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Bioactive constituents of *Spatholobus suberectus* in regulating tyrosinase-related proteins and mRNA in HEMn cells

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Abstract

Spatholobus suberectus Dunn (Leguminosae) is a traditional Chinese herbal medicine used to treat rheumatism, anemia, menoxenia, and other disorders. The extent to which this herbal medicine is useful to skin cells, however, has not been evaluated. Constituents of the 95% ethanol extracts of the dried vine stems of *S. suberectus* were therefore isolated and examined for their skin-whitening capacity. A bio-guided phytochemical investigation, involving use of the mushroom tyrosinase inhibitory system, of active fractions of the extracts resulted in the isolation of 12 constituents. The structures of these constituents, which were characterized by various spectroscopic techniques, consisted of one flavone, three isoflavones, five flavanones, two flavanonols, and one chalcone. Of these constituents 3′,4′,7-trihydroxyflavone (**1**), eriodictyol (**3**), plathymenin (**5**), dihydroquercetin (**6**), butin (**7**), neoisoliquiritigenin (**8**), dihydrokaempferol (**9**), liquiritigenin (**10**), and 6-methoxyeriodictyol (**12**) represented compounds isolated for the first time from *S. suberectus*. These constituents were evaluated their ability to inhibit cellular tyrosinase activity and for their melanin inhibitory activity in human epidermal melanocytes (HEMn). Butin (**7**) was the most efficacious of these constituents and exhibited concentration-dependent effects. Western blot analysis revealed that expression of tyrosinase and tyrosinase-related proteins 1 and 2 (TRP1 and TRP2) was decreased in butin (**7**)-treated HEMn cells. Additionally, quantitative real-time PCR (qRT-PCR) analysis disclosed that expression of mRNAs for tyrosinase, TRP1 and TRP2 was suppressed by butin (**7**). It is concluded that butin (**7**) is the most active of the components of *S. suberectus* in inhibiting pigmentation and that this inhibition is exerted through inhibition of transcription of the genes encoding tyrosinase, TRP1 and TRP2.

Keywords: *Spatholobus suberectus*; Leguminosae; Butin; Tyrosinase; TRP1; TRP2; Quantitative real-time PCR

1. Introduction

Pigment synthesis occurs in melanocytes located in the basal layer between the dermis and epidermis. The major pigments synthesized by these cells are stored in granules termed melanosomes. Specific proteins contained within and forming the melanosomal organelle are considered responsible for the extent of human coloration, and mutations in these proteins may result in albinism (Boissy and Manga, 2004). Melanin pigments of all species are derived by enzymatic oxidation of the amino acid tyrosine, with two types of melanin found in mammalian skin (Nerya et al., 2003). Three major melanosome accessory enzymes of the tyrosinase gene family are involved in melanin biosynthesis. Tyrosinase (EC 1.14.18.1), the rate-limiting enzyme, catalyzes two distinct reactions of melanin synthesis: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone (Tripathi et al., 1992). In the absence of thiol-containing compounds, dopaquinone spontaneously converts initially to dopachrome and then to indole-5,6-quinone or indole-5,6-quinone 2-carboxylic acid. Subsequently tyrosinase-related protein-2 (TRP2/DCT; dopachrome tautomerase; EC 5.3.3.12) and tyrosinase related protein-1 (TRP1; DHICA oxidase) act to produce unstable quinones that undergo further polymerization yielding melanin (Palumbo et al., 1991; Mallick et al., 2005). Genes

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encoding proteins involved in melanogenesis, such as tyrosinase, TRP2/DCT, and TRP1, serve as biomarkers of normal melanocytes.

The human genes encoding the TRPs are often termed TYRPs because of the apparent similarity of human TRP2/DCT and TRP1 proteins to tyrosinase. TYRP1 is currently thought to encode DHICA oxidase activity whereas TYRP2 encodes dopachrome tautomerase (TRP2/DCT) (Camacho-Hubner and Beermann, 2000). Human tyrosinase is encoded by TYR. Thus, three human genes have been identified with the potential for functional polymorphisms that could explain the natural variation in pigmentation phenotypes as well as the existence of several hypopigmented states.

