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Natural Products of Cosmetics: Analysis of Extracts of Plants Endemic to Taiwan for the Presence of Tyrosinase-inhibitory, Melanin-reducing, and Free Radical Scavenging Activities

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ABSTRACT

Non-toxic natural products useful in the formulation of cosmetics are of considerable interest. Recent efforts have focused on the identification of substances that inhibit tyrosinase activity or suppress formation of reactive oxygen species (ROS) in skin cells. Since tyrosinase is the rate-limiting enzyme in the synthesis of melanin, the pigment responsible for the color of human skin, tyrosinase inhibitors may have skin-whitening effects. Since ROS have been implicated in the aging of human skin, agents that suppress the production of ROS may retard such aging. In the present study, ethanol (95%) extracts of 26 plants endemic to Taiwan were examined for their tyrosinase-inhibitory or melanin-reducing activities in human epidermal melanocytes, as well as *in vitro* free radical scavenging activity. Among the preparations tested, extracts of *Pyracantha koidzumii* (M-165) were found to be the least cytotoxic and to possess the highest cellular tyrosinase inhibitory activity (IC_{50}, 54.8 μg/mL). Extracts of *Acer albo-purpurascens* (M-51), *Hygrophila pogonocalyx* (M169), *Machilus japonica* var. *kusanoi* (M-67), and *Eriobotrya deflexa* (M-50) exhibited the most potent free radical scavenging activity against hydroxyl, superoxide, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) anion radicals. The IC_{50} values of M-51, M169, M-67, and M-50 were 3.1, 0.8, 6.6, and 1.8 μg/mL for hydroxyl radical; 5.3, 12.8, 12.4, and 6.1 μg/mL for superoxide radical; 2.4, 7.9, 5.7, and 2.9 μg/mL for ABTS anion radical, respectively. Nevertheless, the phenolic contents were not all correlated with these activities. These plants thus serve as potential sources of ingredients, which could be combined in cosmetic products. Further investigation of the substances responsible for the observed tyrosinase-inhibitory and free radical scavenging activities is therefore warranted.

Key words: plants endemic to Taiwan, tyrosinase, human skin melanocytes, free radical scavenging activity

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase that is widely distributed in nature. The enzyme hydroxylates monophenols to *o*-diphenols and oxidizes *o*-diphenols to *o*-quinones. Quinones are highly reactive compounds that, upon further oxidation, could convert to eumelanin polymers through a radical-coupling pathway. Besides, quinones can polymerize spontaneously, resulting in melanins formation, and can react with amino acids or proteins, thus enhancing the brown color production by the parent compound[(1)].

Melanin synthesis in melanocytes is accompanied by the generation of hydrogen peroxide, which, if inappropriately processed, can lead to the formation of hydroxyl radicals and other ROS[(2)]. These highly reactive free radical compounds are potentially damaging to human tissues. For example, the hydroxyl radical ($HO^{\bullet}$), the superoxide anion radical ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the peroxyl radical ($ROO^{\bullet}$) all have the capacity to generate metabolic products that could attack DNA or lipids in cell membranes. ROS cause biological damage in exposed tissues *via* iron-catalyzed oxidative reactions[(3)]. Oxidative stress is also involved in the pathogenesis of skin disorders[(1)], and ROS scavengers or inhibitors, such as antioxidants, reduce hyper-pigmentation[(5)].

Most of the popular de-pigmenting agents in current use are toxic to melanocytes and have various adverse effects[(6-8)]. Tyrosinase inhibitors involved in the rate-limiting step in the pathway for melanin synthesis, may present safer alternatives to depigmenting compounds. However the well known tyrosinase inhibitor kojic acid, is highly sensitive to skin, and cases of allergic contact dermatitis due to this drug have been reported[(9-11)]. Thus, searching for non-toxic natural products with properties useful in the formulation of cosmetics is desirable.

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Recent efforts have been focused on the identification of plant products that are antioxidant[12-16] or whitening agents[1,17-22].

Because they are easy to culture, human epidermal melanocytes (HEMn cells) are powerful *in vitro* tools for investigating human melanin synthesis and pigmentation. In culture, these cells continue to express the melanogenic phenotype of the skin from which they were derived. The present study was undertaken to investigate whether plants indigenous to Taiwan, contain potentially useful substances for cosmetic purposes. Extracts of Taiwanese plants were screened for tyrosinase-inhibitory activities and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)$^{\bullet-}$ and hydroxyl ($HO^{\bullet}$) radical-scavenging activities using HEMn cells as a model system.

