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# Statistically Designed Enzymatic Hydrolysis for Optimized Production of Icariside II as a Novel Melanogenesis Inhibitor

**Park, Jun-Seong*, Hye Yoon Park, Ho-Sik Rho, Soomi Ahn, Duck Hee Kim, and Ih Seop Chang**

*Skin Research Institute, Amorepacific R&D Center, Yongin 449-729, Korea*

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**Three kinds of prenylated flavonols, icariside I, icariside II, and icaritin, were isolated from an icariin hydrolysate and their effects on melanogenesis evaluated based on mushroom tyrosinase inhibition and quantifying the melanin contents in melanocytes. Although none of the compounds had an effect on tyrosinase activity, icariside II and icaritin both effectively inhibited the melanin contents with an $IC_{50}$ of 10.53 and 11.13 μM, respectively. Whereas icariside II was obtained from a reaction with β-glucosidase and cellulase, the icariin was not completely converted into icariside II. Thus, for the high-purity production of icariside II, the reaction was optimized using the response surface methodology, where an enzyme concentration of 5.0 mg/ml, pH 7, 37.5°C, and 8 h reaction time were selected as the central conditions for the central composite design (CCD) for the enzymatic hydrolysis of icariin into icariside II using cellulase. Empirical models were developed to describe the relationships between the operating factors and the response (icariside II yield). A statistical analysis indicated that all four factors had a significant effect ($p$<0.01) on the icariside II production. The coefficient of determination ($R^2$) was good for the model (0.9853), and the optimum production conditions for icariside II was an enzyme concentration of 7.5 mg/ml, pH 5, 50°C, and 12 h reaction time. A good agreement between the predicted and experimental data under the designed optimal conditions confirmed the usefulness of the model. A laboratory pilot scale was also successful.**

**Keywords:** RSM, central composite design, melanogenesis, icaritin, icariside II

Melanogenesis is the process of melanin synthesis and distribution by a cascade of enzymatic and chemical reactions in melanocytes [6]. Melanin synthesis is principally responsible for skin color and plays a key role in the prevention of UV-induced skin damages. However, increased levels of epidermal melanin synthesis can darken the skin and produce various dermatologic disorders, such as melasma, age spots or liver spots, and actinic damage, resulting in the accumulation of excessive levels of epidermal and dermal pigmentations [3]. Melanin synthesis starts from the conversion of amino acid L-tyrosine into 3,4-dihydroxyphenylalanine (L-DOPA), and then the oxidation of L-DOPA by tyrosinase yields DOPA-quinone [7]. For this reason, melanin production is mainly controlled by the expression and activation of tyrosinase. Accordingly, tyrosinase inhibitors, such as kojic acid and albutin, have been established as important constituents of cosmetic products and depigmenting agents against hyperpigmentation [4].

Recently, the application of naturally occurring products as melanin synthesis inhibitors in cosmetics has attracted much interest [4]. For example, plant polyphenols have been the target of several studies [4, 7], resulting in repeated reviews of their classification, occurrence, structural aspects, reactivity, biochemistry, and biogenesis. Polyphenols is a broad term used in literature on natural products to refer to substances that possess one or more benzene ring(s) and a hydroxyl group, including functional derivatives. Extensive literature is available on the screening of tyrosinase inhibitors among phenolics of plant origin, and polyphenols are currently the target of numerous studies. In particular, several prenylated flavonoids have been demonstrated as potent tyrosinase inhibitors. For example, prenylated flavanones from *Sophora flavenscens*, such as kurarinone and kushenol F, have been reported to be strong noncompetitive tyrosinase inhibitors. Various other prenylated flavonoids, including sophoraflavanone G from the same plant, have even been identified as more potent inhibitors than kojic acid [7]. Therefore, these findings suggest that the prenyl residues in flavonoid molecules may facilitate the potent inhibition of tyrosinase activity. Accordingly, this study examined natural resources originating from *Epimedium* species. Several *Epimedium* species are already used as tonics in

*Corresponding author
Phone: 82-31-280-5878; Fax: 82-31-281-8397;
E-mail: superbody@amorepacific.com

Asian herbal medicine to treat such disorders as hypertension, coronary heart disease, osteoporosis, and menopause syndrome. Although various researchers have already studied the constituents of these plants and isolated a series of prenylated flavonol glycosides, such as icariin and epimedoside A, the affinity with tyrosinase may have been disturbed owing to chemical substitution in the compounds [11, 15, 16].

Microorganisms produce hydrolytic enzymes for the hydrolysis of polysaccharides into metabolizable products. As such, the inorganic and organic chemistry communities have been actively involved in developing models and mimics of hydrolytic enzymes, as their diverse functions, for example glycoside hydrolytic activities with different specificities and modes of action, are required for the efficient modification of natural compounds and plant extracts [1, 8–10, 17].

Response surface methodology (RSM) is a useful statistical technique for the investigation and optimization of complex processes, and the theoretical and fundamental bases of RSM have already been widely reviewed, allowing RSM to be successfully applied in many areas, such as food, chemicals, and biological processes [1, 5, 13, 14].

Therefore, the present work describes the enzymatic hydrolysis of icariin isolated from *Epimedium grandiflorum*. Three kinds of prenylated flavonols were isolated from the icariin hydrolysate and their effect on melanogenesis evaluated based on quantifying the melanin contents in melanocytes. RSM was also used to optimize the operating conditions with cellulase for the production of icariside II as the most effective inhibitor of melanogenesis.

