Korean J. Plant Res. 29(6):690-698(2016)
https://doi.org/10.7732/kjpr.2016.29.6.690

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

In vitro Anti-Tyrosinase Activity of Viscumneoside III and Homoflavoyadorinin B Isolated from Korean Mistletoe (*Viscum album*)

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Abstract - Various bioactive substances are found in mistletoe, including viscumneoside III (1) and homoflavoyadorinin B (2), both which inhibit tyrosinase. These two compounds are mainly found in the EtOAc fraction of the mistletoe extract and demonstrate higher rates of tyrosinase inhibition than ascorbic acid, which was used as a control. Our results suggest that mistletoe extracts can be utilized in skin whitening cosmetics.

Key words - Mistletoe, Viscum album, Tyrosinase inhibitor, Viscumneoside III, Homoflavoyadorinin B

Introduction

Tyrosinase is a copper-containing enzyme that carries out the oxidation of substrate such as phenol and catechol, and is found in mammals, plants, and fungi (Van Gelder *et al*., 1997). The enzyme is involved in the first two stages of melanin biosynthesis, which hydroxylates L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and oxidizes l-tyrosine to *o*-quinone (dopaquinone). Dopaquinones involved in skin pigmentation and is converted to melanin through several steps stages (Manson, 1948). Additionally, in the food industry, tyrosinase is an important enzyme that controls the stability of fruit and vegetables (Mayer, 1986). Therefore, the development of safe and effective tyrosinase inhibitors is a goal of paramount importance in the both the food and cosmetics industries (Seo *et al*., 2003). Currently, many studies are underway to identify whitening substances that suppress the production of melanin by inhibiting tyrosinase (Loizzo *et al*., 2012) Arbutin (Sugimoto *et al.,* 2003), kojic acid (Cabanes, 1994), phenolic compounds (Alam *et al*., 2010), flavonoids (Xie *et al*., 2003), glycolic acid (Usuki *et al*., 2003), and ascorbic acid (Kojima *et al*., 1995) are the most common chemical compounds known to suppress the biosynthesis of melanin by inhibiting tyrosinase.

Many studies have been conducted to extract tyrosinase-inhibiting compounds from various natural sources (Kim *et al*., 2011). Here, we investigated the ability of the flavonoid glucoside, isolated from mistletoe extract, to act as a novel tyrosinase inhibitor.

Mistletoe (*Viscum album* var. *coloratum*) is a photosynthetic plant affiliated with the family Viscaceae, and is a hemiparasitic plant that uses the oak tree, nettle tree, chestnut tree, pear tree, elm tree, as well as others as hosts (Kang *et al*., 2012; Lee *et al*., 2015). Mistletoe has been widely used as a medicinal plant in Korea, as well as many other countries throughout East Asia and Europe. Mistletoe has been reported to have lectin (glucoprotein), viscotoxin (low molecular polypeptide), flavonoid glucoside, and oleanolic acid (triterpenoid) as active components (Fukunaga *et al*., 1989), and is also known for its various benefit including anticancer activity (Hajtó *et al*., 2003), growth promotion of immune cells (Lee *et al*., 2003), antidiabetic activity, antihypertensive activity, antibacterialactivity (Jung *et al*., 2015), and antiviral effects (Choi *et al*., 2013). Lectin from mistletoe is particularly well known in Europe to have excellent anticancer activity, which ensures that this plant is marketed as an agent with anticancer properties.

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Although previous studies have shown that mistletoe extract inhibits tyrosinase (Lee *et al*., 2011), no known active compounds have been identified. Therefore, in this study, tyrosinase-inhibiting substances in mistletoe were extracted, and their chemical structures were determined.

Materials and Methods

Reagents

The mistletoe utilized in our experiment was obtained from an oak tree in Jeongseon, Gangwon-do Province in South Korea in December of 2013, and was dried at room temperature before use. The plant was authenticated by Prof. Yi Sung Shim and Dr. Bo Duk Lee, University of Seoul, Korea. First-class reagents (SK Chemicals, Korea) were used for all solvents including methanol (MeOH), n-hexane (*n*-Hex), methylene chloride (CH_2Cl_2), ethylacetate (EtOAc), and n-butanol (*n*-BuOH). The formic acid (WAKO, Japan) and MeOH (Honeywell Burdick & Jackson, Korea) were selected for the HPLC analysis. 2,2-Diphenyl-L-picrylhydrazyl (DPPH) used for the DPPH radical scavenging activity measurement, and 3,4-dihydroxy-L-phenylalanine was used for the tyrosinase inhibitory activity measurement were both purchased from Alfa Aesar (Haverhill, MA, USA), while Mushroom Tyrosinase (5,771 unit/㎎) was bought from Sigma-Aldrich Korea.

