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Enhanced anti-oxidative effect of fermented Korean mistletoe is originated from an increase in the contents of caffeic acid and lyoniresinol

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Viscum album var. *coloratum* (Korean mistletoe; KM) is an herbal medicine that is used worldwide for the treatment of various immunological disorders and cancers. KM extract showed enhanced anti-oxidative effects in 2,2-diphenyl-1-picrylhydrazyl, Trolox equivalent antioxidant capacity, and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester assays after being fermented with a crude enzyme extract from a soybean paste fungus, *Aspergillus kawachii*. High-performance liquid chromatography analysis showed four increased peaks in enzyme treated KM. The increased peaks were isolated and identified as caffeic acid (**1**), hesperetin (**2**), syringaldehyde (**3**), and lyoniresinol (**4**). Among the four compounds, only **1** and **4** showed strong anti-oxidative activity. Therefore, the fermentation increased the contents of **1** and **4**, which consequently increased the anti-oxidative activity of KM.

1. Introduction

Food processing techniques such as roasting and enzyme treatment (fermentation) have been introduced to enhance nutritional values or improve specific flavors and colors of foods. Furthermore, these processes are mostly aimed at enhancing sensory characteristics such as palatability and organoleptic qualities. Its application was widened to extending the storage period by suppressing microbiological or biochemical induced changes. In previous studies, the quercetin content in the inner layers of onion and the liquiritigenin content in licorice were dramatically increased following the treatment with a crude enzyme extract from *Aspergillus kawachii*.[1,2] In addition, roasting increased the amount of cinnamaldehyde in *Cinnamoni* Cortex.[3] In many studies, the degradation of glycosides and oxidative coupling have been widely investigated in an attempt to enhance the anti-oxidative activity by increasing free phenolic hydroxyl groups.[4,5] Therefore, fermentation, which generally induces glycolysis, might be a promising technique for improving the biological activities of medicinal foods and herbal drugs.

Reactive oxygen species (ROS) is a collective term that includes oxygen radicals and non-radical derivatives of oxygen. The oxygen radicals include superoxide anion ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), peroxy ($ROO^{\bullet}$), alkoxy ($RO^{\bullet}$), and hydroperoxy ($HOO^{\bullet}$) radicals. Non-radical ROS includes ozone (O_3), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$).[6] Normally, ROS play an important role in human health, mainly in homeostasis as well as in the elimination of harmful cell sediments and pathogens.[7] Despite the cellular antioxidant defense mechanisms that protect against oxidative damage induced by ROS, the accumulation of oxidative damage during the cell life cycle promotes the development of age dependent diseases such as cancer, atherosclerosis, arthritis, neurodegenerative disorders, and other conditions.[8] Therefore, antioxidants have been considered as a promising therapy for the prevention and treatment of these diseases. Many phytochemicals in foods and herbs have antioxidant properties and their favorable effects on various risk factors have been reported.[9]

Based on this, several food processing techniques such as heating, roasting, fermentation, and extrusion were attempted to increase the anti-oxidative effect of foods and herbs. In a preliminary experiment, the anti-oxidative effects of 20 commonly used medicinal foods were compared before and after processing. Out of them, the anti-oxidative effect of *Viscum album* var. *coloratum* (Korean mistletoe; KM) was dramatically changed after fermentation. Therefore, the effects of fermentation on the anti-oxidative activity of KM were investigated and the components responsible for the enhanced activity were determined.

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2. Materials and methods

2.1. General

Organic solvents used for extraction and open column chromatography, such as *n*-hexane, ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc), and dichloromethane (CH_2Cl_2), were obtained from Duksan Chemical (Anseong, Republic of Korea). High-performance liquid chromatography (HPLC) grade acetonitrile (MeCN) and acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany). Medium pressure liquid chromatography (MPLC) was carried out using a Reveleris®X2 Flash Chromatography System (Grace, Colombia, MD, USA). Thin layer chromatography silica gel plates, RP-18 F254S (Merck) and silica gel 60 F254 (Merck), were used to monitor the chromatographic pattern. ^{1}H-NMR and ^{13}C-NMR were recorded using an Avance 500 Digital nuclear magnetic resonance (NMR) spectrometer (Bruker, Karlsruhe, Germany) at 500 and 125 MHz, respectively. The chemical shifts were expressed in δ (parts per million) relative to the internal standard, tetramethylsilane. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Amresco (Solon, OH, USA). Fluo-4 AM, and 5-(and-6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) from Invitrogen (Eugene, OR, USA) were used in the flow cytometry analysis. Optical density (OD) was measured using a microplate reader (BioTek Instruments, Winooski, VT, USA).