## MATERIALS AND METHODS

### Materials

Icariin from *Epimedium grandiflorum* was purchased at Zhejiang Chemicals, China, and the following enzyme preparations purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.): β-glucosidase from almonds, cellulase from *Aspergillus niger*, nariginase from *Penicillium decumbens*, and hesperidinase from *Penicillium* sp. The mushroom tyrosinase and L-DOPA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and all the organic solvents used were of analytical grade and purchased from Fisher Scientific U.K. (Loughborough, Leics, U.K.).

### Acid and Enzymatic Hydrolysis of Icariin

For the structure analysis of the hydrolysate, icariin (10 g) was partially hydrolyzed with 1 l of 2 M HCl by heating at 100°C for 15 min in a sealed tube. For the enzymatic hydrolysis, icariin (5 g) in 160 ml of a 0.02 M sodium-acetate buffer (pH 5.0) with 40 ml of several glycolytic enzyme solutions was incubated with stirring at 37°C for 24 h. Each sample and a blank were used as the reaction controls. All the samples were prepared in duplicate. After incubation, each aliquot was extracted with methanol and centrifuged at 4°C for 10 min. To determine the change in the material, it was stored frozen until an HPLC analysis.

### Isolation and Analysis of Icariin Hydrolysate

The analysis of the enzyme reactants was carried out using the following HPLC and TLC methods. The HPLC system consisted of a Waters 2695 separation module, 2996 PDA detector, and Mightysil C18 reverse-phase column (250×4.6 mm). The detector wavelength was set at 263 nm, and the mobile phases used for the sample analysis were a mixture of distilled water (A) and acetonitrile (B) with a gradient elution of 15% to 80% of B in 30 min at a flow rate of 1 ml/min. The HPLC analysis of the icariin hydrolysate extract gave three peaks, compounds 1–3. For the structure analysis of these compounds, the extract (1 g) was further purified using a medium-pressure liquid chromatography (MPLC) system (Yamazen Co., Japan) with an elution system of distilled water (DW) and acetonitrile gradient with an acetonitrile concentration from 30% to 80% in 60 min at a flow rate of 30 ml/min. An ultra pack SI-40D column (40 μm, 50×300 mm; Yamazen Co., Japan) was also used and the detector wavelength set at 270 nm. Ten fractions were collected and monitored by HPLC. The fractions were combined and three compounds obtained: 135 mg of compound 1, 231 mg of compound 2, and 428 mg of compound 3. To determine the structure of the isolated compounds, an LC/MS and NMR analysis was conducted. Acid hydrolysis was also conducted to analyze the carbohydrate residue. Compound 1 or 2 (5 mg) dissolved in 1 ml of 2 M HCl was heated at 100°C for 30 min in a sealed tube, and then analyzed by TLC, where the TLC analysis was performed on a silica gel plate (Merck, Kieselgel 60 $F_{254}$) using n-butanol-acetic acid-water (3:1:1, v/v/v) as the eluent. Next, the hydrolysates separated on the TLC plate were visualized by dipping the plates into 5% (v/v) $H_2SO_4$ in methanol containing 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine, followed by drying and heating for 10 min at 121°C.

### LC/MS and NMR Analysis

The LC-MS-APCI analyses were made using a Thermo Finnigan (Surveyor) HPLC with a PDA detector coupled to a Thermo Finnigan (LCQ Classic) quadrupole ion trap mass spectrometer equipped with an APCI source. The chromatographic separations of the material were performed on a Merck LiChrosphere C18 column (5 μm, 250×4 mm) using a 1 ml/min solvent gradient of 20%–100% aqueous methanol (containing 1% acetic acid) within 40 min. The MS interface was set to a positive-ion mode [Vaporizer tube temperature: 550°C; needle current: 5 μA (approx. 3.6 kV); sheath and auxiliary nitrogen gas pressures: 80 and 20 psi; heated capillary temperature: 150°C]. The mass spectra were acquired within the range of (full) m/z 125–1,200 (MS-MS ion isolation width: 5 Da; MS-MS collision energy: 45%; MS-MS scan range: m/z of parent ion - ca. 1/3 m/z parent ion). To determine the structure of the compounds isolated from the icariin hydrolysate, the $^{1}H$, $^{13}C$-NMR spectra were recorded on a Varian GEMINI-300BB (300 MHz) spectrometer.

*Compound 1*: LC/MS m/z=531 [M+H]; NMR $\delta_H$ (DMSO-*d*6, 300 MHz): 8.23 (2H, d, *J*=8.8 Hz, H-2', 6'), 7.16 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.64 (1H, s, H-6), 5.17 (1H, t, *J*=5.8 Hz, H-12), 4.95 (1H, d, *J*=1.2 Hz, $H1_{Glc}$), 3.87 (3H, s, 4'-OMe), 3.54 (2H, m, H-11, overlapped), 3.83–5.40 (m, sugar protons), 1.83 (3H, s, H-15), 1.70 (3H, s, H-14); NMR $\delta_C$ (DMSO-*d*6, 75 MHz): 176.5 (C-4), 160.6 (C-7), 160.1 (C-5), 158.5 (C-4'), 152.7 (C-9), 146.9 (C-2), 136.2 (C-3), 131.1 (C-13), 129.5 (C-6'), 129.3 (C-2'), 123.4 (C-1'), 122.3 (C-12), 114.3 (C-5'), 114.1 (C-3'), 108.1 (C-8), 104.5 (C-10), 100.5

(C-1"$_{Glu}$), 97.2 (C-6), 77.2 (C-5"), 76.7 (C-3"), 73.4 (C-2"), 69.7 (C-4"), 60.7 (C-6"), 55.4 (4'-OMe), 25.4 (C-14), 21.5 (C-11), 17.9 (C-15). When comparing the data with existing literature [15], compound 1 was identified as icariside I.

