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Inhibitory effects of geranic acid derivatives on melanin biosynthesis

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Synopsis

The effects of geranic acid and its structurally related derivatives (geraniol, citronellic acid, and citronellol) on cell viability and melanin biosynthesis in Melan-a cells were evaluated in this study. Among them, geranic acid evidenced the strongest inhibitory activity on melanin production, coupled with low cell toxicity. Treatment with 500 μ*M* of this compound resulted in a reduction in melanin content of 35.4% as compared to the live cell percentage (91.7%). Moreover, geranic acid also inhibited tyrosinase activity and intracellular tyrosinase expression in a dose-dependent manner. These results show that geranic acid may function as a skin depigmenting agent via the inhibition of tyrosinase activity and expression within melanocytes.

INTRODUCTION

Human skin color is determined largely by the degree of melanin pigment production in melanocytes (1,2). Although melanin is intimately involved with the protection of skin from damage by free radicals and ultraviolet (UV) exposure, melanin overproduction can cause serious hyperpigmentary skin disorders, including freckles, discoloration, and melasma (3,4). In the melanin biosynthesis pathway, tyrosinase is essential for the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-Dopa) and the oxidation of L-Dopa to dopaquinone. Tyrosinase is thought to be the key enzyme in melanogenesis (5). Therefore, tyrosinase inhibitors have long been sought as potential depigmentation agents for use in the treatment of hyperpigmentary skin disorders (6–8).

Citronellol (3,7-dimethyl-6-octen-1-ol), citronellic acid (3,7-dimethyl-6-octenoic acid), geraniol (3,7-dimethylocta-2,6-dien-1-ol), and geranic acid (3,7-dimethyl-2,6-octadienoic acid) are flavor compounds that are used as perfume in certain cosmetics (Figure 1) (9,10). Their compounds are widely distributed aroma components that occur naturally in plants, and are detected principally in *Cymbopogon citratus, Rosa spp*., and *Vitis vinifera* L (11–13). In the bioconversion pathway, the linear monoterpenoids, citronellol and geraniol, oxidize to the corresponding aldehyde, and can thereby induce conversion to citronellic

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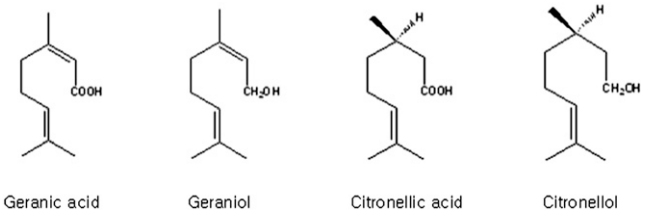

Figure 1. Chemical structure of geranic acid and its derivatives.

acid and geranic acid by subsequent oxidation (14–16). Among these compounds, geranic acid has recently been reported to exert a tyrosinase-inhibitory effect (11). However, no study has yet been conducted on its effects on melanocytes.

The principal objectives of this study were to evaluate the inhibitory effects of these compounds on cell viability and melanin production in melanocytes, and also to assess their effects on the expression of melanin biosynthesis-associated enzymes, including tyrosinase.

EXPERIMENTAL METHODS

MATERIALS

Citronellol, geraniol, citronellic acid, L-Dopa, and kojic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO). Geranic acid was acquired from the Fluka Co. (Buchs, Switzerland). The Melan-a cell line was a gift from Dr. Bennett (St. George's Hospital Medical School, London, UK). Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) medium, and Penicillin–Streptomycin (PS) were purchased from Gibco BRL. (Grand Island, NY)

CELL CULTURE PROCEDURES

The Melan-a cells were cultured in RPMI 1640 medium under 10% FBS and 200-nm phorbol 12-myristate 13-acetate (PMA) conditions. In 100 ml culture dishes, 10 ml of medium was added and then seeded with 5×10^5 cells. The cells were grown to confluence after 3 to 4 days at 37°C in an atmosphere of 5% CO_2, they were seeded with 10^5 cells/well in a 24-well plate, and then incubated for 24 h. Each well was replenished with 990 μl of medium daily, as well as treated with 10 μl of test sample [solvent (v/v): propylene glycol/EtOH/H_2O = 5/3/2] for 3 days; the plate was then incubated for 1 day (17).

CELL VIABILITY

The percentage of viable cells was determined by staining the cell population using a simple crystal violet (CV) staining method to quantitate adherent cell number after treatment (18). After the removal of media from each well, the wells were washed with phosphate-buffered saline (PBS). Two hundred microliters of CV (0.1% CV, 10% EtOH, and the remaining volume as PBS) was then added. The plates were incubated at room temperature for 5 min

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and washed twice with water. After the addition of 1 ml of EtOH, the plates were shaken for 10 min at room temperature. UV absorption was measured at 590 nm (19).

DETERMINING MELANIN CONTENT

After the removal of media from each well, the plate was washed with PBS, followed by the addition of 1 ml of 1 N NaOH to each well to lyse the cells for the release of melanin. The UV absorption was measured at 405 nm. Phenylthiourea (PTU) was employed as a positive control (20).