2.2. Plant material, fermentation, and HPLC analysis

Korean mistletoe was purchased from a local market in Daegu, Korea. The voucher specimens (voucher no. KNUNPM-VA 201302) were deposited at the Natural Products Medicine Laboratory, College of Pharmacy, Kyungpook National University. The methanolic extract of KM (50 mg) was suspended in 1 mL of citrate acid buffer (100 mM, pH 4.6) and 1 mL of crude enzyme from *A. kawachii* (0.276 U mL^{-1}), which was prepared according to a previously reported method,[2] was added to the KM suspension. The mixture was incubated at 30 °C for four days. To the reaction mixture, 2 mL of MeOH was added to precipitate the citric acid. After centrifugation, the supernatant was filtered through a 0.45 μm membrane filter and the filtrate was analyzed using HPLC, in order to compare the chromatographic patterns before and after fermentation. The HPLC analysis was carried out on an Agilent system equipped with a Dionex UltiMate 3000, with diode array detection at a wavelength of 280 nm. The stationary phase was a Waters (Milford, MA, USA) XTerra RP18 reverse phase column (4.6 × 250 mm, 5 μm) with a mobile phase consisting of water (H_2O; solvent A) and MeCN (solvent B) each containing 1% HOAc. After the sample was injected into the column, solvent B was increased from 5% to 65% in 30 min, and then increased to 100% in 5 min, and held at 100% for 3 min. The solvent flow rate was 1 mL min^{-1}. The time course of the HPLC pattern was obtained by analyzing the reactant on days 1, 2, 3, and 4 after onset of fermentation.

2.3. Bulk fermentation, extraction, and isolation

KM (5 kg) was refluxed three times for 3 h with 40 L of MeOH and the solution was then evaporated to dryness (433 g). The methanolic extract (400 g) was suspended in 800 mL of citrate buffer (100 mM, pH 4.6) and then treated with 800 mL of crude enzyme extract from *A. kawachii* (0.276 U mL^{-1}) at 30 °C for 3 days. MeOH (1 L) was added to the reaction mixture in order to precipitate the excess citric acid. After filtration using a filter paper, the filtrate was evaporated under reduced pressure, and the resultant extract was suspended in 1 L of water to be consecutively partitioned with *n*-hexane, CH_2Cl_2, and EtOAc. The EtOAc soluble fraction (8.3 g) of the fermented KM was loaded onto a silica gel column (Merck Grade 7734, 10 × 60 cm) and eluted with CH_2Cl_2–MeOH (100 : 1 to 5 : 1) to obtain five fractions. The MPLC (Reveleris C_{18}, 2.5 × 8.0 cm, H_2O–MeCN = 100 : 0 to 60 : 40, 15 mL min^{-1}) of fraction 4 (245.5 mg) gave 7.0 mg of compound **1**. The CH_2Cl_2 fraction (6.2 g) was further purified using a silica gel column (Merck Grade 7734, 10 × 60 cm, benzene–EtOAc = 100 : 1 to 5 : 1) to yield seven fractions. The MPLC (Reveleris C_{18}, 8 × 20 cm, H_2O–MeOH = 100 : 0 to 60 : 40, 10 mL min^{-1}) of the first fraction (220 mg), isolated 10.0 mg of compound **2**. Compound **3** (5.1 mg) and **4** (130.2 mg) were purified from the third fraction (180 mg) and sixth fraction (420 mg), respectively, using the same method that was used for the first fraction.

2.4. Identification of isolated compounds

Compound **1** (caffeic acid): ^{1}H-NMR (500 MHz), deuterated methanol (CH_3OH-d_4): δ7.52 (1H, d, J = 16.1 Hz, H-3), 7.03 (1H, d, J = 1.9 Hz, H-4), 6.93 (1H, dd, J = 1.9, 8.2 Hz, H-9), 6.78 (1H, d, J = 8.2 Hz, H-8), 6.22 (1H, d, J = 16.1 Hz, H-2).

Compound **2** (hesperetin): ^{1}H-NMR (500 MHz, CH_3OH-d_4): δ 2.72 (1H, dd, J = 17.4, 3.0 Hz, H-3α), 3.04 (1H, dd, J = 17.4, 12.0 Hz, H-3β), 3.88 (3H, s, OMe), 5.32 (1H, dd, J = 12.0, 3.0 Hz, H-2), 5.88 (1H, d, J = 2.0 Hz, H-6), 5.91 (1H, d, J = 2.0 Hz, H-8), 6.93 (1H, dd, J = 9.0, 2.0 Hz, H-6′), 6.94 (1H, d, J = 9.0 Hz, H-5′), 6.95 (1H, d, J = 2.0 Hz, H-2′).

