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ARTICLE

Cosmeceutical bioactivities of isolated compounds from *Ligularia fischeri* Turcz leaves

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Abstract Isolated compounds from the leaves of *Ligularia fischeri* Turcz were evaluated for their cosmeceutical bioactivities. Four novel compounds, quercetin-3-*O*-β-D-glucoside (LF-4), quercetin-3-*O*-rutinoside (LF-5), ethyl-β-D-galactopyranoside (LF-7), and pterodontriol-6-*O*-β-D-glucopyranoside (LF-8), were isolated for the first time from this plant. Additionally, the four known compounds, 3-*O*-caffeoylquinic acid (LF-1), 5-*O*-caffeoylquinic acid (LF-2), 3, 5-di-*O*-caffeoylquinic acid (LF-3), and quercetin-3-*O*-galactoside (LF-6), were also isolated. To demonstrate the value of this plant as a cosmeceutical resource, the antioxidant activity and the inhibitory activity on tyrosinase and elastase were evaluated. The present study is the first time that the elastase inhibition of isolated compounds from *L. fischeri* Turcz has been investigated using the elastase inhibitory assay for anti-wrinkle effect. The ethyl acetate fraction had the highest activities in all the evaluated assays compared with the other fractions. At the compound level, LF-3 had the greatest antioxidant and elastase inhibition effect, and LF-2 showed a high level of tyrosinase inhibition. In addition, quantitative analysis of the fractions showed that the ethyl acetate fraction had the highest amount of isolated cosmeceutical bioactive compounds: LF-2, 0.9 %; LF-3, 5.3 %; LF-5, 2.8 %; and LF-6, 12.8 %.

Keywords Antioxidant · Elastase · *Ligularia fischeri* Turcz · Tyrosinase

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Introduction

Ligularia fischeri Turcz (*L. fischeri* Turcz) is distributed in damp shady regions of Korea, and has long been used as a traditional oriental folk medicine for the treatment of coughs, inflammation, jaundice, scarlet fever, and rheumatoid arthritis. Nine *Ligularia* genuses which are *L. fischeri* Turcz, *Ligularia schmidtii* (Maxim.) Makino, *Ligularia taquetii* (H. Lev. and Vaniot) Nakai, *Ligularia stenocephala* (Maxim.) Matsum. and Koidz., *Ligularia jaluensis* Kom., *Ligularia japonica* (Thunb.) Less., *Ligularia intermedia* Nakai, *Ligularia fischeri* var. *spiciformis* Nakai, and *Ligularia jamesii* (Hemsl.) Kom., are mainly distributed in Korea. The most studied genus is *L. fischeri* var. *spiciformis* Nakai, a well-known wild edible Korean vegetable (Choi et al. 2005; Shang et al. 2010; Yoo et al. 2011); however, the studies on *L. fischeri* Turcs are relatively limited.

Papers relating to the bioactivity of *L. fischeri* Turcz have reported anti-inflammatory effects and inhibition of PTP 1B activity (Lee and Choi 2008; Deng et al. 2009).

With respect to chemical compounds, sesquiterpenoids and phenolic compounds have been isolated from the roots of *L. fischeri* Turcz (Xie et al. 2010; Zhang et al. 2010). However, quercetin derivatives and their quantity have rarely been reported from this plant, the presence of which has been confirmed in other *Ligularia* genuses (Piao et al. 2011).

Moreover, the performance of bioactivity assays of *L. fischeri* Turcz leaves at the fraction and compound level has been insufficient, in particular, activity assays investigating skin protection.

Various environmental stresses including air pollution, water pollution, and ozone depletion have caused skin cancer and photoaging. Continuous exposure to ultraviolet light has caused reactive oxygen species to build up in the body, resulting in wrinkles and a decrease in the elastic properties of the skin. Destruction of elastin is related with wrinkles increasing by elastase promotes (Langton et al. 2010). In addition, overexpression of melanin by activated tyrosinase leads to skin aging and pigmentation (Brenner and Hearing 2008).

In the present study, eight compounds, including quercetin derivatives, were isolated by bioactivity-guided fractionation. The antioxidant activity and the inhibitory activities of tyrosinase and elastase of the constituents from *L. fischeri* Turcz leaves were evaluated for potential cosmetic use. Moreover, the fractions of *L. fischeri* Turcz leaves were quantified by HPLC analysis for isolated compounds. The purpose of this study is to demonstrate the value of *L. fischeri* Turcz leaves in the pharmaceutical and cosmetic industry.

Materials and methods

Chemicals and plant materials

Compound isolation was performed using Diaion HP-20 (Nippon Rensui Co., Tokyo, Japan), silica gel (70–230 mesh, Merck, Darmstadt, Germany), and ODS gel (400–500 mesh, Massachusetts, Waters, USA). HPLC-grade reagents including acetonitrile, methanol, water, and formic acid were purchased from J. T. Baker (Pennsylvania, USA). For the bioactivity assays, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitrotetrazolium blue chloride (NBT), xanthine oxidase, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), mushroom tyrosinase, elastase, and all other relevant chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The leaves of *L. fischeri* Turcz were obtained from Odae Mountain in Gangwon, South Korea. The plant was identified by professor Wan Kyunn Whang, Department of Pharmacy, Chung-Ang University, Korea.

