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Effects of aqueous extracts of *Ecliptae herba*, *Polygoni multiflori radix praeparata* and *Rehmanniae radix praeparata* on melanogenesis and the migration of human melanocytes☆

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ABSTRACT

Ethnopharmacological relevance: *Polygoni multiflori radix praeparata (PMRP), Ecliptae herba (EH)* and *Rehmanniae radix praeparata (RRP)* are the most frequently-used herbs by Traditional Chinese Medicine practitioners for the treatment of vitiligo. Their abilities to stimulate melanogenesis, melanocyte migration and MITF (microphthalmia associated transcription factor) protein expression were evaluated in this study.

Materials and methods: The effects of aqueous extracts of PMRP, EH and RRP on human melanocytes in vitro were examined by MTT assay, tyrosinase activity, melanin synthesis, migration assay and Western blot.

Results: Treatment with EH (at 100 μg/ml and 400 μg/ml) significantly increased intracellular tyrosinase activity in accordance with the elevation of melanin content at the same concentrations. Treatment with RRP (at 100 μg/ml and 400 μg/ml) promoted melanin production but had no stimulatory effect on tyrosinase activity. Treatment with PMRP and EH (at 100 μg/ml) promoted the migration of human melanocytes in a type IV collagen-coated transwell migration assay. Western blot analysis showed MITF protein expression was elevated by PMRP, EH and RRP (at 100 μg/ml).

Conclusion: An aqueous extract of EH has a synergistic effect on melanocytes by up-regulating tyrosinase activity, enhancing melanin synthesis and promoting melanocyte migration as well as elevating MITF protein expression. RRP exhibits a significant stimulating effect on melanogenesis and MITF protein expression. These results suggest that EH and RRP contain substances with direct enhancing effects on melanogenesis and migration, possibly via their effects on MITF protein expression.

1. Introduction

Vitiligo is an acquired skin depigmentation disorder with an estimated prevalence of 0.5–1% among the world's population (Alikhan et al., 2011). It is perhaps the most common depigmenting disorder of the skin and is notoriously difficult to treat (Goldstein et al., 2015). Despite new researches and progress, the etiopathogenesis of vitiligo is still enigmatic and several theories have been proposed (Iannella et al., 2016). The autoimmune theory is the leading hypothesis based on the clinical association of vitiligo with several other autoimmune disorders, such as thyroiditis, rheumatoid arthritis, diabetes mellitus, pernicious anaemia and alopecia areata (Iannella et al., 2016). Antibodies to normal human melanocytes have also been detected. Genome-wide association analyses have identified several susceptibility genes for vitiligo, including loci related to adaptive immunity (eg, HLA class I and II, PTPN22, IL2R α, GZMB, FOXP3, BACH2, CD80, and CCR6), or genes regulate innate immune system (eg, NLRP1, IFIH1 [MDA5], TRIF, CASP7, and C1QTNF6)(Czajkowski and Mecinska-Jundzill, 2014; Jin et al., 2012). Other researches point to the importance of higher level reactive oxygen species and low catalase concentrations in the epidermis which attribute to the anti-melanocyte immune responses (Xie et al., 2016). Neurogenic inflammatory mediators such as NGF and NPY have been demonstrated to be toxic to melanocytes (Ezzedine et al., 2015). Recently, it has been postulated that extracellular matrix molecules may inhibit the adhesion of melanocytes to fibronectin and induce detachment and death of melanocytes. Moreover, β-catenin, the partner of E-cadherin in regulating cell-cell adhesion, is similarly absent or discontinuously

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distributed in melanocyte membranes from nonlesional skin of vitiligo (Wagner et al., 2015). Most authors believe that the loss of functional melanocytes is mostly responsible for its cause, and the ultimate goal of vitiligo treatment is to replenish those melanocytes by promoting the migration of melanocytes from the outer root sheath of hair follicles to the depigmented area and/or to restore the normal function of melanin synthesis by residual melanocytes in the lesional skin (Cui et al., 1991; Goldstein et al., 2015).

Traditional Chinese Medicine (TCM) had gained popularity since ancient times in China, and is, even today, the only available treatment for vitiligo in many rural areas. Therefore, the potential for the application of herb products to ameliorate or prevent vitiligo merits considerable scientific investigations. A recent search of the electronic CNKI database (1955–2008) retrieved 302 herbal prescriptions that have been promising clinical results for vitiligo from 480 articles. The category of kidney-tonifying and liver-strengthening function ranked first, consisting of 92 prescriptions (Jia and AiE, 2010). Of herbs in those categories, *Polygoni multiflori radix praeparata* (PMRP), *Ecliptae herba* (EH) and *Rehmanniae radix praeparata* (RRP) were the most frequently used tonic medicines by TCM practitioners for the treatment of vitiligo (Jia and AiE, 2010).

However, no investigation has elucidated the impact of PMRP, EH or RRP on melanocytic functions. As an initial screen, we aimed to investigate whether aqueous extracts of *PMRP*, *EH* and/or *RRP*, which showed encouraging effects for the treatment of vitiligo, have the ability to stimulate melanogenesis and/or the migration of human melanocytes. Simultaneously, the impact of those extracts on MITF protein expression was evaluated. A positive response would initiate the bioassay-guided fractionation of active constituents of the effective herb(s).