METHODS AND MATERIALS

I. *Reagents*

2,2'-azobis(2-amidino-propane)-dihydrochloride (AAPH), ABTS, l-3,4-dihydroxyphenylalanine (*l*-DOPA), luminol, sodium hydroxide (NaOH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of the highest grade commercially available.

II. *Materials*

All plant materials were selected and collected from the Highlands Experiment Farm, National Taiwan University, Nantou, Taiwan, and were identified by Mr. Chi-Luan Wen, Seed Improvement and Propagation Station, Council of Agriculture, Taiwan. The specimens were deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University, Taiwan.

III. *Preparation of Extracts*

Pulverized dried leaves of plants were extracted twice within 7 days with 95% ethanol. Filtrates were combined, and concentrated under reduced pressure. The concentrates were freeze-dried and stored in a closed container until use. The yields of plant extracts were calculated by the following formula:

Yield (%) = (mass of the extract / mass of the dried raw plant material) × 100.

IV. *Cell Culture*

Cells, culture medium and supplement were obtained from Cascade Biologics, Inc. (Portland, OR, USA). Primary melanocytes derived from normal neonatal human foreskin (HEMn cells; Cat. No. C-102-5C) were propagated in Medium 154 (Cat. No. M-254-500) enriched with Human Melanocyte Growth Supplement (HMGS, Cat. No. S-002-5).

V. *Assay of Cell Viability*

Cells were seeded in 24-well plates (1×10^5 cells/well). After 24 h culture, test materials were added and incubations were continued for another 24 h. Cell viability was determined by the MTT method, which measures the mitochondrial dehydrogenase activity involved in the formation of purple formazan. Cell viability (%) was calculated as (Absorbance at 570 nm of the test sample / Absorbance at 570 nm of the control) × 100.

VI. *Tyrosinase Activity Analysis by DOPA Staining of SDS-PAGE Gels*

Tyrosinase activity was determined by zymography as described[2]. Test materials were added to HEMn cells (1×10^5 / well) in 24-well plates, and cultures were incubated at 37ºC 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. Test supernatants containing (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 37ºC for 30 min, and then subjected to the polyacrylamide gel (10%) electrophoresis. After electrophoresis, gels were washed with 0.1 M PBS (pH 6.8) for 1 h by gentle shaking, and then incubated at 37ºC for 1 h in 0.1 M PBS, pH 6.8, containing 5 mM *l*-DOPA substrate. The tyrosinase activity was calculated for the intensity value (intensity × area) for each band by Quantity One 1-D Analysis Software (Bio-Rad, UK, Europe), and normalized with the intensity value with control.

VII. *Measurement of Melanin Content in Melanocytes.*

Melanin contents were measured as described previously[3]. Cells were treated with test materials for 24 h. Cell pellets were suspended with 1 N NaOH and incubated at 37°C overnight. Samples were clarified by centrifugation for 10 min at 10000 *g*. The optical densities (OD) of supernatants were measured at 450 nm using an ELISA reader.

VIII. *Assay for Hydroxyl Radical Scavenging Activity*

The hydroxyl radical was generated by the Fenton reaction according to a modification of the luminescence method[4]. Reaction mixtures included luminol (40 μM), phosphate buffer (4.17 mM, pH 7.4), Fe^{2+}(4.6 μM)-EDTA (2.3 μM), H_2O_2 (96 mM), and test sample. The chemiluminescent reaction was performed in a KH_2PO_4-NaOH (pH 7.5)-buffer at room temperature. The reaction was initiated by adding Fe^{2+}-EDTA, followed by H_2O_2. Luminescence intensity was monitored over wavelengths ranging from 200 to 900 nm.

IX. *Superoxide Radical Scavenging Activity*

The generation of superoxide radicals by the xanthine/xanthine oxidase system was monitored by the nitro-blue tetrazolium (NBT) procedure with modification as described previously[5]. The reaction mixtures including various concentrations of test samples, 100 μM of xanthine, 15 mU of xanthine oxidase, and 500 μM of NBT were incubated at ambient temperature for 2 min and the reaction was then initiated by adding xanthine oxidase. The samples were dissolved in 100 mM phosphate buffer (pH 7.4). The absorbance (560 nm) of each sample was measured. The effects of the tested compounds on xanthine oxidase were evaluated by following the formation of uric acid from xanthine at room temperature. The same reaction mixtures, except for NBT, were used in the enzymatic assay for superoxide radical-scavenging activity. The absorbance was measured at 295 nm.