Traditionally *S. suberectus* Dunn has been used to "invigorate" the circulation, to purify blood channels and collaterals, to provide nourishment when deficiency and/or stasis of blood lead to menstrual abnormalities, to treat inflammation of peripheral blood vessels, to treat thrombosis, and to manage numbness of the body and limbs (Yen, 1992; Xie and Huang, 1994). The vine plant is also effective for leucopenia induced by radiotherapy (Xie and Huang, 1994). In previous reports, *S. suberectus* was studied for its hematological effects (Su and Chen, 1997; Chen et al., 2004; Liu et al., 2004; Cui et al., 2005), for its capacity to inhibit human immunodeficiency virus type-1 protease (HIV-1 PR) in vitro (Lam et al., 2000), for its anti-inflammatory activity (Li et al., 2003), and for the ability to regulate plasma lipid concentrations (Wang et al., 1991). A limited number of studies have been conducted that describe the biological effects of the components of this herb (Cui et al., 2002; Cheng et al., 2003; Yoon et al., 2004). In certain of these studies, stimulation of the proliferation of hematopoietic progenitor cells was observed (Cui et al., 2005). In the present study, a bioassay-guided system was utilized to identify components of *S. suberectus* active against mushroom tyrosinase. Isolated components were tested for cellular anti-tyrosinase activity, for the ability to inhibit melanin production, and for effects on expression of tyrosinase and TRPs in human epidermal melanocytes (HEMn).

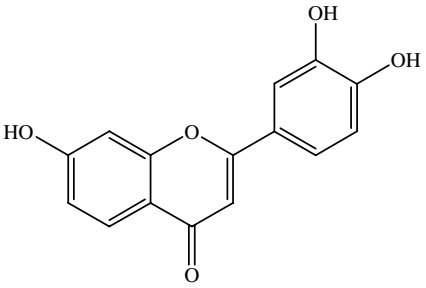
2. Results and discussion

2.1. Isolation and identification of the active constituents of *S. suberectus*

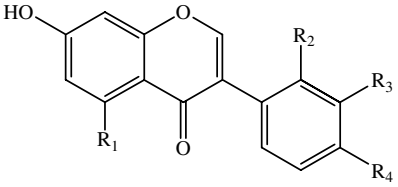
A phytochemical investigation of the 95% ethanol extract of *S. suberectus* was conducted in this study. HPLC-directed isolation was performed and mushroom tyrosinase activity was measured following subjection of extracts to Diaion HP-20, Sephadex LH-20, MCI CHP-20P column chromatography, and semi-HPLC purification. Through correlations with the physical data and NMR spectra reported previously in the literature, 12 components were isolated from the vine stems of *S. suberectus*. Structures of purified compounds were identified by direct comparisons of their retention times, melting points and spectroscopic data (^{1}H-NMR and ^{13}C-NMR) with those of the literature. The structures of these compounds are shown in Fig. 1. The classes of the isolated structures included one flavone: 3′, 4′, 7-trihydroxyflavone (**1**) (van Acker et al., 2000); three isoflavones: formononetin (**2**) (H.M.T.B. Herath et al., 1998), daidzein (**4**) (Fairley et al., 2003), and calycosin (**11**) (Tolleson et al., 2002); five flavanones: eriodictyol (**3**) (Okunade et al., 1994), plathymenin (**5**) (Seikel et al., 1955), butin (**7**) (Kitanaka and Takido, 1992), liquiritigenin (**10**) (Youssef et al., 1998), and 6-methoxyeriodictyol (**12**) (Peter et al., 1988); two flavanonols: dihydroquercetin (**6**) (Agrawal et al., 1980) and dihydrokaempferol (**9**) (Lai and Joseph, 1989); and one chalcone: neoisoliquiritigenin (**8**) (Veitch et al., 2003). Compounds **1**, **3**, **5–10**, and **12** represent substances isolated from this herbal medicine for the first time.

2.2. The cytotoxic, anti-tyrosinase, and melanin inhibitory activities of the isolated constituents in HEMn cells

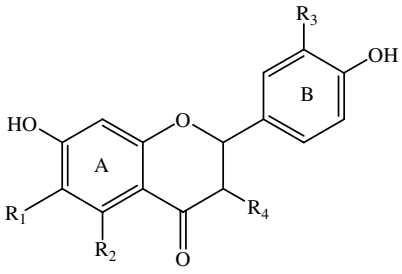
An assessment of the dermal toxicity of the compounds isolated from extracts of *S. suberectus* was considered essential pending use of the plant or its individual components for therapeutic purposes in humans. Before further in vitro studies were conducted, the isolated compounds were tested for their effects on cellular viability and for their anti-tyrosinase actions using HEMn cells as a model system. Each of the 12 components isolated from *S. suberectus* extracts was examined separately at 100 μM for effects on the survival of HEMn cells. As determined with the MTT assay, viabilities were uniformly found to be greater than 80% (Fig. 2). Each component was then examined for the ability to inhibit cellular tyrosinase activity and to reduce cellular melanin content. Findings are presented in Table 1. Butin (**7**) displayed the greatest anti-tyrosinase activity (80.1%), followed in turn by eriodictyol (**3**) (36.0%), plathymenin (**5**) (33.6%), liquiritigenin (**10**) (36.1%), and 6-methoxyeriodictyol (**12**) (44.4%). In this cellular tyrosinase assay, compounds possessing the flavanone skeleton appeared to have the highest tyrosinase inhibitory effects. The isoflavone structures, (**2**), (**4**), and (**11**), showed almost no tyrosinase inhibitory effects in HEMn cells. Other skeletons of the isolated structures from *S. suberectus* exhibited slight effects. It should be noted that several phenolic compounds with tyrosinase-inhibitory properties have been previously found to act as de-pigmenting agents (Boissy and Manga, 2004; Nerya et al., 2004; Khatib et al., 2005; Kim and Uyama, 2005). It has been suggested that the presence of an hydroxyl group located on the para-position in the B ring to an electron donator group is required for a phenolic compound to serve as an alternative substrate for tyrosinase (Briganti et al., 2003; Kim and Uyama, 2005). The extent



