



Spinosin is a flavonoid in the seed of *Ziziphus jujuba* that prevents skin pigmentation in a human skin model



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ABSTRACT

Because tyrosinase catalyzes essential steps in melanin synthesis, tyrosinase inhibitors are widely used for skin-lightening. To reduce the side effects of synthetic compounds, safer tyrosinase inhibitors isolated from natural products have gained attention. Here, we examined the anti-melanogenic effect of the seed of *Ziziphus jujuba* and identified eight major compounds, of which spinosin exhibited the strongest tyrosinase inhibitory activity ($IC_{50} = 47 \mu\text{M}$). When further examined, spinosin suppressed αMSH - or UVB-induced melanogenesis in B16F10 cells without cytotoxicity. The anti-melanogenic effect was also shown in a human skin model. As an underlying mechanism, *in silico* analysis showed that spinosin may bind to and suppress tyrosinase activity by forming multiple hydrogen bonds and hydrophobic interactions with the binding pocket of tyrosinase. Lineweaver-Burk and Dixon plots further revealed that spinosin acts as a competitive inhibitor of tyrosinase (V_{max} , 11.61×10^3 , 11.63×10^3 , and 11.62×10^3 , K_m , 0.139, 0.185, and 0.239 for the con, 10 μM , 50 μM of spinosin, respectively). In conclusion, spinosin, a major compound in the seed of *Ziziphus jujuba*, could be a novel additive for treating pigmentation disorders or skin-lightening cosmetics.

1. Introduction

The skin is a protective barrier against irradiation of ultraviolet (UV) and other environmental pollutants. Although melanin synthesis is a protective mechanism against these harmful factors, uncontrolled melanin accumulation causes pigmentation diseases including melisma, freckles, and senile lentigines (Briganti, Camera, & Picardo, 2003). Furthermore, abnormal changes in skin color cause serious cosmetic problems and eventually degrade the quality of life. Thus, the prevention of abnormal melanogenesis has received great attention for the development of skin-whitening cosmetics (Kang et al., 2015; Lee et al., 2015, 2016).

The melanocyte is a primary cell type that synthesizes melanin in the dermis of the skin. Skin pigmentation occurs by transferring the synthesized melanin to keratinocytes by the dendrites in the melanocytes. Melanin is synthesized from L-tyrosine through hydroxylation and oxidation processes by a copper-containing enzyme called tyrosinase. Tyrosinase catalyzes two rate-limiting steps in melanin synthesis; the monophenolase hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA), and the oxidation of L-DOPA to dopaquinone (Kubo et al., 2000; Olivares, Garcia-Borrón, & Solano, 2002). Therefore, tyrosinase inhibition is an effective strategy to suppress melanin

synthesis and hyperpigmentation. However, cytotoxicity and lack of stability and selectivity have limited the use of many tyrosinase inhibitors in the field of medical products and cosmetics. Thus, numerous efforts have been made to develop more effective tyrosinase inhibitors with no cytotoxicity and improved stability and selectivity.

Ziziphus jujuba Mill. is widely distributed in China, Korea, Europe, southern and eastern Asia, and Australia (Gao, Wu, & Wang, 2013). The fruits and seed of *Ziziphus jujuba* have been popularly used as food and traditional medicine due to their high nutritional values. However, its effect on melanogenesis needs to be further elucidated. Here, we investigated the anti-tyrosinase activity of the seed of *Ziziphus jujuba* and identified spinosin as a major compound that exhibited the strongest inhibitory activity against tyrosinase. We further tested its anti-melanogenic effects using *in silico* analysis, B16F10 cells, and 3D human skin model.

