J. Soc. Cosmet. Scientists Korea
Vol. 40, No. 1, March 2014, 89-94

pISSN 1226-2587
eISSN 2288-9507
http://dx.doi.org/10.15230/SCSK.2014.40.1.89

물푸레나무로부터 분리된 Esculetin와 Esculin의 미백 효능

홍 용 덕† · 남 미 희 · 이 창 석 · 신 송 석† · 박 영 호

(주)아모레퍼시픽 기술연구원

(2013년 10월 7일 접수, 2013년 10월 9일 수정, 2013년 10월 21일 채택)

Depigmenting Effects of Esculetin and Esculin Isolated from *Fraxinus rhynchophyllaHance*

Yong Deog Hong†, Mi Hee Nam, Chang Suk Lee, Song Seok Shin†, and Young Ho Park

Amorepacific Corporation R&D Unit, 314-1, Bora-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-729, Korea
(Received October 7, 2013; Revised October 9, 2013; Accepted October 21, 2013)

요 약: 물푸레나무 수피 추출물은 주요 생활성 물질로서 esculetin과 esculin을 포함하고 있다. 이 가운데, Esculetin은 B16F10 melanoma cells에서 IC_{50} value 2.8 μM의 아주 좋은 melanogenesis 억제 활성을 나타내며, 이를 통해 Melan-A cell에서 melanin 합성을 감소시킨다. 더욱이, esculetin은 mushroom tyrosinase에서도 IC_{50} value 40 μM의 억제 활성을 나타내어 melanin biosynthesis를 저해한다. 위의 결과들로서, 우리는 melanogenic enzyme 활성 조절에 의해 melanin 생성을 저해하는 효과적인 피부미백 물질로서 esculetin을 제안하고자 한다.

Abstract: Stem bark extracts of *Fraxinus rhynchophylla Hance* were found to contain two major bioactive components, esculetin and esculin. Esculetin substantially inhibited melanogenesis in B16F10 melanoma cells, with an IC_{50} value of 2.8 μm, and reduced melanin synthesis in Melan-A cells. Moreover, esculetin suppressed melanin biosynthesis by inhibiting mushroom tyrosinase activity, with an IC_{50} value of 40 μM. Taken together, these results suggest that esculetin could serve as an effective skin-lightening agent that inhibits melanin production by regulating the activity of melanogenic enzymes.

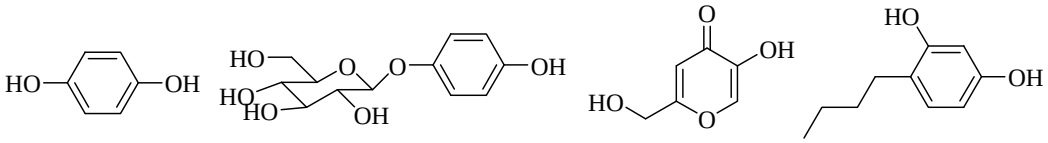
Keywords: *Fraxinus rhynchophylla Hance, melanogenesis, tyrosinase, esculetin, esculin*

1. Introduction

Melanins are unique pigmented biopolymers synthesized by specialized cells known as melanocytes, which exist in skin, hair, eyes, and other areas[1]. The major function of melanin is to protect against the damaging effects of ultraviolet radiation and scavenge free radicals[2]. Human skin color is determined by the quantity of melanin in the skin: an abnormally high level of melanin production by melanocytes can lead to hyperpigmentation in the form of melasma, freckles, and dark spots[3,4]. Melanin synthesis is accelerated by external stimuli such as ultraviolet light and inflammation; regulation of melanin synthesis can be accomplished by reducing exposure to harmful stimuli and blocking signal transmission, as well as suppressing the activity of tyrosinase, a key enzyme in melanin biosynthesis[5-7].

A number of known natural melanin synthesis inhibitors, including hydroquinone, arbutin, rucinol and kojic acid (KA), have been widely studied and are currently being used in cosmetics as skin-lightening agents. However, the carcinogenic potential of KA, coupled with its weak whitening effects, necessitates safer, skn irrita-

† 주 저자 (e-mail: hydhong@amorepcific.com, ssshin@amorepcific.com)



Hydroquinone **Arbutin** **Kojic acid** **Rucinol**

Figure 1. Chemical structures of known depigmenting agents.

