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Lithospermic acid derivatives from *Lithospermum erythrorhizon* increased expression of serine palmitoyltransferase in human HaCaT cells

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ABSTRACT

A MeOH extract of the dry root of *Lithospermum erythrorhizon* showed strong increasing effect on serine palmitoyltransferase (SPT) in normal human keratinocyte cells (HaCaT cells). Bioassay-guided separation on this extract using repeated chromatography resulted in the isolation of lithospermic acid (**1**) and two derivative esters, 9″-methyl lithospermate (**2**) and 9′-methyl lithospermate (**3**). Compounds **1**–**3** significantly increased SPT expressions in the relative quantity (%) of SPT1 mRNA as well as SPT2 mRNA. These constituents also raised the level of SPT protein in HaCaT cells in a dose-dependent manner, with the increased level of SPT protein in HaCaT cells of 55%, 23%, and 81% at the concentration of 100 μg/ml, respectively. This finding suggests that lithospermic acid and its derivatives from *L. erythrorhizon* might improve the permeability barrier by stimulating the protein level of SPT.

The roots of *Lithospermum erythrorhizon* (Boraginaceae) are used in the oriental medicine to treat skin disorders such as healing burn, inflammation, wounds, and used as a dye for food colorants.[1] Naphthoquinones (shikonin derivatives)[1] and caffeic acid oligomers[2] have been reported as major constituents of this plant. These principles have been reported to possess a wide range of biological activities, for example, anti-inflammation,[3] cytotoxicity against human cancer cell lines,[4] and inhibitory effect on cholesterol acyltransferase.[5] Recent studies have suggested that the extracts of *L. erythrorhizon* could be used for the treatment of a variety of problems on human skin such as water loss,[6] the epidermal hyperproliferation,[7] and UVB-induced inflammation.[8] In the present study, we found that one fraction from a MeOH extract of *L. erythrorhizon* exhibited a significant increasing effect on serine palmitoyltransferase (SPT) in HaCaT cells. Serine palmitoyltransferase is a key enzyme for the initial step of sphingolipid biosynthesis,[9] and it is a producer of important components of the epidermal permeability barrier that are decreased in atopic dermatitis and aged skin.[10,11] Therefore, substances that have increasing effect on SPT in HaCaT cells are suggested to possess skin protective activity.[10] Since the MeOH extract of *L. erythrorhizon* showed strong increasing effect on SPT, we set out to study on principle constituents that stimulate the expression levels of SPT in HaCaT cells from this plant.

The bioassay-guided fractionation of the MeOH extract of *L. erythrorhizon* on a HP-20 column eluting with step gradient of MeOH in H_2O revealed a most active fraction LE-A.[12] To characterize the active principles, purification of this active fraction (LE-A), which eluted with 60% MeOH on HP-20 column chromatography, resulted in the isolation of three main compounds **1**–**3** by using repeated chromatography.[12] These isolated compounds (Fig. 1) were identified as lithospermic acid (**1**), 9″-methyl lithospermate (**2**), and 9′-methyl lithospermate (**3**) by comparing their physicochemical and spectroscopic data with published values.[13,14] The active fraction (LE-A) and compounds **1**–**3** showed no cytotoxicity against normal human keratinocyte cell line (HaCaT) at the concentration 50–200 μg/ml in a MTT assay.[15] In order to measure the change in the mRNA expression of SPT in HaCaT cells, the real-time RT-TCR assay was applied.[16] Compounds **1**–**3** showed a significant increase in the levels of SPT expressions with the relative quantity (%) of SPT1 mRNA of 121, 139, and 148, respectively, and the relative quantity (%) of SPT2 mRNA of 151, 139, and 159, respectively (Table 1). To further confirm the effects of isolates on the SPT levels in the HaCaT cells, Western blot analysis was used to examine the protein levels (% of NC).[17] Figure 2 depicts the effects of active fraction (LE-A) and compounds **1**–**3** on expression of SPT protein in HaCaT cells in a dose-dependent manner. Isolates **1**–**3** showed the raising the level of SPT protein in HaCaT cells to 21.7%, 8.9%,

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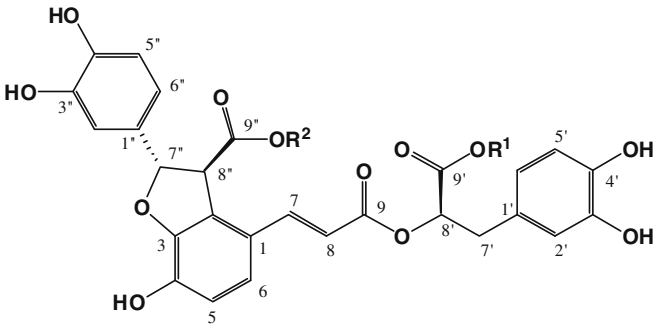

Lithospermic acid (**1**) $R^1 = R^2 = H$

9"-Methyl lithospermate (**2**) $R^1 = H,\ R^2 = CH_3$

9'-Methyl lithospermate (**3**) $R^1 = CH_3,\ R^2 = H$

Figure 1. Structures of isolates **1**–**3** from *L. erythrorhizon*.