Compound **3** (syringaldehyde): ^{1}H-NMR (500 MHz, CH_3OH-d_4): δ9.82 (1H, s), 7.21 (2H, s), 3.85 (6H, s, OMe ×2).

Compound **4** (lyoniresinol): ^{13}C-NMR (125 MHz, CH_3OH-d_4): δ149.09 (C-3′ and 5′), 148.77 (C-5), 147.80 (C-3), 139.44 (C-4 and 1′), 130.27 (C-1), 135.00 (C-4′), 126.36 (C-2), 107.84 (C-6), 106.91 (C-2′ and 6′), 66.87 (C-9), 64.21 (C-9′), 60.26 (3-OMe), 56.85 (3′,5′-OMe), 56.69 (5-OMe), 49.01 (C-8′), 42.43 (C-7′), 40.98 (C-8), 33.71 (C-7).

2.5. DPPH radical scavenging assay

The procedure was performed as previously described.[10] Briefly, 10 μL of samples diluted in dimethyl sulfoxide (DMSO) were added to 190 μl of 150 μM DPPH in EtOH in a 96-well plate. After reacting for 30 min at room temperature in a dark place, the OD value was measured at 517 nm. DPPH radical scavenging activity was expressed as percentages relative to the OD of control which was 10 μl DMSO (0%).

2.6. Trolox equivalent antioxidant capacity (TEAC) assay

The spectrophotometric analysis of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was determined according to a previous method.[11] The ABTS cation radical was produced by the reaction between 7 mM of ABTS in H_2O and 4.9 mM potassium persulfate, stored in the dark at 4 °C for 12 h. Before usage, the ABTS solution was diluted with 80% EtOH to obtain an absorbance of 0.700 ± 0.025 at 734 nm. Then, 190 μl of ABTS solution was added with 10 μl Trolox at different concentrations. After 30 min, the percentage inhibition at 734 nm was calculated for each concentration relative to the absorbance of the blank. The activity of each sample was calculated using the same method and the results were compared with that of Trolox.

2.7. Cell culture and measurement of intracellular ROS production

Mouse derived hippocampal neuronal cells (HT22) were donated by Professor Dong-Seok Lee (College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Welgene, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) (*v*/*v*), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Welgene) at 37 °C under 5% carbon dioxide. Then the cells were subcultured every two days. Cells were incubated with 5 μM CM-H_2DCFDA in phenol red free DMEM for 15 min. The intracellular ROS generation was estimated by the analysis of the green fluorescence (530 nm) intensity. For each sample, 10 000 cells were collected and analyzed.

3. Results

3.1. Fermentation, HPLC analysis, and isolation of increased peaks

In the HPLC analysis, four new peaks appeared after fermentation with the crude enzyme extract from *A. kawachii* (Fig. 1). However, several peaks at retention time (R_t) 5–9 min were diminished. In order to optimize the fermentation time, the KM extract was fermented over the time course at 30 °C. Three days after the onset of fermentation, the four peaks that showed an increase had attained their maximum levels (Fig. 2). Based on this preliminary experiment, a large amount of KM extract (400 g) was fermented for three days to isolate the increased compounds. Repeated chromatography of the fermented KM using silica gel and C_{18} columns gave increased amounts of compounds **1–4**. Compound **1** showed typical unsaturated phenylpropanoid signals in ^{1}H-NMR and was identified as caffeic acid by comparing its ^{1}H-NMR data with that of a reference.[12] ^{13}C-NMR spectra of **2** and **3** corresponded to the reference data of hesperetin and syringaldehyde,

