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A traditional herbal formula composed of 'white' herbs down regulates tyrosinase-related proteins in B16F10 melanoma cells

Chae Young Bang[1], Min Kyoung Kim[1], Su-Hyun Kim[2], Dong-Il Kim[2], Se Young Choung[1,3*]& Young Pyo Jang[1,4*]

[1]Department of Life and Nanopharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea; [2]Department of OB & GY, College of Korean Medicine, Dong-Guk University, Dong-Guk University International Hospital, 27 Donkguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do, Korea; [3]Department of Preventive Pharmacy and Toxicology, College of Pharmacy, Kyung Hee University, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea; [4]Division of Pharmacognosy, College of Pharmacy, Kyung Hee University, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea

Young Pyo Jang: Division of Pharmacognosy, College of Pharmacy, Kyung Hee University, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea

E-mails: ypjang@khu.ac.kr; sychoung@khu.ac.kr

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The traditional Chinese medicine (TCM) theory postulates the pharmacological targets of white-coloured herbs include skin. To explore the therapeutic implications of this theory in skin disorders, especially for excess pigmentation, a series of anti-melanogenic evaluations were performed using a selected herbal formulation, WASAP-H. Ultra-performance liquid chromatography-electrospray ionization mass spectrometry (UPLC-ESI-MS) analysis was performed to determine the major components of WASAP-H. Effects of WASAP-H on tyrosinase activity, melanin content, and the expression of melanogenic proteins such as tyrosinase, tyrosinase related protein (TRP)-1, and TRP-2 was evaluated in B16F10 melanoma cells. WASAP-H decoction significantly inhibited tyrosinase activity and reduced melanin contents in B16F10 melanoma cells. The key enzymes for melanin synthesis such as tyrosinase, TRP-1, and TRP-2 were significantly down regulated by WASAP-H in a dose-dependent manner. The results suggested that the anti-melanogenic activity of WASAP-H was directly linked to the inhibition of tyrosinase activity and the down regulation of melanogenic enzymes. The scientific translation of traditional knowledge in this study will provide a platform for the modernization of traditional herbal medicines and the development of evidence-based herbal medicinal products.

Keywords: TCM theory, Traditional herbal formula, Tyrosinase, Tyrosinase-related proteins
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Theories such as the yin-yang theory and the five-element theory have been commonly employed to explain the rationale behind the use of traditional Chinese medicine (TCM); the five-element theory has been well established in traditional herbal medicine and defines the relation between colour of herbs and their pharmacological target organs[1]. For example, white-coloured herbs have been used in TCM for patients with lung and skin disorders since the theory postulates that the lung and skin are the target organs of these herbs[2]. While the five-element theory has been used for more than two-thousand years, more specific evidence is required for the translation of this oriental theory into scientific models, which are experimentally reproducible. To validate this theory with regard to skin disorders, an herbal prescription, WASAP-H was selected for biochemical evaluation. The herbal formula WASAP-H is derived from the Korean traditional medical classic, Hyang-Yak-Jip-Seong-Bang[3,4,5] and is composed of four medicinal herbs, *Angelica dahurica* Benth. et Hook. f., *Aconitum koreanum* Raymond, *Ampelopsis japonica* Makino, and *Bletilla striata* (Thunberg) Reichenbach Fil., all categorized as white according to the five-element theory. Some of these herbs were known to have an inhibitory effect on tyrosinase activity; however, the antimelanogenesis effect of the formula as a whole and its underlying mechanism of action have not been elucidated[6,7]. In this study, the biological efficacy of a white-coloured herbal formula WASAP-H in skin whitening was evaluated by using a melanoma cell-based biomolecular approach. Tyrosinase inhibition, melanin contents, and the expression of tyrosinase, tyrosinase related protein-1 (TRP-1), and TRP-2 were evaluated to corroborate the relation between oriental herbal medicine theory and its biological articulation.