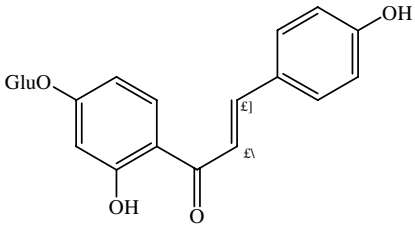
3',4',7-Trihydroxyflavone (**1**)



	R_1	R_2	R_3	R_4
Formononetin (**2**)	H	H	H	OCH_3
Daidzein (**4**)	H	H	H	OH
Calycosin (**11**)	H	H	OH	OCH_3



	R_1	R_2	R_3	R_4
Eriodictyol (**3**)	H	OH	OH	H
Plathymenin (**5**)	OH	H	OH	H
Dihydroquercetin (**6**)	H	OH	OH	OH
Butin (**7**)	H	H	OH	H
Dihydrokaempferol (**9**)	H	OH	H	OH
Liquiritigenin (**10**)	H	H	H	H
6-Methoxyeriodictyol (**12**)	OCH_3	OH	OH	H



Neoisoliquiritigenin (**8**)

Fig. 1. The structures of isolated compounds from *S. suberectus*.

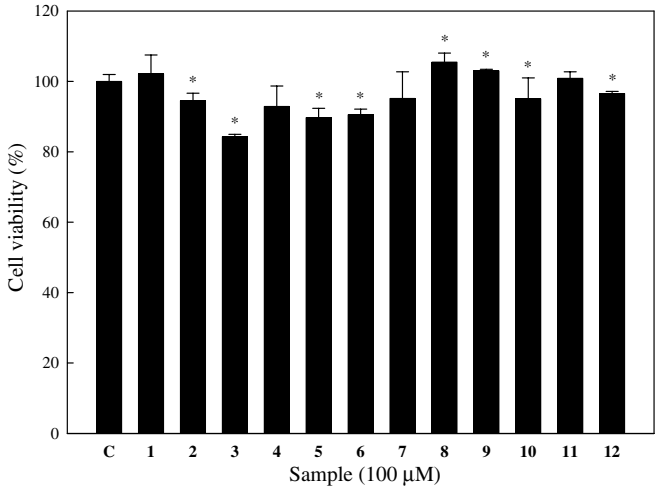


Fig. 2. Cytotoxicities of components of *S. suberectus* to human epidermal melanocytes (HEMn). Data was considered to indicate statistical significance (P-value < 0.5) by means of the non-parametric Mann–Whitney U-test.

of reduction in melanin contents, following treatment with these compounds, varied as shown in Table 1. More specifically, the tyrosinase inhibition of the compounds did not correlate with melanin reduction. This observation may be due to the possibility that melanin biosynthesis in HEMn cells occurs via multiple steps. Besides tyrosinase, these compounds may be influenced by other factors, such as dopachrome tautomerase or DHICA oxidase (Lamoreux et al., 2001).

Table 1
The inhibition of cellular tyrosinase activity and melanin content of the isolated 12 compounds

Compound	Inhibitory activity (%) (100 μM)	
	Tyrosinase ± SD	Melanin ± SD
Arbutin	43.51 ± 3.17[a]	7.06 ± 2.00[a]
1	15.32 ± 4.85	26.59 ± 3.11
2	5.09 ± 4.53	21.12 ± 1.34
3	36.00 ± 1.53	9.31 ± 4.50
4	8.75 ± 1.59	20.87 ± 4.26
5	33.61 ± 3.95	20.10 ± 2.33
6	22.25 ± 4.02	−1.58 ± 2.43
7	80.07 ± 1.88	29.26 ± 1.34
8	20.36 ± 5.29	26.34 ± 1.53
9	15.18 ± 6.47	1.58 ± 7.77
10	36.13 ± 3.58	26.72 ± 2.70
11	3.57 ± 3.99	24.55 ± 3.86
12	44.36 ± 3.02	25.19 ± 3.64

Arbutin, positive control.
[a] The concentration is 2.5 mM.