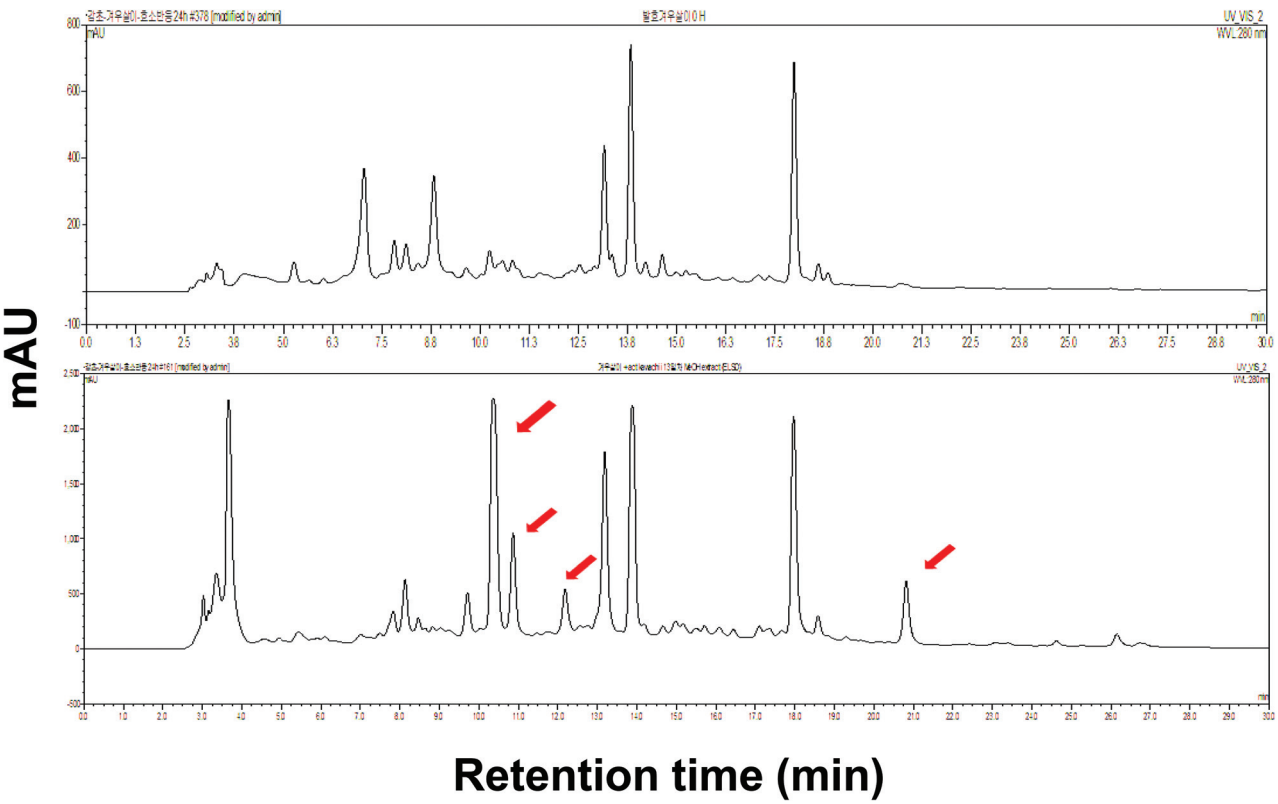

Fig. 1 Changes in HPLC profile before and after fermentation of KM with a crude enzyme extract from *A. kawachii*. Upper: inactivated enzyme-treated control. Lower: day 4 after onset of fermentation. Arrows indicate the increased peaks after fermentation.