Extraction and partition

Naturally dried mistletoe (100 g) was pulverized with a pulverizer (HMF-3250s, Hanil electric, Korea), and added to MeOH (500 ㎖) in order to perform the 3-time reflux extraction at 80ºC, for 12 h. The MeOH extract was vacuum filtered using Celite 545 (Samchun chemicals, Korea), and concentrated by a rotary vacuum evaporator (Hei-Vap Advantage Rotary evaporator, Heidolph, Germany), to obtain 22.01 g of MeOH crude extract. The crude extract was suspended in the 90% MeOH solution (200 ㎖), and added to *n*-Hex (200 ㎖) to perform the solvent fraction 3 times. The fractioned solution was vacuum concentrated to obtain 2.01 g of *n*-Hex. After removing MeOH by vacuum concentrating the MeOH/H_2O mixed solution, it was suspended in 200 ㎖ of water. The aqueous suspension was extracted three times using 200 ㎖ of CH_2Cl_2, EtOAc, and *n*-BuOH in that order. The extracts were vacuum concentrated to following amounts: *n*-Hex (2.01 g), CH_2Cl_2 (2.39 g), EtOAc (1.24 g), and *n*-BuOH (5.78 g).

HPLC analysis of EtOAc fraction

To analyze flavonoids in the EtOAc fraction, HPLC (YL-9100, Young Lin instruments, Korea) was used and conditions utilized are listed in Table 1.

Purification of EtOAc extract

The EtOAc had the highest tyrosinase inhibitory activity and DPPH radical elimination ability. Using this fraction, a silica gel 60 (230-400 mesh, Merck) column chromatography (2.5×24 ㎝) was run to obtain three fractions (Fraction 1; 90.2 mg, R_f = 0.75, Fraction 2; 50 mg, R_f = 0.5, Fraction 3; 263.6 mg, R_f = 0.12, developing solvents MeOH/CH_2Cl_2 = 1/5). The mixture was then separated into the active compounds viscumneoside III (**1**, 20.3 ㎎), and homoflavoyadorinin B (**2**, 35.4 ㎎) using preparative HPLC (YL-9100, Young Lin instruments, Korea).

Structure analysis

To confirm the chemical structure of the compounds separated with the Prep-HPLC, a 400 MHz FT-NMR spectrometer (Bruker Advance 400 MHz spectrometer,

Table 1. Parameters used for HPLC

Instrument	YL-9100 (Young Lin instruments, Korea)
Column	TC-C18 (250 ㎜ × 4.6 ㎜, particle size 5 ㎛, Agilent, USA)
Flow rate	0.8 ㎖/min
Column Temperature	30°C
Detection	UV 270 ㎚
Solvent	0.1% formic acid (A), MeOH (B)
Gradient elution	0-20 min, 40-52% B; 20-0 min, 52-80% B; 5 min, 40% B

Table 2. Parameters used for Prep-HPLC

Instrument	YL-9100s (Young Lin instruments, Korea)
Column	Prep-C18(21.2 ㎝ × 4.6 ㎜, particle size 10 ㎛, Agilent, USA)
Flow rate	8.5 ㎖/min
Detection	UV 270 ㎚
Solvent	0.1% formic acid (A), MeOH (B)
Gradient elution	0–20 min, 40–52% B; 20–0 min, 52–80% B; 5 min, 40% B

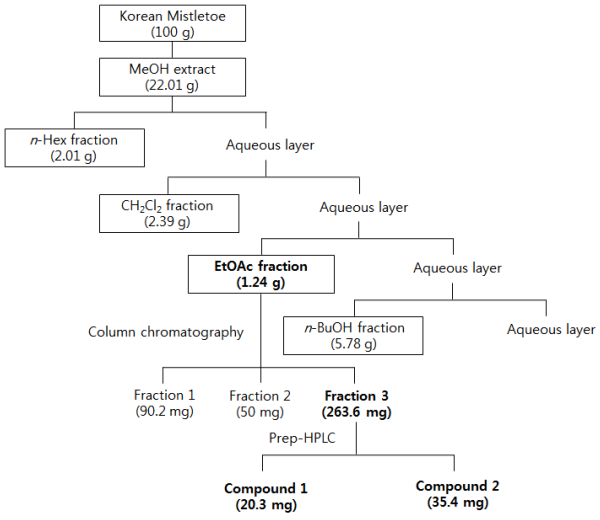

Fig. 1. Isolation and purification scheme for compounds **1** and **2** from the mistletoe MeOH extract.

