Melanogenesis Inhibitory Effect of Fatty Acid Alkyl Esters Isolated from Oxalis triangularis

Sungran Huh,a,# Young-Sooh Kim,a,∗ Eunsun Jung,a Jihee Lim,b Kwang Sun Jung,b Myeong-Ok Kim,b,c Jongsung Lee,a and Deokhoon Parka,a

a Biospectrum Life Science Institute; 101–701 SK Ventium, 522 Dangjiung Dong, Gunpo, Gyunggi Do 435–833, Republic of Korea; b SkinCure Life Science Institute, Jeju Bio-industry Development Center; Jeju, Jeju Do 690–121, Republic of Korea; and c Department of Cosmetic & Beauty, Sookmyung Women’s University; Seoul 140–742, Republic of Korea.

Received November 23, 2009; accepted March 31, 2010; published online April 16, 2010

Ten fatty acid alkyl esters isolated from Oxalis triangularis, were evaluated for the effects on melanogenesis using mouse B16 melanoma cells. Treatment of methyl linoleate, methyl linolenate, ethyl linoleate and ethyl linolenate significantly blocked forskolin-induced melanogenesis and inhibited tyrosinase activity. In addition, we found that they inhibited cAMP production, suggesting that their anti-melanogenic effect is mediated by the inhibition of cAMP production. We concluded that methyl/ethyl linoleate and linolenate isolated from Oxalis triangularis have pigment inhibition activity. These compounds may be useful as the cosmetic agent to stimulate skin whitening.

Key words Oxalis triangularis; melanogenesis; fatty acid alkyl ester

Oxalis triangularis (purple shamrock or purple clover) in the family Oxalidaceae is an edible perennial plant that is easily cultivated. The leaves of Oxalis triangularis are especially appreciated due to their sour and exotic taste. Oxalis triangularis has intensely purple leaves with a monomeric diglucoside basis, which makes them a potential source of anthocyanin content of 195 mg/100 g on a malvidin 3,5-triangulis (High Pressure Liquid Chromatography) was conducted using a Combiflash Companion instrument with an UV/Vis detector (Teledyne ISCO, Inc., U.S.A.). Preparative HPLC was conducted on reversed phase preparative HPLC (Phenomenex Luna 250 mm, 5 μm film thickness, Hewlett-Packard, Palo Alto, CA, U.S.A.) connected to a 5890 gas chromatograph fitted with a fused silica capillary column (HP-5, 0.25×30 m, 0.25 μm film thickness, Hewlett-Packard). The GC-conditions were as follows: on-column injection mode, He 1 ml/min; oven temperature 60 °C for 2 min; and thermal gradient, 10 °C/min to 260 °C. Medium pressure liquid chromatography (MPLC) was conducted using a Combiflash Companion instrument with an UV/Vis detector (Teledyne ISCO, Inc., U.S.A.). Preparative HPLC (High Pressure Liquid Chromatography) was conducted using a Prep LC 2000 and 2487 Dual λ Absorbance detector (Waters). Silica gel (230—400 mesh, Merck) was used for the column chromatography. All HPLC-grade organic solvents and bulk organic solvents were purchased from J.T. Baker and Duksan Company, South Korea.

Plant Material Naturally-grown Oxalis triangularis were collected from Jeju Island, Korea, from June to August 2008. A voucher sample has been deposited at the Jeju Bio Diversity Research Institute of the Jeju Hi-Tech Industry Development Institute.