*Compound 2*: LC/MS m/z=515 [M+H]; NMR $\delta_H$ (DMSO-*d*6, 300 MHz): 7.91 (2H, d, *J*=8.8 Hz, H-2', 6'), 7.17 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.33 (1H, s, H-6), 5.40 (1H, br s, H-1$_{Rha}$), 5.15 (1H, t, *J*=5.8 Hz, H-12), 3.82 (3H, s, 4'-OMe), 3.50 (2H, m, H-11, overlapped), 3.00–5.40 (m,sugar protons), 1.68 (3H, s, H-15), 1.59 (3H, s, H-14), 0.77 (3H, d, *J*=6.0 Hz, H-6$_{Rha}$); NMR $\delta_C$ (DMSO-*d*6, 75 MHz): 177.3 (C-4), 160.7 (C-7), 160.6 (C-5), 159.2 (C-4'), 154.5 (C-2), 153.1 (C-9), 133.7 (C-3), 131.3 (C-13), 130.8 (C-6'), 129.8 (C-2'), 122.2 (C-12), 122.1 (C-1'), 114.3 (C-5'), 113.3 (C-3'), 106.9 (C-8), 104.6 (C-10), 101.5 (C-1"$_{Rha}$), 97.2 (C-6), 71.0 (C-4"), 70.6 (C-3"), 70.4 (C-5"), 69.8 (C-2"), 55.6 (4'-OMe), 25.5 (C-14), 21.8 (C-11), 17.9 (C-15), 17.5 (C-6"). When comparing the data with existing literature [15], compound 2 was identified as icariside II.

*Compound 3*: LC/MS m/z=369 [M+H]; NMR $\delta_H$ (DMSO-*d*6, 300 MHz): 8.12 (2H, d, *J*=8.70 Hz, H-2', 6'), 7.10 (2H, d, *J*=8.70 Hz, H-3', 5'), 6.25 (1H, s, H-6), 5.14 (1H, t, *J*=6.0 Hz, H-12), 3.80 (3H, s, 4'-OMe), 3.26 (2H, d, *J*=6.0 Hz, H-11), 1.80 (3H, s, H-15), 1.64 (3H, s, H-14). When comparing the data with existing literature [16], compound 3 was identified as icaritin.

**Mushroom Tyrosinase Assay**

The tyrosinase activity was determined using the method of Pomerantz with a minor modification [8]. Twenty-five µl of 0.5 mM L-DOPA, 875 µl of a 50 mM phosphate buffer (pH 6.5), and 25 µl of the test sample solution were mixed, and then 50 µl of mushroom tyrosinase (1,600 U/ml) was added. The amount of dopachrome produced in the reaction mixture was determined against a blank (solution without any enzyme) at 475 nm (OD475) using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

**Melanin Synthesis in Mouse Melanoma Cells**

The Mel-Ab cell lines were C57BL/6 mouse-derived spontaneously immortalized melanocyte cell lines that were grown on plastic tissue culture flasks. The media used for the Mel-Ab cells were Dulbecco's Minimal Essential Media (DMEM) supplemented with 10% fetal bovine serum (Gibco Life Tech.), 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (Sigma), 1 nM cholera toxin (Sigma), 0.001% streptomycin (Gibco Life Tech.), and 10,000 U/l penicillin. Confluent Mel-Ab cultures were removed from the plastic flasks using 0.25% trypsin/EDTA, and then the cells were placed into Falcon 24-well plastic culture plates at a density of $10^5$ cells/well and incubated for 24 h in media prior to treatment with the designated compound. After 24 h, the media were replaced with 990 ml of a fresh medium, and 10 ml of the test compounds (dissolved in a vehicle composed of 50% propylene glycol, 30% ethanol, and 20% water) added to duplicate wells. The control wells were treated with just the vehicle. The feeding and agent treatment was repeated daily for three days with no additional treatment on the fourth day. Following four days of treatment, the remaining adherent cells were assayed. One well out of duplicate wells was used to determine the melanin content, whereas the other well was used to observe the cell viability. The melanin content of melanocytes after treatment was determined by the addition of 1 ml/well of 1 N NaOH to the cells. The crude cell extracts were then assayed using a spectrophotometer at 400 nm (ELX 800, Bio-TEK Instrument Inc., U.S.A.). The cell viability was measured by dyeing live cells with crystal violet.

**Experimental Design and Analysis**

For the enzymatic hydrolysis of icariin into icariside II, the important factors considered were the pH, temperature, enzyme concentration, and reaction time, and the response surface methodology was used to optimize these reaction factors. To predict the optimal point, a second-order polynomial function was fitted to the experimental results. Thus, for the four factors, the equation was