Germany) was used. Both CD_3OD and DMSO-d_6 were used as solvents, while tetramethylsilane (TMS) was used as an internal standard, which recorded δ (ppm).

Assay of DPPH radical-scavenging activity

To confirm the antioxidative activity of the separated substances, DPPH was used to measure the radical scavenging ability (Blois, 1958). DPPH was dissolved in 100% EtOH, and stirred for 2 h to produce 0.15 mM DPPH solution. Onto a 96-well plate, 150 ㎕ of 0.15 mM DPPH solution, and 50 ㎕ of the sample at concentrations ranging from 0.1-5 ㎎/㎖ were added, and stirred for 30 min in a dark room. The Versamax Absorbance Microplate Reader (Molecular Devices LLC, USA) was used to measure absorbance at 532 ㎚, with ascorbic acid as the control. The DPPH radical scavenging ability was calculated using the following formula:

DPPH radical scavenging ability (%)
= {1-(A sample/A control)} × 100

Assay of mushroom tyrosinase inhibitory activity

To confirm the tyrosinase inhibitory activity of the samples, the DOPA chrome created from the tyrosinase reaction was measured using the colorimetric method (Jung *et al*., 1995). Specifically, 40 ㎕ 0.1 M phosphate buffer (pH 6.8), and 40 ㎕ 5 mM L-DOPA solution were added to a 96-well plate. After that, 100 ㎕ of viscumneoside III, and homoflavoyadorinin B at concentrations ranging from 0.1-5.0 ㎎/㎖ were added to the solution. After that, 20 ㎕ tyrosinase (250 unit/㎖) was added, and allowed to react for 10 min at 35°C, after which the absorbance was measured at 475 ㎚ using the Versamax Absorbance Microplate Reader (Molecular Devices LLC, USA). Ascorbic acid was used as the control. Tyrosinase inhibition was calculated using the formula:

Tyrosinase inhibition (%) = {1-(A sample/A control)} × 100

Statistical analysis

All measured values are displayed as the mean ± SD of more at least 3 replicates. The statistical analyses were conducted using IBM SPSS 22.0. The comparison between different cells was first completed using a one-way ANOVA, after which the Dunnett test was also conducted, with significant values considered at $p < 0.05$.

Results and Discussion

Antioxidative activity of solvent-fractionated layers

Although many studies have reported the antioxidative

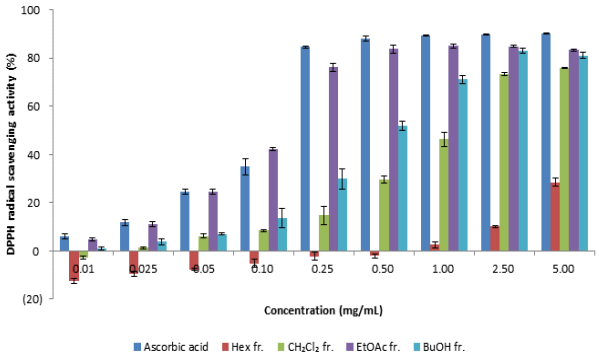

Fig. 2. DPPH radical-scavenging activities of various fractions by solvent extraction.

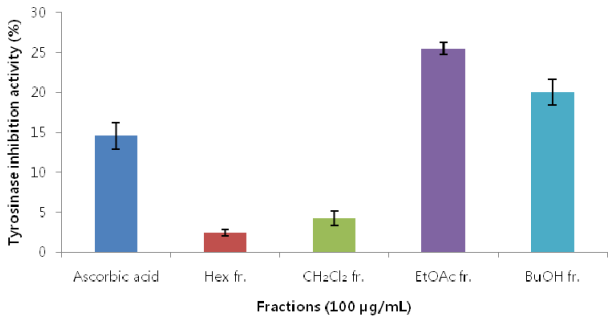

Fig. 4. Effect of total methanol extract and each subsequent fraction of Korean Mistletoe extract on tyrosinase inhibitory activity.