Extraction and fractionation

Ligularia fischeri Turcz leaves (2 kg) were extracted with 70 % methanol (3 × 5 L) for 3 days at room temperature by the optimized extract condition for 4 bioactive components as phenolic compounds (Figs. 1, 2). The pooled extraction solutions were concentrated under a vacuum using a rotary evaporator at 50 °C, which yielded 400 g. The concentrate was dissolved in water and successively partitioned with hexane (3 × 1.5 L), ethyl acetate (3 × 1.5 L), and *n*-butanol (3 × 1.5 L). Each fraction was concentrated, resulting in a hexane fraction (230.3 g), an ethyl acetate fraction (20.1 g), an *n*-butanol fraction (45.5 g), and a water fraction (85.1 g), respectively.

The ethyl acetate and *n*-butanol fractions were selected as described in the "Activity assay" section and were subsequently subjected to isolation.

The ethyl acetate fraction was loaded onto silica gel using a $CHCl_3$–CH_3OH–H_2O solvent system (50:10:0 → 70:30:4), resulting in fractions 1-8. For further isolation, fractions 4, 5, 6, and 7 were separated by ODS gel using a CH_3OH–H_2O solvent system. LF-1 (20.0 mg) was isolated from subfraction 4-6-1 eluted with CH_3OH–H_2O (35:65). LF-2 (93.1 mg) was obtained from subfraction 5-3-2 eluted with CH_3OH–H_2O (45:55). Fraction 6 was eluted with CH_3OH–H_2O (35:65 → 15:85). Subfractions 6-5-2, 6-7-4, and 6-8-3 were obtained, which contained LF-3 (14.2 mg), LF-4 (9.6 mg), and LF-5 (12.1 mg), respectively. LF-6 (10.2 mg) was isolated from subfraction 7-4-4 eluted with CH_3OH–H_2O (25:75).

The *n*-butanol fraction was loaded on Diaion HP-20P and first eluted with water before sequential elution with 30, 60, and 100 % methanol. Fractions 1-4 were obtained, and fraction 2 was separated by ODS gel using CH_3OH–H_2O (30:70 → 25:75). Subfractions 2-2 and 2-3, contained LF-7 (49.7 mg) and LF-8 (14.0 mg), respectively. The structures of the isolated compounds were elucidated and

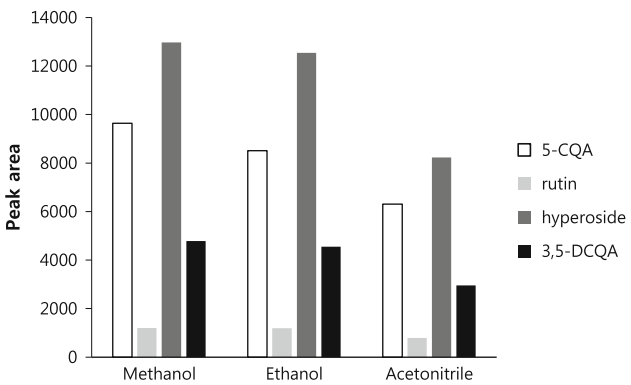


Fig. 1 Phenolic compounds comparing of extraction solvent from *L. fischeri* Turcz leaves (peak area, $n = 3$)

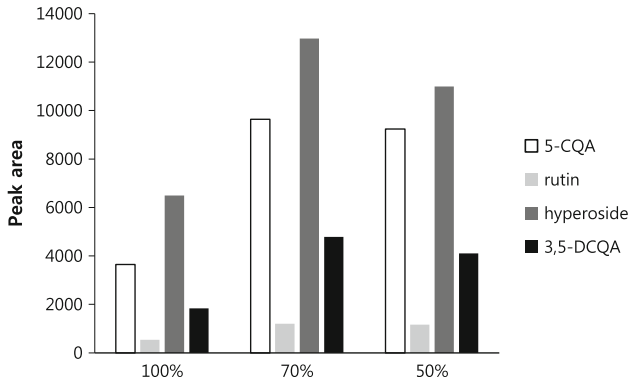


Fig. 2 Phenolic compounds comparing of 100, 70, 50 % MeOH extractions from *L. fischeri* Turcz leaves (peak area, $n = 3$)

confirmed by nuclear magnetic resonance (NMR), using VNS 600 MHz (Varian, Palo Alto, USA), and fast atom bombardment mass spectrometry, (FAB-MS) using JMS-700 (JEOL, Tokyo, Japan).

LF-1; brown amorphous powder; FAB-MS *m/z* 355 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 2.1(2H, m, H-2), 5.3(1H, m, H-3), 3.8(1H, dd, $J = 9.7$, 3.0 Hz, H-4), 4.2(1H, d, $J = 2.8$ Hz, H-5), 2.1(2H, m, H-6), 7.2(1H, d, $J = 1.5$ Hz, H-2′), 6.9(1H, d, $J = 8.1$ Hz, H-5′), 7.1(1H, dd, $J = 1.5$, 8.1 Hz, H-6′), 7.6(1H, d, $J = 15.9$ Hz, H-7′), 7.6(1H, d, $J = 15.9$ Hz, H-8′)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 78.3(C-1), 41.3(C-2), 73.4(C-3), 75.5(C-4), 73.6(C-5), 40.1(C-6), 178.1(C-7), 126.8(C-1′), 117.8(C-2′), 144.8(C-3′), 147.6(C-4′), 119.6(C-5′), 125.7(C-6′), 148.7(C-7′)].