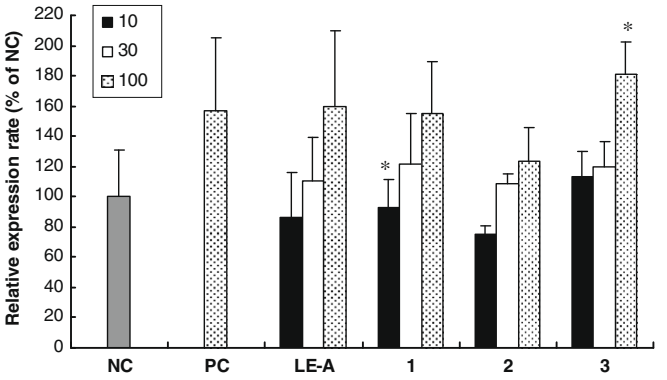

Figure 2. Effects of compounds **1**–**3** in expression of SPT protein in HaCaT cells. Compounds were treated at 10, 30, and 100 μg/ml for 9 h and the levels of SPT were analyzed by image quantitative analysis software.[17] NC, negative control; PC, positive control (nicotinamide, 100 μM); LE-A, the active fraction of *L. erythrorhizon*. Data are expressed as means ± SD of triplicate experiments. $^{*}p < 0.05$ versus the negative control.

and 20.2%, respectively, at the concentration of 30 μg/ml, and to 55%, 23%, and 81%, respectively, at 100 μg/ml. Meanwhile, the positive control nicotinamide increased about 57.3% the level of SPT protein at 100 μg/ml. The active fraction (LE-A) and compounds **1**–**3** were tested for their effects on NF-κB activity[18] because increased de novo cerimide synthesis by SPT enzyme could be associated with cell damages as the major factor in lipoapoptosis[19] or inflammatory cascades.[20] The result in Figure 3 showed that relative NF-κB reporter activity was not significantly altered by LE-A as well as compounds **1**–**3**. Western blot anaysis[21] also indicated that LE-A and compounds **1**–**3** at 100 μg/ml do not showed any change on the p65 nuclear transcription, a major protein subunit of NF-κB complex, until 12 h treatment (data not shown). These observations demonstrate that the increasing in SPT expressions in HaCaT cells by LE-A and its principles is not associated with NF-κB activation as well as inflammation response.[22] Taken together, it could be conclude that lithospermic acid derivatives (**1**–**3**) exhibited stimulatory expression of SPT in HaCaT cells, which is a novel action of these natural compounds.

The epidermal permeability barrier is the outmost layer of the epidermis, its disruption causes an increase in trans-epidermal water loss (TEWL), aging in skin, and epidermal hyperproliferation. For the recovery of normal epidermal permeability, therapeutic methods by steroids combined with UVB or retinoids have been used until now. Furthermore, the control of hyperproliferation by small molecules without adverse effects has been struggled in difficulty of skin delivery. Previous studies have demonstrated that organic or water extracts of *L. erythrorhizon* reversed the epidermal

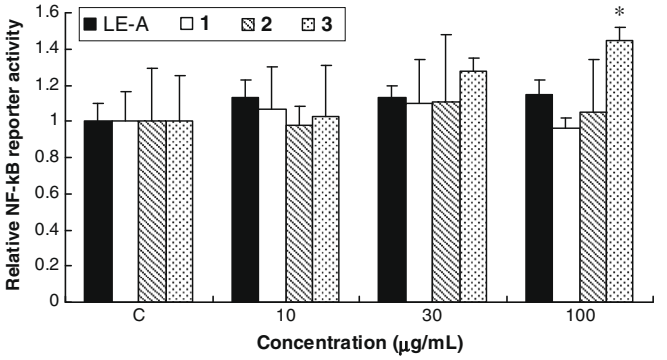

Figure 3. Effect of active fraction (LE-A) and compounds **1**–**3** on the basal NF-κB activity in HaCaT cells. A dual-luciferase reporter gene assay was performed on lysed cells co-transfected with pNF-κB-Luc plasmid (firefly luciferase) and phRL-SV (*hRenilla* luciferase). Reporter gene activations were expressed as changes relative to *hRenilla* luciferase activity. The results shown represent means ± SD of three separate sample. $^{*}p < 0.05$ versus the control (C).