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{11}X^2_1+b_{22}X^2_2+b_{33}X^2_3 +b_{44}X^2_4+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+b_{23}X_2X_3 +b_{24}X_2X_4+b_{34}X_3X_4 \quad (1)$$

where Y, the predicted response, stands for the icariside II production or icariside yield; $b_0$ is the intercept; $b_1$–$b_4$ are the linear coefficients; $b_{11}$, $b_{22}$, $b_{33}$, and $b_{44}$ are the quadratic coefficients; $b_{12}$, $b_{13}$, $b_{14}$, $b_{23}$, $b_{24}$, and $b_{34}$ are the cross-product coefficients; and $X_i$ is the coded independent variable. The yield was obtained from experiments with a central composite design (CCD), consisting of 16 experiments with $2^4$ factorial components, eight (2×4) axial points, and five center point replicates for a total of 29 runs. The center point replicates provided an estimate of the pure error. The relative percentage conversion (yield) was then determined by the ratio of the product concentration ([P]) to its initial substrate concentration ([$S_o$]) as follows:

$$Y=[P]/[S_o]\times 100$$

and estimated using the peak area integrated by the online Waters Empower software. Design-Expert trial ver 6.0.0 (Stat-Ease, Inc.) was used to fit the quadratic response surface model to the experimental data and create contour plots based on holding two variables of the model constant. The optimization of the reaction conditions in terms of the enzyme concentration, pH, temperature, and reaction time was calculated using the predictive models from the RSM. The production of icariside II was then carried out using the predicted optimal conditions, and the observed response obtained under these conditions analyzed and compared with the predictive value.

## RESULTS AND DISCUSSION

### Isolation and Analysis of Icariin Hydrolysate

Icariin samples from *Epimedium grandiflorum* were analyzed by HPLC and the chromatogram is given in Fig. 1A, which shows one intense peak, indicating icariin with a purity of 90%. The icariin was partially hydrolyzed to investigate any possible products before enzymatic hydrolysis. After the reaction, an HPLC analysis of the icariin hydrolysate gave three new peaks, as shown in Fig. 1B. For the structural analysis of these compounds, the icariin hydrolysate was further purified by MPLC with distilled water (DW) and acetonitrile as the mobile phase using an Ultra pack SI-40D column. After being monitored by HPLC, the fractions were combined, resulting in 135 mg of compound 1, 231 mg of compound 2, and 428 mg of compound 3. To

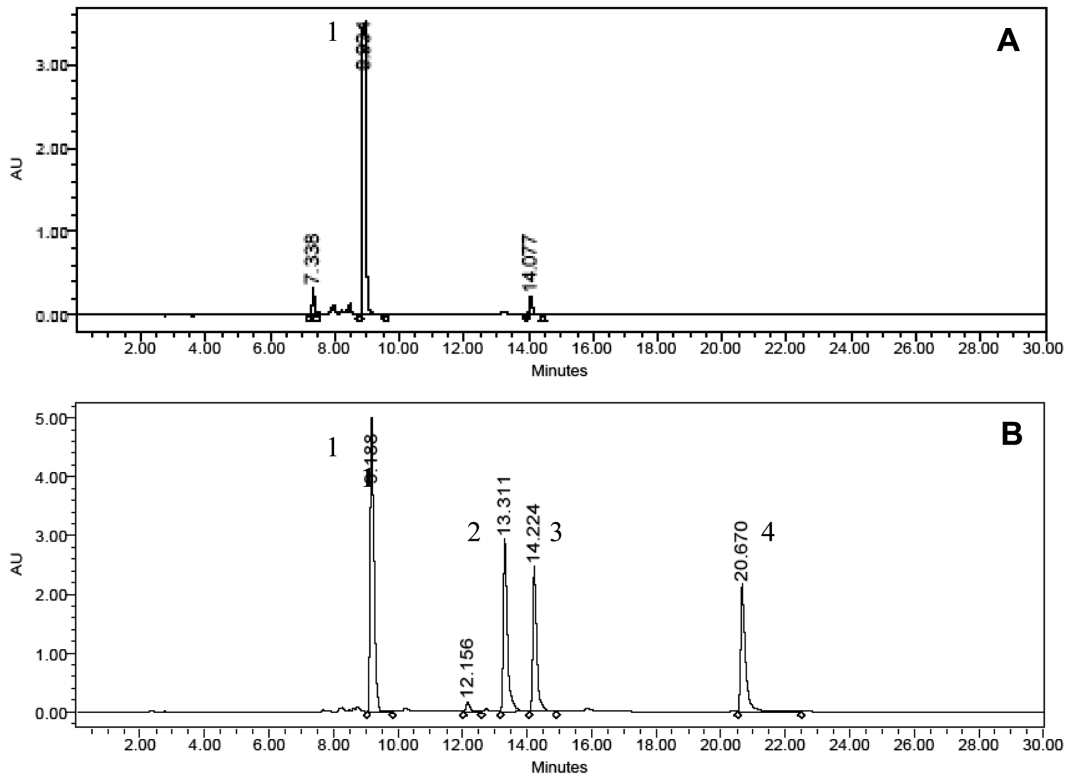
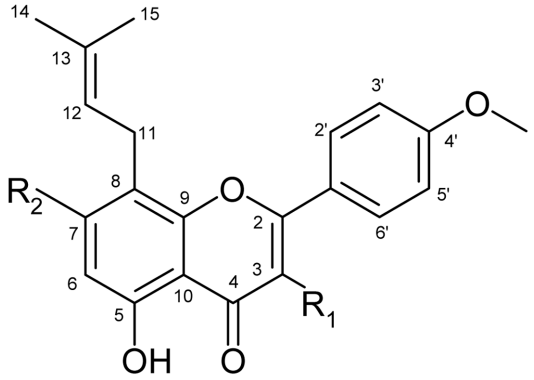



**Fig. 1.** HPLC profiles of icariin from *Epimedium grandiflorum* (**A**) and partial acid hydrolysate of icariin (**B**).
Key to peak identity: 1, icariin; 2, compound 1; 3, compound 2; 4, compound 3.