X. *Assay for ABTS Radical Anion Scavenging Activity*

Reaction mixtures contained 1 mM AAPH, 2.5 mM ABTS as the diammonium salt, 100 mM potassium phosphate buffer (pH 7.4), and 150 mM NaCl. After 15 min of incubation at 68°C, the blue-green $ABTS^{\bullet-}$ containing solution was adjusted to an absorbance of 0.650 at 734 nm. Samples of interest (20 μL) were added to 180 μL of the ABTS radical solution in 96-well plates. Mixtures were incubated at 37°C for 10 min in the dark, followed immediately by measurement of absorbance at 734 nm[6].

XI. *Determination of Total Phenolics*

The total phenolic content of each extract was determined by the Folin-Ciocalteu method[7]. The sample solution (50 μL) was mixed with an equal volume of 1 N Folin-Ciocalteu reagent-20% sodium carbonate (Na_2CO_3). After 25 min of incubation at room temperature, the reaction mixture was clarified by centrifugation at 3500 *g* for 10 min. The absorbance of the supernatant was measured at 730 nm. The amount of total phenolics was expressed as gallic acid equivalents (GAE) in grams per gram dry plant extract.

XII. *Statistical Analysis*

Data are presented as the mean ± standard deviation (SD) for measurements conducted in triplicate. Differences between groups were tested for significance by means of the non-parametric Mann-Whitney U-test. A *P*-value < 0.5 indicated statistical significance.

RESULTS AND DISCUSSION

Whitening cosmetics that contain naturally occurring, as opposed to synthetic, substances are currently desirable. Twenty-six Taiwanese plants were therefore evaluated as potential sources of skin-whitening and antioxidant ingredients. Ethanol extracts of these plants were prepared with yields ranging from 1.4% to 14.7% (Table 1).

Among many factors contribute to the color of mammalian skin, the most important ones are the degree and distribution of melanin pigmentation. Melanin, one of the most widely distributed pigments in animals, is secreted by melanocytes distributed in the epidermis. Inhibition of melanin synthesis by tyrosinase inhibitors represents a main strategy for developing new whitening agents. HEMn cells were therefore employed to examine the anti-tyrosinase and melanin-decreasing activities of plant extracts. Since a cosmetic additive must have a low cytotoxicity, the effect on viability was examined first. Of the 26 extracts investigated, those from *B. chitoensis* (M-151), *P. koidzumii* (M-165), and *P. lucida* (M-143) were least cytotoxic (viability > 80%) after 24 h treatment at concentration of 100 μg/mL (Figure 1). Tyrosinase catalyzes two key reactions in the melanin biosynthesis pathway, and substrates for both reactions have been used to assay tyrosinase[8]. In the present study, *l*-DOPA zymography was used to detect tyrosinase inhibition by extracts of *B. chitoensis* (M-151), *P. koidzumii* (M-165), and *P. lucida* (M-143) in HEMn cells (Figure 2). The effect of extracts on tyrosinase activity was semi-quantified relative to that of medium alone. Tyrosinase inhibition by arbutin (used as a positive control) was dose-dependent between 2 mM and 10 mM. Tyrosinase inhibitory activity was expressed as the intensity value (intensity × area) for each protein band visualized by Image Analysis Software (Quantity One, Bio-Rad) and normalized to the intensity value for control. Of the three extracts tested at concentrations of 20 μg/mL, 40 μg/mL, 80 μg/mL, or 100 μg/mL, only the *P. koidzumii* (M-165) extract exhibited dose-dependent inhibitory activity. Extracts of *B. chitoensis* (M-151) and *P. lucida* (M-143) were minimally inhibitory. Measurement of the melanin content of cells exposed to each of the three extracts revealed that *P. koidzumii* (M-165) extract significantly and dose-dependently reduced melanin content ($p < 0.05$, Figure 3). The other two plant extracts had almost no effect on melanin content of HEMn cells. This observation may be due to that melanin biosynthesis in HEMn cells occur *via* multiple steps. Compounds in these extracts may also affect enzymes besides tyrosinase, such as dopachrome tautomerase or DHICA oxidase[9].