2. Materials and methods

2.1. Preparation of the water and ethanol extract of the seed of *Ziziphus jujuba*

The seed of *Ziziphus jujuba* was purchased from Yeongcheon

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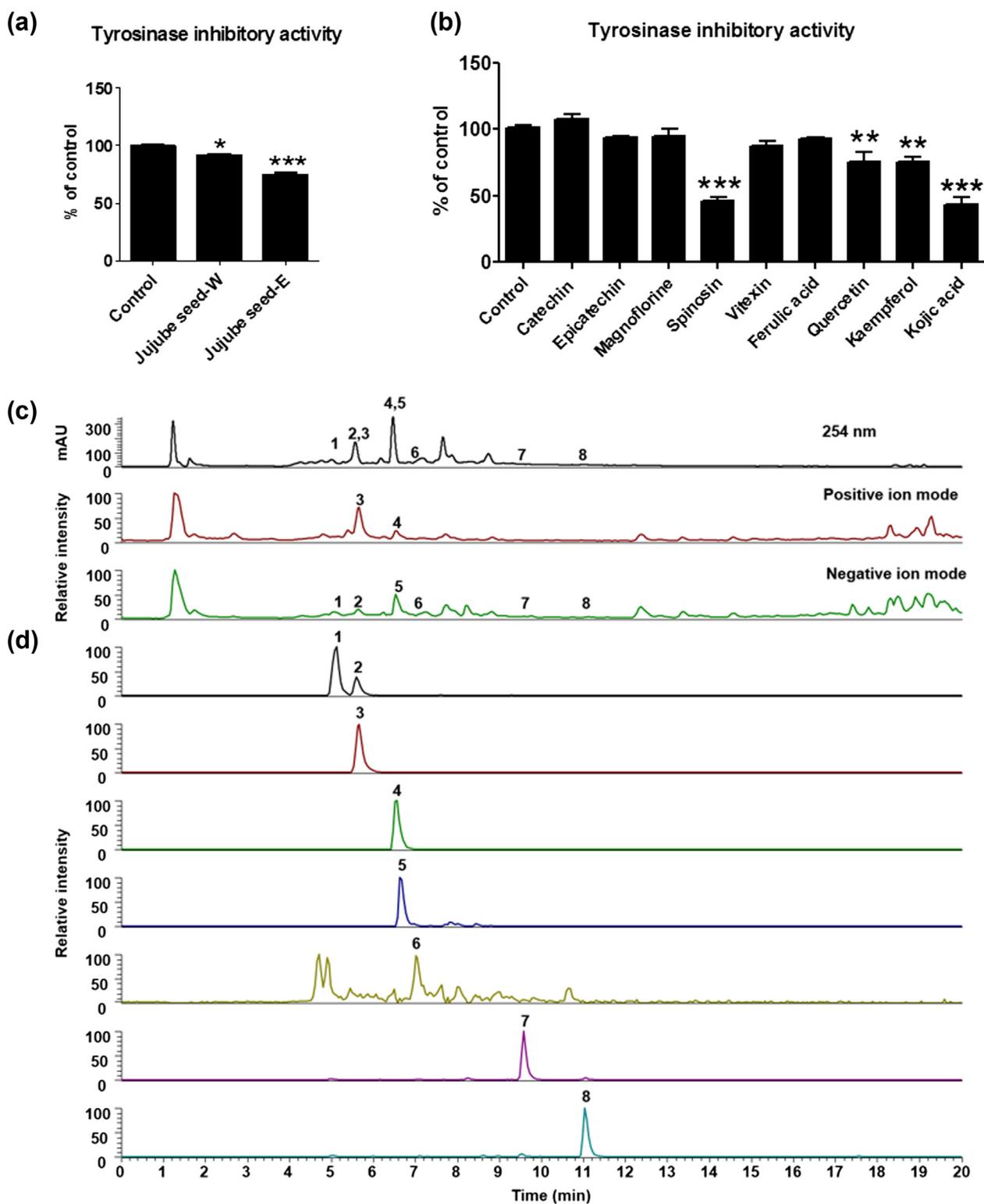


Fig. 1. UPLC-DAD-MS of identified phytochemicals in the ethanol extract of the seed of *Ziziphus jujuba*. (a) Tyrosinase activity assay was performed in test tubes using the water and ethanol extract of the seed of *Ziziphus jujuba*. (b) Tyrosinase activity assay was performed in test tubes using the major compounds in the ethanol extract of the seed of *Ziziphus jujuba*. (c) UPLC-UV and total ion chromatogram of the ethanol extract of the seed of *Ziziphus jujuba*. (d) The ion chromatogram of the identified compounds from the ethanol extract of the seed of *Ziziphus jujuba*. Catechin (1), epicatechin (2), magnoflorine (3), spinosin (4), vitexin (5), ferulic acid (6), quercetin (7), kaempferol (8).

Oriental Herbal Market (Yeongcheon, Korea) and verified by Professor Ki Hwan Bae in the College of Pharmacy at Chungnam National University (Daejeon, Korea). The seed of *Ziziphus jujuba* was stored in the herbal bank of Korean Medicine Application Center (Daegu, Korea). For the water extraction, the dried seed of *Ziziphus jujuba* (50 g) was

placed in 1 L D.W and heat-extracted at 100 °C for 3 h. The extract powder was filtered through a testing sieve (150 μm, Retsch, Haan, Germany), freeze-dried, and stored in desiccators at −20 °C prior to use. For the ethanol extraction, the dried and fragmented seed of *Ziziphus jujuba* (50 g) was mixed with 300 ml of 70% ethanol for 24 h in

Table 1Phytochemicals identified in *Zizyphus jujuba* by ultra-performance liquid chromatography-ultraviolet detector-mass spectrometry.

Compound ^a	R _t (min)	Chemical formula	Adduct	Calculated (m/z)	Measured (m/z)	Error (ppm)	MS/MS fragments (m/z)
1 Catechin	5.09	C ₁₅ H ₁₄ O ₆	[M–H] [–]	289.0718	289.0714	–1.312	289.0713, 245.0813, 137.0230
2 Epicatechin	5.60	C ₁₅ H ₁₄ O ₆	[M–H] [–]	289.0718	289.0714	–1.312	289.0713, 245.0811, 137.0230
3 Magnoflorine	5.67	C ₂₀ H ₂₄ NO ₄	[M] ⁺	342.1700	342.1693	–1.864	342.1696, 297.1117, 256.0855
4 Spinosin	6.57	C ₂₈ H ₃₂ O ₁₅	[M+H] ⁺	609.1814	609.1806	–1.308	351.0859, 237.0858, 297.0754
5 Vitexin	6.68	C ₂₁ H ₂₀ O ₁₀	[M–H] [–]	431.0984	431.0978	–1.443	431.0978, 341.0658, 311.0557
6 Ferulic acid	7.06	C ₁₀ H ₁₀ O ₄	[M–H] [–]	193.0506	193.0497	–4.907	178.0259, 165.0544, 134.0358
7 Quercetin	9.59	C ₁₅ H ₁₀ O ₇	[M–H] [–]	301.0354	301.0349	–1.453	301.0349, 178.09975, 151.0023
8 Kaempferol	11.03	C ₁₅ H ₁₀ O ₆	[M–H] [–]	285.0405	285.0400	–1.477	285.0399, 255.0283, 239.0766

^a Compared with the retention time (R_t) and MS spectral data of an authentic standards.