WESTERN IMMUNOBLOTTING ANALYSIS

The Melan-a cells were harvested and extracted in a triple-detergent lysis buffer [50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.5% sodium deoxycholate, 100 μg/ml of phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml of aprotinin]. The protein content was then measured with a protein assay kit (Bio-rad, Hercules, CA). Next, 50 μg of the protein was separated on 8% SDS-polyacrylamide gel and transferred to a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 5% skim milk and incubated with tyrosinase (Santa Cruz Biotech, Santa Cruz, CA 1/250 dilution) primary antibody or dopachrome tautomerase (TRP-2, Santa Cruz Biotech, 1/300 dilution) primary antibody, and anti-goat secondary antibodies. Detection was performed using ECL (Amersham Pharmacia Biotech, Piscataway, NJ). The Western blot results obtained by the scanner were photographed (HP, Palo Alto, CA). Density of protein bands was measured using Image J program by National Institute of Health (NIH) (21).

MEASURING INHIBITORY EFFECT ON TYROSINASE ACTIVITY

Tyrosinase activity was measured by its dopa-oxidase activity, using a slightly modified version of the method reported by Shono and Toda (22). Each concentration (1 m*M*, 500 μ*M*, 100 μ*M*, and 10 μ*M*) of the test substance was dissolved in MeOH. Next, 120 μl of L-Dopa (5 m*M*, dissolved in a 67 m*M* phosphate buffer, pH 6.8) and 40 μl of either the same buffer or the test sample were added to a 96-well microplate, after which 40 μl of mushroom tyrosinase (125 U) was added. The quantity of dopachrome in the reaction mixture was measured after 20 min of incubation at 37°C. Based on the optical density at 490 nm (OD 490), the inhibitory activity of the sample was expressed as the concentration required to effect an inhibition of enzyme activity of 50% (IC_{50}). Kojic acid was utilized as the reference material (23).

STATISTICAL ANALYSIS

The data are expressed as the means ± S.D. from three independent experiments. Statistical comparisons between the different treatments were conducted via analysis of variance.

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RESULTS

EFFECTS ON CELL VIABILITY AND MELANIN PRODUCTION

To evaluate the depigmenting ability of the geranic acid derivatives, Melan-a cells were employed in this study. Melan-a cells are highly pigmented melanocytes and provide an excellent parallel non-tumorigenic cell line derived from C57BL/6 mice (24). Although Melan-a cell is not a human melanocyte, it is widely used for studies of melanin biosynthesis regulation in the skin. As shown in Table I, treatment with geranic acid from 5 to 500 μ*M* reduced melanin contents in a dose-dependent manner, with low cell toxicity.

Table I
Effects of Geranic Acid Derivatives on Cell Growth and Melanin Production in Melan-a Cells

Samples	Concentrations (μ*M*)	Cell viability (%)	Melanin content (%)	Depigmenting effect (%)[a]
Geranic acid	5	96.2 ± 0.9	92.7 ± 5.9	3.5
	50	96.7 ± 1.6	89.6 ± 4.4	7.1
	500	91.7 ± 3.4	66.1 ± 6.7	25.6
Geraniol	5	99.6 ± 2.6	98.8 ± 5.1	0.8
	50	90.8 ± 2.6	91.5 ± 3.9	−0.7
	500	61.4 ± 5.5	34.9 ± 4.3	26.5
Citronellic acid	5	98.2 ± 1.2	99.2 ± 2.8	−1.0
	50	98.2 ± 4.3	96.1 ± 3.1	2.1
	500	90.6 ± 4.7	84.2 ± 9.3	6.4
Citronellol	5	97.2 ± 4.1	96.6 ± 3.2	0.6
	50	91.4 ± 2.6	87.1 ± 3.0	4.3
	500	47.0 ± 8.7	34.8 ± 7.5	12.2
Kojic acid	5	97.5 ± 2.3	95.6 ± 4.7	1.9
	50	94.1 ± 3.6	90.6 ± 5.8	3.5
	500	84.3 ± 3.1	79.2 ± 5.1	5.1
PTU	5	96.4 ± 5.0	91.1 ± 2.9	5.3
	50	79.0 ± 5.5	40.2 ± 6.5	38.8
	500	68.8 ± 4.9	21.7 ± 5.3	47.1

Each value is expressed as the means ± S.E. of three experiments. Kojic acid and PTU were used as reference materials.
[a]Depigmenting effect was expressed as the difference between the percentage of cell viability and the melanin content [Cell viability (%) − Melanin content (%)].

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Geranic acid suppressed only 8.3% of viable cells, but this compound reduced melanin production by 43.9% at 500 μ*M*. By way of contrast, geraniol and citronellol evidenced high cell toxicity at above 50 μ*M*, and citronellic acid did not inhibit melanin production at any concentration.

INHIBITORY EFFECTS ON TYROSINASE ACTIVITY

For many years, mushroom tyrosinase has been studied for its use in cosmetics as well as in food industries (25). The inhibitory effects of geranic acid derivatives against the dopa-oxidase activity of tyrosinase are shown in Figure 2. According to our results, geranic acid significantly inhibited tyrosinase activity with a 77% inhibition at 500 μ*M*. Citronellic acid and geraniol also evidenced tyrosinase-inhibitory activity in a dose-dependent manner, but to a lesser degree than was noted with geranic acid. The IC_{50} of geranic acid, citronellic acid, and geraniol were measured as 195.4, 645.9, and 950.4 μ*M*, respectively.