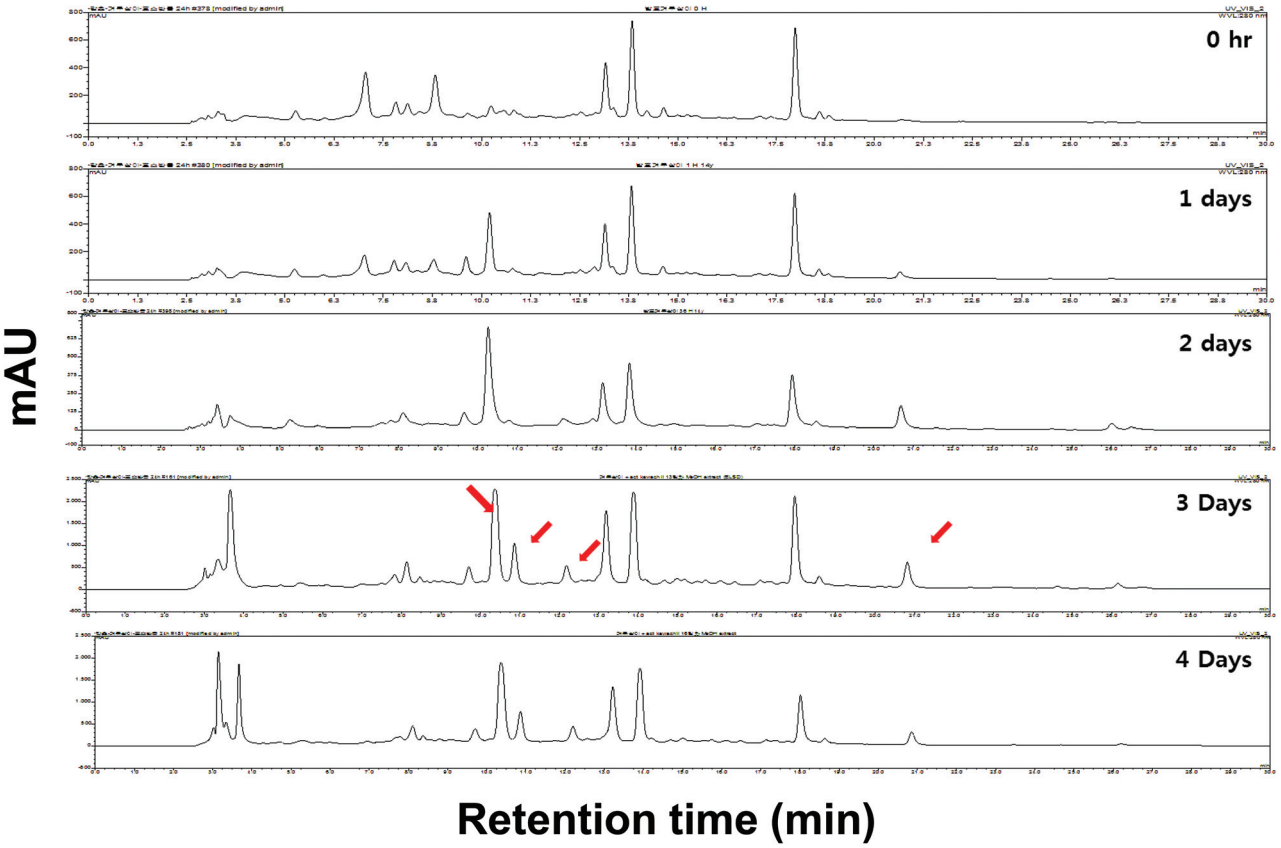

Fig. 2 Time course changes in HPLC chromatograms of fermented KM. From top to bottom, HPLC chromatograms on day 0, 1, 2, 3, and 4 after fermentation of KM extracts commenced. Arrows indicate increased peaks after fermentation.

respectively.[13] Compound **4**, which showed two identical pairs of methoxyl groups in the ^{1}H-NMR spectra, was identified as lyoniresinol.[14] The chemical structures of the increased compounds are presented in Fig. 3. The retention times of the isolated compounds **1–4** were identical to those of the increased peaks in the fermented KM (Fig. 4). The retention times were confirmed by co-injection of fermented KM extract with each isolated standard compound (data not shown).

3.2. DPPH and ABTS radical scavenging activity

The DPPH scavenging activity was increased after fermentation (Fig. 5A). At 500 ppm (mg L^{-1}), the KM extract removed only about 20% of the DPPH radical compared to the control, however, the scavenging activity was increased up to 60% at the same concentration after fermentation. In addition, although the KM extract did not show an anti-oxidative effect in the ABTS assay, the fermentation dramatically raised the anti-oxidative activity (Fig. 5B). The DPPH radical scavenging activities of the four compounds with increased activity were compared to determine the compounds responsible for the enhanced anti-oxidative effect of the fermented KM. Out of all the compounds, **1** and **4** showed much stronger activities than **2** and **3** did (Fig. 6A), therefore indicating that the elevated anti-oxidative activity after fermentation might have occurred because of the increase in the content of compounds **1** and **4** (Fig. 6B).

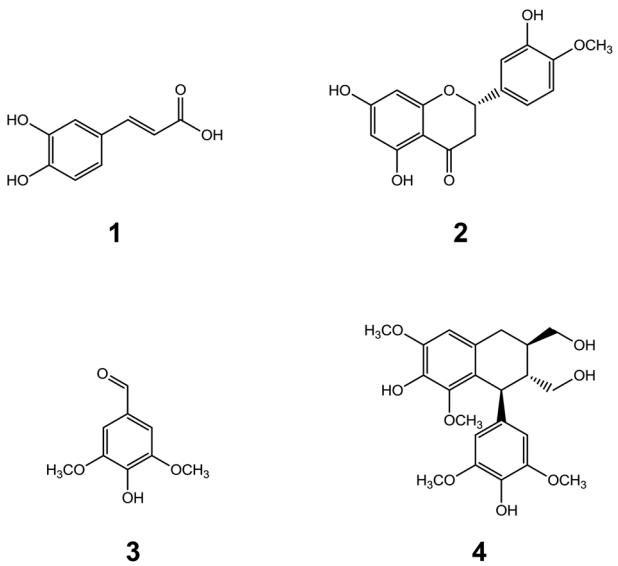

Fig. 3 Chemical structures of increased compounds. **1**, caffeic acid. **2**, hesperetin. **3**, syringaldehyde, **4**, lyoniresinol.