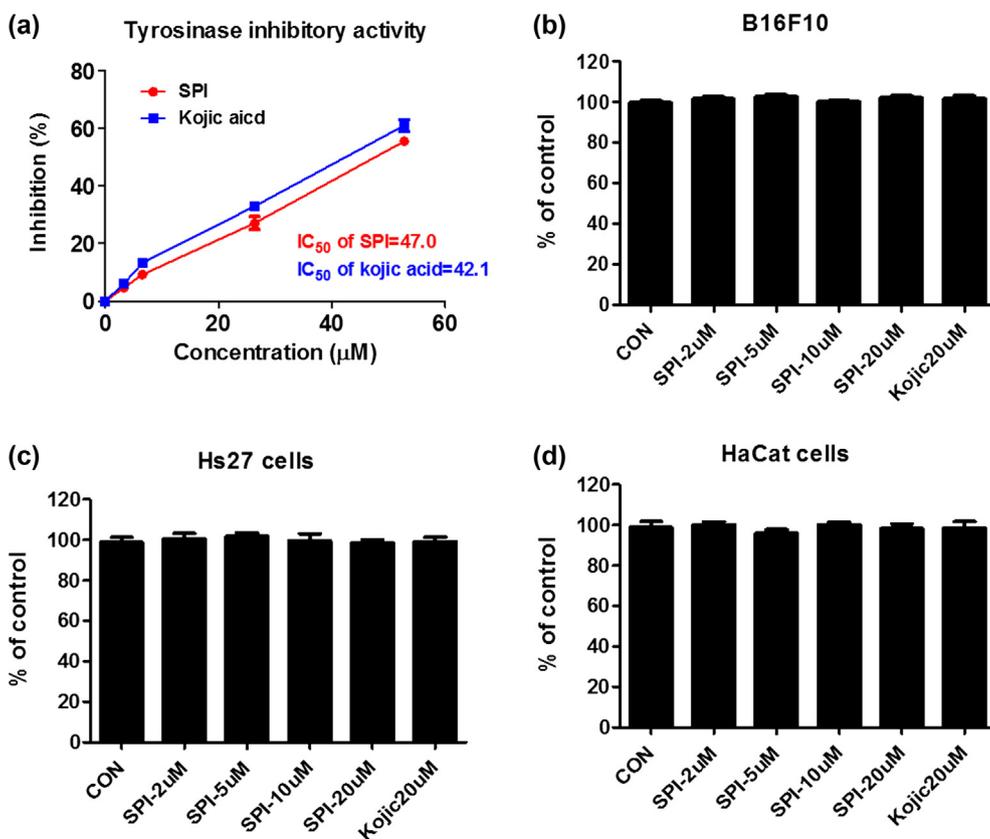


Fig. 2. IC₅₀ and cellular toxicity of spinosin. (a) Tyrosinase activity was measured in test tubes using L-tyrosine and mushroom tyrosinase. Based on this, the IC₅₀ values of kojic acid and spinosin were calculated. (b) B16F10 (mouse melanoma), (c) Hs27 (human fibroblast), or (d) HaCat (human keratinocyte) cells were treated with various concentrations of spinosin for 48 h. MTT assay was performed (n = 4/group) to measure cellular toxicity of spinosin.

a shaking incubator (100 rpm, 40 °C). The extract was subjected to filtration by 150 mm filter paper (Whatman, Piscataway, NJ, USA) and concentration through a rotary vacuum evaporator (Buchi, Tokyo, Japan). The extract was freeze-dried and stored in desiccators at 4 °C before use. The extract powder was dissolved in D.W. and centrifuged at 14,000 rpm for 10 min, and the resulting supernatant was filtered (0.2-µm pore size) and stored at 4 °C until analysis.

2.2. Tyrosinase activity measurement using mushroom tyrosinase

Tyrosinase activities of natural compounds were evaluated using a previously described method (Lee et al., 2017; Moon et al., 2018). Spinosin was purchased from ChemFaces (CFN99600, Purity ≥ 98%). Spinosin or kojic acid (50 µM) was loaded onto a 96-well microplate (Nunc, Denmark) in tyrosinase buffer (200 µL) containing mushroom tyrosinase (1000 U), 1 mM L-tyrosine solution, and 50 mM phosphate buffer (pH 6.5). The plate was incubated at 37 °C for 15 min and dopaquinone was evaluated by spectrophotometry (450 nm). Based on the measurement, the IC₅₀ was calculated using log-linear curves and their equations.