Extraction and Isolation Whole bodies of Oxalis triangularis (1.6 kg dry weight) were homogenized and extracted with 80% ethanol (301×3). The extracts were then concentrated in vacuo, after which they were re-extracted with chloroform (21×3). After reducing to dryness in vacuo, the chloroform fraction (138 g) was solvent-partitioned between n-hexane and 80% methanol (21×3). The concentrated n-hexane soluble fraction (57.58 g) was then purified by silica gel column chromatography (100 g, Merck) and eluted stepwise with chloroform containing 0, 1, 2, 10 and 100% methanol (11 each). The fractions eluted in 0% and 1% methanol in chloroform were combined and concentrated in vacuo (17.32 g), after which they were subjected to MPLC (RediSep, silica 40 g, 30×140 mm; detection, UV at 254 nm; flow rate, 40 ml/min). The elution was performed stepwise in chloroform–methanol (0%, 2%, 10%, 100% methanol) for 10 min each to give 40 subfractions. Fraction 4 was subjected to reversed phase preparative HPLC (Phenomenex Luna C18(2), 21.2×250 mm, 5 μm) and eluted with 40% acetonitrile in water for 10 min, then in a gradient to 100% acetonitrile for 20 min, and finally in 100% acetonitrile for 20 min. The samples were eluted at a flow rate of 17 ml/min and the fractions were collected every minute. HPLC fractions 42—43 (methyl linolenate), 45 (ethyl linolenate), 47 (methyl linoleate) and 51 (ethyl linoleate) showed biological activity; therefore, these fractions were analyzed by GC-MS and 1H- and 13C-NMR.

Methyl Linoleate 1H-NMR (500 MHz, CDCl3) δ:

# These authors contributed equally to this work.
∗ To whom correspondence should be addressed. e-mail: pdh@biospectrum.com © 2010 Pharmaceutical Society of Japan
5.40—5.30 (4H, m, H-9, 10, 12, 13), 3.66 (3H, s, −OCH3), 2.77 (2H, t, J = 6.5 Hz, H-11), 2.30 (2H, t, J = 7.5 Hz, H-2), 2.07—2.03 (4H, m, H-8, 14), 1.65—1.59 (2H, m, H-3), 1.38—1.26 (14H, m, H-4, 5, 6, 7, 15, 16, 17), 0.89 (3H, t, J = 6.5 Hz, H-18); 13C-NMR (500 MHz, CDCl3) δ: 174.4 (C-1), 130.4 (C-13), 130.3 (C-9), 128.3 (C-12), 128.2 (C-10), 51.7 (−OCH2−), 34.3 (C-2), 31.7 (C-16), 29.8 (C-7), 29.6 (C-15), 29.4 (C-14), 29.3 (C-3), 29.3 (C-5), 29.3 (C-4), 27.4 (C-14), 27.4 (C-15), 26.0 (C-8), 25.8 (C-11), 25.2 (C-3), 22.8 (C-17), 14.5 (C-18).

6.50—6.45 (4H, m, H-9, 10, 12, 13), 4.13 (2H, m, −OCH2−), 2.28 (2H, t, J = 7.5 Hz, H-2), 2.08—2.03 (4H, m, H-8, 14), 1.61—1.60 (2H, m, H-3), 1.39—1.31 (14H, m, H-4, 5, 6, 7, 15, 16), 1.23—1.23 (3H, n, H-20), 0.89 (3H, t, J = 6.3 Hz, H-18); 13C-NMR (500 MHz, CDCl3) δ: 174.1 (C-1), 130.4 (C-13), 130.3 (C-9), 128.3 (C-12), 128.2 (C-10), 60.4 (−OCH2−), 34.6 (C-2), 31.8 (C-16), 29.8 (C-7), 29.6 (C-14), 29.3 (C-5), 29.3 (C-4), 27.4 (C-14), 27.4 (C-8), 25.8 (C-11), 25.2 (C-3), 22.8 (C-17), 14.5 (C-18), 14.3 (C-18).

Ethyl Linolenate

1H-NMR (500 MHz, CDCl3) δ: 5.42—5.39 (6H, m, H-9, 10, 12, 13, 15, 16), 4.12 (2H, m, −OCH2−), 2.28—2.27 (4H, m, H-8, 14), 1.65—1.60 (2H, m, H-3), 1.39—1.31 (8H, m, H-4, 5, 6, 7, 15, 16), 1.28—1.27 (3H, m, H-20), 1.32—1.29 (3H, t, J = 7.5 Hz, H-18); 13C-NMR (500 MHz, CDCl3) δ: 174.0 (C-1), 132.2—127.4 (6C, C-9, 10, 12, 13, 15, 16), 51.6 (−OCH3), 34.3 (C-2), 31.7 (C-16), 29.8 (C-7), 29.6 (C-15), 29.4 (C-14), 29.3 (C-5), 29.3 (C-4), 27.4 (C-7), 25.8 (C-14), 25.8 (C-11), 25.2 (C-3), 22.8 (C-17), 14.5 (C-18), 14.3 (C-18).