2.3. Tyrosinase inhibitory effects of butin (7) as measured by l-DOPA zymography and by an anti-tyrosinase assay

The mechanism through which butin (**7**), the component with the greatest anti-tyrosinase activity, exerted its inhibitory action was explored. The effects of butin (**7**) on tyrosinase activity as measured by L-DOPA zymography are shown in Fig. 3. As compared to treatment with medium only (untreated condition), treatment with butin (**7**) at concentrations ranging from 10 to 100 μM resulted in increasing degrees of inhibition of tyrosinase in HEMn cells. When the cellular tyrosinase inhibitory activity of butin (**7**) was examined, a similar dose-dependent inhibition was observed (Fig. 4). The IC_{50} value for butin (**7**) was found to be 35.9 μM. By contrast, the IC_{50} value for arbutin, a positive control, was found to be 2.9 mM in this assay system.

2.4. Expression of pigmentation-related proteins in butin (7)-treated human epidermal melanocytes

Human melanocytes are known to express tyrosinase, TRP1, and TRP2/DCT (Kushimoto et al., 2001; Kushimoto et al., 2003). These proteins constitute a specific family of membrane proteins that are structurally related but with distinct enzymatic functions (Negroiu et al., 2000). The degree of expression of these proteins following 24 h of treatment with butin (**7**) was evaluated by Western blotting. Treatment with various concentrations (10, 25, 50, and 100 μM) of butin (**7**) resulted in dose-dependent decreases in tyrosinase and TRP2/DCT expression, although a weaker correlation was observed with respect to TRP1 expression and butin (**7**) concentration (Fig. 5). Inhibition of expression of tyrosinase and TRP2/DCT by arbutin at 2.5 mM were comparable to those observed in response to butin (**7**) at 50–100 μM (Fig. 5). Tryosinase and TRP activities in human melanosomes are believed to serve as the chief determinants of phenotypic regulation of pigmentation. Therefore butin (**7**) may adversely affect the process of melanin synthesis in HEMn.

2.5. Expression of pigmentation-related genes in butin (7)-treated human epidermal melanocytes

It was considered important to ascertain whether decreased transcription of the TYR, TYRP1, and TYRP2 genes was responsible for the observed decrease in expression of tyrosinase, TRP1 and TRP2/DCT in butin (**7**)-treated HEMn cells. Therefore, the degree of expression of mRNAs for these proteins in butin (**7**)-treated cells was measured by using qRT-PCR. The gene for GAPDH served as the housekeeping gene. Levels of mRNAs encoding tyrosinase, TRP1 and TRP2/DCT were found to be down-regulated by butin (**7**) in a dose-dependent manner. As compared to the untreated control values, expression was decreased by 0.20, 0.21, 0.59 and 0.75 fold for the TYR gene, by 0.15, 0.32, 0.59 and 0.77 fold for the TYRP1

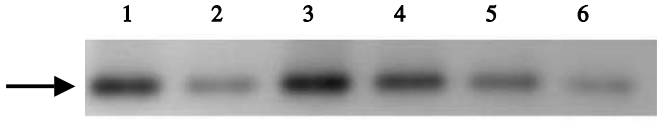


Fig. 3. Inhibition of tyrosinase activity of human epidermal melanocytes (HEMn) by butin (**7**) and arbutin. Tyrosinase activity was determined by L-DOPA zymography as described in Section 4 . Lanes 1: medium; 2: 2.5 mM arbutin; 3: 10 μM butin (**7**); 4: 25 μM butin (**7**): 5: 50 μM butin (**7**): 6: 100 μM butin (**7**).

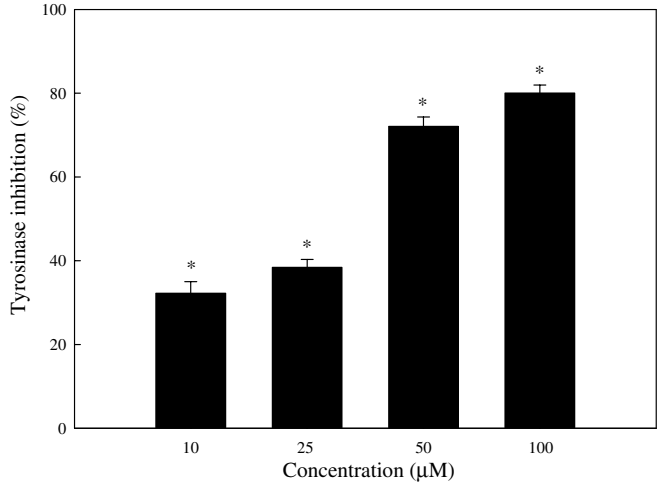


Fig. 4. Butin (**7**) concentration-dependent inhibition of tyrosinase in human epidermal melanocytes (HEMn). Cell viability was determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method after 24 h of treatment with test compounds. Data was considered to indicate statistical significance (P-value < 0.5) by means of the non-parametric Mann–Whitney U-test.