2.3. Identification of phytochemical constituents

The standard compounds derived from the seed of *Zizyphus jujuba* were purchased from ChemFaces Biochemical. The Dionex UltiMate 3000 system (Dionex Corp., Sunnyvale, CA, USA) equipped with Thermo Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) was used to analyze the phytochemical constituents of the seed of *Zizyphus jujuba*. The ethanol extract of the seed of *Zizyphus jujube* was separated on a Waters Acquity UPLC BEH C₁₈ analytical column (2.1 × 100 mm, 1.7 µm) and mobile phase consisted 0.1% formic acid in water (A, v/v) and acetonitrile (B). Gradient elution was set as following: 3.0% B, 0.0–1.0 min; 3.0–15.0% B, 1.0–2.0 min; 15.0–50.0% B, 2.0–13.0 min; 50–100% B, 13.0–20.0 min; 100–100% B, 20.0–23.0 min; 3–3% B, 23.0–27.5 min with the flow rate at 0.25 mL/min. UV chromatogram acquired at a wavelength of 320 nm. The operation state in the mass spectrometry were programmed as follows: ionization mode, negative and positive ion-switching mode; spray voltage, 3.8 kV; capillary temperature, 320 °C; sheath gas pressure, 40 arbitrary units; auxiliary gas pressure, 10 arbitrary units; ion scans, 100–1500 m/z; resolution of MS scans, 70,000. The samples were solubilized in methanol, filtered through a 0.22 µm filter membrane, and analyzed by ultra-performance

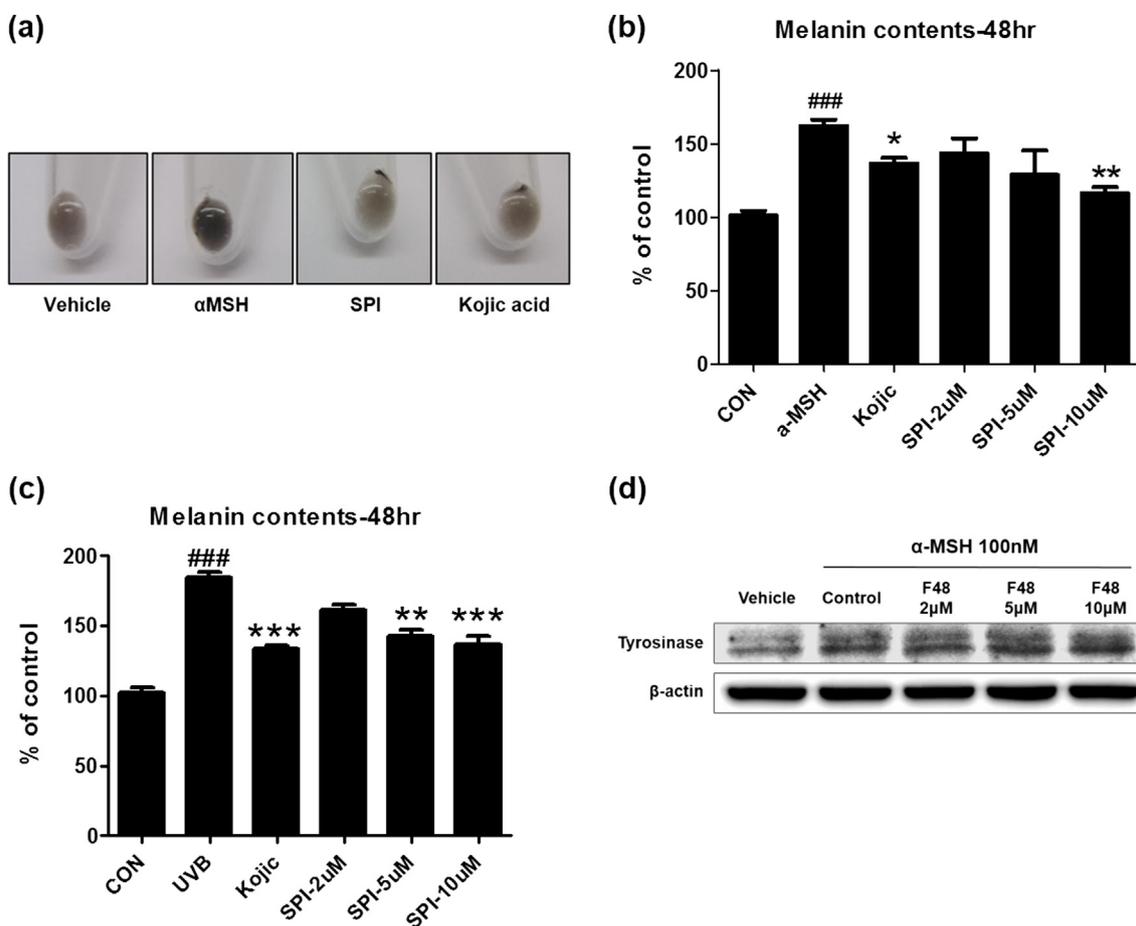


Fig. 3. Anti-melanogenic effect of spinosin on UVB or α MSH-treated B16F10 cells. B16F10 melanoma cells were pretreated with spinosin or kojic acid for 1 h and the cells were stimulated with (a–b) α MSH (500 nM) or (c) UVB (8 mJ) for additional 48 h ($n = 4$ /group) with spinosin or kojic acid treatment. Melanin concentrations were determined using a microplate reader (405 nm). (d) Representative western blotting images of tyrosinase ($n = 3$). Each value was expressed as mean \pm SEM. Data were analyzed using one-way ANOVA followed by Bonferroni test. ^{###} $p < 0.001$ vs. a control group without α MSH or UVB exposure; ^{*} $p < 0.05$, ^{**} $p < 0.01$ and ^{***} $p < 0.001$ vs. the α MSH or UVB-exposed group.

liquid chromatography-ultraviolet detector-mass spectrometry (UPLC-UV-MS).