*Corresponding author

Methodology

Preparation of WASAP-H decoction

The raw material for WASAP-H comprised of *Angelica dahurica* Benth. et Hook. f. (Umbelliferae, root, voucher specimen number: DUMCKM2015-031, 40 gm dry weight), *Aconitum koreanum* Raymond (Ranunculaceae, tuberous root, voucher specimen number: DUMCKM2015-097, 30 gm), *Ampelopsis japonica* Makino (Vitaceae, tuberous root, voucher specimen number: DUMCKM2015-028, 40 gm), and *Bletilla striata* (Thunberg) Reichenbach Fil. (Orchidaceae, root-like stem, voucher specimen number: DUMCKM2015-098, 20 gm). The herbal medicines were Korean Food and Drug Administration (KFDA)-certified and purchased from a local herbal market in South Korea; their botanical authenticity was confirmed by Professor Dong-Il Kim, College of Korean Medicine, Dong-guk University, Ilsan, Korea. Voucher specimens for these herbal medicines were deposited at the Herbarium of the College of Korean Medicine, Dong-guk University, Ilsan, Korea. The raw materials of WASAP-H were extracted with 250 mL of distilled water at 100 ℃ for 4 hrs, using a Soxhlet extractor. The extract was filtered with a filter paper (Hyundai Micro Co., Ltd., Seoul, Korea) and the filtrate was freeze-dried to yield 28.76 gm of WASAP-H and stored at 4 °C.

UPLC-ESI-MS analysis

WASAP-H (30 mg) was dissolved in 1 mL of water and filtered through 0.2-μm Whatman syringe filter (Whatman International Ltd., Maidstone, Kent, UK) before being subjected to ultra-performance liquid chromatography (UPLC) analysis. The UPLC system consisted of Waters model ACQUITY Quaternary Solvent Manager, ACQUITY Sample Manager-FTN, and ACQUITY PDA detector operated by Empower software (Waters, Milford, MA, USA). The Brownlee SPP C18 column (100 × 3.0 mm i.d.; 2.7 μm) was selected for the analysis. The UV/Vis detection wavelength was set to 280 nm. The mobile phase comprised of acidified acetonitrile with acetic acid (0.1 %, solvent A) and acidified water with acetic acid (0.1 %, solvent B). The gradient program was 0 min, 2 % of solvent A; 14 min, 3% of solvent A; 15 min, 5 % of solvent A; 20 min, 10 % of solvent A; 40 min, 30 % of solvent A; 49 min, 100 % of solvent A at a flow rate of 0.3 mL per min. The injection volume was 3 μL. AccuTOF® single-reflectron time-of-flight mass spectrometer was equipped with an ESI source and was operated with Mass Center System version 1.3.7b (JEOL, Tokyo, Japan). In the positive ion mode, the atmospheric pressure interface potentials were typically set to the following values: orifice 1 = 80 V and ring lens and orifice 2 = 10, 5 V, respectively. The ion guide potential and detector voltage were set to 1500 V and 2300 V, respectively. ESI parameters were set as follows: needle electrode = 2000 V, nitrogen gas was used as a nebulizer and for desolvation and the flow rates were 1 and 3 L/min, respectively, de-solvating chamber temperature = 250 °C, and orifice 1 temperature = 80 °C. Mass scale calibration was accomplished with YOKUDELNA calibration kit (JEOL, Tokyo, Japan) for accurate mass measurements and calculations of the elemental composition. MS acquisition was set at a scan range of *m/z* 100 to 1500.

B16F10 melanoma cell culture

B16F10 melanoma cells derived from C57BL/6J mouse were purchased from ATCC (Manassas, VA, USA). Cells were incubated in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 ℃ in 5% CO_2.

Cell viability assay

To determine optimal concentration range of WASAP-H for bioactivity experiments, cell viability was measured by the MTT assay after treatment with various concentrations of WASAP-H (0, 10, 50, 100, 200, and 400 μg/mL). B16F10 melanoma cells were incubated in 24-well plates at a density of 10^4 cells/well for 24 hrs. On the second day, cells were exposed to various concentrations of WASAP-H for 48 hrs. Then, the media was removed and the cells were washed with phosphate-buffered saline (PBS, *p*H 7.4) and incubated with 0.5 mg/mL MTT prepared in PBS, at 37 ℃. Four hours later, the MTT reagent was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) solution; then absorption values were read at 540 nm using an ELISA microplate reader (Bio-Tek instruments Inc., Winooski, VT, USA).

