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Activity and Mechanism of Natural Resorcinol-Type Phenolics from the Twigs of Cudrania tricuspidata as Skin Whitening Agents

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Melanin is a substance that protects the skin from harmful environmental factors such as UV radiation and toxic chemicals. However, excess accumulation of melanin causes hyperpigmentation, which is associated with a number of unhealthy conditions including solar lentigines, melasma, freckles and post-inflammatory hyperpigmentation [1]. Melanin is produced in melanocytes, which are responsible for the pigmentation of skin. Melanocytes are phenotypically prominent but histologically inconspicuous skin cells which comprise 5% to 10% of the cells in the basal layer of the epidermis. The process by which melanocytes produce melanin is known as melanogenesis. This is a complex process with a series of pathways leading to long-lasting pigmentation [2]. Briefly, in human epidermal melanocytes, α-melanocyte stimulating hormone (α-MSH) is required for development of pigmentation. It acts as a melanogen towards mammalian epidermal melanocytes through the signaling pathway of protein kinase A, a class of cyclic AMP (cAMP) -dependent enzymes. α-Melanocyte stimulating hormone binds to its specific receptor MC1R, increasing cAMP via the G protein. cAMP exerts its effect in part through protein kinase A. Protein kinase A phosphorylates and activates the cAMP-response element binding protein that binds to the cAMP-response element present in the M promoter of the microphthalmia-associated transcription factor (MITF) gene[3]. MITF contains a basic helix-loop-helix and leucine-zipper structure and consists of many isoforms with different N-termini. The MITF-M isoform is expressed exclusively in melanocytes and melanoma cells [4]. The resulting transient increases MITF expression and leads to the upregulation of melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and 2 (TRP-2) [5].

The tyrosinase family of enzymes mediates the conversion of L-tyrosine to melanin. Tyrosine, one of the 20 amino acids used by cells to synthesize proteins, is the precursor of pigment melanin. During this catalyzing process tyrosinase catalyzes the two initial steps: the hydroxylation of L-tyrosine to L-3-(3, 4-dihydroxyhpeyl)-alanine (L-dopa), and the oxidation of L-dopa to dopaquinone [6]. Dopaquinone is a highly reactive intermediate yielding two different kinds of melanin, eumelanin and pheomelanin, under different conditions. In the absence of thiol compounds, dopaquinone spontaneously converts to dopachrome. TRP-2/dopachrome tautomerase catalyzes conversion of dopachrome to 5, 6-dihydroxyindole-2-carboxylic acid [7]. Subsequently, TRP-1 catalyzes the oxidation of 5, 6-dihydroxyindole-2-carboxylic acid to indole-5, 6-quinone-2-carboxylic acid. TRP-2/dopachrome tautomerase and TRP-1 have related structures, which finally yielding eumelanin by further polymerization [8]. However, with the intervention of thiols, such as in the presence of L-cysteine, this process gives rise to the thiol adducts 5-S-cysteinyldopa and 2-S-cysteinyldopa. Further oxidation of these cysteinyldopa isomers leads to the production of pheomelanin instead of eumelanin [9]. Most of the melanin pigments present in the skin are complex heteropolymers made up of both eumelanin and pheomelanin building blocks.

Cudrania tricuspidata, a deciduous tree growing in East Asia, is rich in xanthones and flavonoids [10]. According to previous research in our laboratory, the twig extract of Cudrania. tricuspidata has strong inhibitory effects on mushroom tyrosinase. Electrospray ionization mass spectrometry and NMR spectral data showed that of the twenty-eight phenolic compounds isolated from the twigs of Cudrania. Tricuspidata, only trans-dihydromorin and oxyresveratrol showed potent inhibitory activity against mushroom tyrosinase (Figure 1).