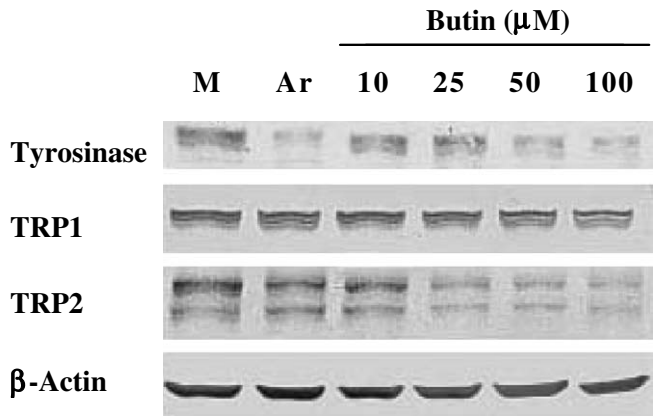


Fig. 5. Expression of tyrosinase, TRP1, and TRP2/DCT in butin (**7**)- and arbutin-treated human epidermal melanocytes (HEMn). Cultured melanocytes were treated with medium or test compounds for 24 h. Cells were then harvested and lysates (10 μg protein) were subjected to polyacrylamide gel (10%) electrophoresis, followed by electroblotting and immunostaining with antibodies to tyrosinase, TRP1, and TRP2/DCT. M: medium only; Ar: 2.5 mM arbutin; 10: 10 μM butin (**7**); 25: 25 μM butin (**7**); 50: 50 μM butin (**7**); 100: 100 μM butin (**7**).

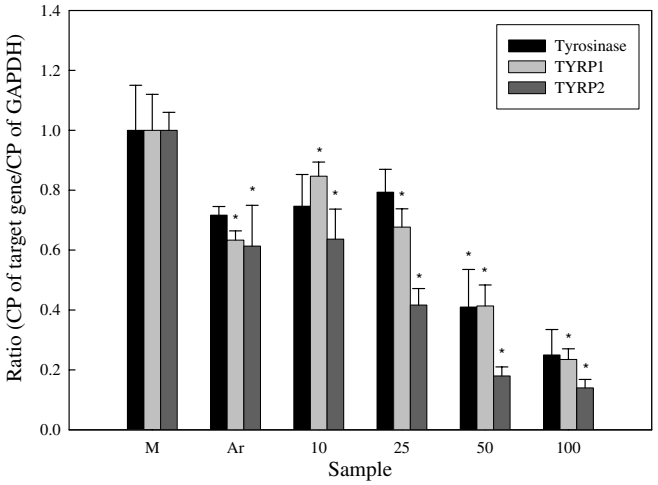


Fig. 6. Expression of TYR, TYRP1, and TYRP2 mRNAs by butin (**7**)- and arbutin-treated human epidermal melanocytes (HEMn). Findings were normalized to the expression of GAPDH mRNA. Measurements were conducted in triplicate, and mean expression values for test samples relative to mean expression values for negative controls are indicated. M: medium only; Ar: 2.5 mM arbutin; 10: 10 μM butin (**7**); 25: 25 μM butin (**7**); 50: 50 μM butin (**7**); 100: 100 μM butin (**7**). Data was considered to indicate statistical significance (P-value < 0.5) by means of the non-parametric Mann–Whitney U-test.

gene, and by 0.36, 0.58, 0.82 and 0.86 fold for the TYRP2 gene at concentrations of 10, 25, 50, and 100 μM butin (**7**), respectively (Fig. 6).

Tyrosinase and the TRPs serve to catalyze different steps in a multi-step melanogenic pathway (Jimenez-Cervantes et al., 1994; Fang et al., 2001). Regulation of the genes encoding these enzymes is a hallmark of cells of the mammalian melanocytic linkage (1). The remarkably similar degrees of inhibition of expression of the genes encoding tyrosinase, TRP1 and TRP2/DCR reported here for butin (**7**)-treated HEMn cells strongly support the proposal these genes are subject to coordinate regulation in human melanocytes. Such coordinate regulation may be essential for the normal synthesis of melanin.