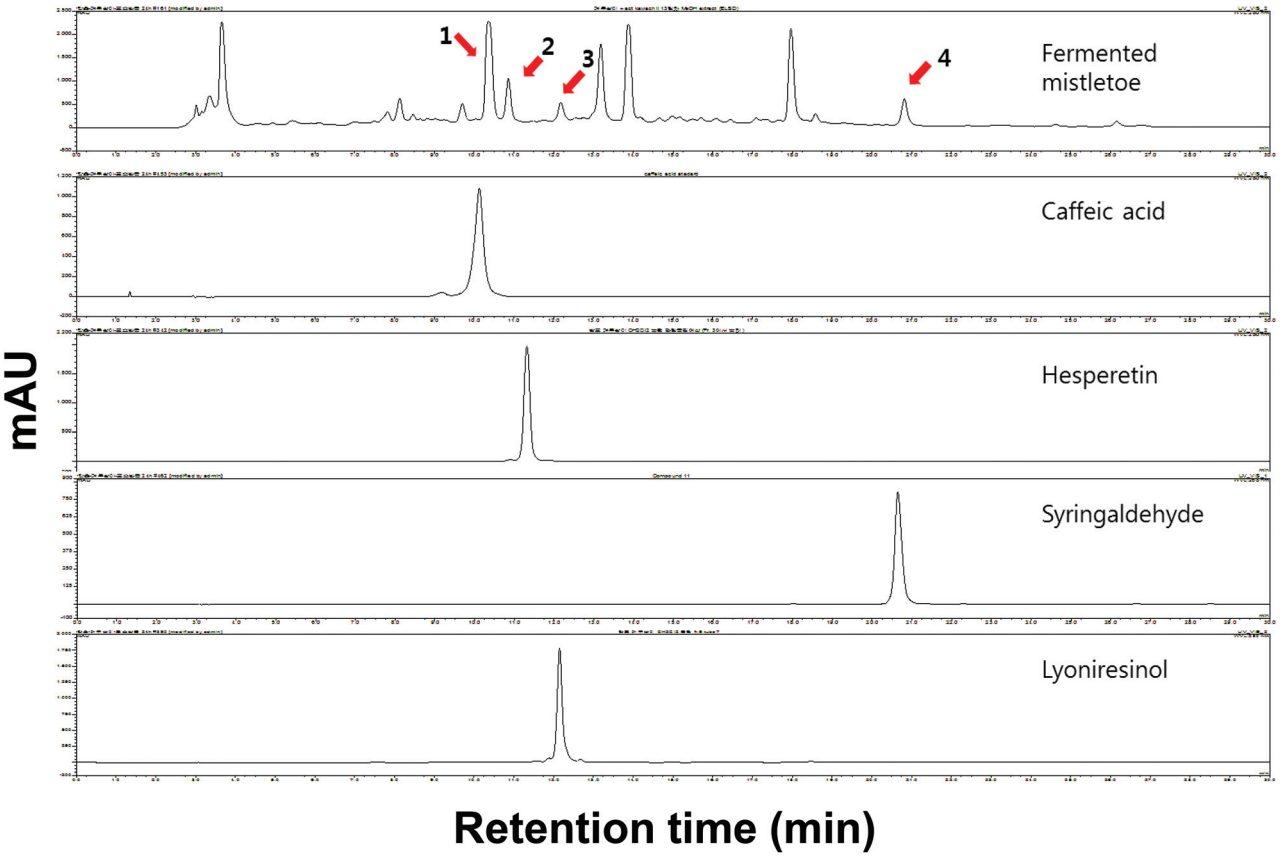

Fig. 4 Comparison of retention times of increased peaks with isolated compounds. **1**, caffeic acid. **2**, hesperetin. **3**, syringaldehyde. **4**, lyoniresinol.