Ethyl Linoleate

1H-NMR (500 MHz, CDCl3) δ: 5.42—5.39 (6H, m, H-9, 10, 12, 13, 15, 16), 4.12 (2H, m, −OCH2−), 2.28—2.27 (4H, m, H-8, 14), 1.65—1.60 (2H, m, H-3), 1.39—1.31 (8H, m, H-4, 5, 6, 7, 15, 16), 1.28—1.27 (3H, m, H-20), 0.38 (2H, m, −CH2−), 1.32—1.29 (3H, t, J = 7.5 Hz, H-18); 13C-NMR (500 MHz, CDCl3) δ: 174.1 (C-1), 130.4 (C-13), 130.3 (C-9), 128.3 (C-12), 128.2 (C-10), 60.4 (−OCH2−), 34.6 (C-2), 31.8 (C-16), 29.8 (C-7), 29.6 (C-14), 29.3 (C-5), 29.3 (C-4), 27.4 (C-14), 27.4 (C-8), 25.8 (C-11), 25.2 (C-3), 22.8 (C-17), 14.5 (C-18), 14.3 (C-18).

Cell Culture

B16 mouse melanoma cells were purchased from the Korean Cell Bank (Seoul, Korea). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified, 5% CO2 controlled incubator.

Melanin Content Assay

The melanin content of cultured B16 cells was determined following the method described by Kinoshita et al. Briefly, cells were washed with phosphate-buffered saline (PBS) and then dissolved in 1 N NaOH for 1 h at 60 °C. The absorbance of each sample at 420 nm was then read. The intensity of the color was inversely proportional to the concentration of melanin in the cell lysates.

Statistical Evaluation

The means ± S.E.M. were calculated and compared among groups using a t-test for independent samples. Values of *p < 0.05 were considered to be significant [ED highlight – consider deleting this, e.g., “A p < 0.05 was considered to be significant.” The asterisk would be useful if there were 2 values (e.g. *p < 0.05, **p < 0.01) or in a figure legend].

RESULTS AND DISCUSSION

Whole bodies of Oxalis triangularis were extracted with 80% aqueous ethanol, followed by chloroform. The extract was then solvent-partitioned between n-hexane and 80% methanol, after which the concentrated n-hexane soluble fraction was purified by silica gel column chromatography and subjected to MPLC. The GC-MS analysis of the MPLC fraction 4 of Oxalis triangularis revealed the presence of five fatty acid methyl esters (FAMEs) and five fatty acid ethyl esters (FAEEs). Based on a comparison with authentic FAMEs and FAEEs, these compounds were identified as methyl palmitate, ethyl palmitate, methyl linoleate, methyl linolenate, methyl oleate, methyl stearate, ethyl linolenate, ethyl oleate, ethyl linoleate, and ethyl stearate (Fig. 1). The endogenous levels of FAMEs and FAEEs in Oxalis triangularis were also determined based on comparison with authentic FAMEs and FAEEs. Specifically, the levels were found to be 248.19 (methyl palmitate), 367.99 (ethyl palmitate), 149.09 (methyl linoleate), 124.10 (methyl linolenate), 186.31 (methyl oleate), 54.36 (methyl stearate), 327.75 (ethyl linolenate), 173.93 (ethyl oleate), 488.55 (ethyl linoleate) and 66.58 (ethyl stearate) μg/g fr. weight. For purification, MPLC...
fraction 4 was subjected to reversed phase preparative HPLC. As a result, HPLC fractions 42 and 43 (methyl linolenate), 45 (ethyl linolenate), 47 (methyl linoleate) and 51 (ethyl linoleate), were obtained.

To determine the effects of these 5 FAMEs and 5 FAEEs isolated from *Oxalis triangularis* on melanogenesis, we investigated the levels of melanin content in cultured B16 melanoma cells. We found that methyl linoleate, methyl linolenate, ethyl linoleate and ethyl linolenate reduced the levels of melanin content in a concentration-dependent manner. Specifically, methyl linoleate, methyl linolenate, ethyl linoleate and ethyl linolenate decreased the level of melanin content by 82—54%, 80—25%, 92—47%, and 91—30%, respectively, when compared with forskolin-treated control cells (Fig. 2A). However, there is the possibility that the reduction of melanin content was induced by a cytotoxic effect of these compounds. Therefore, we conducted an MTT assay in B16 melanoma cells. The results revealed that methyl/ethyl linoleate and linolenate had no cytotoxic effect when administered at 100 μM (Fig. 2B).