Skin has the largest surface area of any tissue of the human body and is constantly exposed to oxidative stress induced by ROS generated both from endogenous enzymes and external pro-oxidant stimuli. ROS-mediated oxidative damage to skin can be extensive, resulting in modification of DNA, lipid peroxidation, and release of inflammatory cytokines. The hydroxyl radical is one of the most reactive and damaging ROS[10]. To deal with harmful effects by such radicals, mammalian skin is equipped with antioxidant scavenging defense mechanisms. Some plant extracts can also scavenge hydroxyl

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radicals and thereby protect cellular lipids against oxidative damage by free radicals[11]. The $HO^{\bullet}$ quenching activity of the 26 extracts was determined using the Fenton reaction system in combination with a chemiluminescence (CL) assay. This approach has been used widely in the fields of biology[12], medicine[13-14], and food science[15]. All but the extracts of *C. osmophloeum* (M-82) and *Ophiopogon formosanum* (M-162) owned potent scavenging activity (IC_{50} > 10 μg/mL).

The superoxide radical was generated by an xanthine/xanthine oxidase enzymatic system. Xanthine oxidase catalyzes the oxidation of xanthine in the presence of molecular oxygen to yield uric acid and superoxide anion. This is an important physiological pathway in humans[16]. Extracts of *A. albopurpurascens* (M-51), *H. pogonocalyx* (M169), *Machilus japonica* var. *kusanoi* (M-67), *E. deflexa* (M-50), *P. lucida* (M-143), and *P. lucida* (M-130) showed potent scavenging activity (IC_{50} < 100 μg/mL) and no inhibitory effect on xanthine oxidase at a concentration of 100 μg/mL (data not shown).

ABTS radical anion has recently become a popular tool for measuring radical-scavenging activity. ABTS-based methods are often employed to screen complex mixtures, such as plant extracts, beverages, and biological fluids, for scavenging activity and for estimating the antioxidant activity of lipophilic and water-soluble materials [6,17]. Analytical methods involving $ABTS^{\bullet-}$ may be preferable to other methods for evaluating the total antioxidant capacity of various chemicals and food substances[18-21]. By using an ABTS-based method, potent scavenging activity (IC_{50} 2.43-9.48 μg/mL) was detected in extracts of *A. albopurpurascens* (M-51), *S. euphlebium* (M-150),

Table 1. Ethno-botanical data, extract yields, phenolic contents of extracts, and IC50 values for extracts relevant to their free radicals scavenging activities for 26 selected Taiwanese plants.