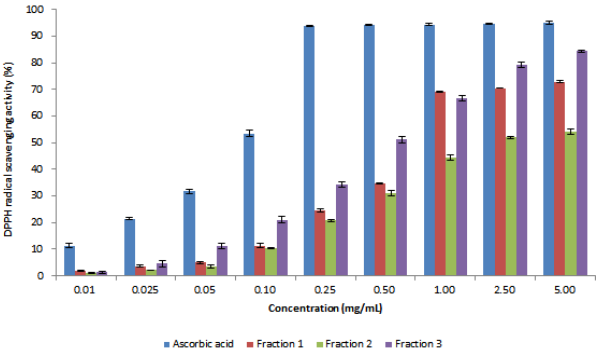

Fig. 3. DPPH radical-scavenging activities of various fractions by column chromatography.

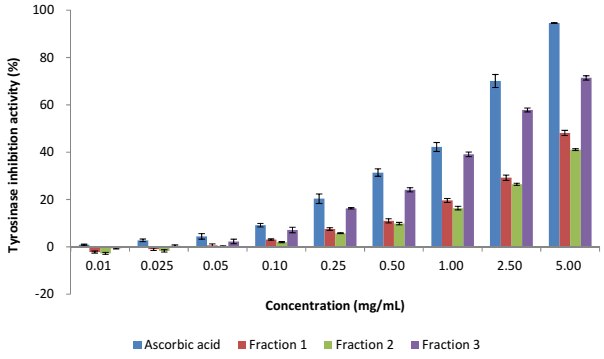

Fig. 5. Tyrosinase inhibition activities of various fractions by column chromatography.

effects of mistletoe extract, its activity differs greatly depending on various conditions such as geographic location, host tree, extraction method, and solvent used (Kim *et al*., 2012). In this study, the antioxidative performance of the organic MeOH extract of mistletoe was inferred using the measurement of the DPPH radical scavenging ability. During the measurement of DPPH radical scavenging of each concentration, conducted to confirm the antioxidative activity of each mistletoe extract, the EtOAc fraction had the highest antioxidative activity, followed by the BuOH, CH_2Cl_2, and *n*-Hex. fractions (Fig. 2.). Notably, the radical scavenging was 42.3% at 0.1 ㎎/㎖, the inhibition activity was higher than that of ascorbic acid (35.0%), the positive control.

Tyrosinase inhibition activities of various mistletoe extracts

There have been previous reports regarding the tyrosinase inhibitory activity of mistletoe extract; however, no known study has been conducted examining the active low molecular weight compounds in the extracts (Lee *et al*., 2011). In this study, to identify the compounds responsible for tyrosinase inhibition in mistletoe, the activity of each solvent fraction was investigated. We found that at 100 ㎍/㎖, the EtOAc fraction had the highest inhibitory activity (25.5%), followed by BuOH (20.1%), CH_2Cl_2 (4.3%), and *n*-Hex (2.5%), (Fig. 4). These results demonstrated that the EtOAc and BuOH fractions had higher tyrosinase inhibitory activity than ascorbic acid (14.6%) at 100 ㎍/㎖, which was the control material. Therefore, it can be inferred that the main compounds responsible for tyrosinase inhibition are contained in the EtOAc fraction.

Isolation of active compounds with tyrosinase inhibitory activity from the EtOAc layer

To confirm the antioxidative and tyrosinase inhibition abilities of the active material in mistletoe, the low-molecular substances in the EtOAc fraction, which were responsible for