determine the structure of the isolated compounds, acid hydrolysis, LC/MS, and an NMR analysis were conducted. Compound 1 was obtained as a pale yellow solid and exhibited an [M+H] ion peak at m/z 531 in the positive-ion LC-MS, indicating a molecular weight of 530. The $^{1}$H NMR spectrum showed three kinds of aromatic protons [6.64 (1H, s, H-6), 8.23 (2H, d, *J*=8.8 Hz, H-2', 6'), and 7.16 (2H, d, *J*=8.8 Hz, H-3', 5')], three kinds of allylic proton [3.54 (2H, m, H-11, overlapped), 1.83 (3H, s, H-15) and 1.70 (3H, s, H-14)], and one vinyl proton [5.17 (1H, t, *J*=5.8 Hz, H-12)], indicating an icaritin skeleton with one anomeric proton [4.95 (1H, d, *J*=1.2 Hz, $H1_{Glc}$)]. Furthermore, 27 carbon signals were observed in the $^{13}$C NMR spectrum, where 21 carbon signals were assigned to icaritin and one carbon signal [100.5 (C-1"$_{Glu}$)] assigned to the anomeric carbon of the sugar moiety. In the acid hydrolysis of compound 1, compound 3 (icaritin) and glucose were identified by TLC. Compound 2 was also obtained as a pale yellow solid and exhibited an [M+H] ion peak at m/z 515 in the positive-ion LC-MS, indicating a molecular weight of 514. The acid hydrolysis of compound 2 gave icaritin and rhamnose. The $^{1}$H NMR spectrum showed three kinds of aromatic protons [7.91 (2H, d, *J*=8.8 Hz, H-2', 6'), 7.17 (2H, d, *J*=8.8 Hz, H-3', 5'), and 6.33 (1H, s, H-6)], three kinds of allylic protons [3.50 (2H, m, H-11, overlapped), 1.68 (3H, s, H-15), and 1.59 (3H, s, H-14)], and one vinyl proton [5.15 (1H, t, *J*=5.8 Hz, H-12)], indicating an icaritin skeleton with one anomeric proton [5.40 (1H, br s, H-1$_{Rha}$)] and -$CH_3$ proton [0.77 (3H, d, *J*=6.0 Hz, H-6$_{Rha}$)]. In addition, 27 carbon signals were observed in the $^{13}$C NMR spectrum, where 21 carbon signals were assigned to icaritin and one carbon signal [101.5 (C-1"$_{Rha}$)] assigned to the anomeric carbon of the sugar moiety. Therefore, the LC-MS, $^{1}$H NMR, and $^{13}$C NMR spectrum data suggested that compounds 1 and 2 were the known flavonol glycosides icariside I and II, respectively [15]. Compound 3 (icaritin) was obtained as a pale yellow solid and exhibited an [M+H] ion peak at m/z 369 in the positive-ion LC-MS, indicating a molecular weight of 368. The $^{1}$H NMR spectrum showed three kinds of aromatic protons [8.12 (2H, d, *J*=8.70 Hz, H-2', 6'), 7.10 (2H, d, *J*=8.70 Hz, H-3', 5'), and 6.25 (1H, s, H-6)], three kinds of allylic protons [3.26 (2H, d, *J*=6.0 Hz, H-11), 1.80 (3H, s, H-15), and 1.64 (3H, s, H-14).], one vinyl proton [5.14 (1H, t, *J*=6.0 Hz, H-12)], and one -$OCH_3$ proton [3.80 (3H, s, 4'-OMe)]. In the $^{1}$H NMR spectrum, all signals were very close to published data for icaritin in *Epimedium wushanense* [16]. Moreover, the aglycone signals for compound 3 showed a great similarity with those published for icaritin glycosides [11, 15]. Thus, the LC-MS and $^{1}$H NMR spectrum data suggested that compound 3 was the known flavonol compounds, icaritin.



| Compound | $R_1$ | $R_2$ |
|---|---|---|
| Icariin | Rha | Glc |
| Compound 1 | H | Glc |
| Compound 2 | Rha | H |
| Compound 3 | H | H |

**Fig. 2.** Structure of compounds isolated from the enzymatic-hydrolyzed product of icariin; Rha: rhamnopyranoside, Glc: glucopyranoside.

Fig. 2 shows the structure of the compounds isolated from the partial acid hydrolysates of icariin.

### Effect of Icariin Hydrolysate on Melanin Synthesis in Mel-Ab Cells and Tyrosinase Inhibition

As melanin formation is the most important factor determining mammalian skin color, the inhibition of melanin formation may reduce darkened skin colors [3]. Several flavonoid derivatives have already been found to exhibit varying degrees of inhibitory activity toward tyrosinase. In particular, several prenylated flavonoids have been shown to be potent tyrosinase inhibitors. Therefore, these findings suggest that the prenyl residues in flavonoid molecules may be responsible for the potent inhibition of tyrosinase activity [7]. Dooley *et al.* [4] previously described a general strategy for the discovery and development of novel topical skin-lightening products. A desirable skin-lightening agent should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase, exhibit low cytotoxicity, and be nonmutagenic. Table 1 presents the assessment of the icariin hydrolysates in Mel-Ab cells. To investigate the effect of the icariin hydrolysates, the $IC_{50}$ values for the inhibition of cultured Mel-Ab cell pigmentation due to melanin synthesis were compared for each compound. Icariside II and icaritin were both very effective compounds, with an $IC_{50}$ of 10.53 and 11.13 μM, respectively. To determine the inhibitory effects of the icariin hydrolysates on tyrosinase activity, mushroom tyrosinase and various concentrations of each sample were incubated with L-DOPA as the substrate, and then the formation of dopachrome was measured. None of the samples had any significant effect on the mushroom tyrosinase activity (Table 1). However, albutin inhibited the mushroom tyrosinase with an $IC_{50}$ of 32.4 μM. Therefore, the present results using mushroom tyrosinase demonstrated