2.4. Protein-ligand docking simulation

AutoDock Vina was used for the *in silico* protein-ligand docking simulation (Lee et al., 2017). The crystal structure of *Agaricus bisporus* (PDB ID: 2Y9X) was used to obtain the three-dimensional structure of tyrosinase. The predefined binding site of tyrosine was applied as a docking pocket. After docking simulations between tyrosinase and spinosin or kojic acid, the LigandScout 3.0 software was used to predict binding residues between different compounds and tyrosinase.

2.5. The analysis of enzyme kinetics

L-DOPA was prepared at concentrations of 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 mM, and spinosin was prepared at 0, 10, and 50 μ M. The reaction mixture solution was prepared in a 96-well plate, in which 20 μ L of tyrosinase substrate (L-DOPA), 10 μ L of an aqueous mushroom tyrosinase solution (200 U), and 50 mM potassium phosphate buffer (pH 6.5) were included. The dopachrome production rate of the reaction mixture was measured at a wavelength of 450 nm using a microplate reader. The tyrosinase inhibition mode of spinosin was then calculated using a Lineweaver-Burk and Dixon plot analyses. The Michaelis constant (K_m) and maximal velocity (V_{max}) were also calculated by Lineweaver-Burk plots with different concentrations of L-DOPA

substrate (Kang et al., 2015).

2.6. Cell culture and viability assay

B16F10 melanoma cells were purchased from the Korea Cell Line Bank. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS), and 1% penicillin, streptomycin, L-glutamine, and sodium pyruvate. The cells were maintained at 37 °C in a humidified 95% air/5% CO₂ atmosphere. For the cell viability assay, B16F10 melanoma cells were seeded in 96-well plates. The cells were treated with spinosin at various concentrations for 48 h. Ez-Cytox (10 μ L) was added to each well and incubated for 2 h. The formazan crystals formed was measured by spectrophotometry at 450 nm. Cell viability was shown as the percentage of the control group without spinosin treatment.

2.7. Melanin content in B16F10 cells

Melanin level was measured using the method previously described with slight modifications (Lee et al., 2016). B16F10 cells were allowed to grow to 70–80% confluence in 6-well plates. The cells were pretreated with spinosin or kojic acid for 2 h. Then, the cells were treated with α MSH or UVB and incubated for 48 h. After washing with PBS, the cells were detached using trypsin and dissolved in 90 μ L of 1 N NaOH solution containing DMSO (5%). After incubation at 60 °C for 1 h, melanin content was determined by measuring absorbance at 405 nm.