3. Conclusions

Selected components of *S. suberectus*, a traditional Chinese herbal medicine, display anti-tyrosinase and melanin-reducing properties. The presence of a flavanone skeleton appears to be required for the anti-tyrosinase activity of these compounds. None of the components of *S. suberectus* is significantly cytotoxic to human epidermal melanocytes. Butin (**7**) is the most efficacious of the components of *S. suberectus* in reducing tyrosinase activity of human melanocytes. The anti-tyrosinase action of butin (**7**) is attributable to inhibition of TYR gene transcription. The observation that transcription of the TYR gene and transcription of the TYRP genes are inhibited to comparable degrees by butin (**7**) supports the hypothesis that these genes are subject to coordinate regulation. Butin (**7**) may prove to be a novel and effective whitening agent.

4. Experimental

4.1. General experimental procedures

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DRX-500 MHz (^{1}H at 500 MHz; ^{13}C at 125 MHz), and chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. Two dimensional spectra were obtained through ^{1}H, ^{1}H-COSY, HMQC, HMBC, and NOESY experiments. Optical rotation was measured on a JASCO P-1020 polarimeter (Tokyo, Japan). HPLC was conducted with a Hitachi L-7100 pump and an L-7420 UV–VIS detector with a reversed phase column (Biosil ODS-W, 4.6 mm × 250 mm, 10 mm × 250 mm; Biotic Chemicals, Taipei, Taiwan). UV spectra were recorded on a UV-1601 (Shimadzu, Tokyo, Japan) spectrophotometer. All solvents were distilled before use. Solvents were removed from extracts by rotary evaporation under reduced pressure at temperatures upto 40 °C. Silica gel (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany) was used to perform thin layer chromatography (TLC).

4.2. Materials

The vine stems of *S. suberectus* Dunn (Leguminosae) were purchased from the Chinese drug market in 2004 at Taipei, Taiwan. Stems were identified by Dr. H.C. Chang, Bureau of Food and Drug Analysis, Department of Health, Taiwan. A reference specimen (Number M-99) was deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University, Taiwan.

4.3. Reagents

Triton X-100, L-3,4-dihydroxyphenylalanine (L-DOPA), mushroom tyrosinase, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), L-tyrosine, polyacrylamide, aprotinin, and leupeptin were purchased from Sigma (St. Louis, MO). The other chemicals and reagents used in the study were high-grade commercial products.

4.4. Isolation and identification of active components of *S. suberectus*

The dried vine stems of *S. suberectus* (12 kg) were cut into pieces and refluxed three times with 95% ethanol for 8 h. After filtration and combination of the filtrates, the EtOH was evaporated under reduced pressure. The aqueous concentrate was partitioned with *n*-hexane, EtOAc, and water-saturated *n*-BuOH, respectively. All four fractions were subjected to bioactive evaluation with

the mushroom tyrosinase inhibitory model. The EtOAc extract (110 g) showed the strongest activity (60.4 ± 0.7%, 100 μg/ml). Repeated chromatography of the fractions followed by using bio-guided phytochemical assay led to the isolation of active constituents. The EtOAc extract was subjected to chromatography on Diaion HP-20, followed by elution with a gradient solvent system of methanol in H_2O ($H_2O \rightarrow 20\%$ MeOH $\rightarrow 40\%$ MeOH $\rightarrow 60\%$ MeOH $\rightarrow 100\%$ MeOH) to obtain seven fractions (SS-1 to SS-7). After monitoring by HPLC and bio-activity evaluation, SS-4 was subjected to Sephadex LH-20 column chromatography. The column was developed with H_2O–MeOH in a stepwise gradient mode (40% MeOH to 100% MeOH), yielding ten fractions (SS-4-1 to SS-4-10). SS-4-7 was submitted to C-18 column chromatography followed by elution with a gradient solvent system of MeOH in H_2O (45% MeOH to 100% MeOH) and purification with a semi-preparative HPLC (column: Biosil 5 ODS-W, 10 × 250 mm; solvent system: 30% CH_3CN; flow rate: 3 ml/min; detector: 280 nm) to obtain compounds **5** (42.0 mg), **6** (9.5 mg), **7** (30.0 mg), **8** (3.5 mg), **9** (9.3 mg), **10** (11.5 mg), **11** (2.0 mg), and **12** (2.5 mg). SS-4-8 was also subjected to C-18 column chromatography and elution with a gradient solvent system of MeOH in H_2O (45% MeOH to 100% MeOH) to obtain seven fractions (SS-4-8-1 to SS-4-8-7). SS-4-8-3 and SS-4-8-4 were then purified by semi-preparative HPLC (column: Biosil 5 ODS-W, 10 × 250 mm; solvent system: 55% CH_3CN; flow rate: 3 ml/min; detector: 280 nm) to obtain compounds **1** (3.0 mg) and **4** (6.5 mg), respectively. SS-4-8-6 was also purified by semi-preparative HPLC with 65% CH_3CN to obtain compound **2** (1.0 mg). The purification procedures for SS-4-9 were similar to those for SS-4-7 and yielded compound **3** (5.2 mg).