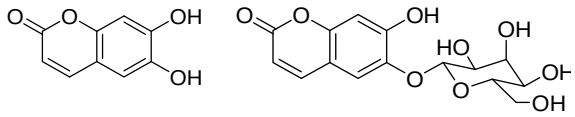


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Figure 2. Chemical structures of esculetin (1) and esculin (2).

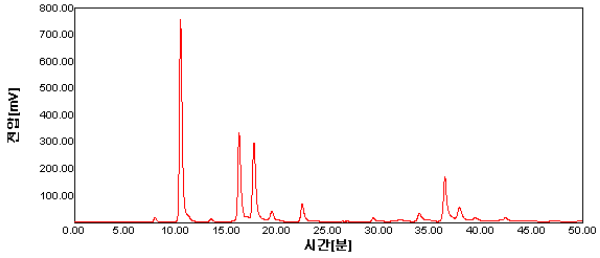

Figure 3. HPLC of an extract of the medicinal tree *Fraxinus rhynchophylla Hance* revealed two major bioactive components, esculin and esculetin.

tion and more effective alternatives[8,9]. Accordingly, the cosmetics industry has focused considerable attention on the identification and use of natural products that ha ve the same effects without the undesirable side effects[10-13].

Fraxinus rhynchophylla Hance (FR), a tree belonging to the Oleaceae family, is commonly used as a Chinese herbal drug and is officially listed in the Chinese Pharmacopoeia. FR is generally used to remove toxins and eliminate excess heat from the blood (to treat dysentery) and from the liver to improve visual acuity. It is also widely used in the treatment of inflammatory diseases, tumors, and various cardiovascular diseases, and is recognized for its radical-scavenging and neuroprotective properties [14.15].

In the course of an *in vitro* screen to identify anti-melanogenic compounds from extracts of various medicinal herbs, an ethanolic extract of the stem bark of FR was found to have melanogenesis-inhibitory activity (70% at 10 ppm). The major bioactive components of FR were identified by high performance liquid chromatography (HPLC) and mass spectrometry as esculetin and esculin (Figures 2 and 3).

This study evaluated the anti-melanogenic / depigmenting potential of esculetin and esculin. The ability of these compounds to inhibit melanogenesis and tyrosinase activity was determined using *in vitro* assays. In addition, the structure-activity relationship between these compounds and mushroom tyrosinase was examined in docking studies.

2. Materials and Methods

2.1. Extraction and purification of coumarins

Ten gram of stem bark was obtained from FR and extracted three times with a 1 : 1 ethanol : water mixture. The combined extracts were evaporated under reduced pressure. Two gram of the gummy residue were dissolved in 5 mL of methanol, and purified by column chromatography using a silica gel (methanol : chloroform = 1 : 9). Esculetin and esculin were obtained as white solid materials (10 and 37 mg, respectively). Esculetin and esculin were also purchased from Sigma.

2.2. Cell culture

B16F10 melanoma cells were grown in culture medium (13.4 mg/mL Dulbecco's Modified Eagle's medium; 10 mM HEPES; 143 U/mL benzylpenicillin potassium; 100 μg/mL streptomycin sulfate; 24 mM $NaHCO_3$, pH 7.1) containing 10% FBS at 37 ℃ in 5% CO_2. Cells were dissociated from culture dishes using 0.05% trypsin and 0.5 mM EDTA in PBS.

2.3. Determination of melanin synthesis

B16 cells (1×10^6 cells/mL) were incubated in the presence or absence of esculetin and esculin, followed by incubation for 72 h with 100 nM alpha-melanocyte-stimulating hormone (α-MSH). After two PBS washes, samples were dissolved in 100 μL of 1 N NaOH at 60 ℃ for 2 h. Total melanin content was estimated by measuring the absorbance at 405 nm, and comparing the obtained readings with a standard curve generated for melanin.

2.4. Measurement of mushroom tyrosinase inhibition

Mushroom tyrosinase and L-tyrosine were purchased from Sigma. The reaction mixture for determining the inhibition of mushroom tyrosinase activity consisted of 150 μL of 0.1 MPB (pH 6.5), 3 μL of sample solution, 8 μL of mushroom tyrosinase (2,100 units/mlin 0.05 MPB, pH 6.5), and 36 μL of 1.5 mM L-tyrosine. After incubation at 37 °C for 20 min, tyrosinase activity was determined by measuring the optical density at 490 nm using a Model 3550 Microplate Reader (Bio-Rad, Richmond, USA). The inhibitory activity was expressed as the concentration that inhibited 50% of the enzymatic activity (IC_{50}).