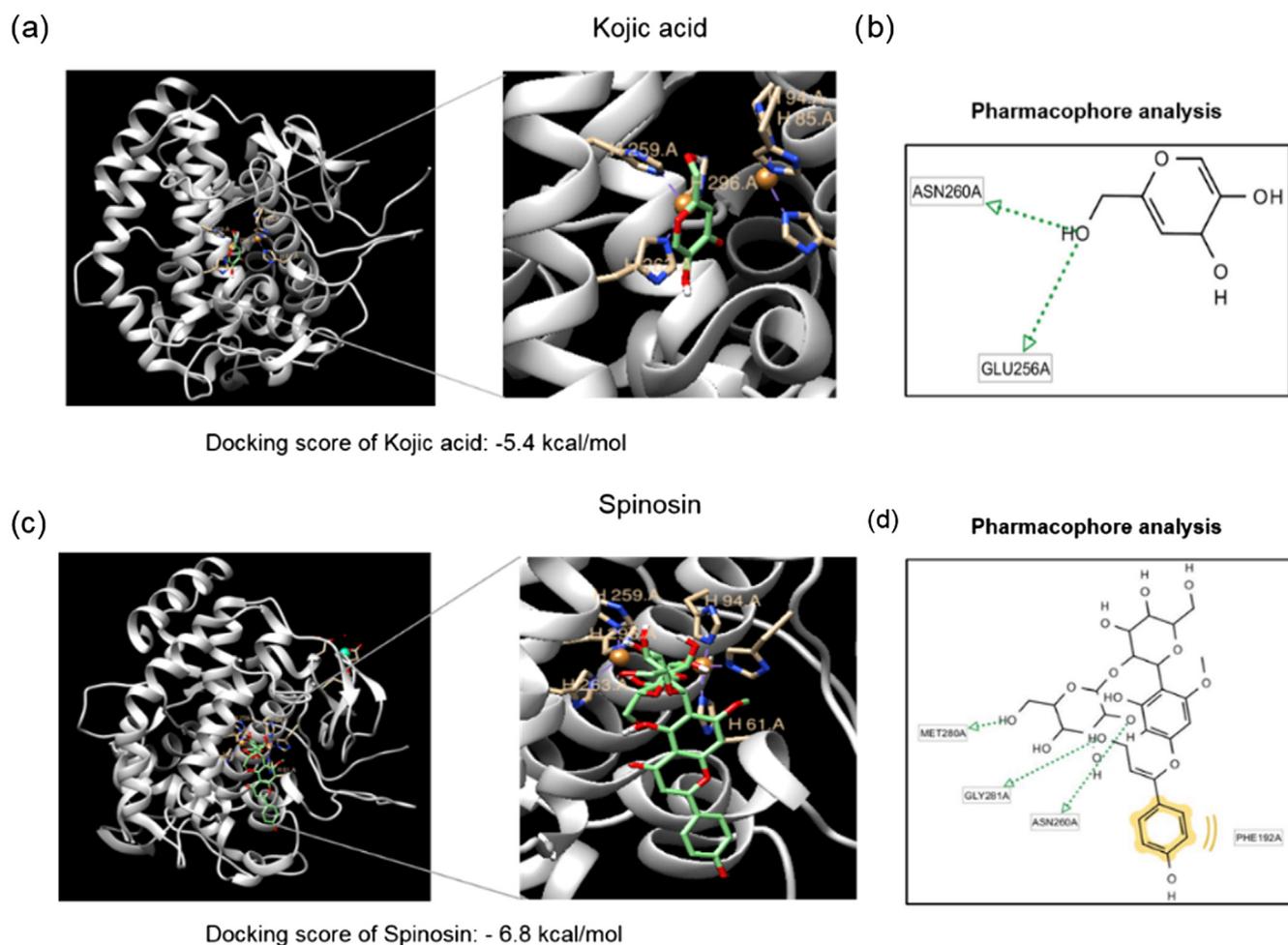


Fig. 4. Spinosin may bind to and inhibit tyrosinase. Protein docking simulation was performed using AutoDock Vina to predict binding affinity of (a) kojic acid or (c) spinosin with tyrosinase (*Agaricus bisporus*, PDB ID: 2Y9X). LigandScout 3.1 software was used to analyze binding residues of tyrosinase that interact with (b) kojic acid or (d) spinosin.

2.8. The measurement of melanogenesis in a human skin model

A viable, reconstituted, three-dimensional human epidermis (Neoderm-ME, Tego Science) was used to examine the anti-melanogenic effect of spinosin in a human skin model. The human skin model was pretreated with DMSO (vehicle) or spinosin for 1 h and cultured in the maintenance media provided by the company for 5 d with DMSO or spinosin treatment. Microscopic analysis was performed at day 1 to day 5 to observe skin pigmentation. The microscopic images were analyzed by Image J software to semi-quantify the darkening of the skin. For the Fontana-Masson staining, skin samples were fixed in 4% paraformaldehyde overnight at room temperature and the samples were analyzed by a commercially available company (Garam Meditech, South Korea).

2.9. Statistical analysis

All data are expressed as mean \pm SEM. Experimental groups were compared using one-way analysis of variance followed by the Bonferroni post-test. $P < 0.05$ was considered statistically significant.

3. Result and discussion

3.1. Spinosin is a compound in the seed of *Ziziphus jujuba* that exhibits the strongest tyrosinase inhibitory activity

To examine the anti-melanogenic activity of the seed of *Ziziphus jujuba*, tyrosinase activity was measured using the water and ethanol extract of the seed of *Ziziphus jujuba*. Compared to the control group, the water and ethanol extract inhibited mushroom tyrosinase activity by 8.2 and 23.2% respectively (Fig. 1a). Thus, we further analyzed the ethanol extract by UPLC-DAD-MS to identify major compounds that showed the tyrosinase inhibitory activity. One alkaloid (i.e., magnoflorine), six flavonoids (i.e., catechin, epicatechin, kaempferol, quercetin, spinosin, vitexin), and one phenylpropanoid (i.e., ferulic acid) were identified from the ethanol extract in comparison with retention time and mass spectrum of authentic standards (Fig. 1c–d and Table 1). The retention time of catechin, epicatechin, magnoflorine, spinosin, vitexin, ferulic acid, quercetin, and kaempferol were 5.09, 5.60, 5.67, 6.57, 6.68, 7.06, 9.59, and 11.03, respectively (Fig. 1c–d), which were consistent with the previous report (Gao et al., 2013). We further tested the inhibitory effects of these compounds the data showed that spinosin, quercetin, kaempferol significantly inhibited tyrosinase activity, of which spinosin exhibited the strongest inhibitory activity (Fig. 1b). Therefore, we focused on spinosin for further analysis.