2. Materials and methods

2.1. Human melanocytes in culture

All human foreskin specimens were obtained under written informed consent of the donors, in accordance with the Ethical Committee approval process of the Affiliated Hospital of Nanjing University of Chinese Medicine. Skin specimens were briefly sterilized in iodine, minced and then treated with dispase II overnight at 4 °C. The epidermis was separated and placed in a solution containing 0.25% trypsin for 5 min at 37 °C. After vigorous pipetting, cells were pelleted and resuspended in growth medium, which is composed of Medium 254 (Life Technologies, USA) and human melanocyte growth supplement (HMGS)(Life Technologies, USA). Cell cultures were incubated at 37 °C in a humidified incubator supplemented with 5% CO_2. Melanocytes at the third or fourth passage were used in these experiments.

2.2. Preparation of herbal extracts

Raw PMRP, EH and RRP were purchased from Henan, Jiangsu and Henan Corp, respectively. All raw materials were authenticated by Professor Gu Wei of the Nanjing University of Chinese Medicine and the herbal drugs were in accordance with the Chinese pharmacopoeia (Chinese Pharmacopoeia, 2010). The raw materials of PMRP, EH and RRP (500 g each) were refluxed with 5 L ultrapure water for 40 min. The filtrates were collected and the residues were refluxed again in 4 L ultrapure water for 30 min. The filtrates were combined and freeze-dried, and 46 g of PMRP, 25 g of EH and 87 g of RRP extracts were harvested. Each final extract was redissolved at a concentration of 0.5 g/ml, filtered through a 0.22 μ membrane, then stored at −20 °C until used for study.

2.3. Ultra high performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS), instrument and conditions

Chromatographic analysis was performed on an Acquity UPLC system (Waters Corp, USA), consisting of a binary pump solvent management system, an online degasser and an autosampler. An Acquity UPLC BEH C18 column (100 mm×2.1 mm, 1.7 μm) was employed and the column temperature was maintained at 35 °C. The mobile phase was composed of A (0.1% formic acid) and B (acetonitrile) using a gradient elution of 5–10% B at 0–1 min, 10–30% B at 1–6 min, 30–40% B at 6–7 min, 40–95% B at 7–8 min and 95–5% B at 8–10 min with a flow rate of 0.4 ml min^{-1}. The autosampler was conditioned at 4 °C and the injection volume was 6 μl.

Mass spectrometry detection was performed using a Xevo Triple Quadrupole MS (Waters Corp, USA) equipped with an electrospray ionization source (ESI). The ESI sources were both set in positive ionization mode. The parameters in the source were set as follows: capillary voltage 3.5 kV; source temperature 150 °C; desolvation gas flow 1000 L h-1; desolvation temperature 550 °C; cone gas flow 50 L h-1. The MS/MS analysis was performed using multiple reaction monitoring (MRM), and the cone voltage and collision energy were optimized for each analyte and selected values are reported in Table 1. Dwell time was automatically set by MassLynx (Waters Corp, USA).

2.4. Melanocyte proliferation assay

The proliferation rates of melanocytes treated with PMRP, EH and RRP were determined using a colorimetric assay with MTT. Exponentially growing melanocytes were trypsinized, harvested and equal numbers of cells (2×10^5 cells/ ml) in 100 μl medium were plated in 96-well microplates. After overnight incubation, 100 μl of different concentrations (25, 100, 400, 1600 μg/ml) of each extract of PMRP, EH and RRP was added for 3 days to the wells. The untreated controls were exposed to fresh medium. Following this, 50 μl 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (KeyGEN BioTECH, China) solution was added to each well and incubated for 4 h. After aspirating the culture medium, the resulting formazan was dissolved with 150 μl dimethylsulfoxide (Sigma, USA). The plates were then placed on a shaker for 5 min and read immediately using a Multimode Plate Reader (Perkin Elmer, U.S.A) at a wavelength at 490 nm. The assays were performed in 3 independent experiments, and triplicate wells were used for each concentration each extract.

2.5. Tyrosinase activity assay

Tyrosinase activity was determined by measuring the rate of oxidation of L-DOPA to dopachrome according to a previously described method (Abdel-Malek et al., 1994) with a slight modification. The cells were cultured at a density of 2×10^5 cells/ml in 96-well

Table 1
Cone voltage and collision energy and each analyte selected values of MS/MS.

	MS/MS	Cone Voltage	Collision energy	formula/ Mass
Scutellarein	287.16→123.05	42	32	286
Luteolin	287.16→153.00	50	32	286
Apigenin	271.16→153.00	46	30	270
Diosmetin	301.22→286.12	34	26	300
Kaempferol	301.22→229.32	40	38	300
Verbascoside	623.35→160.98	44	38	624
Tetrahydroxystilbene glucoside	407.29→245.14	12	14	406
Emodin	271.16→115.10	36	42	270