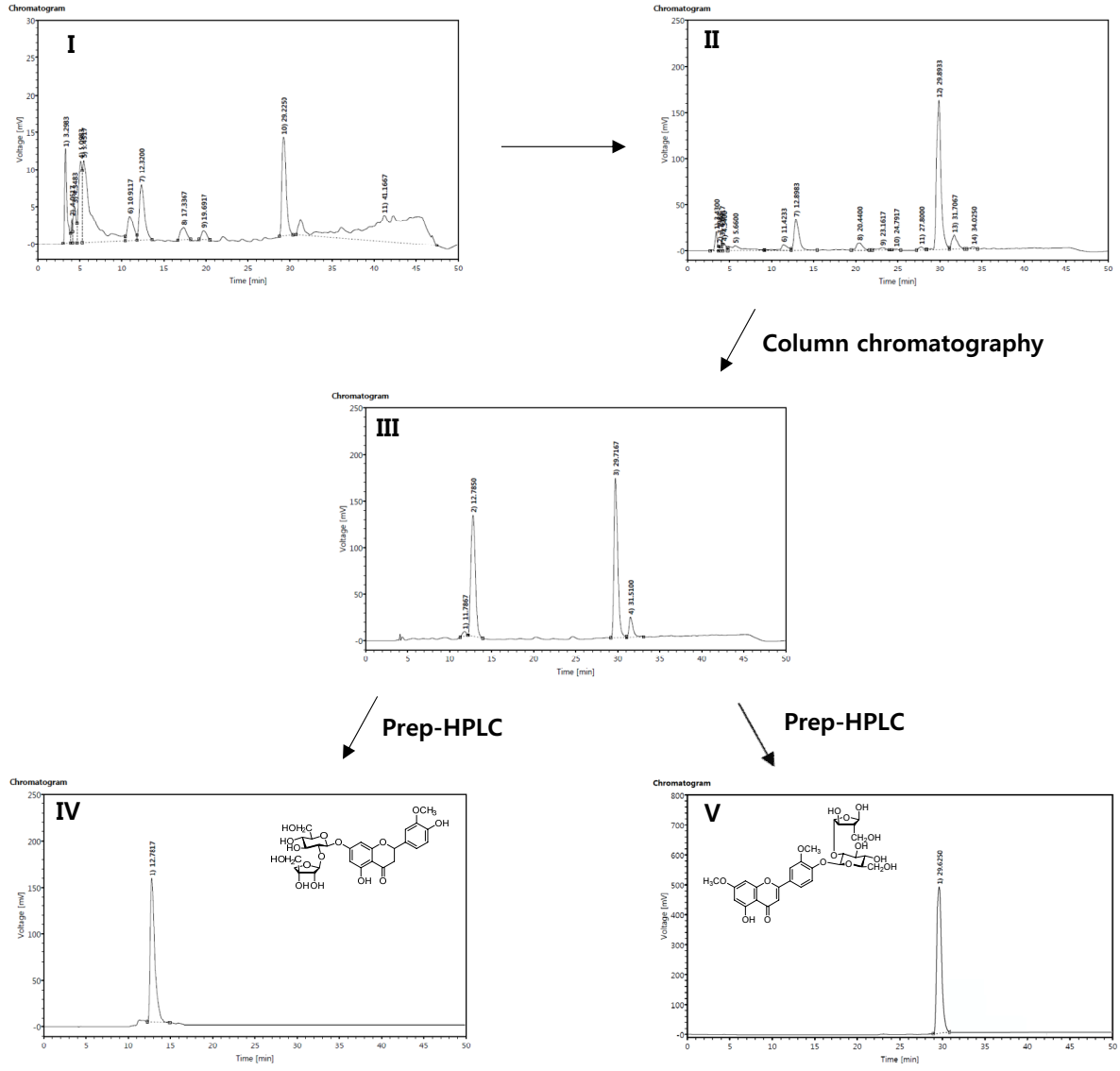

Fig. 6. HPLC chromatogram (I : MeOH extract, II : EtOAc fraction, III : Fraction 3, IV : Viscumneoside III, V : Homoflavoyadorinin B).

the highest rates of both tyrosinase inhibition and DPPH radical scavenging, were subjected to separation. By using the HPLC to analyze the EtOAc fraction, we identified two major main compounds. The TLC (Thin layer chromatography) analysis revealed that the two materials had a $R_f = 0.12$, when using the developing solvent composed of MeOH:CH_2Cl_2 at a ratio 1:5. Through silicagel column chromatography, the major material with the R_f = 0.12, under the developing solvent composed of MeOH:CH_2Cl_2 at a ratio of 1:5 was separated, and prep-HPLC was used to separate the materials at each main peak.

Identification of the isolated compounds

To confirm the chemical structure of the two major components contained in the EtOAc fraction of the mistletoe extract, ^{1}H-NMR analysis as well as ^{13}C-NMR analysis were conducted. By comparing the spectra, we confirmed that viscumneoside III (**1**) and homoflavoyadorinin B (**2**) were in the EtOAc fraction of the mistletoe extract and identified their chemical structures (Fig. 7) (Nhiem *et al*., 2013).

Viscumneoside III : Green powder. ^{1}H-NMR(CD_3OD, 400 MHz), δ : 7.08 (1H, s), 6.92 (1H, d, J = 7.57 Hz), 6.80 (1H, d, J = 7.56 Hz), 6.19 (1H, d, J = 1.76 Hz), 6.16 (1H, d, J = 2.08 Hz), 5.42 (1H, d, J = 1.4 Hz), 5.37 (1H, s), 5.03 (1H, m), 3.98

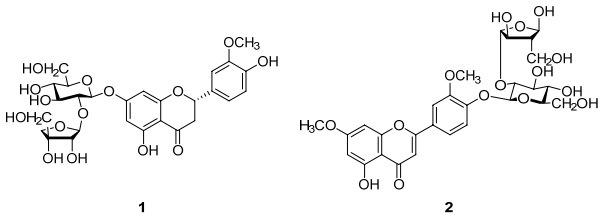

Fig. 7. Chemical structures of viscumneoside III (**1**) and homoflavoyadorinin B (**2**).