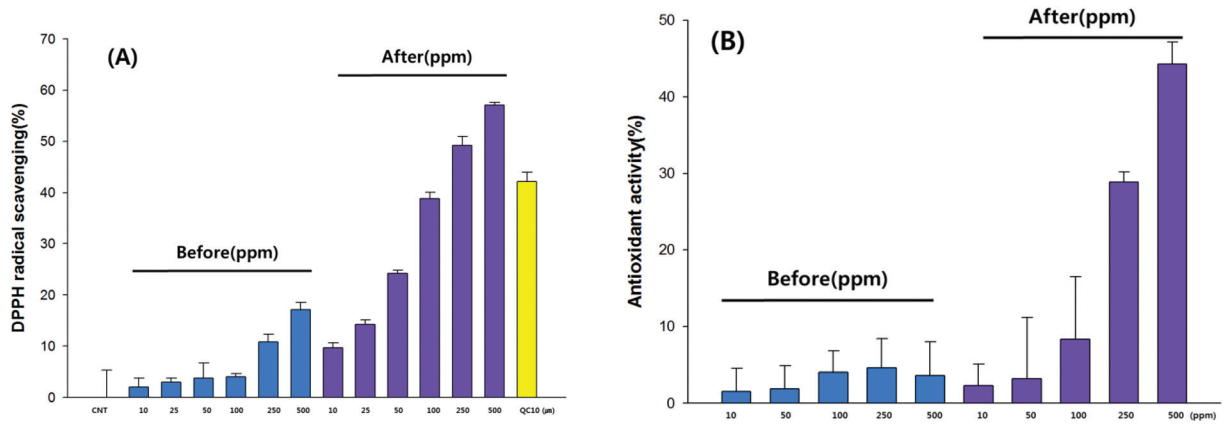

Fig. 5 DPPH (A) and ABTS (B) assays before and after fermentation of KM. Quercetin (10 μM) was used as a positive control in the DPPH assay.

3.3. Effect of 1 and 4 on glutamate induced intracellular ROS generation and neuroprotection in HT22 cells

Compounds **1** and **4** effectively reduced the intracellular ROS in glutamate treated HT22 cells (Fig. 7A). When HT22 cells were treated with **1** or **4**, the cell morphology was recovered effectively at concentrations from 50 to 100 μM, and the cell viability was significantly increased up to 98 ± 5% and 79 ± 1%, respectively, in a dose dependent manner. Furthermore, treatment with glutamate decreased the viability to 46 ± 1% (Fig. 7B).

4. Discussion

Mistletoe is a kind of hemiparasitic plant growing on deciduous trees.[15] Korean mistletoe has been suggested as a promis-

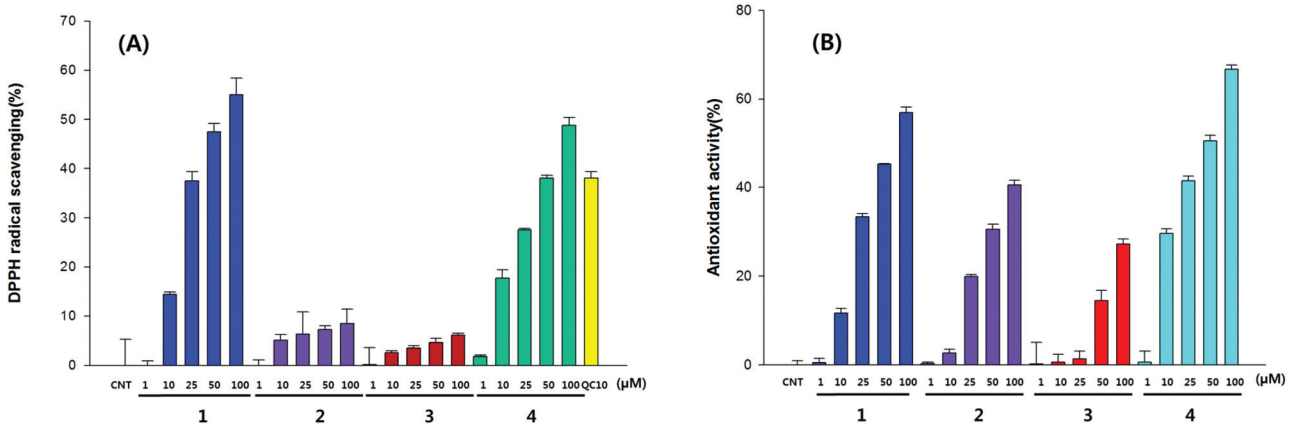

Fig. 6 Comparison of anti-oxidative effect of increased compounds by DPPH (A) and ABTS (B) assays. **1**, caffeic acid. **2**, hesperetin. **3**, syringaldehyde. **4**, lyoniresinol. Quercetin (10 μM) was used as a positive control in the DPPH assay.