Measurement of tyrosinase activity

B16F10 cells, plated at 5×10^4 cells in a 6-well culture plate, were treated with various concentrations of WASAP-H (0, 50, 100, and 200 μg/mL) and incubated for 48 hrs. Cells were washed with ice-cold PBS and lysed with phosphate buffer (*p*H 6.8)

containing 1% Triton X-100. Then, the cell lysates were disrupted by repeated freezing and thawing and whole cell lysates were centrifuged at 10,000 gm for 5 min. After quantifying protein levels and adjusting protein concentrations with lysis buffer, 90 µL of each lysate was placed in each well of a 96-well plate and 10 µL of 10 mM L-DOPA was added. Control wells contained 90 µL of lysis buffer and 10 µL of 10 mM L-DOPA. Following incubation at 37 ℃ for 15 min, optical absorbance of dopachrome was measured using an ELISA reader at 475 nm.

Melanin content measurement

B16F10 melanoma cells were treated with various concentrations of WASAP-H (0, 50, 100, and 200 µg/mL) for 48 hrs. The cells were suspended in 0.2 mL of 1 N NaOH and boiled for 30 min. The melanin content was determined using an ELISA microplate reader (Bio-Tek instruments Inc.) at 460 nm and normalized to the protein level of cell lysate.

Tyrosinase, TRP-1, and TRP-2 protein expression

The effects of WASAP-H on tyrosinase, TRP-1, and TRP-2 expression were investigated using the western blot technique. To determine the total protein content of each supernatant, the Bradford assay (Bio-Rad, Richmond, CA, USA) was used. B16F10 melanoma cells were lysed in cell lysis buffer [62.5 mM Tris-HCl, *p*H 6.8; 2% SDS; 5% *β*-mercaptoethanol; protease inhibitor (CompleteTM, Roche, Mannheim, Germany); 2 mM PMSF; 10 mM EDTA; 50 mM NaF; and 1 mM Na_3VO_4). Ten micrograms of protein per lane was separated by 8% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membranes, which were then blocked with 5% dried milk in Tris-buffered saline containing 0.1% Tween 20. Blots were exposed to specific primary antibodies at a dilution of 1:1000 overnight at 4 °C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 2 hrs at room temperature. The blots were developed using an enhanced chemiluminescence detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Statistical analysis

Results are presented as the mean ± S.E.M of at least three independent experiments. Statistical analysis was performed using t-test. The Student's t-test and paired were performed to assess the differences between the means. P-values less than 0.05 were considered statistically significant.

Results

UPLC profile and the identification of major components of WASAP-H

Chemical analysis of multi-component herbal samples provides critical information on chemical complexity and major components; chemical fingerprinting of the sample yields reproducible results. To establish the standard chromatogram of WASAP-H for data reproducibility and quality assurance of the sample, we adopted UPLC analysis. The representative chromatogram of WASAP-H is shown in Fig. 1. Although WASAP-H is a decoction of the mixture of 4 different herbal medicines, the chromatogram showed relatively simple profiles of chemical components. This could be attributed to the simple chemical profile of each herbal medicine or because each herb comprised many non-UV absorbing constituents. Among the UPLC peaks, three peaks were identified by direct comparison of mass data and UV/Vis spectra from UPLC-ESI-MS study with those previously reported[8, 9]. The UPLC-MS data of other peaks were not specific enough to be identified as single compound. The retention time, observed mass, mass difference, and proposed compounds corresponding to the three peaks are listed in Table 1. Three compounds xanthotoxol, aviprin, and byakangelicin were identified as the components of *Angelica dahurica*.

