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Anti-Pigmentation Effects of Eight *Phellinus linteus*-Fermented Traditional Crude Herbal Extracts on Brown Guinea Pigs of Ultraviolet B-Induced Hyperpigmentation

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We have previously found that mycelia culture broth of eight kinds of traditional herbal extracts fermented with *Phellinus linteus* (previously named as 8-HsPLCB) not only inhibited melanin and tyrosinase activity, but also reduced the contents of melanogenesis-related proteins, including tyrosinase and microphthalmia-associated transcription factor, in 3-isobutyl-1-methylxanthine-stimulated B16F0 melanoma cells. For a further study, the effect of 8-HsPLCB against skin pigmentation in brown guinea pigs with ultraviolet B (UVB)-induced hyperpigmentation was investigated. 8-HsPLCB (3%) and arbutin (2%) as positive controls were applied topically twice daily for 4 weeks to the hyperpigmented areas. 8-HsPLCB showed skin-lightening effect as effective as arbutin, one of the most widely used in whitening cosmetics. Melanin index values as the degree of pigmentation showed a significant reduction week by week post 8-HsPLCB treatment and then substantially reduced by 4 weeks. The degree of depigmentation after 4 weeks of topical application with 8-HsPLCB was 32.2% as compared with before treatment (0 week). Moreover, using Fontana-Masson staining and hematoxylin-eosin staining, 8-HsPLCB reduced melanin pigmentation in the basal layer of the epidermis and epidermal thickness changes exposed to the UV-B irradiation as compared with non-treatment and vehicle treatment. The intensity of the skin-lightening effect of 8-HsPLCB was similar to arbutin. These results suggest that the skin-lightening effect of 8-HsPLCB might be resulted from inhibition of melanin synthesis by tyrosinase in melanocytes. To conclude, 8-HsPLCB treatment showed reduction of the melanin pigment and histological changes induced by UV irradiation in brown guinea pigs.

Keywords: Fermentation, melanin index, skin-whitening effect, UV radiation, epidermal thickness

Introduction

Melanin is an exclusive pigmented biopolymer and is a major determinant of skin color [1]. Melanin plays an important role in the prevention of skin injury, such as hyperpigmentation diseases [2]. Melanin pigments are known to be biosynthesized in the melanosomes of melanocytes, moved to keratinocytes, stored in the basal layer of the epidermis of human skin [3]. Tyrosinase is a major melanocyte-specific enzyme in melanogenesis that catalyzes the rate-limiting reaction of the melanogenic process [4, 5]. Melanin production depends mainly on the expression and activation of tyrosinase [4, 5]. Therefore, the inhibitors of tyrosinase enzyme and melanin biosynthesis could be used for cosmetic skin whitening. The study of melanogenesis-inhibiting and skin-lightening substances from natural sources has become a hot topic in the skin-whitening cosmetics field [6, 7]. Many traditional herbs have been utilized as natural substances for developing whitening cosmetics because of their relatively few side

effects [6, 8, 9]. These traditional herbal extracts having skin-whitening effect prevent melanin biosynthesis by directly inhibiting tyrosinase activity [7].

We have screened melanin biosynthesis inhibitors from the extracts of traditional herbs, marine algae, and natural product and found strong tyrosinase inhibitors using mushroom tyrosinase inhibitory assay in vitro [10, 11]. Fermented products of traditional herb extracts by fungi and bacteria also showed strong tyrosinase inhibitory activities [10, 12, 13, 14]. Recently, our study also found that mycelial culture broth of eight traditional herbal extracts fermented with *Phellinus linteus* (8-HsPLCB) inhibited melanin production and tyrosinase activity, and also reduced the contents of melanogenesis-related proteins, including tyrosinase and microphthalmia-associated transcription factor (MITF) in B16F0 melanoma cells [15]. However, the effect of 8-HsPLCB had not been determined in brown guinea pigs, whose skin serves as an excellent pigmentation model because it is similar to human skin [16].

Thus, this current study investigated the effect of 8-HsPLCB on skin pigmentation in brown guinea pigs by the light microscopy examination using the Fontana-Masson staining and hematoxylin-eosin (H&E) staining method.