LF-2; brown amorphous powder; FAB-MS *m/z* 355 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 1.7(2H, m, H-2), 3.7(1H, m, H-3), 3.4(1H, m, H-4), 5.1(1H, m, H-5), 2.2(2H, m, H-6), 6.9(1H, d, $J = 2.0$ Hz, H-2′), 6.7(1H, d, $J = 7.8$ Hz, H-5′), 6.8(1H, dd, $J = 1.8$, 7.8 Hz, H-6′), 7.4(1H, d, $J = 15.6$ Hz, H-7′), 6.2(1H, d, $J = 15.6$ Hz, H-8′)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 76.1(C-1), 38.2(C-2), 71.3(C-3), 73.5(C-4), 72(C-5), 38.8(C-6), 175.4(C-7), 127.8(C-1′), 115.2(C-2′), 146.8(C-3′), 149.6(C-4′), 116.5(C-5′), 123(C-6′), 147.1(C-7′), 115.3(C-8′), 168.7(C-9′)].

LF-3; yellow amorphous powder; FAB-MS *m/z* 517 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 2.1(2H, m, H-2), 5.3(1H, m, H-3), 3.9(1H, dd, $J = 4$, 7.8 Hz, H-4), 5.4(1H, m, H-5), 2.3(2H, m, H-6), 7(1H, d, $J = 1.9$ Hz, H-2′), 6.7(1H, d, $J = 7.8$ Hz, H-5′), 6.9(1H, dd, $J = 1.8$, 7.8 Hz, H-6′), 7.5(1H, d, $J = 15.6$ Hz, H-7′), 6.2(1H, d, $J = 15.6$ Hz, H-8′), 7.2(1H, d, $J = 1.9$ Hz, H-2″), 6.7(1H, d, $J = 7.8$ Hz, H-5″), 6.8(1H, dd, $J = 1.8$, 7.8 Hz, H-6″), 7.4(1H, d, $J = 15.6$ Hz, H-7″), 6.2(1H, d, $J = 15.6$ Hz, H-8″)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 74.7(C-1), 36(C-2), 72.5(C-3), 70.5(C-4), 72.1(C-5), 38.6(C-6), 177.3(C-7), 127.9(C-1′), 115.1(C-2′), 146.8(C-3′), 149.6(C-4′), 116.5(C-5′), 123.1(C-6′), 147.3(C-7′), 115.5(C-8′), 168.9(C-9′), 127.8(C-1″), 115.3(C-2″), 146.9(C-3″), 149.5(C-4″), 116.1(C-5″), 123(C-6″), 147(C-7″), 115.2(C-8″), 168.3(C-9″)].

LF-4; yellow amorphous powder; FAB-MS *m/z* 465 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 6.2(1H, d, $J = 2.0$ Hz, H-6), 6.4(1H, d, $J = 2.0$ Hz, H-8), 7.5(1H, d, $J = 2.0$ Hz, H-2′), 6.8(1H, d, $J = 8.0$ Hz, H-5′), 7.4(1H, dd, $J = 8.0$, 1.8 Hz, H-6′), 5.4(1H, d, $J = 7.8$ Hz, H-1 glu), 3.3–3.8 (5H, m, H-2 ∼ 6 glu)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 156.7(C-2), 133.8(C-3), 177.9(C-4), 161.7(C-5), 99.1(C-6), 164.5(C-7), 93.9(C-8), 156.6(C-9), 104.4(C-10), 121.6(C-1′), 115.6(C-2′), 145.2(C-3′), 148.9(C-4′), 116.6(C-5′), 121.5(C-6′), 101.3(C-1 glu), 74.5-61.4(C-2 ∼ 6 glu)].

LF-5; yellow amorphous powder; FAB-MS *m/z* 611 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 6.2(1H, d, $J = 1.8$ Hz, H-6), 6.3(1H, d, $J = 1.8$ Hz, H-8), 7.5(1H, d, $J = 2.4$ Hz, H-2′), 6.8(1H, d, $J = 8.4$ Hz, H-5′), 7.6(1H, dd, $J = 1.8$, 8.4 Hz, H-6′), 5.3(1H, d, $J = 7.2$ Hz, H-1 rha), 3.3–3.8(5H, m, H-2 ∼ 6 rha), 4.3(1H, d, $J = 1.8$ Hz, H-1 glu), 3.2–3.7(4H, m, H-2 ∼ 5 glu), 0.9(1H, d, $J = 0.6$ Hz, H-6 glu)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 157.3(C-2), 134.1(C-3), 178.2(C-4), 157.5(C-5), 99.5(C-6), 164.9(C-7), 94.5(C-8), 162.1(C-9), 104.8(C-10), 122.5(C-1′), 116.1(C-2′), 145.6(C-3′), 149.3(C-4′), 117.1(C-5′), 122(C-6′), 102.2(C-1 rha), 69.1-73.3(C-2 ∼ 5 rha), 101.6(C-1 glu), 67.9–77.3(C-2 ∼ 6 glu), 18.6(C-6 glu)].

LF-6; yellow amorphous powder; FAB-MS *m/z* 465 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 6.1(1H, d, $J = 2.0$ Hz, H-6), 6.3(1H, d, $J = 2.0$ Hz, H-8), 7.5(1H, d, $J = 1.9$ Hz, H-2′), 6.3(1H, d, $J = 8.9$ Hz, H-5′), 7.5(1H, dd, $J = 1.89$, 8.9 Hz, H-6′), 5.3(1H, d, $J = 7.7$ Hz, H-1 gal), 3.3–3.6(5H, m, H-2 ∼ 6 gal)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 156.3(C-2), 133.5(C-3), 177.5(C-4), 161.2(C-5), 98.7(C-6), 164.2(C-7), 93.5(C-8), 156.3(C-9), 103.9(C-10), 121.1(C-1′), 115.2(C-2′), 144.8(C-3′), 148.5(C-4′), 115.9(C-5′), 122(C-6′), 101.8(C-1 gal), 60.2–75.8(C-2 ∼ 6 gal)].