4.4.1. The active compound – butin (7)

Yellow powder; FAB-MS *m/z* 272.9 $[M+H]^+$; $[\alpha]^{22}$–22.0 (*c* 0.1, MeOH); UVλ_{max} (MeOH) nm (log ε) 311 (3.8), 277 (4.2); IR (KBr) υ_{max} 3422, 1636, 1015 cm^{-1}; ^{1}H-NMR (CD_3OD, 500 MHz), δ 2.67 (1 H, *dd*, *J* = 16.9, 2.7 Hz, H3a), 2.97 (1H, *dd*, *J* = 16.9, 12.9 Hz, H3b), 5.28 (1H, *dd*, *J* = 12.9, 2.7 Hz, H2), 6.34 (1H, *d*, *J* = 1.1 Hz, H8), 6.47 (1H, *dd*, *J* = 8.7, 1.1 Hz, H6), 6.77 (2H, *bs*, H5′, H6′), 6.91 (1 H, *bs*, H2′), 7.70 (1H, *d*, *J* = 8.7 Hz, H5); ^{13}C-NMR (CD_3OD, 125 MHz) δ 44.8 (C3), 80.9 (C2), 103.8 (C8), 111.7 (C6), 114.7 (C2′), 114.9 (C10), 116.2 (C5′), 119.2 (C6′), 129.8 (C5), 131.9 (C1′), 146.4 (C4′), 146.7 (C3′), 165.5 (C9), 166.7 (C7), 193.6 (C4).

4.5. Mushroom tyrosinase purified enzyme assay

Tyrosinase activity was measured by a method modified from that of Pomerantz (Masuda et al., 2005). Tyrosinase was reconstituted in 50 mM potassium phosphate buffer, pH 6.8, at 1000 U/ml and stored at −20 °C prior to use. The reaction mixture consisted of 2.5 mM L-tyrosine, 500 U/ml mushroom tyrosinase, and test sample. After incubation for 30 min at 37 °C, the absorbance was measured at 475 nm in a model μQuant microplate reader (Bio-tek Instruments, INC).

4.6. Cell culture

Primary cultures of HEMn cells (Cascade Cat. C-102-5 C) from neonatal foreskin were propagated in Medium 254 (Cat. M-254-500) supplemented with Human Melanocyte Growth Supplement (HMGS, Cat. S-002-5, Cascade Biologics, Inc., Portland, OR, USA).

4.7. Assay of cell viability

Cells were plated at 1×10^5/well (24-well plates). Twenty-four hours after plating, test samples were added and cultures were incubated for an additional 24 h. Viability was determined using the 3-(4,5-dimethyl-thiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) method, a colorimetric assay involving formation of purple formazan by mitochondrial dehydrogenase of active mitochondria.

4.8. Tyrosinase activity analysis by 3,4-dihydroxyphenylalanine (DOPA) staining of SDS-PAGE gels

Analysis of tyrosinase activity by zymography was performed as described (Li et al., 2000). Test compounds were added to HEMn cells (1×10^5 cells/well; 24-well plates), and cultures were incubated at 37 °C with 5% CO_2 in a humidified incubator for 1 day. The protein contents of culture supernatants were determined with a Bio-Rad protein assay kit. Equal amounts of each test supernatant (40 μg protein) were incubated with a five-fold excess of sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol) at 37 °C for 30 min. Samples were then subjected to polyacrylamide gel (10%) electrophoresis. After electrophoresis, gels were washed with 0.1 M PBS (pH 6.8) for 1 h by gentle shaking. The gels were then incubated at 37 °C for 1 h in 0.1 M PBS, pH 6.8, containing 5 mM L-DOPA substrate, and were subsequently stained with Coomassie Blue. Gels were then destained in 30% methanol containing 10% glacial acetic acid to visualize clear areas corresponding to regions of protein degradation.