(2H, dd, J = 13.41, 3.76 Hz), 3.93 (1H, s), 3.88 (3H, s, -OCH_3), 3.85 (1H, s), 3.76 (1H, d, J = 9.57 Hz), 3.64 (1H, m), 3.62 (1H, d, J = 4.96 Hz), 3.56 (2H, s), 3.46 (1H, m), 3.31 (1H, m), 3.20 (1H, m), 2.73 (1H, d, J = 4.96 Hz). ^{13}C-NMR(CD_3OD, 100 MHz), δ: 198.5, 166.8, 164.9, 164.6, 149.1, 148.2, 131.4, 120.7, 116.1, 111.4, 111.3, 110.8, 104.9, 99.8, 97.9, 96.7, 80.9, 80.7, 78.6, 78.4, 78.1, 75.4, 71.1, 65.9, 62.3, 56.5, 44.3. MS m/z = 597.17 $[M+H]^+$ for

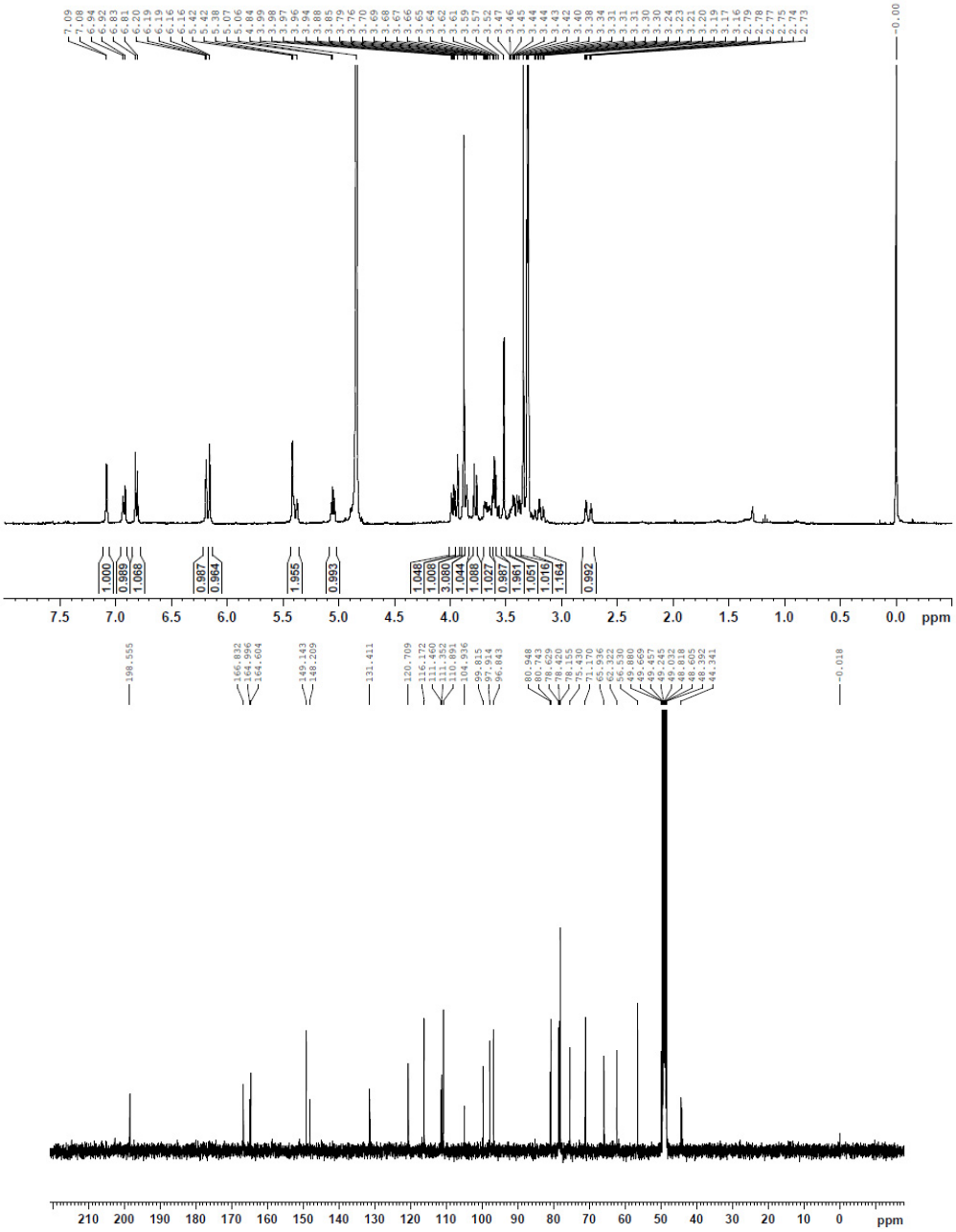

Fig. 8. NMR spectrum of viscumneoside III (**1**).

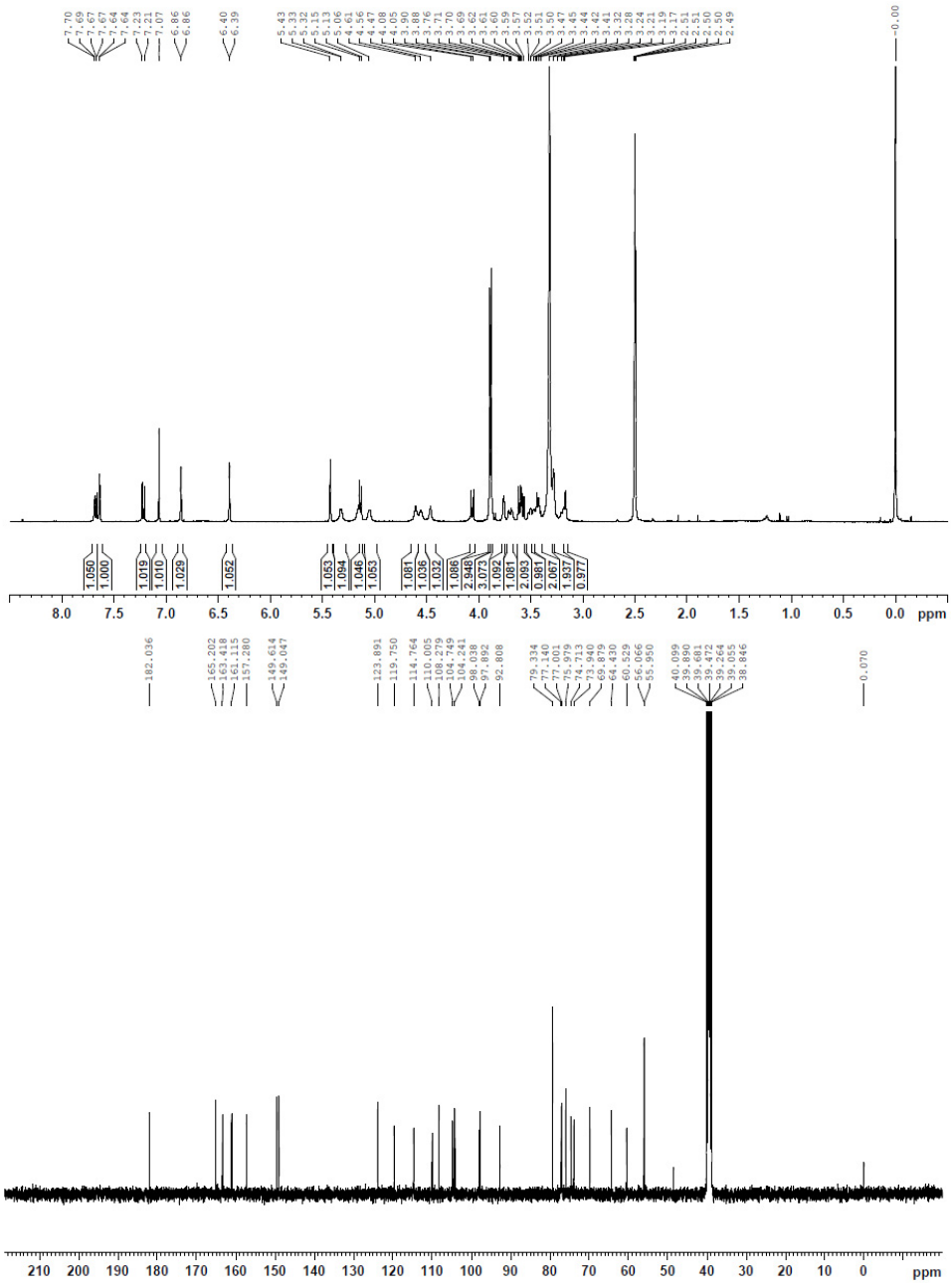

Fig. 9. NMR spectrum of homoflavoyadorinin B (**2**).