Effects of WASAP-H on tyrosinase activity and melanin synthesis

Tyrosinase is a critical and rate-limiting enzyme in melanin synthesis[10]. The biosynthesis of melanin is

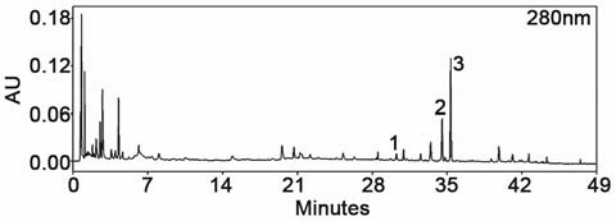

Fig. 1—Representative UPLC profile of WASAP-H. The peaks identified by UPLC-MS study were marked numerically. Peak 1-xanthotoxol, peak 2-aviprin, peak 3-byakangelicin.

Table 1—The observed and calculated mass numbers of UPLC peaks of WASAP-H

Peak No.	Rt (min)	Theoretical mass $[M+H]^+$	Observed mass $[M+H]^+$	Mass difference (mmu)	Identification
1	30.28	203.03388	203.02838	-5.50	Xanthotoxol
2	34.53	305.10195	305.09416	-7.79	Aviprin
3	35.34	335.11251	335.11231	-0.20	Byakangelicin

initiated by the catalytic oxidation of tyrosine to DOPA by tyrosinase in a reaction that requires DOPA as a cofactor. As shown in Fig. 2A, WASAP-H reduced tyrosinase activity in a dose-dependent manner, with 88.2 %, 80.7 %, and 77.5 % inhibition at 50, 100 and 200 μg/mL, respectively. To evaluate whether WASAP-H affected the melanin content in melanoma cells, B16F10 cells were treated with various concentrations of WASAP-H (0, 10, 50, 100, 200, and 400 μg/mL). WASAP-H did not show significant toxicity at concentrations up to 200 μg/mL (data not shown). As shown in Fig. 2B, WASAP-H significantly and dose-dependently decreased melanin contents from the concentration of 100 μg/mL; this effect was not significant when compared to the positive control kojic acid[11].

WASAP-H decreased the expression of tyrosinase, TRP-1, and TRP-2 proteins

In the process of melanogenesis, three major enzymes are involved as key factors, tyrosinase, TRP-1, and TRP-2[12]. These proteins were quantitated in the absence and presence of WASAP-H treatment to evaluate its effects on the expression of these enzymes. The down regulation of tyrosinase was prominent after treatment with WASAP-H at all tested concentrations and the protein levels of TRP-1 and TRP-2 were significantly reduced by WASAP-H at concentrations above 100 μg/mL (Fig. 3). In the case of TRP-1 and TRP-2, the effect of WASAP-H was dose-dependent. The results suggested that the anti-melanogenic activity of WASAP-H is directly linked to the suppression of the three critical melanogenesis-related enzymes.

Discussion

Modernization or scientific translation of the TCM theory is a critical area of research to exploit this ancient medicinal heritage. One of the straightforward methods to prove the pharmacological efficacy of the TCM theory is the biologically relevant evaluation of the herbal formula prescribed based on the theory. In this pilot study, a model herbal medicinal formula based on the theory of 5-elements was selected to verify the pharmacological action proposed by the TCM theory. The prescription comprised herbal medicines categorized as white according to the TCM theory and demonstrated significant anti-melanogenic activity in B16F10 melanoma cells; this was evidenced with the inhibition of tyrosinase and the reduction of melanin along with concurrent down