LF-7; white powder; FAB-MS *m/z* 209 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 5.3(1H, d, $J = 3.7$ Hz, H-1), 4.6(1H, dd, $J = 3.7$, 9.9 Hz, H-2), 4.4(1H, dd, $J = 3.7$, 9.9 Hz, H-3), 4.6(1H, dd, $J = 1.4$, 3.5 Hz, H-4), 4.4(1H, m, H-5), 4.3(2H, t, $J = 6.5$ Hz, H-6), 3.5(2H, m, H-1′), 1.0(3H, t, 7.0 Hz, H-2′)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 100.5(C-1), 72.7(C-2), 71.8(C-3), 70.6(C-4), 71(C-5), 63.5(C-6), 62.7(0C-1′), 15.4(C-2′)].

LF-8; white powder; FAB-MS *m/z* 419 $[M+H]^+$; ^{1}H-NMR (600 MHz, CD_3OD) [δ 3.6(1H, m, H-1), 1.8–1.9 (2H, m, H-2), 1.8–1.9(H, m, H-3), 2.3(1H, d, $J = 11.4$ Hz, H-5), 5.0(1H, dd, $J = 11.4$, 4.4 Hz, H-6), 2.2(1H, m, H-7), 1.6–1.8(2H, m, H-8), 1.5–1.9(2H, m, H-9), 2.4(1H, m, H-11), 0.9(3H, d, $J = 6.5$ Hz, H-12), 1.4(3H, d,

$J = 6.5$ Hz, H-13), 1.2(3H, s, H-14), 1.7(3H, s, H-15), 5.1(1H, d, $J = 6.5$ Hz, H-1′), 3.9(1H, t, $J = 8.3$ Hz, H-2′), 4.2(1H, t, $J = 8.3$ Hz, H-3′), 4.1(1H, t, $J = 8.3$ Hz, H-4′), 4.0(1H, m, H-5′), 4.3–4.5(2H, m, H-6′)]; ^{13}C-NMR (151 MHz, CD_3OD) [δ 79.3(C-1), 29.3(C-2), 41(C-3), 72.4(C-4), 51.3(C-5), 78.6(C-6), 41.7(C-7), 23.3(C-8), 36.5(C-9), 42.4(C-10), 25.9(C-11), 22.9(C-12), 23.6(C-13), 14.6(C-14), 24.5(C-15), 100.3(C-1′), 75.8(C-2′), 78.9(C-3′), 72.1(C-4′), 78.6(C-5′), 63.2(C-6′)].

Activity assays

The antioxidant activities (using DPPH, ABTS, and superoxide anion scavenging assays) and inhibitory activities of tyrosinase were performed on the extract and fractions at different concentrations (12.5–100 μg/mL). Isolated compounds were evaluated for their antioxidant activities and inhibition activities of tyrosinase and elastase. Isolated compounds were dissolved in dimethyl sulfoxide to a final concentration of 6.25–100 μM. All assay results were measured with a Sunrise microplate reader (Tecan, Männedorf, Switzerland), and expressed using the IC_{50} flowing formula $[1 - (A_{experiment} - A_{blank})/A_{control}] \times 100$.

DPPH assay

The free radical scavenging activity was measured using the stable DPPH radical according to a modification of Hatano et al. (1997). The sample solution (20 μL) was added to 0.1 mM DPPH ethanol solution (180 μL). The mixture was vortexed for 10 s and incubated at 37 °C for 15 min. The activity was measured at 517 nm, with ascorbic acid as a positive control.

ABTS radical scavenging assay

ABTS radical scavenging activity was measured according to a modification of the method described by Arnao et al. (2001). The reaction mixture was prepared with 7.4 mM ABTS and 2.6 mM potassium persulfate buffer (pH 7.5), and incubated for 12 h at room temperature in the dark. The reaction mixture was diluted with methanol until the absorbance was 0.8–1.2 at 732 nm. The reaction mixture (950 μL) and sample solutions (50 μL) were then mixed and incubated at 37 °C for 10 min. The ABTS radical scavenging assay was evaluated by measuring the absorbance at 732 nm, with Trolox as a positive control.

Superoxide anion scavenging assays

Superoxide anion scavenging activity was evaluated by nitrotetrazolium blue chloride (NBT) reduction (Choi et al. 2002). 160 μL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.4), 0.6 mM hypoxanthine, 0.2 mM NBT, and 1 mM EDTA was added to the sample solution (20 μL). 0.2 unit/mL xanthine oxidase (20 μL) was subsequently added, and the reaction mixture was incubated for 10 min at 37 °C. The activity was measured as the absorbance of NBT at 560 nm. NBT could be reduced to mono- and di-formazans with an absorbance maximum at approximately 560 nm, by superoxide anions generated in the biological system by hypoxanthine and by xanthine oxidase at pH 7.4. Allopurinol was used as a positive control.

Tyrosinase inhibition assay

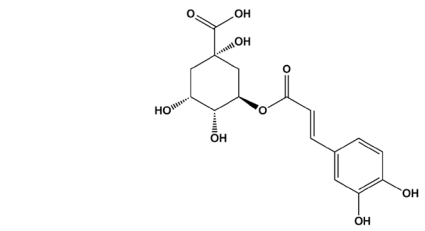
Tyrosinase inhibition was assessed following a modification of the method by Jo et al. (2012). In brief, 120 μL reaction mixture containing 0.1 mM L-tyrosine and 10 mM sodium phosphate buffer (pH 6.8) was added to the sample solution (20 μL). 100 unit/mL mushroom tyrosinase (70 μL) was subsequently added. The reaction mixture was incubated for 20 min at 37 °C before measuring the absorbance at 490 nm. Kojic acid was used as a positive control.