formula $C_{27}H_{32}O_{15}$

Homoflavoyadorinin B : Yellow powder. ^{1}H-NMR(DMSO-d_6, 400 MHz), δ : 7.68 (1H, dd, J = 8.6, 2.12 Hz), 7.65 (1H, s), 7.21 (1H, d, J = 8.81 Hz), 7.09 (1H, s), 6.87 (1H, d, J = 2.2 Hz), 6.40 (1H, d, J = 2.2 Hz), 5.43 (1H, s), 5.32 (1H, br, s, -OH), 5.14 (1H, d, J = 7.73 Hz), 5.06 (1H, br, s, -OH), 4.61 (1H, br, s, -OH), 4.56 (1H, br, s, -OH), 4.47 (1H, br, s, -OH), 4.06 (1H, d, J = 9.49 Hz), 3.90 (3H, s, -OCH_3), 3.88 (3H, s, -OCH_3), 3.76 (1H, s), 3.68 (1H, m), 3.59 (2H, m), 3.50 (1H, m), 3.45 (1H, m), 3.43 (1H, m), 3.28 (2H, s), 3.17 (1H, m). ^{13}C-NMR(DMSO-d_6, 100 MHz), δ : 182.0, 165.2, 163.4, 161.1, 157.2, 149.6, 149.0, 123.8, 119.7, 114.7, 110.0, 108.2, 104.7, 104.2, 98.0, 97.8, 92.8, 79.3, 77.1, 77.0, 75.9, 74.7, 73.9, 69.8, 64.4, 60.5, 56.0, 55.9. MS m/z = 609.18 $[M+H]^+$ for formula $C_{28}H_{32}O_{15}$

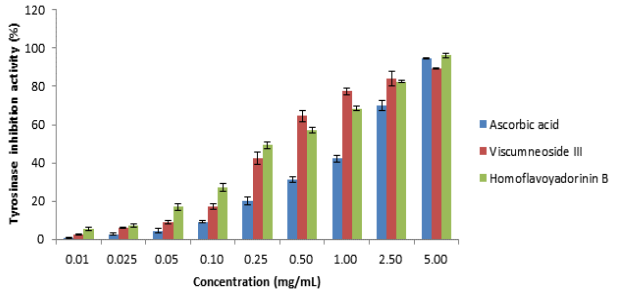

Fig. 10. Tyrosinase inhibitory activities of and viscumneoside III (**1**) and homoflavoyadorinin B (**2**).

Table 3. IC_{50} value of viscumneoside III (1) and homoflavoyadorinin B (**2**)

Compounds	IC_{50} (mM)
Ascorbic acid	7.50
Viscumneoside III	0.50
Homoflavoyadorinin B	0.51

Tyrosinase inhibition activities of the isolated compounds

Two major extracts separated from mistletoe were investigated for tyrosinase inhibition activity. We found that viscumneoside III (**1**) and homoflavoyadorinin B (**2**) had higher inhibition activities at concentrations lower than that used for ascorbic acid, which was the control. In particular, at a concentration of 1.0 mg/ml, the inhibition activities of viscumneoside III (**1**) and homoflavoyadorinin B (**2**) were 77% and 68%, respectively, and this activity was greater than that determined for ascorbic acid (42%) (Fig. 10).

Various bioactive substances are found in mistletoe, including a substance that inhibits tyrosinase activity, leading to skin whitening. To verify that the tyrosinase inhibitory substance was found in the mistletoe, the EtOAc organic solvent fraction was confirmed to have the highest activity. Viscumneoside III (**1**) and homoflavoyadorinin B (**2**) were confirmed to be the major constituents, after separation and purification using the prep-HPLC. The tyrosinase inhibition of the two substrates showed higher inhibitory activity than the ascorbic acid, the control group. From such result, it can be considered that viscumneoside III (**1**) and homoflavoyadorinin B (**2**), the tyrosinase inhibitory substances, exist within the EtOAc fraction of the mistletoe, and that they can be used as materials for cosmetics with whitening function.

Acknowledgments

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the Export Promotion Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (313011-03).

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(Received 7 September 2016 ; Revised 2 October 2016 ; Accepted 21 November 2016)