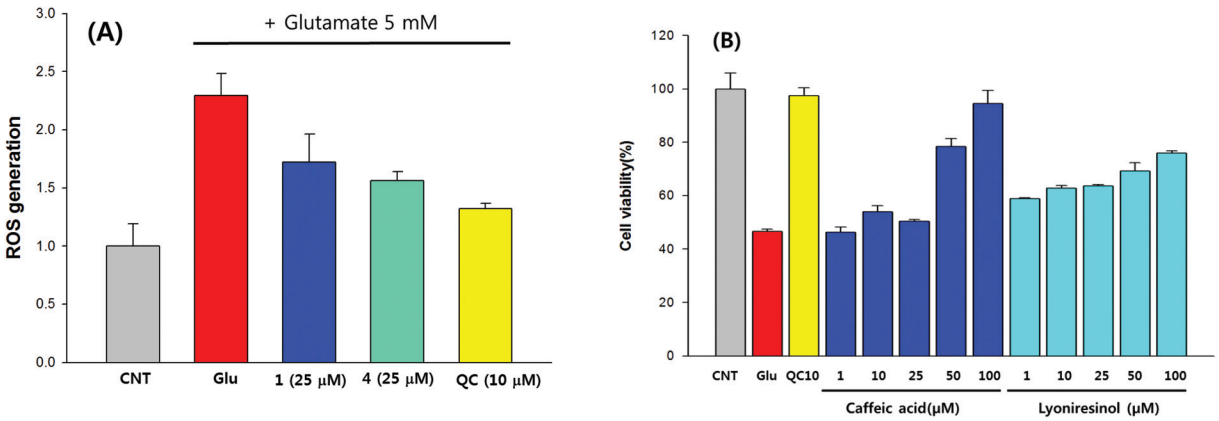

Fig. 7 Intracellular ROS scavenging (A) and cytoprotective (B) effects of compound **1** and **4** in HT22 cells. **1**, caffeic acid. **4**, lyoniresinol. Quercetin (10 μM) was used as a positive control.

ing agent for treating colon cancer,[16] hepatoma,[17,18] and immunomodulation.[19,20] These biological activities were mainly attributed to lectins consisting of A (31 kDa) and B (34 kDa) chains that usually bind to galactose and *N*-acetyl-D-galactosamine.[21–23] In contrast, other compounds that were isolated from KM involving *epi*-oleanolic acid,[24] viscothionin,[25] and alkaloids[26] having anti-tumor activities, and homo-flavoyadorinin-B as an antioxidant,[27] have only been investigated to a small extent compared to the lectins. The neuroprotective effects of KM extract against H_2O_2 [28] and amyloid β protein (25–35)[29] were investigated in a previous study by the present authors; however, the active component was not identified.

In this study, the anti-oxidative activities of the extract of KM were enhanced through simple fermentation using the crude enzyme extract derived from *A. kawachii* which was known to have the β-glucosidase activity,[2,11] and then the active compounds having the properties of antioxidants and neuroprotectants were identified. As a result, it was found that the key materials from fermented KM extract were caffeic acid (**1**) and lyoniresinol (**4**). Even the reduced compounds were not isolated, the origin of hesperetin (**2**) and **4** were easily deduced because their corresponding glycosides (2*S*)-eriodictyol-7-*O*-β-D-glucopyranoside[30] and eleutheroside E[31] had been isolated from mistletoe. In addition, the glycosides of **1** and syringaldehyde (**3**) have not been previously reported from mistletoe, however, the reduction of peaks at R_t 5–9 min suggested that they also presumably originated from their corresponding glycosides because many **1** and syringin derivatives have been found in mistletoe.[30–33] Therefore, the enhanced anti-oxidative effect is presumably occurring because of an increase in free phenolic OH groups by the cleavage of the glycosidic bond.

Compound **1** is known to be one of the anti-oxidative phenolic acids in mistletoe.[34,35] Huang *et al.* reported the protective effects of **1** and caffeic acid phenethyl ester against acrolein induced neurotoxicity in HT22 mouse hippocampal cells.[36] Compound **4**, which is relatively rare in nature, has been reported to be a cytoprotective and anti-oxidative compound obtained from *Berberis vulgaris*[37] and *Eurya japonica*;[38] however, this is the first report of them as a constituent of KM and as a neuroprotectant.

The simple fermentation technique for KM extract and its functional compounds **1** and **4** would be useful for the development of other compounds for preventing and treating the various neurodegenerative disorders.

5. Conclusions

The results of this research are intriguing, because simple fermentation using the crude enzyme extract from *A. kawachii* was able to enhance the health value of Korean mistletoe by increasing the biologically active constituents such as caffeic acid (**1**) and lyoniresinol (**4**).

Abbreviations

ABTS	2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
CM-H_2DCFDA	5-(and-6-)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
HPLC	High-performance liquid chromatography
KM	Korean mistletoe
MPLC	Medium pressure liquid chromatography
MPTP	1-Methyl-4-phenyl-1,2,3,4-tetrahydroptridine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
ROS	Reactive oxygen species
R_t	Retention time
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TMS	Tetramethylsilane

Conflict of interest

None declared.

Acknowledgements

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