Elastase inhibition assay

Elastase inhibitory activity was measured using a modification of the method described by Nema et al. (2011). 80 μL 100 mM Tris–HCl buffer (pH 8.0), 20 μL 3 mM N-Succinyl-(Ala)3-p-nitroanilide, and 40 μL sample solution were mixed and incubated for 10 min at 25 °C, followed by the addition of 20 μL 0.2 unit/mL elastase. Lastly, the solution was incubated for 10 min at 25 °C and the absorbance was measured at 410 nm. Oleanolic acid, a known anti-wrinkle ingredient, was used as a positive control.

Statistical analysis

Statistical significance was tested using the statistical package for social science (SPSS for Windows ver. 19.0) program. An ANOVA test was carried out for all experiments. The data are expressed as the mean ± standard deviation (SD) for triplicate determinations. A p value of <0.05 was considered statistically significant.



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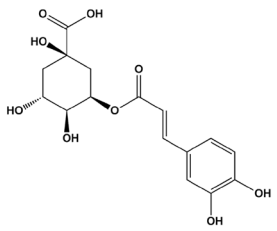

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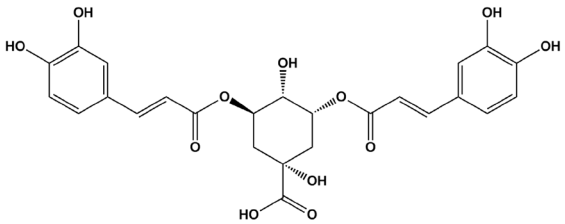

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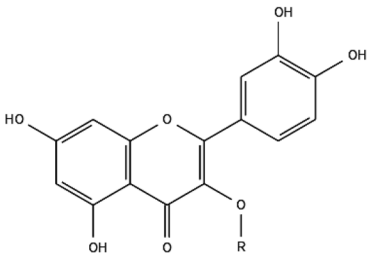
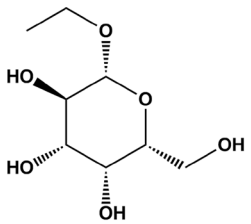


4: quercetin-3-O-β-D-glucoside(R=glucose)
5: quercetin-3-O-rutinoside(R=rutinoside)
6: quercetin-3-O-galactoside(R=galactose)



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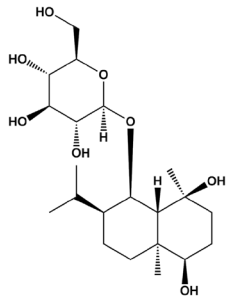

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Fig. 3 Chemical structures of compounds **1–8** from *L. fischeri* Turcz leaves

HPLC analysis

All fractions were analyzed by HPLC for the quantification of LF-2, 3, 5, and 6, which were suggested as marker compounds. LF-1, 4, 7, and 8 were excluded as markers due to the fact that LF-1 and 4 were contained in small amounts in *L. fischeri* Turcz leaves, and LF-7 and 8 were not detected by UV according to their structural characteristics. Each 50 μg/mL fraction was separated on an Kromasil 100-5 C18 column (4.6 × 250 mm 5 μm, phenomenex, California, USA) using an Alliance HPLC System (Waters, Massachusetts, USA) equipped with a UV detector. The mobile phase consisted of solvent A (0.2 % aqueous formic acid) and solvent B (acetonitrile). The gradient conditions used were as follows: 0–10 min, solvent B 10–15 %; 10–15 min, solvent B 15–16 %; 15–20 min, solvent B 16–17 %; and 20–30 min, solvent B 17–15 %. The flow rate was 1 mL/min and the injection volume was 10 μL. The UV detection was performed at 320 nm.

Calibration preparation

The content profile for each fraction was calculated from the regression equations based on the calibration curve. The standards were dissolved in methanol to a final concentration of 2–200 μg/mL. Calibration curves of the five concentrations were presented as the slope, intercept, correlation coefficient, limit of detection (LOD), and limit of quantification (LOQ). The measurements were conducted

Table 1 IC_{50} values of the fractions in the DPPH, ABTS, and superoxide anion radical scavenging assays

Fraction	Antioxidant activity (IC_{50}, μg/mL)		
	DPPH	ABTS	Superoxide anion radicals
Methanol extract	673.2 ± 21.9*	313.6 ± 2.3*	>1000
Hexane	789.3 ± 26.9**	678.1 ± 22.5*	>1000
Ethyl acetate	269.2 ± 16.6**	202.4 ± 1.7**	458.3 ± 44.5**
n-Butanol	530.2 ± 25.6*	247.1 ± 3.5*	670.4 ± 43.7**
Water	848.9 ± 12.4**	628.7 ± 20.3**	>1000

Values are expressed as the mean ± SD (* $p < 0.05$, ** $p < 0.01$, $n = 3$)

Table 2 IC_{50} values of the compounds in the DPPH, ABTS, and superoxide scavenging radical assays