**Table 1.** *In vitro* assessment of compounds isolated from the icariin hydrolysate on the inhibitory activity of melanin formation and mushroom tyrosinase activity.

| Compounds | Melanin synthesis $IC_{50}$ (μM) | Tyrosinase activity $IC_{50}$ (μM) |
|---|---|---|
| Icariin | N.E. | N.E. |
| Compound 1 (icariside I) | 49.04 | N.E. |
| Compound 2 (icariside II) | 10.53 | N.E. |
| Compound 3 (icaritin) | 11.13 | N.E. |
| Arbutin | 108.6 | 32.4 |

N.E., not examined.

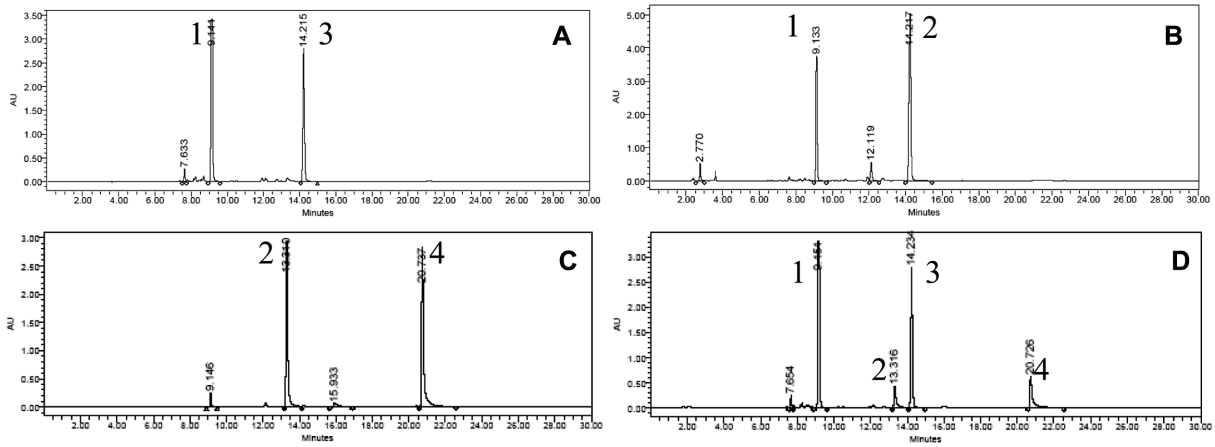



**Fig. 3.** HPLC profile of hydrolysate of icariin using several glycolytic enzymes; reaction with beta-glucosidase (**A**), cellulase (**B**), nariginase (**C**), and hesperidinase (**D**).
Key to peak identity: 1, icariin; 2, compound 1; 3, compound 2; 4, compound 3.

**Table 2.** Operating variables, levels, and experimental data used in central composite design (CCD).

| Expt no. | Enzyme conc. (mg/ml) | pH | Temp (°C) | Reaction time (h) | Yield (%) |
|---|---|---|---|---|---|
| 1 | 2.5 | 5 | 25.0 | 4 | 48.74 |
| 2 | 7.5 | 9 | 25.0 | 12 | 59.74 |
| 3 | 5.0 | 5 | 37.5 | 8 | 67.40 |
| 4 | 2.5 | 9 | 50.0 | 12 | 80.35 |
| 5 | 2.5 | 7 | 37.5 | 8 | 57.63 |
| 6 | 2.5 | 9 | 25.0 | 12 | 55.40 |
| 7 | 7.5 | 5 | 25.0 | 4 | 66.72 |
| 8 | 5.0 | 7 | 37.5 | 8 | 67.97 |
| 9 | 7.5 | 5 | 50.0 | 4 | 72.40 |
| 10 | 2.5 | 9 | 50.0 | 4 | 42.90 |
| 11 | 7.5 | 7 | 37.5 | 8 | 70.14 |
| 12 | 2.5 | 9 | 25.0 | 4 | 31.40 |
| 13 | 7.5 | 9 | 25.0 | 4 | 41.50 |
| 14 | 5.0 | 7 | 37.5 | 8 | 68.40 |
| 15 | 2.5 | 5 | 50.0 | 12 | 78.40 |
| 16 | 5.0 | 7 | 37.5 | 8 | 67.80 |
| 17 | 2.5 | 5 | 50.0 | 4 | 45.60 |
| 18 | 5.0 | 7 | 50.0 | 8 | 72.40 |
| 19 | 5.0 | 7 | 37.5 | 12 | 75.92 |
| 20 | 7.5 | 9 | 50.0 | 4 | 68.50 |
| 21 | 5.0 | 9 | 37.5 | 8 | 61.70 |
| 22 | 7.5 | 9 | 50.0 | 12 | 90.90 |
| 23 | 5.0 | 7 | 37.5 | 8 | 68.51 |
| 24 | 5.0 | 7 | 37.5 | 4 | 53.10 |
| 25 | 2.5 | 5 | 25.0 | 12 | 66.70 |
| 26 | 7.5 | 5 | 50.0 | 12 | 89.70 |
| 27 | 5.0 | 7 | 25.0 | 8 | 55.80 |
| 28 | 5.0 | 7 | 37.5 | 8 | 67.90 |
| 29 | 7.5 | 5 | 25.0 | 12 | 79.58 |