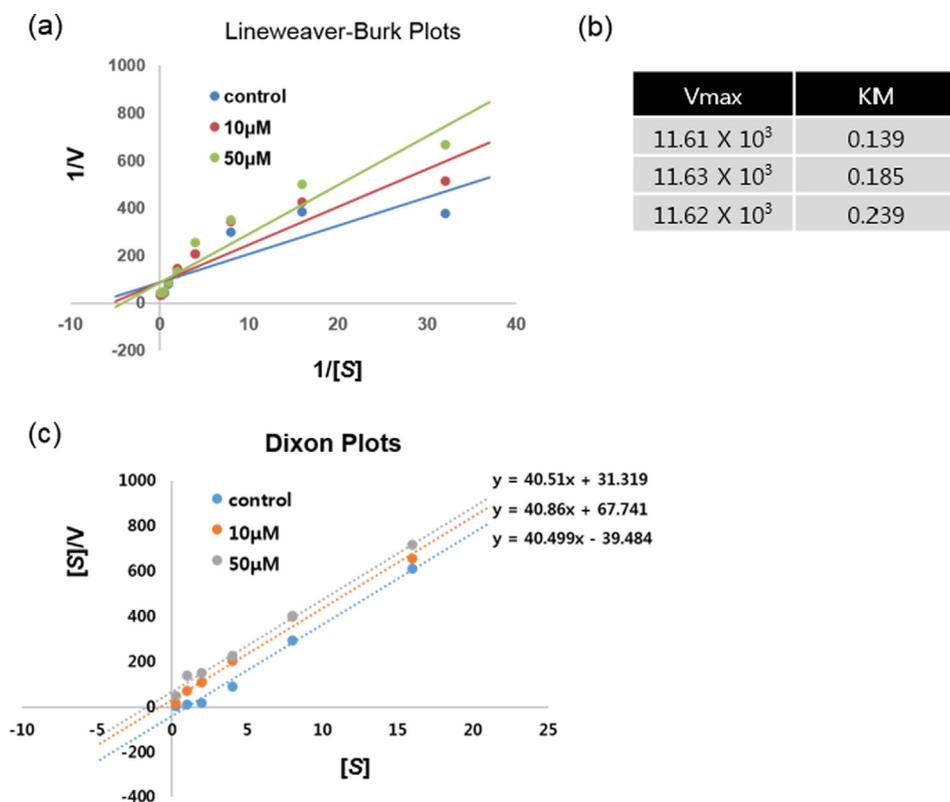


Fig. 5. Spinosin is a competitive inhibitor of tyrosinase. (a) The Lineweaver-Burk plot analysis was performed to test the mode of inhibition by spinosin. (b) Km is the Michaelis–Menten constant and Vmax is the maximum reaction velocity based on Lineweaver–Burk plot analysis. (c) The Dixon plot analysis was performed to confirm the mode of inhibition by spinosin. [S] represents the substrate of tyrosinase (L-DOPA).

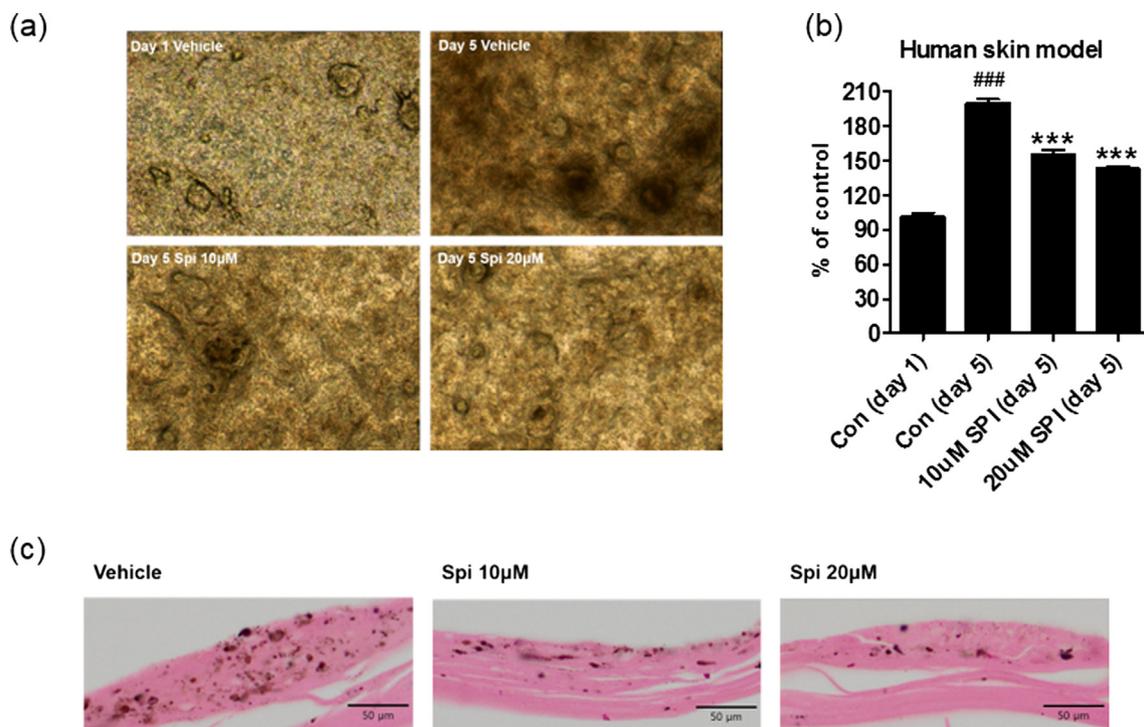


Fig. 6. Spinosin blocks melanin accumulation in a human skin model. A viable, reconstituted, three-dimensional human epidermis (Tego Science) was used to examine the anti-melanogenic effect of spinosin. The human skin model was pretreated with DMSO (vehicle) or spinosin for 1 h and cultured in the maintenance media provided by the company for 5 d with DMSO or spinosin treatment. (a) Representative microscopic images of the human skin with or without spinosin treatment. (b) The darkness of the skin was analyzed by Image J software. (c) Fontana-Masson staining showing the epidermis of human skin section with or without spinosin treatment. Each value was expressed as mean \pm SEM. Data were analyzed using one-way ANOVA followed by Bonferroni test. ### p < 0.001 vs. the day 1 group without any treatment, *** p < 0.001 vs. the day 5 group (control treated with DMSO).

3.2. Spinosin is a tyrosinase inhibitor without cellular toxicity

We further tested a concentration-dependent inhibitory effect of spinosin on tyrosinase to calculate IC_{50} . When comparing with kojic acid ($IC_{50} = 42.1$), a known tyrosinase inhibitor, spinosin exhibited a strong inhibitory effect on tyrosinase ($IC_{50} = 47.0$) (Fig. 2a). Before applying spinosin to cell models, we examined the cytotoxicity of spinosin using multiple cell lines including HaCat (human keratinocyte), B16F10 (mouse melanoma), and Hs27 (human fibroblast). Spinosin did not show cytotoxicity in these cell lines up to 20 μM (Fig. 2b–d).