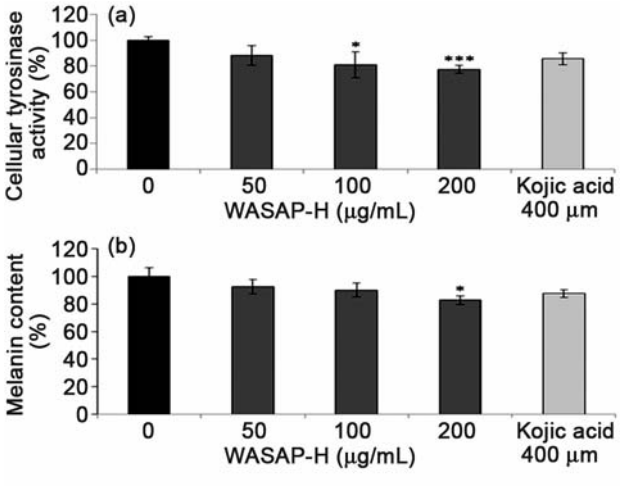

Fig. 2—Inhibitory effects of WASAP-H on tyrosinase activity (A) and melanin contents (B) in B16F10 melanoma cells. Each determination was performed in triplicate: the data represent mean ± S.E. $^{*}P<0.05$ and $^{***}P<0.001$ vs. without WASAP-H.

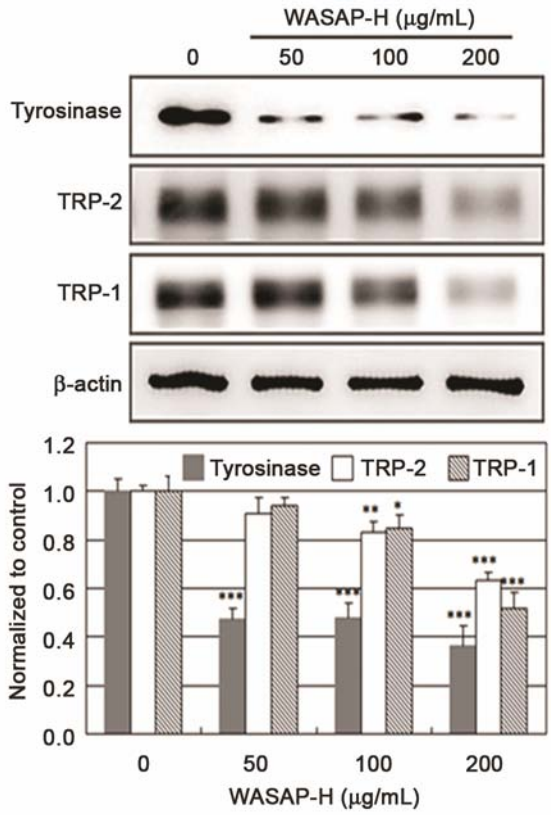

Fig. 3—The effects of WASAP-H on the protein expressions of tyrosinase, TRP-1, and TRP-2 in B16F10 melanoma cells. Each determination was performed in triplicate: the data represent mean ± S.E. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs. without WASAP-H.

regulation of TRP-1 and TRP-2. Melanin plays a crucial role in protecting the skin by absorbing and scattering ultraviolet light from the sun, neutralizing

free radicals, and scavenging toxic chemicals[13-15]. However, excessive synthesis and accumulation of melanin darken the epidermis and induce a number of skin hyperpigmentation conditions such as melasma, freckles, age spots, and solar lentigo[16-19]. Tyrosinase, a copper-containing metalloenzyme, is a key enzyme in the melanogenic processes and catalyses two rate-limiting steps in the synthesis of melanin, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and the oxidation of DOPA to dopaquinone[20-22]. Dopaquinone is further catalysed by tyrosinase-related protein-1 (TRP-1) and TRP-2 to eumelanin, which has a brown or black colour, or non-enzymatically polymerized to pheomelanin, which has a yellow to reddish-brown colour[23]. Therefore, these enzymes are known to be the targets of anti-melanogenic agents; hence, biological assay of these enzymes was performed. A well-established bioassay confirmed the efficacy of herbs that were classified as having a white color by the TCM theory, in skin whitening. This pilot study with the herbal medicines with a specific characteristic in traditional theory could be applied to the translation of various theoretical characteristics of traditional herbs into the relevant pharmacological action. Considering that most of the testing samples were discarded as non-positive ones in the conventional random screening systems, this traditional knowledge-based approach of sample selection for the bioassay could be a promising method that will increase the hit rate while reducing significant monetary costs in the discovery stages.

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