of *Z. piperitum* showed anti-inflammatory activity and another study on *Z. shinifolium* revealed several skin care potential. This study successfully identified active compounds and skin care benefit of *Z. piperitum*. Evaluation of the PIP stimulating activity showed that extracts of branches and fruits can be developed as anti-aging agents as well, which is also in good correspond to the previous work on *Z. rhetsa* [21].

Of the isolated compounds, hydroxy-*α*-sanshool (**3**) and *α*-sanshool (**4**) are aliphatic amides that were originally isolated from *Z. piperitum*, and known to spice with anti-cancer activity [22-24]. In this study, hydroxy-*α*-sanshool (**3**) and *α*-sanshool (**4**) displayed strong anti-inflammatory effect, and it is the first report with a strong biological significance.

CONCLUSION

Extracts of *Z. piperitum* were confirmed to possess a range of skin care activity. Also, hydroxy-*α*-sanshool (3) and *α*-sanshool (4) isolated from *Z. piperitum* had a strong anti-inflammatory effect. Thus, the results suggest that *Z. piperitum* may be a valuable resource with various benefits in the ever-demanding natural skin care market.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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