Table 1
Effects of compounds **1**–**3** on mRNA expression of SPT in HaCaT cells

Treatment[a]	Relative quantity (%) of SPT1 mRNA	Relative quantity (%) of SPT2 mRNA
NC	100 ± 13.8	100 ± 9.1
PC	127 ± 11.0	128 ± 17.0
LE-A	107 ± 10.7	165 ± 4.2**
1	121 ± 14.6	151 ± 7.1**
2	139 ± 16.1*	139 ± 7.1*
3	148 ± 16.4**	159 ± 2.8*

[a] All the compounds were treated at 100 μg/ml. Data are expressed as means ± SD of duplicate experiments and the significance of mean is analyzed by *t*-test versus negative control. NC, negative control; PC, nicotinamide was used as a positive control; LE-A, the active fraction of *L. erythrorhizon*.

* $p < 0.05$.

** $p < 0.01$ versus negative control.

hyperproliferation in animal models,[6] and the EtOH extract of roots of this plant had moisturizing effect on human skin.[7] Kim et al. suggested that water extract of *L. erythrorhizon* reversed the epidermal hyperproliferation by dietary supplement and significantly increased the ceramide synthesis.[6] Moreover, extracts of the roots of *L. erythrorhizon* protected the UVB-induced damage in normal human epidermal keratinocytes (NHEK).[8] It has been shown that shikonin derivatives are active constituents showing the increase of cell viability in UVB-irradiated NHEK.[8] However, this study reports for the first time that lithospermic acid and its derivatives (**1**–**3**) from *L. erythrorhizon* increased expression of SPT in HaCaT, suggesting that these compounds might improve the permeability barrier by stimulating SPT. Further investigation and optimization of this property of lithospermic acid derivatives might useable to development of new protective skin agents.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.052.