Materials and Methods

Preparation of Mycelial Culture Broth of Eight *Phellinus linteus*-Fermented Traditional Crude Herbal Extracts

For the current study, *P. linteus* KCTC 6190 was purchased from the Korean Collection for Type Cultures (Korea). Eight kinds of traditional Korean herbs (*Glycyrrhiza glabra*, *Broussonetia kazinoki*, *Angelica gigas*, *Atractylodes macrocephala*, *Poria cocos*, *Morus alba* root bark, *Paeonia albiflora*, and *Ligusticum officinale*) used in this study were purchased from Hyomin Pharma. Ind. Co., Ltd. (Korea). Mycelial culture broth of the eight kinds of traditional crude herbal extracts fermented with *P. linteus* (previously named as 8-HsPLCB) was prepared by cultivating at 28°C for 9 days according to a previous study [15].

Establishment of Animal Model of Ultraviolet B (UVB)-Induced Hyperpigmentation

Brown guinea pigs (5-weeks-old male) were purchased from the Oriental Yeast Co. (Japan) and were individually housed in stainless steel cages at an air-conditioned room with an automated heating system at a controlled temperature of 20–23°C under a 12 h light/12 h dark cycle. Animals were allowed free access to a standard diet for 1 week and were then randomly divided into four experimental groups ($n = 6$ animals per group). The UVB-induced hyperpigmentation was performed on the backs of the guinea pigs, using a slightly modified method by Kong *et al.* [17]. To develop pigmentation, the guinea pigs were anesthetized with zoletil (30 mg/kg) and then the back of each guinea pig was cleanly shaved with electric clippers. Four areas (1 cm × 1 cm) on the back of each were irradiated with a UVB lamp (Waldmann UV 800, Herbert Waldmann GmbH, Philips TL/12 lamp emitting 280–305 nm). The animals were exposed to the UVB irradiation once a week for three consecutive weeks at an intensity of 2 mW/cm^2 and a total irradiation dose of 900 mJ/cm^2. Then, 8-HsPLCB (3% in propylene glycol:ethanol:water = 5:3:2) was applied topically to the UV-irradiated areas (10 µl/square) twice a day for 4 weeks. Arbutin (2%) and vehicle (eight traditional crude herbal extracts) were used as control application. The degree of pigmentation was measured once a week for 4 weeks using a Mexameter MX18 (Courage-Khazaka Electronic, Germany). At the end of the experimental period, the guinea pigs were sacrificed by withdrawing blood from the abdominal aorta under light ether anesthesia, and samples of the dorsal skin were removed. Skin biopsy specimens were collected from spots of the treatment area, and fixed in 10% formalin. Then they were applied to H&E stain for evaluating the skin thickness of epidermis and Fontana-Masson stain for melanin. Animal care followed the National Institute of Health guidelines on the care and use of laboratory animals, and the study was approved by the Institutional Animal Care and Use Committee of our institution (JBMI IACUC 2015003).

Result

Depigmentating Effect on UVB-Induced Hyperpigmentation of Animal Skin

8-HsPLCB was topically applied twice daily for 4 weeks to the dorsal skin of brown guinea pigs, which was exposed to UV irradiation once a week for three consecutive weeks. Arbutin (2%) and vehicle were used as a control. In the photograph of dorsal skin (Fig. 1), 8-HsPLCB showed depigmenting effects on UV-induced hyperpigmentation after 4 weeks of topical application, similar to arbutin, which is one of the widely used skin-lightening agents in cosmetics, compared with the non-treated control or vehicle treatment.

The melanin index, which is a parameter influenced by the melanin contents (high melanin index interpreted as more pigmentation), was represented with the degree of pigmentation measured by the Mexameter MX18 (Fig. 2). A significant reduction of melanin index by 8-HsPLCB was observed week by week for 4 weeks, similar to arbutin, compared with the non-treated control or vehicle treatment. However, reduction of the melanin index by vehicle treatment (herbal extracts) occurred at 4 weeks, probably due to ingredients in the extracts. The melanin index rate (%) after 4 weeks of topical application with 8-HsPLCB and