3.3. Spinosin inhibits melanin accumulation in B16F10 cells

We investigated the anti-melanogenic effect of spinosin using B16F10 cells pre-treated with kojic acid or spinosin followed by α MSH or UVB exposure, the strongest melanogenic inducers. When the cells were stimulated with α MSH, melanin accumulation was notably elevated (Fig. 3a–b). Spinosin treatment decreased cellular melanin content in a dose-dependent manner (Fig. 3b) and the anti-melanogenic effect was stronger than that of kojic acid (Fig. 3b). Consistently, the pictures of the cell pellets indicated the strong inhibitory activity of spinosin on α MSH-induced melanogenesis (Fig. 3a). We further examined the inhibitory effect of spinosin on UVB-induced melanogenesis and the results showed that spinosin also decreased UVB-induced melanin accumulation in a dose-dependent manner (Fig. 3c). To study mechanisms underlying the spinosin-mediated anti-melanogenic effect, we performed western blotting to measure the protein level of tyrosinase. As expected, α MSH increased protein level of tyrosinase; however, spinosin showed no effect on the protein level (Fig. 3d). Together, these data indicate that spinosin is a strong anti-melanogenic compound without affecting the protein level of tyrosinase.

3.4. Spinosin may bind to and inactivate tyrosinase

Because spinosin showed no effect on the protein level of tyrosinase, we hypothesized that spinosin binds to and suppresses tyrosinase activity. Protein docking simulation was performed using AutoDock Vina to predict binding affinity. The 3D structure of the tyrosinase (*Agaricus bisporus*) was obtained from the protein data bank (PDB ID: 2Y9X). The binding affinity of kojic acid to tyrosinase was -5.4 kcal/mol, whereas that of spinosin was -6.8 kcal/mol (Fig. 4a–c), indicating that spinosin may bind to tyrosinase with stronger affinity than kojic acid does. To examine the binding residues of tyrosinase that interact with spinosin or kojic acid, LigandScout 3.1 software was used. Kojic acid can bind to tyrosinase mainly via hydrogen bonds with the ASN260 or GLU256 residue of tyrosinase (Fig. 4b). Spinosin forms three hydrogen bonds with the MET280, GLY281, and ASN260 residues of tyrosinase and hydrophobic interaction with the PHE192 residues of tyrosinase (Fig. 4d), which likely contributes to the stronger inhibitory effect of spinosin on tyrosinase.

3.5. Spinosin is a competitive inhibitor of tyrosinase

Because a predefined binding site of tyrosine was applied as a docking pocket in the protein-ligand docking simulation (Kang et al., 2015), it is likely that spinosin may competitively bind to tyrosinase with tyrosine. We performed a Lineweaver-Burk and Dixon plot analyses (Fig. 5a–c) to examine the mode of inhibition by spinosin. As the concentration of spinosin increased, K_m values also increased while V_{max} values were unchanged (Fig. 5b). Furthermore, Dixon plot showed that the slopes ($[S]/V$) at different concentrations of spinosin are parallel (Fig. 5c). These data suggest that spinosin is a competitive inhibitor of tyrosinase.

3.6. Spinosin suppresses melanin accumulation in a human skin model

To test the anti-melanogenic effect of spinosin on a human skin model, we cultured a viable, reconstituted, three-dimensional human epidermis consisting of human melanocytes and keratinocytes (Neoderm®-ME, Tegoscience Co) that has been shown to undergo spontaneous melanogenesis with time (Lee et al., 2017; Park, Lee, Lee, Myung, & Hwang, 2016). The human skin model was pretreated with spinosin for 1 h and cultured in the maintenance media (Tegoscience Co) for 5 d. Compared to the day 1, we observed the darkening of the epidermis at day 5 and spinosin treatment significantly decreased it in a concentration-dependent manner (Fig. 6a–b). Consistently, the Fontana-Masson staining showed that melanin level in the epidermis was decreased by spinosin treatment (Fig. 6c), indicating that spinosin also blocks melanin accumulation in the human skin model.

Although spinosin is a major compound in the fruit and the seed of jujube that has been used as food and traditional medicine (Yang, Yang, Chen, Hua, & Jiang, 2013); the current study did not provide crucial evidence on the safety of spinosin for its application to human skin. Nevertheless, we showed that spinosin did not show toxicity in multiple cell lines including B16F10 (mouse melanoma), Hs27 (human fibroblast), and HaCat (human keratinocyte). Furthermore, we did not find visible signs of toxicity including cell detachment and cell debris formation based on microscopic observation when spinosin was applied to the human skin model with the concentrations that did not exhibit cellular toxicity. Because safety is an important issue for the application of many tyrosinase inhibitors to the cosmetics or medical products, *in vivo* examinations are necessary to further confirm the safety of spinosin as a skin whitening agent.

In conclusion, we identified that spinosin is a compound in the ethanol extract of the seed of *c* that showed the strongest inhibitory activity against tyrosinase. Spinosin suppressed melanin synthesis up to a satisfactory limit both in the cell and human skin models presumably by binding to and competitively inhibiting tyrosinase. Spinosin may be a novel additive to suppress skin pigmentation.

Author contribution

Lee B and Moon KM designed and performed experiments and wrote the manuscript. Hwang YH analyzed major compounds within *Ziziphus jujuba* and involved in the revision of the manuscript and Yang JH performed experiments using the 3D human skin model.

Acknowledgment

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Conflict of interest statement

There are no conflicts of interest to declare

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