Compound	Antioxidant activity (IC_{50}, μM)		
	DPPH	ABTS	Superoxide anion radicals
1	54.4 ± 3.8*	80.1 ± 3.6**	108.7 ± 3.2**
2	54.7 ± 2.1*	70.3 ± 2.6 *	121.8 ± 8.4*
3	37.4 ± 1.2*	50.3 ± 5.7**	63.5 ± 1.5*
4	52.4 ± 1.1*	88.4 ± 5.4*	92.5 ± 1.9*
5	51.2 ± 0.9**	74.7 ± 1.3	78.2 ± 5.4*
6	53.6 ± 1.4*	80.8 ± 2.0*	102.5 ± 7.5*
7	387.6 ± 1.4	–	230.5 ± 10.4*
8	687.6 ± 1.1	–	399.7 ± 7.2
Positive control	48.4 ± 1.5**	89.9 ± 0.9*	97.7 ± 3 2*

Values are the mean ± SD (* $p < 0.05$, ** $p < 0.01$, $n = 3$)

Table 3 IC_{50} values of the fractions in the tyrosinase inhibitory activity

Fraction	IC_{50} (μM)
Methanol extract	–
Hexane	>1000
Ethyl acetate	428.7 ± 64.9*
n-Butanol	621.0 ± 43.1*
Water	>1000

Values are the mean ± SD (* $p < 0.05$, $n = 3$)

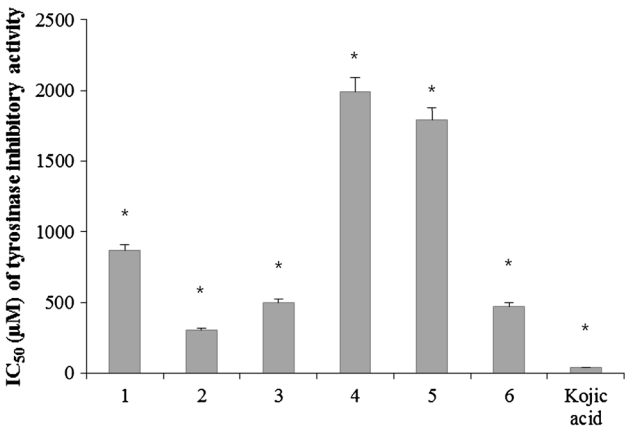

Fig. 4 Tyrosinase inhibition assay of compounds **1–6**. Values are expressed as the mean ± SD (* $p < 0.05$, $n = 3$). No effect on tyrosinase inhibition was observed with 7 or 8. Kojic acid was used as a positive control

by plotting the peak area versus the concentration in triplicate. The LOD and LOQ for the four standards were assessed based on the standard deviation of the response and the slope.

Results and discussion

Structure elucidation of the novel compounds

Bioassay-guided fractionation afforded four compounds: LF-4, 5, 7, and 8, which is the first instance that these compounds have been isolated from *L. fischeri* Turcz leaves.

Four known compounds were also isolated and identified as 3-*O*-caffeoylquinic acid (LF-1), 5-*O*-caffeoylquinic acid (LF-2), 3,5-di-*O*-caffeoylquinic acid (LF-3) (Hyun et al. 2010), and Quercetin-3-*O*-galactoside (LF-6) (Pereira et al. 2012), on the basis of comparison of their NMR data

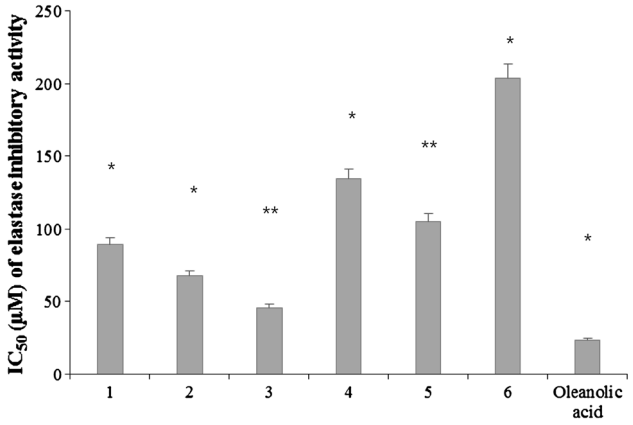

Fig. 5 Elastase inhibitory activity of the isolated compounds. Values are expressed as the mean ± SD (* $p < 0.05$, ** $p < 0.01$, $n = 3$). **7** and **8** compounds showed no effect on elastase inhibition

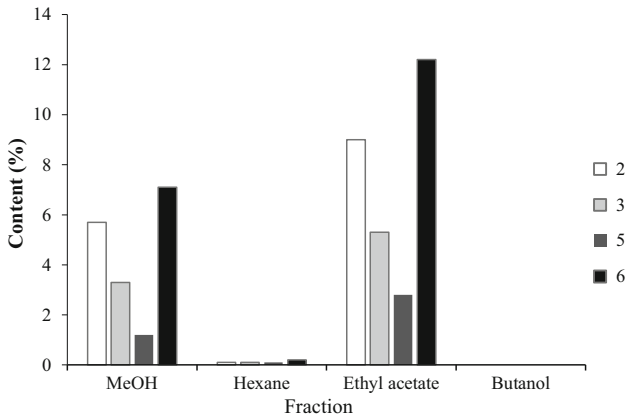

Fig. 6 Content profile of the fractions from *L. fischeri* Turcz leaves

and molecular weight with those reported in the literature. Figure 3 shows the structure of the isolated compounds.