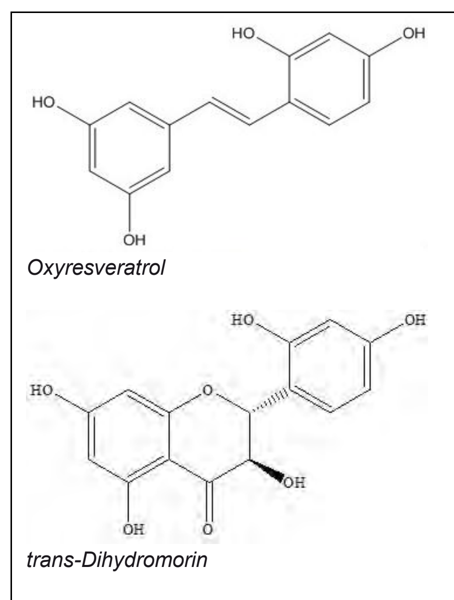

Fig. 1 Chemical structures of oxyresveratrol and trans-dihydromorin

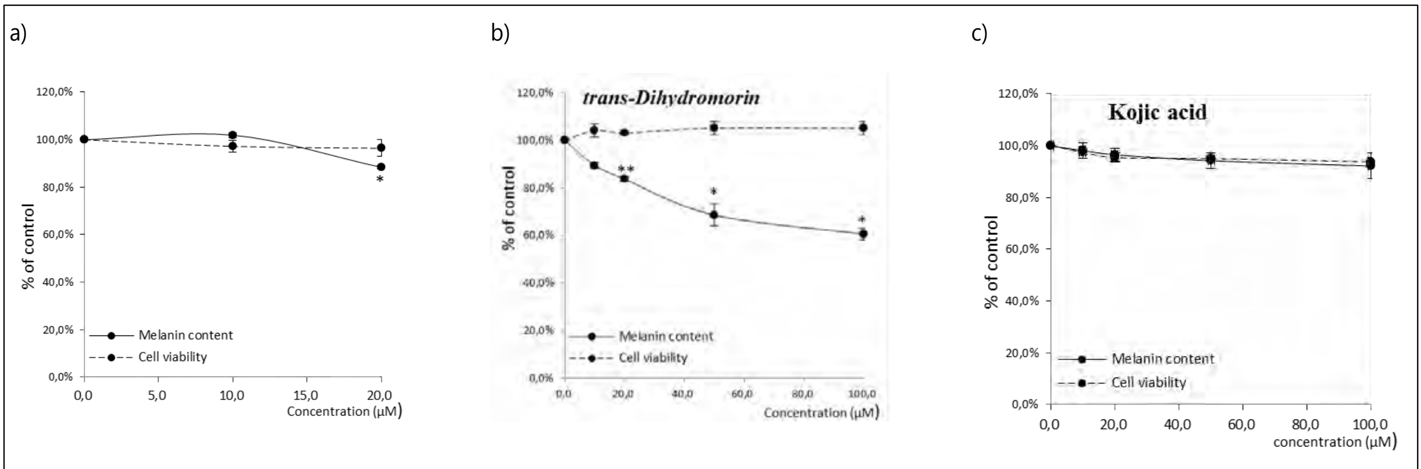

Fig. 2 Effects of oxyresveratrol (a), trans-dihydromorin (b) and kojic acid (c) on melan-a cell viability and melanin content after 72 h of treatment, as determined by the CCK-8 assay and SRB combined assay, respectively.
Cells were treated with α-MSH plus different concentrations of agents for 72 h. Each value is presented as the mean ± S.D. from triplicate independent experiments.
*Significant is defined as $p<0.05$ vs. control. ** Significant is defined as $p<0.01$ vs. control.

They might effectively inhibit the melanogenesis process in melanocytes [11]. Oxyresveratrol is a stilbene derivative and trans-dihydromorin is a flavanone derivative. They are both resorcinol-type phenolics and have similar structures, with two hydroxyl group substitutions at the 2′ and 4′ positions of the B-ring. Stilbenes and flavonones are considered to be safe and largely free from adverse side effects, but safety and efficacy studies of these compounds in mammalian melanocyte cultures are rare. In this study, we evaluated their effects in melan-a cells, an immortalized non-tumorigenic mouse melanocyte cell line which is highly pigmented. Results showed that both oxyresveratrol and trans-dihydromorin had a negligible toxic effect in a concentration range of 0-20 µM in both cell lines. Moreover, trans-dihydromorin was non-toxic in a concentration range of 0-100 µM, indicating that trans-dihydromorin is less toxic than oxyresveratrol. We also found that oxyresveratrol and trans-dihydromorin decreased the amount of melanin at concentrations of 10 and, 20 µM and 10, 20, 50 and 100 µM, respectively, in a dose-dependent manner. They both showed much stronger depigmenting effects than kojic acid (Figure 2).

However, the inhibitory effect of trans-dihydromorin was much stronger than that of oxyresveratrol.

As tyrosinase is the key regulatory enzyme for melanin synthesis, measuring its cellular activity is always the first step in investigating the hypopigmentation mechanism of selected chemical agents [12]. However, at a concentration of 20 µM, trans-dihydromorin presented remarkable inhibitory results on tyrosinase activity, while the suppression by oxyresveratrol was negligible (Figure 3).