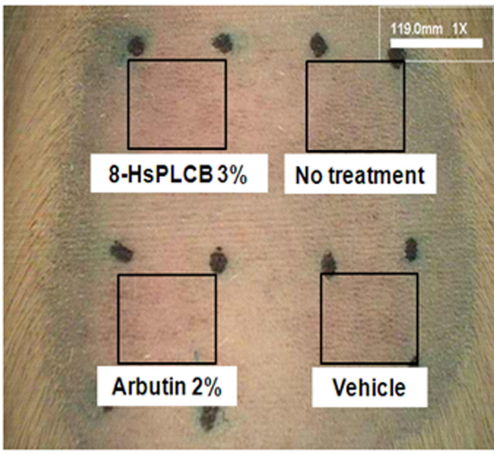

Fig. 1. Photograph of the dorsal skin area showing depigmenting effects after 4 weeks of topical application on UV-induced pigmentation in brown guinea pigs.
8-HsPLCB, mycelial culture broth of eight *Phellinus linteus*-fermented traditional herbal extracts; Arbutin, skin-lightening agent as positive control; no treatment, medium; vehicle, eight traditional herbal extracts.

arbutin were 32.2% and 32.3% as compared with the degree of depigmentation before topical application (last irradiation), respectively, whereas that of non-treated control or vehicle treatment was not significantly changed (Fig. 3). This result indicates that 8-HsPLCB showed a potent skin-lighting effect that was similar to arbutin as a positive control.

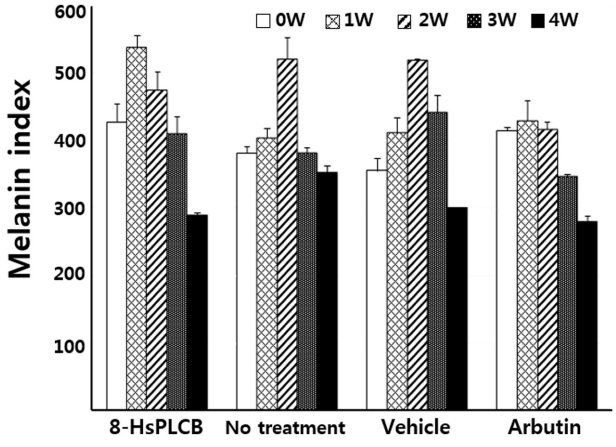

Fig. 2. Changes of melanin index values after daily topical application for 4 weeks.
Melanin index values were measured using the Mexameter MX18 (Courage-Khazaka Electronic GmbH, Germany). 8-HsPLCB, mycelial culture broth of eight *Phellinus linteus*-fermented traditional herbal extracts; Arbutin, skin-lightening agent as positive control; no treatment, medium; vehicle, eight traditional herbal extracts.

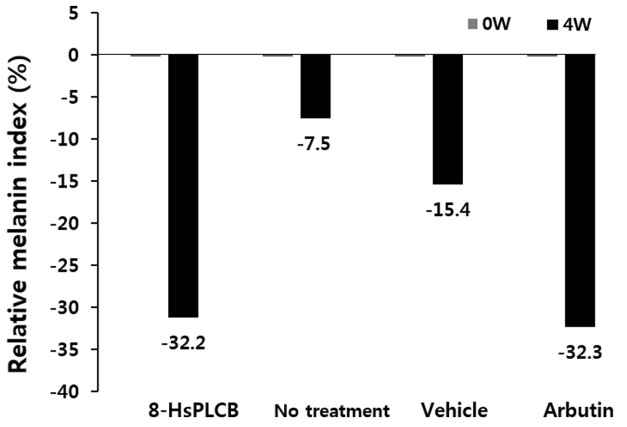

Fig. 3. Degree of depigmentation before and after daily topical application for 4 weeks.
Melanin index values were measured using the Mexameter MX18 (Courage-Khazaka Electronic GmbH, Germany). 8HsPLCB, mycelial culture broth of eight *Phellinus linteus*-fermented traditional herbal extracts; Arbutin, skin-lightening agent as positive control; no treatment, medium; vehicle, eight traditional herbal extracts.

Melanin Detection and Epidermal Thickness Changes on UVB-Induced Hyperpigmentation of Animal Skin

To detect melanin formation, skin biopsy specimens were obtained after 4 weeks from topical application sites and were processed for light microscopy examination of Fontana–Masson silver stain, which stains melanin granules/melanosomes black. Although 8-HsPLCB- and arbutin-treated sites showed the presence of melanin, they displayed a significantly decreased level of melanin pigment in the basal layer of the epidermis as compared with non-treated or vehicle-treated sites (Fig. 4). In the comparison of epidermal thickness changes in UVB-induced hyperpigmentation of skin determined by H&E staining, 8-HsPLCB and arbutin also significantly reduced epidermal thickness changes as compared with non-treatment and vehicle treatment (Fig. 5), indicating that 8-HsPLCB might prevent the morphological changes of UVB-induced photodamage in the epidermis with a similar effect to arbutin.