2.5. Molecular modeling

Molecular modeling study was carried out on linux system using SYBYL-X version 1.2. To prepare the tyrosinase structure, the crystal structure of the oxy form of S. castaneoglobisporus tyrosinase was taken from the Protein Data Bank (PDB cod 1 wx2) because there is no crystal structure of mushroom tyrosinase published yet. The caddie protein and water molecules were removed. Hydrogen atoms were added to the enzyme using the SYBYL. For the molecular docking method, Surflex-Dock version 2.5 was used using standard parameters and allowing the hydrogen of protein movement.

Table 1. Inhibitory Effects of Various Compounds on Melanogenesis

Compound	Melanin synthesis IC_{50} (μM)[a]	Clog*P*[b]
esculetin	2.8	1.214
esculin	326	−0.731
kojic acid	500	−1.387
4-n-butylresorcinol	16	2.365

[a]Concentration at which 50% of activity is inhibited
[b]Clog*P* values calculated using ChemDraw 12.0 V

2.6. Statistical analysis

Data are presented as the mean ± S.D. Significant differences between samples were determined using the *t*-test, *P*-values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Effects of esculetin and esculin on melanogenesis

A B16F10 melanoma 4A5 cell system was used to evaluate the melanogenesis-inhibiting (*i.e.*, depigmenting) activity of esculetin and esculin, two bioactive compounds purified from FR extract. Rucinol and KA were used as positive controls. Melanin production was stimulated by incubating the cells with α-MSH; results from the assay are summarized in Table 1. Esculetin, which lacks a glucose group on the coumarin backbone, exhibited strong anti-melanogenic activity with an IC_{50} of 2.8 μM, making it over six times more potent than rucinol, and 180 times more potent than kojic acid (KA). Incontrast, esculin—in which glucose masks the free -OH group—did not inhibit melanogenesis.

Lipophilicity with respect to glucose was a significant factor in the depigmenting ability of coumarin compounds; the Clog*P* value indicated that anti-melanogenic activity was directly proportional to lipophilicity (esculetin: IC_{50} = 2.8 μM, Clog*P* = 1.214; esculin: IC_{50} = 326 μM, Clog*P* = −0.731).

Table 2. Inhibitory Effects of Various Compounds on Mushroom Tyrosinase Activity

Compound	Mushroom tyrosinase IC_{50} (μM)[a]
esculetin	40
esculin	> 500
KA	30

[a]Concentration at which 50% of activity is inhibited; values were determined from log concentration-inhibition curves and represent the means of three experiments.

3.2. Effects of esculetin and esculin on mushroom tyrosinase activity

Melanin is derived from the precursor dopaquinone, which is formed by tyrosinase oxidation of L-tyrosine; tyrosinase activity is the rate-limiting step in melanin biosynthesis. Esculetin had an inhibitory effect comparable to KA on mushroom tyrosinase activity (IC_{50} = 40 μM; Table 2)[14], while esculin had no effect on activity (IC_{50} > 500 μM).

Taken together, these data confirmed the presence of two anti-melanogenic compounds in FR extracts, namely, esculetin, and esculin. The depigmenting ability of these compounds was evaluated by measuring their melanogenesis- and tyrosinase-inhibiting activities. The results demonstrate that esculetin has strong depigmenting activity, suggesting that it could serve as an effective, naturally occurring skin-lightening agent.

3.3. Tyrosinase modeling study

To elucidate the binding modes of esculetin and KA with mushroom tyrosinase, docking studies were performed using Surflex-Dock[17] v.2.51 in SYBYL-X-1.2 (Tripos, L.P., St. Louis, USA). The crystal structure of the oxy form of tyrosinase was downloaded from the Protein Data Bank (PDB code 1wx2)[18]. The caddie protein (ORF378) and water molecules were removed, and hydrogen atoms were added using the SYBYL software. The docked conformations with the highest docking scores were selected for binding mode analyses. The binding modes of esculetin and KA were similar to

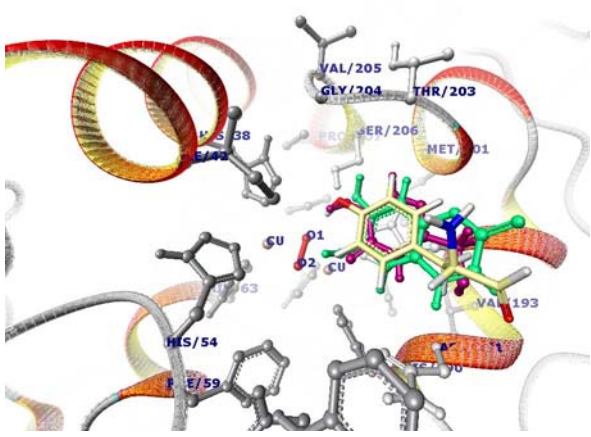

Figure 4. Docking pose of esculetin (green sticks) and KA (yellow sticks), with superimposed L-tyrosine (purple sticks) from Tyr98 of the caddie protein (ORF378).