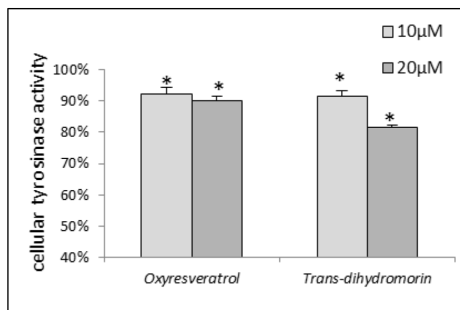

Fig. 3 Inhibitory effects of cellular tyrosinase in the melan-a cell line by oxyresveratrol and trans-dihydromorin. Cells were treated with α-MSH plus 10 µM and 20 µM of agents for 72 h, and the percentage inhibition of tyrosinase activity was determined. Each value is presented as the mean ± S.D. from triplicate independent experiments.
*Significant is defined as $p<0.05$ vs. control.
** Significant is defined as $p<0.01$ vs. control.

In addition, we used Western blot analysis to investigate also whether oxyresveratrol and trans-dihydromorin regulate melanogenesis genes. The protein levels of MITF, TRP-1 and TRP-2 were found to be significantly suppressed by trans-dihydromorin at a concentration of 20 µM (Figure 4).

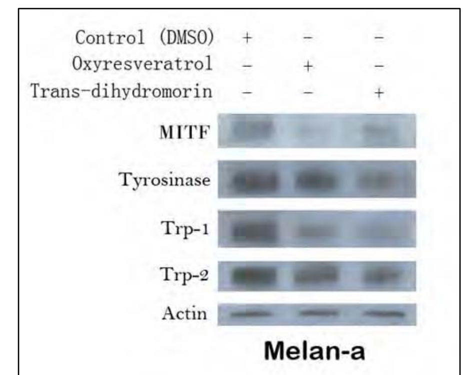

Fig. 4 Expression of MITF, tyrosinase, TRP-1 and TRP-2 was analyzed by Western blot. Total cellular proteins (20 µg/lane) were subjected to 10% SDS-PAGE gels. Protein loading variation was determined by blotting with anti-actin antibody.

Subsequent measurement of the mRNA level by the real-time reverse transcription polymerase chain reaction explained the decrease in production of these proteins. Trans-dihydromorin significantly decreased the mRNA level of tyrosinase, TRP-1 and TPR-2 with a reduction of MITF. Oxyresveratrol also exhibited the same action mechanism, but the inhibitory effect was weaker than with trans-dihydromorin (Table 1).

Inhibition rate	*oxyresveratrol*	*trans-Dihydromorin*
mRNA level	MITF 51.41% **	MITF 68.76% **
	Tyr 23.77% **	Tyr 72.33% **
	TRP1 19.87% *	TRP1 53.1% *
	TRP2 21.1% **	TRP2 28.64% **

Table 1 mRNA Levels of Melanogenesis-Related Proteins.
(Relative mRNA levels were analyzed by quantitative real-time PCR and the 22DDCT Method after treated with oxyresveratrol and trans-dihydromorin. Gadph was used as an internal standard. Each value is presented as mean±S.D. from triplicate independent experiments.)

*Significant is defined as $p<0.05$ vs. control.
** Significant is defined as $p<0.01$ vs. control.

In conclusion, both oxyresveratrol and trans-dihydromorin are useful for skin lightening and treatment of hyperpigmentation, with trans-dihydromorin showing a better depigmenting effect than oxyresveratrol. However, an *in vivo* study or artificial skin model based study is still required to substantiate their hypopigmentation effects.

METHODS SUMMARY

Oxyresveratrol and trans-dihydromorin were purified from the twigs of Cudrania tricuspidata. Murine melan-a cells were maintained according to institutional regulations. The CCK-8 assay was used to measure the cell viability after 72 hours of treatment. The melanin content was measured using a previously described SBR combined assay [13]. Cellular tyrosinase activity was determined by measuring the oxidation of L-dopa to dopachrome in cell lysates[8]. Expressions of MITF, tyrosinase, TRP-1 and TRP-2 were determined by Western blot analysis. Their mRNA levels were measured using quantitative reverse transcription-polymerase chain reaction. Significant differences between treatment groups were determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test using the Graphpad Prism 5 software package (GraphPad Software, San Diego, CA). A p value < 0.05 was considered statistically significant.

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