In the ^{13}C-NMR of LF-4, the ketone carbon signal at δ_C 177.9 and the flavone-3-ol carbon signal at δ_C 133.8 suggested a flavonol backbone. In the ^{1}H-NMR of LF-4, signals at δ_H 7.5, 7.4, and 6.8, showing the ABX coupling system of the B-ring, indicated a quercetin derivative. The proton signals at δ_H 3.3-3.8 indicated a glucose moiety. The anomeric proton at δ_H 5.5 was related to the β-linkage with the aglycone at the C-3 position. Based on the data above, LF-4 was identified as quercetin-3-glucoside in comparison with previously reported data (Song et al. 2007).

The ^{13}C-NMR data of LF-5 indicated that there were 15 carbon signals typical for a quercetin, and the others were assigned to glycosides. By comparison with the quercetin, the C-3 was shifted upfield, demonstrating glycosylation at that position. The ^{1}H-NMR spectra of LF-5 supported the presence of one rhamnose and one glucose moiety, with the rhamnose anomeric proton signal at δ_H 5.3 and the glucose H-1 signal at δ_H 4.3. The coupling constant of the anomeric proton ($J = 7.2$ Hz) confirmed a β-linkage with aglycone. LF-5 was identified as quercetin-3-rutinoside by comparison with the authentic reference (Gohari et al. 2009).

The NMR spectra of LF-7 showed anomeric proton signals at δ_H 4.3-5.3 and an anomeric carbon signal at δ_C 100.5, indicating a galactopyranoside. Proton signals at δ_H 3.5 and 1.0 showed the existence of an ethyl. LF-7 was identified as ethyl-β-D-galactopyranoside by comparison with an authentic reference (Kim Tuyen et al. 2007).

The ^{1}H-NMR spectra of LF-8 showed the existence of four methyl groups at δ_H 0.9, 1.2, 1.4, and 1.7, and two oxygenated methine proton signals at δ_H 3.6 and 5.0. In the ^{13}C-NMR of 8, 15 carbon signals indicated a eudesmane sesquiterpene skeleton. In HMBC, a proton signal at δ_H 100.3 corresponded to δ_C 78.6, indicating a linkage of a glucosyl unit with an aglycone at the C-6 position. Compared with an authentic reference (Hua et al. 2004), LF-8 was identified as pterodontriol-6-*O*-β-D-glucopyranoside.

Antioxidant assay

The antioxidant activity (using the DPPH, ABTS, and superoxide anion radical scavenging assays) was assessed by IC_{50} values. A lower IC_{50} value indicates a better antioxidant activity. The antioxidant activity of the fractions showed that the ethyl acetate fraction had a significantly higher antioxidant level, followed by the *n*-butanol fraction.

The data from the antioxidant assays of the isolated compounds showed a similar or higher antioxidant effect compared with the positive controls, with the exception of LF-7 and 8, with which low antioxidant activities were

Table 4 Regression equation, correlation coefficient (r^2), range, LOD, and LOQ of the four compounds

Compound	Regression equation[a]	Correlation coefficient (r^2)	Range (μg/mL)	LOQ (μg/mL)	LOD (μg/mL)
2	$Y = 6862.7X - 4106.1$	1	2–200	1.6	1.8
3	$Y = 2977.4X - 7990.7$	0.9999	10–200	3.3	1.3
5	$Y = 16696X - 148{,}904$	0.9997	2–200	1.2	0.3
6	$Y = 9930.1X + 17{,}987$	0.9999	2–200	1.4	0.5