No.	Vouch species	Family	Botanical name	Yield (%)	Total phenolic contents (g GAE/g extract)	IC50 (μg/mL)		
						$HO^{\bullet}$	$O_2^{\bullet-}$	$ABTS^{\bullet-}$
1	M-51	Aceraceae	*Acer albopurpurascens* Hayata	8.9	5.38	3.07	5.29	2.43
2	M-150	Myrtaceae	*Syzygium euphlebium* (Hayata) Mori	2.8	1.31	1.72	> 100	9.48
3	M-151	Begoniaceae	*Begonia chitoensis* Liu & Lai	1.4	1.33	0.13	> 100	17.53
4	M-169	Acanthaceae	*Hygrophila pogonocalyx* Hayata	3.1	0.80	0.78	12.75	7.94
5	M-141	Euphorbiaceae	*Gelonium aequoreum* Hance	2.5	0.25	0.61	> 100	89.65
6	M-146	Ericaceae	*Rhododendron oldhamii* Maxim.	3.9	2.17	0.94	> 100	7.37
7	M-156	Iridaceae	*Iris formosana* Ohwi	3.1	0.32	0.40	> 100	> 100
8	M-82	Lauraceae	*Cinnamomum osmophloeum* Kanehira	10.3	7.37	10.62	> 100	20.42
9	M-67	Lauraceae	*Machilus japonica* Sieb& Zucc. var. *kusanoi* (Hayata) Liao	4.5	12.50	6.60	12.36	5.67
10	M-52	Lauraceae	*Machilus zuihoensis* Hayata	7.3	23.20	1.96	> 100	2.46
11	M-149	Lauraceae	*Cinnamomum brevipedunculatum* C. E. Chang	2.3	1.64	5.04	> 100	7.64
12	M-140	Lauraceae	*Litsea krukovii* Kosterm	4.0	1.13	5.03	> 100	12.69
13	M-162	Liliaceae	*Ophiopogon formosanum* Ohwi	2.8	0.22	13.26	> 100	83.01
14	M-142	Liliaceae	*Musa formosana* (Warb.) Hayata	7.1	0.38	7.47	> 100	33.58
15	M-167	Moraceae	*Ficus pumila* L. var. *awkeotsang* (Makino) Corner	4.9	0.19	6.57	> 100	13.95
16	M-191	Myrtaceae	*Syzygium formosanum* (Hatata) Mori	14.7	0.78	0.67	> 100	7.61
17	M-50	Rosaceae	*Eriobotrya deflexa* (Hemsl) Nakai	8.8	18.30	1.82	6.14	2.87
18	M-144	Rosaceae	*Rhaphiolepis indica Lindl.* var. *tashiroi* Hayata ex Matsum & Hayata	3.5	0.58	3.57	> 100	24.18
19	M-119	Rosaceae	*Pyracantha koidzumii* (Hay.) Rehder (Leaves)	6.3	3.55	0.90	> 100	4.65
20	M-165	Rosaceae	*Pyracantha koidzumii* (Hay.) Rehder (Fruit)	7.1	0.23	8.16	> 100	76.09
21	M-143	Rosaceae	*Pourthiaea lucida* Decaisine (Leaves)	8.4	1.30	4.60	21.03	13.82
22	M-130	Rosaceae	*Pourthiaea lucida* Decaisine (Branches)	5.1	0.46	8.33	69.81	26.89
23	M-161	Staphyleaceae	*Turpinia formosana* Nakai	6.1	0.96	0.86	> 100	10.63
24	M-36	Styracaceae	*Styrax formosana* Matsum	13.5	14.46	0.13	> 100	4.56
25	M-166	Valerianaceae	*Patrinia formosana* Kitamura	4.7	0.44	5.50	> 100	37.32
26	M-168	Zingiberaceae	*Alpinia shimadae* Hayata var. *shimdae*	4.2	0.97	7.25	> 100	11.11

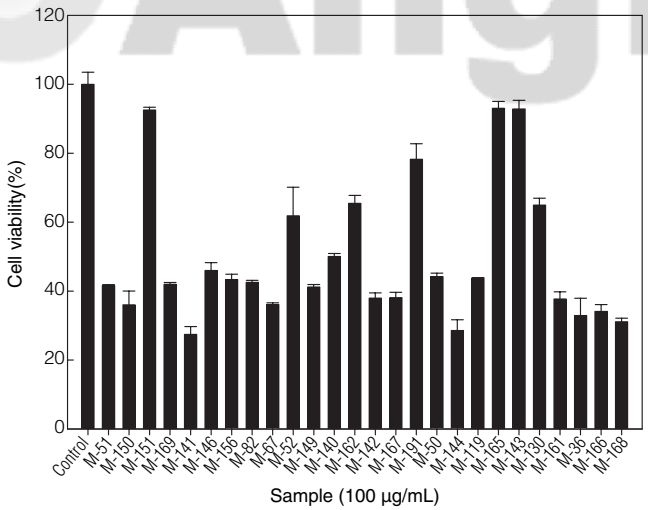

Figure 1. The viabilities of human epidermal melanocytes treated with ethanol extracts derived from each of 26 selected Taiwanese plants.

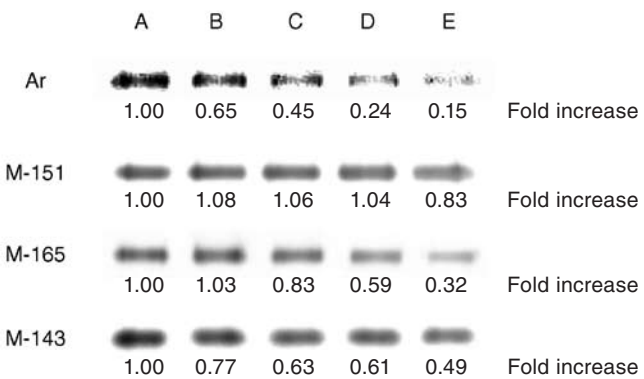

Figure 2. *l*-DOPA zymography analysis of the inhibition of human epidermal melanocyte tyrosinase by extracts of *B. chitoensis* (M-151), *P. koidzumii* (M-165), and *P. lucida* (M-143). (A) medium only; (B) 2 mM arbutin (Ar), 40 μg/mL test extract; (C) 4 mM arbutin, 60 μg/mL test extract; (D) 6 mM arbutin, 80 μg/mL test extract; (E) 10 mM arbutin, 100 μg/mL test extract.