The melanogenesis-inhibitory activities of the four compounds were further evaluated by determining their half maximal inhibitory concentration (IC₅₀), which is a measure of the effectiveness of a compound at inhibiting biological function. The IC₅₀ of these compounds was determined using a melanin content assay. As shown in Table 1, while the IC₅₀ of methyl and ethyl linoleates was 245 μM and 325 μM, respectively, the IC₅₀ of methyl and ethyl linolenates was 60 μM and 70 μM, respectively. These results indicate that methyl and ethyl linolenates have more potent anti-melanogenic activity than methyl and ethyl linoleates. In a tyrosinase luciferase reporter assay, all of the compounds inhibited tyrosinase promoter activity induced by forskolin, which was consistent with the results of the melanin content assay (Fig. 3). In addition, the compounds reduced forskolin-induced cAMP production (Fig. 4). These findings suggest that these com-

---

**Fig. 1.** GC-Chromatogram of MPLC Fraction 4 from *Oxalis triangularis* (A) and Authentic FAMEs and FAEEs (B)
1, Methyl palmitate (17.70 min); 2, ethyl palmitate (18.36 min); 3, methyl linoleate (19.35 min); 4, methyl linolenate (19.40 min); 5, methyl oleate (19.41 min); 6, methyl stearate (19.61 min); 7, ethyl linolenate (19.95 min); 8, ethyl oleate (20.01 min); 9, ethyl linoleate (20.02 min); 10, ethyl stearate (20.21 min).

**Fig. 2.** Melanin Content Assay (A) and MTT Assay (B) of FAMEs and FAEEs from *Oxalis triangularis*
Methyl palmitate (MP), methyl stearate (MS), methyl oleate (MO), methyl linoleate (MLA), methyl linolenate (MLN), ethyl palmitate (EP), ethyl stearate (ES), ethyl oleate (EO), ethyl linoleate (ELA), ethyl linolenate (ELN). Medium only (M), Forskolin 5 μM (F), and arbutin 500 μM (A) were used for control (Cont).
pounds inhibit melanogenesis by operating upstream of the cAMP production step.

Many investigations have focused on the specific mechanisms involved in melanogenesis to develop new therapeutic agents for skin pigmentation abnormalities. Most popular whitening agents, including hydroquinone (HQ), kojic acid and arbutin (HQ β-D-glucopyranoside), act as tyrosinase inhibitors. We found that Oxalis triangularis contains melanin biosynthesis inhibitors, which were isolated and identified as methyl linoleate, ethyl linoleate, methyl linolenate and ethyl linolenate. It has been reported that unsaturated fatty acids such as oleic acid, linoleic acid and α-linolenic acid decrease melanin synthesis and tyrosinase activity, and that these inhibitory effects occur in proportion to the number of unsaturated bonds. Accordingly, melanogenesis is inhibited most effectively by α-linolenic acid, followed by linoleic acid and then oleic acid. Consistent with these findings, the results of the present study revealed that methyl and ethyl linolenates exerted higher inhibitory activity against melanin synthesis than methyl and ethyl linoleates. In addition, these results suggest that methyl and ethyl group may act as a negative factor in the anti-melanogenic effect.

Collectively, FAMEs and FAEEs isolated from Oxalis triangularis were characterized and evaluated for their inhibitory effects against melanogenesis in B16 melanoma cells. Among them, methyl/ethyl linoleate and linolenate have a depigmenting activity and may be introduced as a possible therapeutic agent for hyperpigmentation or as a cosmetic lightening agent.

Acknowledgements This work was supported by a grant from the Small and Medium Business Administration (S1040155) in Korea.

REFERENCES

![Fig. 3. Effects of Methyl/Ethyl Linoleate and Linolenate on Tyrosinase Promoter Activity](image1)

![Fig. 4. Effects of Methyl/Ethyl Linoleate and Linolenate on cAMP Production](image2)