that the depigmentation effect of the icariin hydrolysates was not associated with inhibiting tyrosinase activity. Several melanogenesis inhibitors, such as linoleic acid and terrain, have also recently been shown to inhibit melanin production by another pathway of melanogenesis. Thus, icariside II and icaritin could be involved in such an alternate pathway. Although further examination is required to clarify the mechanism in detail, at least the direct inhibition of tyrosinase by icariside II and icaritin can be excluded. According to Curto *et al*. [3], a potentially efficacious skin depigmentation agent is one that inhibits tyrosinase with an $IC_{50}$<25 μg/ml and inhibits melanocyte pigmentation with an $IC_{50}$<100 μg/ml. Thus, all the compounds tested in the present study, except for icariin, satisfied these requirements, where icariside II was the most effective.

**Enzyme Screening for Production of Icariside II**

As conventional chemical hydrolysis inevitably produces side reactions, glycolytic enzymes are normally used for the deglycosylation of products [1]. Thus, several commercially useful glycolytic enzymes, including β-glucosidase, cellulase, nariginase, and hesperidinase, were tested for the concurrent bioconversion of icariin into icariside II. All the reactions were monitored by HPLC. As shown in Fig. 3, several hydrolyzed compounds were produced after the reactions with β-glucosidase (Fig. 3A), cellulase (Fig. 3B), naringinase (Fig. 3C), and hesperidinase (Fig. 3D). Although icariside II was formed in the reaction with β-glucosidase and cellulase, the icariin was not completely converted into icariside II. Thus, for a high-purity production of icariside II, the response surface methodology was applied to optimize the production of icariside II by cellulase, as it

**Table 3.** Analysis of variance for quadratic model.

| Source | Sum of squares | DF | Mean Square | F-Value | *P*-value |
|---|---|---|---|---|---|
| Model | 5,373.55 | 14 | 383.82 | 67.21 | <0.0001 |
| Residual | 79.96 | 14 | 5.71 | | |
| Lack of Fit | 79.55 | 10 | 7.96 | 78.82 | <0.0004 |
| Pure Error | 0.40 | 4 | 0.10 | | |
| $R^2$ | 0.9853 | | | | |

was more effective than β-glucosidase among the two enzymes.

**Process Optimization**

To optimize the production process of icariside II from icariin using cellulase, the important factors considered were the pH, temperature, enzyme concentration, and reaction time. Thus, a 5.0 mg/ml enzyme concentration (EC), pH of 7, temperature of 37.5°C, and 8 h reaction time were chosen as the central conditions of the CCD to optimize the production process. The responses were obtained by an HPLC analysis of the samples after the reactions were performed under the designed conditions. The experimental design was repeated twice. The coefficients of the response surface model given by Eq. (1) were evaluated by fitting to the experimental data, where the responses and variables were fitted with multiple regressions. Table 2 shows the experimental conditions and results for the icariside II production and yield according to the factorial design. A good fit was obtained, and no outliers were observed. An ANOVA (analysis of variance) indicated that the model was highly appropriate for the prediction, owing to its high *F* model value (67.21) and small significance *P* (0.0001). Moreover, a statistical analysis revealed no lack of fit, and the coefficient of determination ($R^2$) of the model was 0.9853, indicating an adequate precision and that the model could be used to navigate the design space. (Table 3). The statistical analysis also showed that all four factors had a significant effect on the enzymatic hydrolysis of icariin into icariside II (Table 4); however, the most relevant variable ($P<0.01$) as regards the icariside II yield was the

**Table 4.** Regression coefficients of variables.

| Variables | Coefficient estimate | DF | Standard error | Prob>F |
|---|---|---|---|---|
| $b_0$ | 66.41 | 1 | 0.78 | |
| $b_1$ | 7.34 | 1 | 0.56 | <0.0001 |
| $b_2$ | −4.60 | 1 | 0.56 | <0.0001 |
| $b_3$ | 7.53 | 1 | 0.56 | <0.0001 |
| $b_4$ | 11.44 | 1 | 0.56 | <0.0001 |
| $b_{11}$ | −1.11 | 1 | 1.49 | 0.4677 |
| $b_{22}$ | −0.44 | 1 | 1.49 | 0.7693 |
| $b_{33}$ | −0.89 | 1 | 1.49 | 0.5569 |
| $b_{44}$ | −0.48 | 1 | 1.49 | 0.7493 |
| $b_{12}$ | −1.15 | 1 | 0.60 | 0.0752 |
| $b_{13}$ | 1.81 | 1 | 0.60 | 0.0090 |
| $b_{14}$ | −2.59 | 1 | 0.60 | 0.0007 |
| $b_{23}$ | 4.39 | 1 | 0.60 | <0.0001 |
| $b_{24}$ | 1.32 | 1 | 0.60 | 0.0439 |
| $b_{34}$ | 2.31 | 1 | 0.60 | 0.0017 |