CHANGES OF INTRACELLULAR LEVEL OF TYROSINASE-RELATED PROTEINS

Based on the results of our depigmenting activity measurements, the regulatory effects of geranic acid on the expression of melanin generation-related protein in Melan-a cells were assessed via Western immunoblotting analysis. Both tyrosinase and dopachrome tautomerase (TRP-2) are melanin generation-related enzymes, and perform a central role in the melanogenesis pathway. Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-Dopa and the oxidation of L-Dopa to o-dopaquinone in the first two melanogenetic steps. The yield of o-dopaquinone is spontaneously converted to dopachrome, which is processed either into 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid

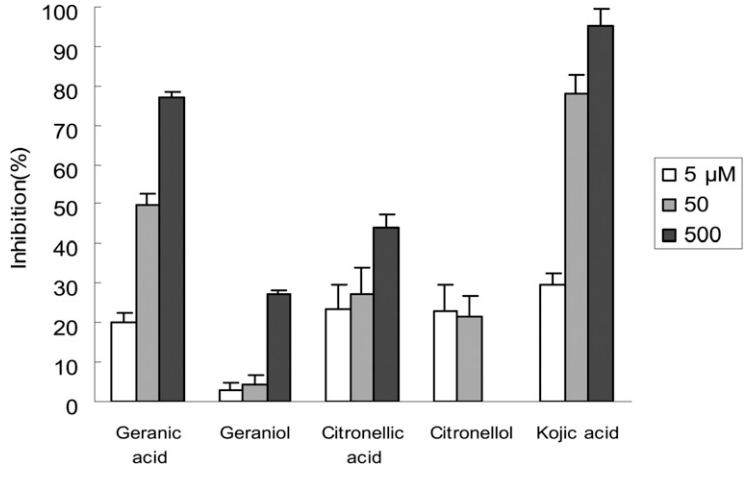

Figure 2. The inhibitory effects of geranic acid derivatives against the dopa-oxidase activity of tyrosinase. Kojic acid was utilized as a positive control. Each value is expressed as the mean ± S.E. of three experiments. The inhibitory effects of 500 μ*M* citronellol could not be determined because of its absorbance at 490 nm.

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(DHICA). TRP-2 catalyzes the tautomerization of dopachrome to DHICA, and the future oxidation of DHICA by DHICA oxidase (TRP-1) gives rise to the DHICA-eumelanin (26,27).

As is shown in Figure 3, treatment with 500 μ*M* of geranic acid for 3 days resulted in a significant reduction in the generation of intracellular tyrosinase. However, the level of intracellular TRP-2 was not altered by geranic acid treatment. Therefore, geranic acid acts specifically to reduce the expression of tyrosinase.

DISCUSSION

Based on previous reports of the tyrosinase-inhibitory effects of geranic acid (11), the inhibitory activity of geranic acid and three geranic acid derivatives on the biosynthesis of melanin pigment in Melan-a cells was evaluated. According to our results, geranic acid inhibited relative melanin contents by 25.6% as compared to the cell viability level at 500 μ*M*. Additionally, geranic acid evidenced inhibitory effects on tyrosinase activity and intracellular expression. However, the inhibitory activities of geranic acid derivatives on melanin formation were found to be weaker than that of geranic acid. Geraniol, an alcohol analog of geranic acid, evidenced more profound cell toxicity than was observed with geranic acid. Citronellol and citronellic acid evidenced no significant melanin synthesis-inhibitory activity at any tested concentration.

Geranic acid is a colorless to pale yellow clear oily liquid, with a typical flavor. It is frequently used as a perfuming agent in the cosmetics industry. Although the tyrosinase-inhibitory activity of geranic acid has been recently reported, its depigmenting properties in melanocytes were first reported, to the best of our knowledge, in this study. Overall, these results indicated that geranic acid may prove useful not only as a perfuming agent but also as a skin depigmentation agent. Although Melan-a cells are commonly used in such studies, experiments using normal human melanocytes would be an appropriate next step.

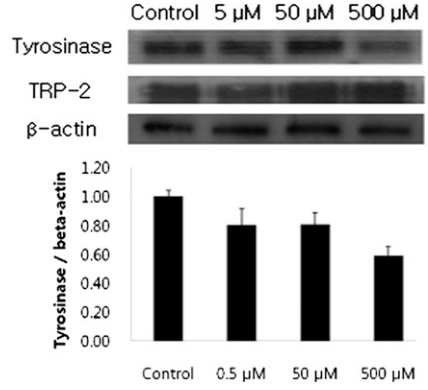

Figure 3. Effects of geranic acid on tyrosinase and dopachrome tautomerase expression in Melan-a cells. The Melan-a cells were treated with geranic acid for 3 days. TRP-2 (tyrosinase-related protein-2); dopachrome tautomerase.

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