Discussion

The skin-whitening cosmetics using traditional herbs are popular with East Asian women. We have previously found that fungus-fermented traditional herbs, including *Angelica gigas* and *Morus alba* root bark, have a much greater effect in inhibiting mushroom tyrosinase activity than those extracts only due to high contents of the phenolics and

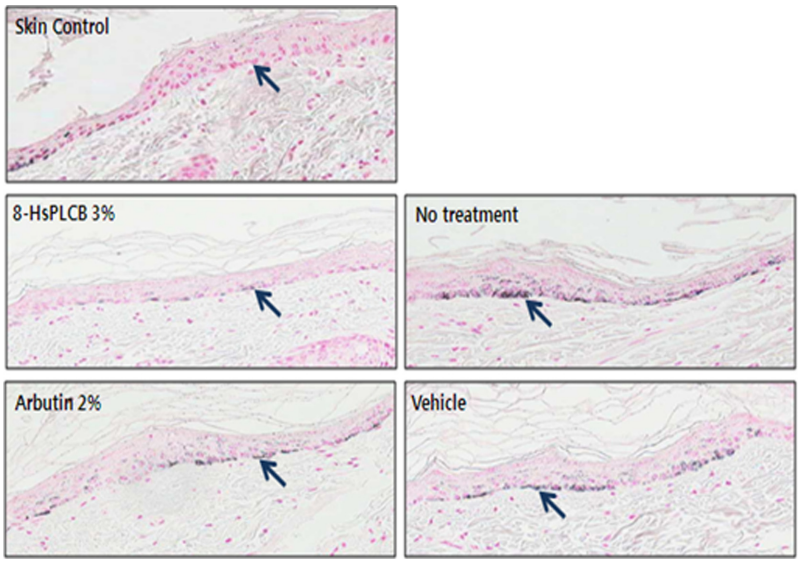

Fig. 4. Morphological distribution of melanin pigment in the epidermis of brown guinea pig skin.
Biopsy specimens after 4 weeks of topical application were processed for light microscopy examination of Fontana–Masson silver stains. Arrows represents melanin. Pink, nuclear fast red; black, Fontana-Masson-stained melanin. Magnification 10×. 8-HsPLCB, mycelial culture broth of eight *Phellinus linteus*-fermented traditional herbal extracts; Arbutin, skin-lightening agent as positive control; no treatment, medium; vehicle, eight traditional herbal extracts.

flavonoids [10, 15]. Recently, our study also found that mycelial culture broth of eight kinds of traditional herbal extracts fermented with *Phellinus linteus* (8-HsPLCB) inhibited melanin production and tyrosinase activity, and also reduced the contents of melanogenesis-related proteins, including tyrosinase and MITF, in B16F0 melanoma cells [15].

In the present study, therefore, we investigated the in

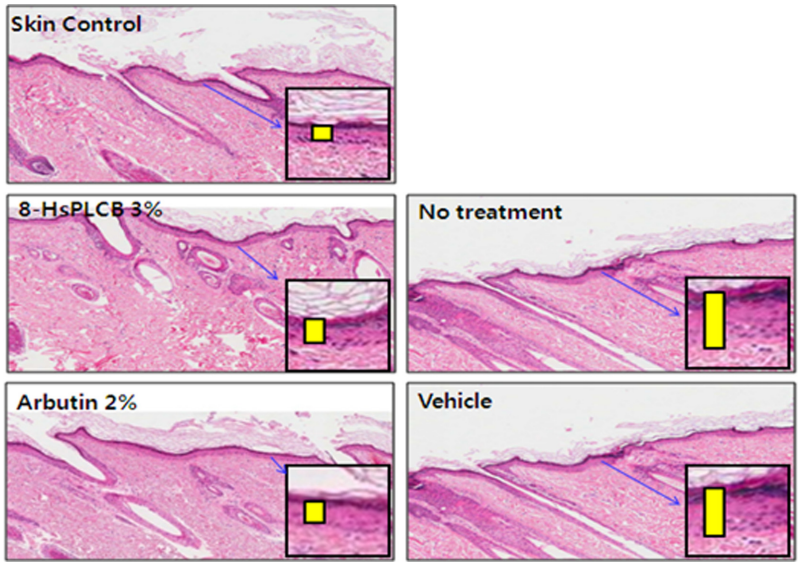

Fig. 5. Epidermal thickness of the dorsal skin in brown guinea pig.
Biopsy specimens after 4 weeks of topical application were processed for light microscopy examination of hematoxylin-eosin staining. Magnification 10×. 8-HsPLCB, mycelial culture broth of eight *Phellinus linteus*-fermented traditional herbal extracts; Arbutin, skin-lightening agent as positive control; no treatment, medium; vehicle, eight traditional herbal extracts.