[a] Y peak area; X concentration

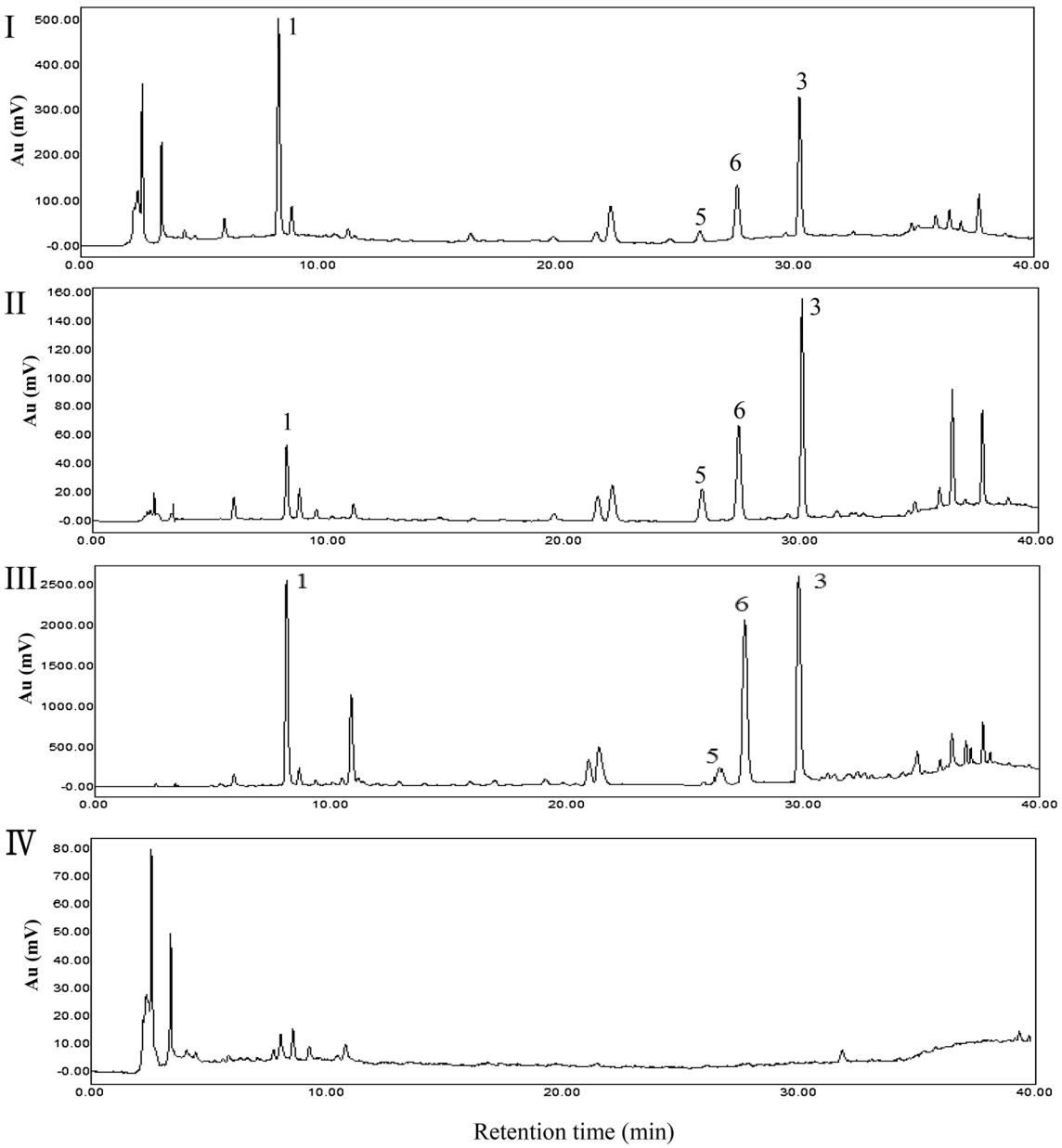

Fig. 7 HPLC chromatogram of the extracts and fractions of *L. fischeri* Turcz leaves. I, 70 % methanol extract; II, hexane fraction; III, ethyl acetate fraction; IV, butanol fraction; 1, LF-1; 3, LF-3; 5, LF-5; 6, LF-6

observed. Phenolic compounds such as LF-1, 2, 3, 4, 5, and 6 had a better antioxidant activity than the non-phenolic compounds, LF-7 and 8. Although the *n*-butanol fraction showed the second highest antioxidant activity, LF-7 and 8 isolated from the *n*-butanol fraction showed no antioxidant activity. This result suggests that the *n*-butanol fraction may contain other bioactive materials, or that LF-7 and 8 may interact with a coexisting substance in the *n*-butanol fraction to result in the antioxidant reaction.

The results of the antioxidant assays of the fractions and compounds are shown in Tables 1, 2, respectively.

Tyrosinase inhibitory activity

The whitening effect as seen by the tyrosinase inhibitory activity assay was screened using L-tyrosine as a substrate and kojic acid as a positive control. The tyrosinase inhibitory activity is expressed as IC_{50} values in Table 3. The tyrosinase inhibition of the fractions showed that the ethyl acetate fraction had the lowest IC_{50} (428.7 μg/mL), followed by the *n*-butanol fraction (621.0 μg/mL).

At the compound level, the tyrosinase inhibitory activity of the compounds showed that most of the caffeic acid derivatives displayed a higher level of tyrosinase inhibition than the flavonoid derivatives. The IC_{50} values of LF-1, 2, and 3, which are caffeic acid derivatives, were 868.0, 302.7, and 496.5 μM, respectively. The IC_{50} values of LF-4, 5, and 6, which are flavonoid derivatives, were 1992.5, 1789.3, and 470.8 μM, respectively (Fig. 4). LF-7 and 8 showed no tyrosinase inhibitory activity.

Elastase inhibitory activity

This is the first time that the elastase inhibition of isolation compounds from *L. fischeri* Turcz was investigated using oleanolic acid as a positive control in order to inquire about the anti-wrinkle effect. The elastase inhibitory activity is expressed as IC_{50} values in Fig. 5. The caffeic acid derivatives showed a more effective elastase inhibition activity than the flavonoid derivatives. The IC_{50} values of LF-1-6 were 89.8, 67.8, 45.9, 134.6, 105.2, and 203.6 μM, respectively. LF-3 contained two caffeoyl moieties and had the strongest elastase inhibition activity compared with the other compounds, while LF-7 and 8 showed no elastase inhibitory activity.

Content profiles of the fractions

The calibration results showed that the correlation coefficient of the best-fitting lines was obtained by regression, which was >0.9999 for all. The LOD and LOQ were <3.3 and <1.8 μg/mL, respectively, indicating that the method had suitable sensitivity. Table 4 shows the results of linearity for LOD and LOQ.

In order to screen the quantity of compounds in each fraction, the hexane, ethyl acetate, and butanol fractions were analyzed for LF-2, 3, 5, and 6 as marker compounds. The four compounds were mainly included in the ethyl acetate fraction: LF-2, 9.0 %; LF-3, 5.3 %; LF-5, 2.8 %; and LF-6, 12.2 % (Fig. 6). The butanol fraction, which contained LF-7 and 8, seemed to contain no compounds by HPLC analysis because LF-7 and 8 were not detected at UV 320 nm. Copies of the HPLC chromatograms are available as shown in Fig. 7.

In conclusion, our study reveals bioactive components and their quantities from *L. fischeri* Turcz leaves. The caffeic acid and quercetin derivatives from this plant are considered potent material as antioxidants and tyrosinase and elastase inhibitors. Our data support the use of *L. fischeri* Turcz leaves as a cosmeceutical resource.

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