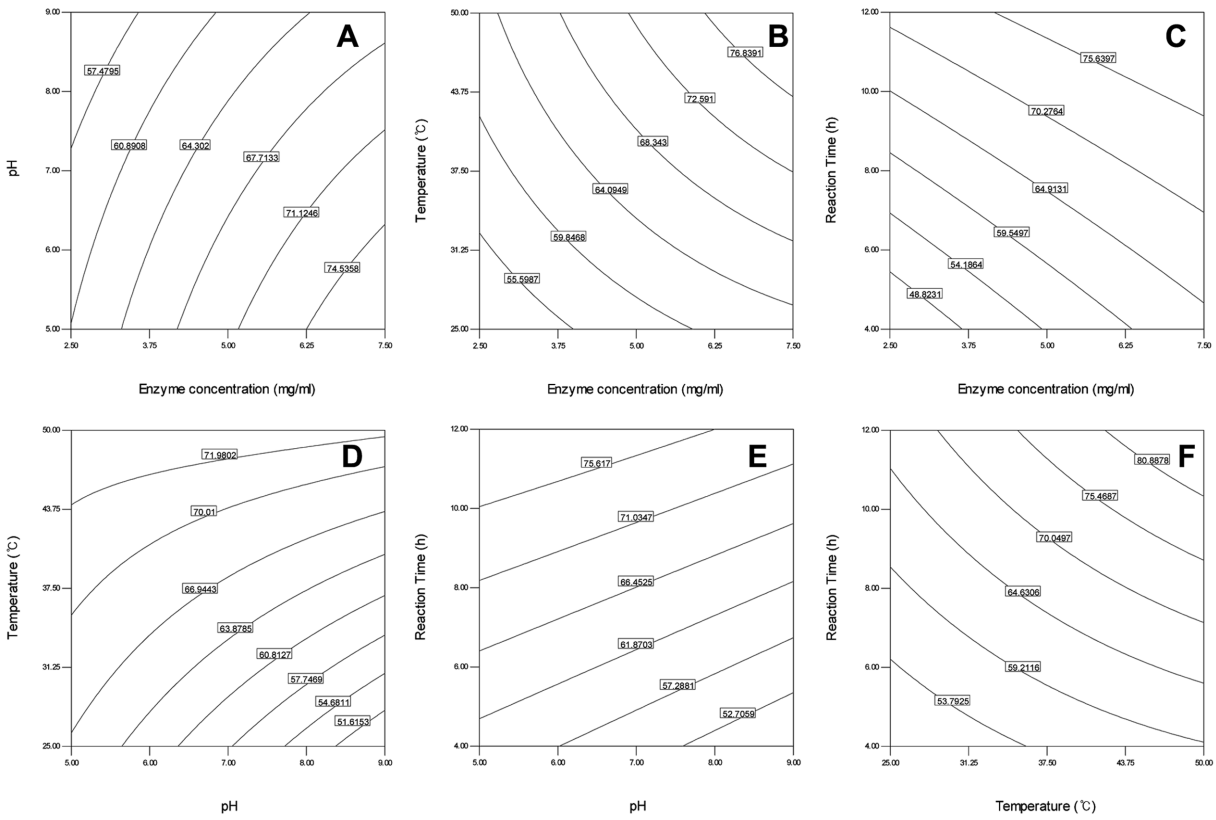



**Fig. 4.** Contour plots between any two factors for yield of icariside II: Other two factors were kept at the central point.

reaction time (RT). After eliminating the nonsignificant interaction coefficients ($P$-value>0.05), the reduced models could be expressed as follows:

$$Y = 66.41+7.34X_1-4.6X_2+7.53X_3+11.44X_4+1.81X_1X_3 -2.59X_1X_4+4.39X_2X_3+1.32X_2X_4+2.31X_3X_4 \quad (2)$$

where X1, X2, X3, and X4 represent the enzyme concentration (mg/ml), pH, temperature (°C), and reaction time (h), respectively.

Fig. 4 shows the contour plots between any two factors for the yield of icariside II calculated from Eq. (2). Besides showing the effects of the variables on the icariside II yield, the contour plots also indicate several optimal variable combinations that can be selected by the manufacturer to obtain a higher yield. Thus, according to the optimization based on the contour plots, the recommended conditions were an enzyme concentration of 7.5 mg/ml, pH 5, 50°C, and 12 h reaction time, which predicted an icariside II yield of 91.35%. When experiments were conducted using these optimal conditions to validate the RSM model, the resulting icariside II yield was 94.14%, thereby validating the values predicted by the RSM model. A scaled-up experiment was then conducted in a 500-ml reactor using the optimized conditions with 50 g of icariin and 3.75 g of cellulase. As a result, 45.48 g of icariside II was produced, representing a yield of 90.97%, which was also close to the predicted value for the scale-up reaction.

Three prenylated flavonols, icariside I, icariside II, and icaritin, were isolated from an icariin hydrolysate, and their effect on melanogenesis examined by quantifying the melanin content in melanocytes. Icariside II and icaritin were both found to be very effective as regards inhibiting melanogenesis, with an $IC_{50}$ of 10.53 and 11.13 μM, respectively. Thus, for icariside II production, enzymatic bioconversion methods were conducted using several glycolytic enzymes, and cellulase was eventually selected for the high-purity production of icariside II, where the reaction was optimized using the response surface methodology. A statistical analysis indicated that four factors, the enzyme concentration, pH, temperature, and reaction time, had a significant effect ($p$<0.01) on icariside II production. The coefficient of determination ($R^2$) was good for the model (0.9853). The optimum production conditions for icariside II were an enzyme concentration of 7.5 mg/ml, pH 5, 50°C, and 12 h reaction time. Under these conditions, the icariside II yield was estimated to be 91.35%, whereas the experimental yield was 94.14%. A laboratory pilot scale (500 ml) was also successful, with a yield of 90.97%.

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