4.9. Assay of cellular tyrosinase activity

Tyrosinase activity was measured as described previously with minor modifications (Nagata et al., 2004). HEMn cells were cultured in 24-well plates. After treatment with individual test samples for 24 h, the cells were washed with PBS and lysed with phosphate buffer, pH 6.8, containing 1% Triton X-100. The cells were disrupted by freezing and thawing, and lysates were clarified by cen-

trifugation at 10,000*g* for 10 min. After determination of protein content with a Bio-Rad protein assay kit, lysates were adjusted with lysis buffer to contain equal amounts of protein (40 μg). These lysates were then added to wells (96-well plates) containing 2.5 mM L-DOPA in 0.1 M phosphate buffer, pH 6.8. After incubation at 37 °C for 1 h, the absorbance of samples was measured at 450 nm using an ELISA reader.

4.10. Measurement of melanin content of melanocytes

Melanin contents were measured as described previously with slight modifications (Nagata et al., 2004). Cells were treated with test substances for 24 h and then harvested by centrifugation. Cell pellets were incubated in 1 N NaOH at 37 °C for 16 h and suspensions were clarified by centrifugation for 10 min at 10,000*g*. The optical densities (OD) of supernatants were measured at 450 nm using an ELISA reader.

4.11. Western blot analysis

Western blot analysis was performed as described previously (Lee and Kang, 2003) to determine the extent of expression of tyrosinase and the TRPs. Cells (1×10^6) were lysed with PBS containing 1% TritonX-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, and 10 mg/ml leupeptin, and lysates were subjected to centrifugation at 12,000*g* for 10 min. The total protein content of each supernatant was determined with a Bio-Rad protein assay kit. Samples (approximately 10 μg of protein) were added to equal volumes of SDS sample buffer, and proteins were separated by polyacrylamide gel (10%) electrophoresis. Following electrotransfer to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA), the membranes were incubated overnight with PBS containing 5% non-fat dry milk, 0.1% Tween 20 and 0.1% NaN_3. Anti-tyrosinase (C-19), anti-TRP1 (G-17), and anti-TRP2 (D-18) antibodies (Santa Cruz Biotechnology, Inc., Europe) were added at a 1:1000 dilution, and membranes were incubated at room temperature for 3 h. After extensive washes, the blots were incubated for 2 h at room temperature with alkaline phosphatase-conjugated anti-goat IgG (Santa Cruz Biotechnology) diluted 1:5000 in PBS containing 5% non-fat dry milk, 0.1% Tween 20, and 0.1% NaN_3. After washing, protein-bound alkaline phosphatase activity was detected with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate. The extent of protein loading was evaluated by Western blotting with antibody to β-actin.

4.12. RNA extraction and reverse transcription

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). The quality of the total RNA sample was evaluated by determination of the A260/A280 ratio. To prepare a cDNA pool from each RNA sample, total RNA (1 μg) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Each cDNA pool was stored at −20 °C until real-time PCR analysis was performed.

4.13. PCR primers

Specific oligonucleotide primer pairs to be used for quantitative real-time PCR (q-RT PCR) were selected from the Roche Universal ProbeLibrary. The sequences of the primers used are as follows:

TYR forward primer: CATTCTTCTCCTCTTGGCAGA
and TYR reverse primer: CCGCTATCCCAGTAAGTGGA;
TYRP1 forward primer: GCTTTTCTCACATGGCACAG
and TYRP1 reverse primer: GGCTCTTGCAACATTTCCTG;
TYRP2 forward primer: CGACTCTGATTAGTCGGAACTCA
and TYRP2 reverse primer: GGTGGTTGTAG TCATCCAAGC;
GAPDH forward primer: AGCCACATCGCTCAGACAC
and GAPDH reverse primer: GCCCAATACGACCAAATCC.

4.14. Quantitative real-time PCR (q-RT PCR)

Quantitative real-time PCR reactions were performed on the Roche LightCycler Instrument 2.0 using LightCycler® TaqMan Master (Roche Cat. 04 535 286 001). ProbeFinder software (www.universalprobelibrary.com) was used to design the optimal assay comprised of the respective labeled probe of the Universal ProbeLibrary Set and human and gene-specific primers. Briefly, 20 μl reactions contained 5 μl cDNA template, 4 μl Master Mix, 0.2 μl of 10 μM probe, 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reverse primer, and 10 μl water. The RT-PCR program was 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 72 °C for 1 s, and 40 °C for 30 s. At the end of the program a melt curve analysis was performed. At the end of each RT-PCR run, the data were automatically analyzed and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler4 Data analysis software automatically calculates the CP value (crossing point: the turning point corresponds to the first maximum of the second derivative curve) which indicates the beginning of exponential amplification. The mRNA level was normalized with reference to the amount of housekeeping gene transcript (GAPDH mRNA).

4.15. Statistical analysis

Differences between the groups were tested for significance by means of the non-parametric Mann–Whitney *U*-test. A P-value < 0.5 was considered to indicate statistical significance.

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