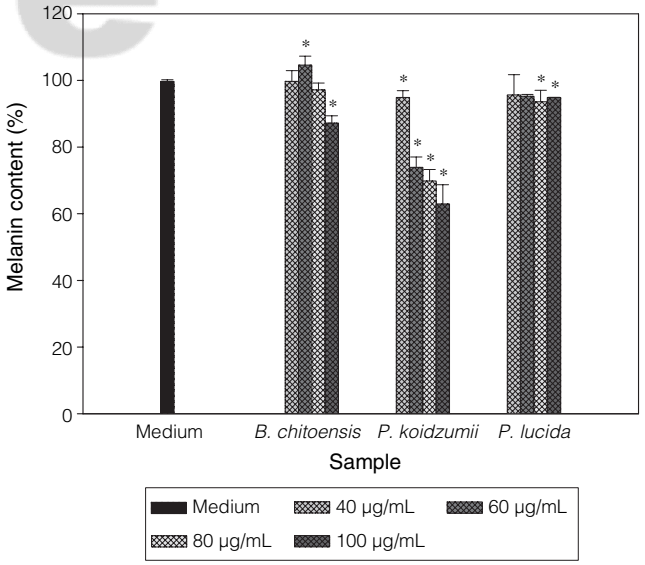

Figure 3. The melanin content of human epidermal melanocytes treated with ethanol extracts of three selected Taiwanese plants. Differences between control and treatment groups were tested for significance using the non-parametric Mann-Whitney *U*-test. A *P*-value < 0.05 indicated statistical significance.

H. pogonocalyx (M169), *R. oldhamii* (M146), *Machilus japonica* var. *kusanoi* (M-67), *M. zuihoensis* (M-52), *Cinnamomum brevipedunculatum* (M-149), *S. formosanum* (M-191), *E. deflexa* (M-50), *Pyracantha koidzumii* (M-119), and *Styrax formosana* (M-36).

Plant phenolic compounds have diverse properties including antioxidant[22-24], antiviral[25], antibacterial[26-28], and anti-tumor activities[29-32]. Total phenolic content may reflect the total antioxidant activity[33]. The total phenolic content of extracts is presented in Table 1. Phenolics were abundant (more than 2 g GAE/g extract) in *A. albopurpurascens* (M-51), *R. oldhamii* (M146), *Cinnamomum osmophloeum* (M-82), *M. japonica* var. *kusanoi* (M-67), *M. zuihoensis* (M-52), *E. deflexa* (M-50), *P. koidzumii* (M-119), and *S. formosana* (M-36).

Few reports have addressed the commercial value of plants endemic to Taiwan. The present study found that extracts of *P. koidzumii* (M-165) were non-toxic and had significant tyrosinase-inhibitory and melanin-reducing activity in HEMn cells. Nevertheless, extracts of this plant were not enriched in phenolics and not effective in scavenging superoxide and ABTS radicals. *P. koidzumii* (M-165) extracts may therefore contain non-phenolic products that effectively reduce melanin synthesis by unique mechanisms. Oxidative stress induces ROS and other free radicals and plays a role in the pathogenesis of skin disorders. Therefore, free radical scavengers or antioxidants are commonly included in skin care formulations. The present study found that extracts of *A. albopurpurascens* (M-51), *H. pogonocalyx* (M169), *M. japonica* var. *kusanoi* (M-67), and *E. deflexa* (M-50) exhibited the most potent free radical scavenging activities, including activities against hydroxyl, superoxide, and ABTS anion radicals. Due to their potent free radical scavenging activities, these extracts can be combined in the cosmetic formulation, and because of their cytotoxicity, their concentration needs to be reduced in the formulation. Not all of these activities correlate with phenolic components of the plant extracts. Some activity may be due to other secondary metabolites contained in the active plants. Therefore, compounds from a variety of Taiwanese plants are potentially useful in the formulation of cosmetics.

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