Table 3. Docking Scores and Contributions of Polar Interactions in a Docking Model of Mushroom Tyrosinase

Compound	Surflex-Dock score[a]	Polar contribution
esculetin	4.19	1.99
KA	4.67	1.83

[a]Represents the binding affinity expressed in units of $-\log K_d$

that of a superimposed L-tyrosine from Tyr98 of the caddie protein (ORF378), as shown in Figure 4.

An interaction was observed between the oxygen atoms in both of the docked ligands and the copper atom (Cu in Figure 4) located within a distance of 3 Å. Moreover, both docked conformations engaged in hydrogen (H)-bonding with peroxide in the active site (Figure 5). In the binding pocket, H-bonding occurred between both docked ligands and Ser206. Specific H-bonding with Asn191 was found in the docked conformation of KA, while H-bonding with Thr203 was found in the docked conformation of esculetin. Esculetin and KA had similar polar interactions with tyrosinase (Table 3); however, esculetin had more H-bonding interactions than KA, specifically with peroxide. The docking scores concurred with the observed *in vitro* data. In addition, a π-πinteraction between both ligands and His194 was observed, and the positions of the aromatic rings in esculetin and

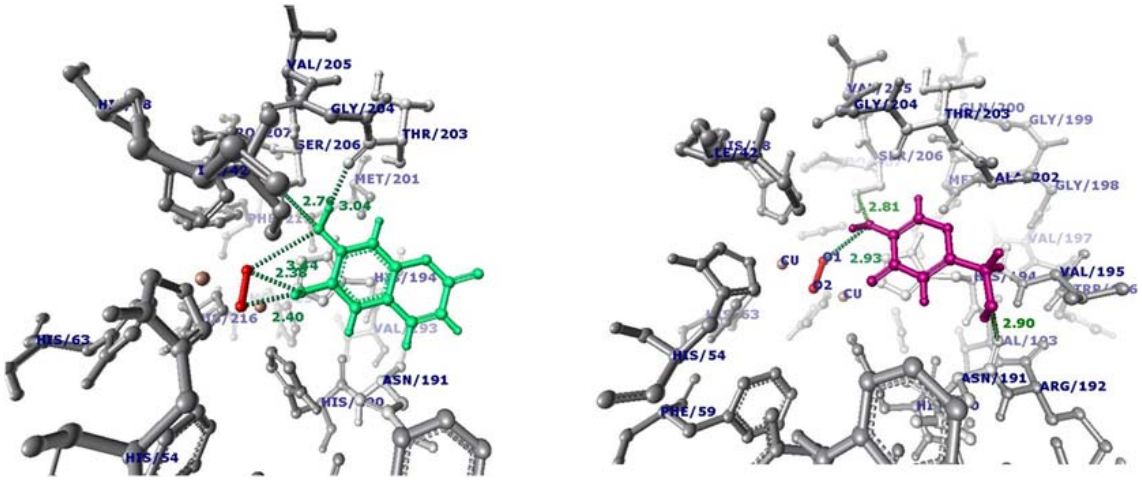

Figure 5. H-bonding between residues in the tyrosinase binding pocket, and esculetin (left, green sticks) and KA (right, purple sticks).

KA overlapped with those of L-tyrosine.

In summary, we found potent anti-melanogenic from medicinal herb, *Fraxinus rhynchophylla Hance*, and isolated to two major bioactive components, esculetin and esculin. Esculetin substantially inhibited melanogenesis in B16F10 melanoma cells, with an IC_{50} value of 2.8 μM (kojic acid 500 μM and rucinol 16 μM). Moreover, esculetin also observed inhibiting mushroom tyrosinase activity, with an IC_{50} value of 40 μM (kojic acid 10 μM). As results, we suggest that *Fraxinus rhynchophylla Hance* containing esculetin could serve as an effective skin-lightening agent.

Acknowledgments

The tyrosinase modeling study was supported by the Department of Chemistry at Hangyang University (Seoul, Korea).

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