vivo effect of 8-HsPLCB on skin pigmentation in brown guinea pigs. 8-HsPLCB (3% in propylene glycol:ethanol:water = 5:3:2) and arbutin (2%) or vehicle (herbal extracts) as control were applied topically twice a day for 4 weeks to the hyperpigmented areas of brown guinea pig skin exposed to UVB irradiation. 8-HsPLCB showed skin-lightening effect as effective as arbutin, one of the most widely used skin-whitening cosmetics, on UV-induced pigmentation after 4 weeks of topical application (Fig. 1). The color in the dorsal skin of brownish guinea pigs returned to its original color after 8-HsPLCB treatment for 4 weeks.

Mexameter (Courage-Khazaka Electronic GmbH, Germany), which is a pigment-measuring device, has been widely used for most commercially available epidermal melanin concentration determination [18]. The melanin index was also obtained as the degree of UV-induced skin pigmentation in the present study. The melanin index on the dorsal skin of brown guinea pigs by 8-HsPLCB treatment was reduced week by week after topical application and then substantially reduced by 4 weeks. As expected, arbutin, which is a glycosylated hydroquinone extracted from the bearberry plant (*Arctostaphylos* species) [19], gradually reduced without initial increasing of UV-induced pigmentation. However, the sudden reduction of melanin index at 4 weeks by vehicle treatment was probably due to other ingredients in the extracts.

Melanin index values as the degree of UV-induced skin pigmentation on the dorsal skin of brown guinea pigs after 4 weeks of topical application with 8-HsPLCB and arbutin were 32.2% and 32.3% as compared with the degree of pigmentation before application, respectively (Fig. 3). According to recent study, arbutin inhibits melanin production through the inhibition of tyrosinase activity in B16 cells induced with alpha-MSH and also inhibits melanin production in brown guinea pig and human skin tissues [20]. This result indicates that the skin-whitening effect by 8-HsPLCB might be similar to the mode of arbutin.

In the histological examination, 8-HsPLCB-treated sites displayed a decreased total melanin pigment (black spot) in the basal layer of the epidermis of UV-irradiated guinea pigs under light microscopy examination of Fontana-Masson staining, compared with the non-treated or vehicle-treated sites (Fig. 4). These results suggest that the skin-whitening effect of 8-HsPLCB was probably due to inhibition of the melanin synthesis by tyrosinase in melanocytes. The production and distribution of melanin pigment is a major determinant of skin and hair color. The degree of reduction of the epidermal thickness, which is concomitant with pigmentation by UV radiation, was also observed in the dorsal skin of UV-irradiated guinea pigs treated with 8-HsPLCB by using H&E staining unlike those of non-treatment or vehicle-treatment (Fig. 5).

Tyrosinase is the key enzyme in the pathway of melanogenesis and plays a regulatory role in the production of melanin [6]. Therefore, melanin production depends mainly on the activation and expression of tyrosinase. Polyphenols are good inhibitors of tyrosinase activity and melanin production in melanoma cells. They are considered as a potential source of skin-whitening agents [21]. 8-HsPLCB inhibited intercellular tyrosinase and decreased melanin production in IBMX-treated B16F0 melanoma cells, presumably due to highly contained phenolics and flavonoids [15]. 8-HsPLCB also induced downregulation of melanogenesis through decreased phosphatidylinositol-3-kinase (PI3K) / Akt/ glycogen synthase kinase-3beta (GSK3β) phosphorylation, leading to a reduction in MITF protein expression and consequently decreased tyrosinase protein expression and melanin production in melanoma cells [15]. This pigmentation-inhibiting effect by 8-HsPLCB was also shown in brown guinea pigs, as expected from the results obtained using B16F0 melanoma cells.

In conclusion, 8-HsPLCB might be an effective inhibitor of hyperpigmentation caused by UV irradiation.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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