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12. *Bioassay-guided isolation of active compounds*. The dry roots of *Lithospermum erythrorhizon* were purchased from an herb market in Gwangju, April 2007. The voucher specimen (CU-KOR-02) was identified by Prof. Won Keun Oh and deposited at the College of Pharmacy, Chosun University. The roots (2 kg) were extracted with MeOH (10 L) by sonication for 4 h at room temperature and repeated two times. The extracts were combined and evaporated under reduced pressure to yield 390 g dry residue. The residue was suspended in H_2O (2 L) and then partitioned with $CHCl_3$ (2 L × 3 times) to afforded a $CHCl_3$ layer and a H_2O layer. Dianion HP-20 column chromatography (10 × 40 cm) of the H_2O layer eluting with the solvent MeOH in H_2O (0%, 20%, 40%, 60%, 80%, 100%, each 10 L) and separated into five fractions. The most active fraction (LE-A, 17.3 g, eluted with 60% MeOH) was chromatographed on RP-18 column (4.5 × 30 cm) using MeOH/H_2O step gradient (1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 1:0; 500 ml each). A part (500 mg) of the selected fraction (eluted with MeOH/H_2O 1:2, 1:1; 3.2 g) was separated by repeated HPLC [Gilson system, YMC column (20 × 150 mm, particle size 4 μm), UV detection at 280 and 320 nm] using MeCN in H_2O as mobile phase (0 min, 29%; 35 min, 31%; 50 min, 50%, 65 min, 100%), resulted in the isolation of three major compounds **1** (10.2 mg, t_R = 29 min), **2** (15 mg, t_R = 34 min), and **3** (21 mg, t_R = 42 min).
13. Physicochemical and spectroscopic data of compounds **1**–**3**. *Lithospermic acid* (**1**): yellow gum; $[\alpha]_D^{22}$ +56.5 (*c* 0.5, MeOH); UV (MeOH): λ_{max} 252, 286, 304, 332 nm; IR (KBr): ν_{max} 3340, 2970, 1720, 1610, 1510, 1170, 1090 cm^{-1}; ^1H and ^{13}C NMR, see Table S1 (Supplementary data). *9″-Methyl lithospermic acid* (**2**): brown yellow gum; $[\alpha]_D^{22}$ +67.3 (*c* 0.5, MeOH); UV (MeOH): λ_{max} 255, 291, 305, 330 nm; IR (KBr): ν_{max} 3335, 2960, 1725, 1610, 1520, 1250, 1190 cm^{-1}; ^1H and ^{13}C NMR, see Table S1 (Supplementary data). *9′-Methyl lithospermic acid* (**3**): brown yellow gum; $[\alpha]_D^{22}$ +92.1 (*c* 0.5, MeOH); UV (MeOH): λ_{max} 252, 291, 302, 329 nm; IR (KBr): ν_{max} 3340, 2975, 1725, 1610, 1515, 1270, 1100 cm^{-1}; ^1H and ^{13}C NMR, see Table S1 (Supplementary data).
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15. *Cell, cell culture, and cytotoxic assay*. Cell culture reagents were purchased from Invitrogen, Cambrex, Gibco, and Welgene. The spontaneously transformed normal human keratinocyte cell line (HaCaT) was kindly provided by Dr. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics (100 streptomycin and 100 IU penicillin per ml) and maintained at 37 °C in 5% CO_2. Cells were harvested and analyzed 9 or 24 h after each treatment by reverse transcriptase-polymerase chain reaction (RT-PCR) or Western blot analysis. The cytotoxic activity of compounds against HaCaT cells was examined by a MTT method (Mosmann, T. *J. Immunol. Method* **1983**, *65*, 55).
16. *Real-time PCR*. The serine palmitoyltransferase (SPT) mRNA was quantified with real-time PCR by using a QuantiTect RT-PCR kit (Qiagen Cat. No. 204343, Valencia, CA, USA) with Corbett Rotor-Gene RG3000 (Applied Biosystems, Foster city, CA) according to the manufacturer's instructions. Primers and two labeled probes for human SPT and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were purchased for Assays-on-Demand™ Gene expression products (Applied Biosystems cat# 4326317E, Cat. No. 212543, Foster city, CA). Briefly, PCR mixture contained 8 μl of DNA template, 10 μl of Taqman-Master mix, and 0.5 mM primer, 0.2 mM probe, respectively. The PCR was performed in 20 μl (final volume) and the conditions for thermal cycling were as follows: initial denaturation for 10 min, followed by 55 amplification cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 8 s. Real-time RT-PCR was used to measure the mRNA expression after treatment with 100 ppm each molecule for 9 h.[11] *Statistical analysis*. The statistical analyses were performed using the SPSS program (SPSS 12.0). One sample *t*-test was used to examine the difference between groups. *p* Values of 0.05 were considered to be statistically significant.
17. *Western blot analysis*. HaCaT cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) and the proteins were quantified with a BCA™ Protein Assay (PIERCE, Rockford, IL, USA). Twenty-five micrograms of protein was electrophoresed on NuPAGE® gel in an MES buffer system (Invitrogen, Carlsbad, CA, USA) and transferred to a nitrocellulose membrane (LC2001; Invitrogen, USA). SPT was detected with a rabbit polyclonal antibody (1:500 in 0.1% Tween 20 and nonfat milk) (abcam Cat. No. ab23696, Cambridge, UK) and ECL™ anti-rabbit IgG-HRP (Amersham Bioscience, Piscataway, NJ). Actin was detected with a rabbit anti-actin antibody (Sigma). Blotting proteins were visualized by enhanced chemiluminescence with ECL+ (Amersham Bioscience, Piscataway, NJ). Image analysis was performed using Image J version 1.34 (NIH, USA).[12,13]
18. *NF-κB reporter gene assay*. Cells were plated at in 12-well plate and transfected at 70% confluency. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were transiently transfected with pNF-κB-luciferase plasmid and p*h*RL-SV plasmid using the Hillymax reagent (Dojindo Molecular Technologies, Gaitherburg, MD). The cells were then incubated in culture medium without serum for 18 h. The firefly and *hRenilla* luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies, Bad Wildbad, Germany). The relative luciferase activity was calculated by normalizing the promoter-driven firefly luciferase activity to the *hRenilla* luciferase activity (Majumdar, S.; Lamothe, B.; Aggarwal, B. B. *J. Immunol.* **2002**, *168*, 2644).
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21. Preparation of p65 nuclear extract and Western blot analysis: Cells were removed using a cell scraper and centrifuged at 2500*g* for 5 min at 4 °C, then swollen with 100 μl of lysis buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride]. Tubes were vortexed to disrupt cell membranes, and samples were incubated for 10 min on ice and then centrifuged for 5 min at 4 °C. Pellets containing crude nuclei were resuspended in 100 μl of extraction buffer [20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride], incubated for 30 min on ice, and centrifuged at 15,800*g* for 10 min; the supernatants containing the nuclear extracts were collected and stored at −80 °C until required. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and immunoblot analyses were performed.[22] Cell lysates were fractionated by 10% gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently incubated with primary antibody, and then horseradish peroxidase-conjugated secondary antibodies. Finally, the membranes were developed using an ECL chemiluminescence detection kit.[22]
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