J Food Sci Nutr
Vol 10, p 244~250 (2005)

J Food Science and Nutrition

Tyrosinase Inhibitory and Antioxidant Activities of Korean Mistletoe (*Viscum album* var. *coloratum*) Extract and Its Fractions

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Abstract

The copper-containing enzyme, tyrosinase, catalyzes the oxidation of tyrosine into dihydroxy phenylalanine (DOPA) and subsequently DOPAquinone. It is responsible, not only for the pigment melanin biosynthesis in human skin, but also for browning in foods. In the present study, tyrosinase inhibitory and antioxidant activities of Korean mistletoe extract and its fractions were investigated. As a result, both water and methanol (MeOH) extracts inhibited the tyrosinase activity. Among the fractions, the fraction eluted with 95% MeOH significantly inhibited the tyrosinase activity. The fraction was further purified, and the purified fraction C strongly inhibited the enzyme activity up to 92%. In addition, water and methanol extracts exerted radical scavenging effects. The fractions eluted with 70% MeOH and 95% MeOH showed high radical scavenging activities. In conclusion, these results suggest that Korean mistletoe extract and its fractions might be useful for the treatment of various dermatological disorders such as epidermal hyperpigmentation and for improving food quality.

Key words: mistletoe, tyrosinase, melanin, inhibition, antioxidant

INTRODUCTION

Tyrosinase (monophenol monooxygenase, E:C:1.14. 18.1), also known as polyphenol oxidase (1) is a copper-containing enzyme that is widely distributed in nature. This enzyme is mainly involved in the first two steps of melanin biosynthesis, which consist of the hydroxylation of L-tyrosine (monophenolase activity) and the oxidation of the product of this reaction, the L-Dopa (diphenolase activity), to the corresponding *O*-quinone (2). Tyrosinase is responsible for enzymatic browning in plants, producing undesirable changes in colour, flavour and nutritive values of plant-derived foods and beverages (3,4). In addition, tyrosinase is responsible for melanin biosynthesis in human skin. Clinically, various dermatological disorders, such as freckles and age spots, result in epidermal hyperpigmentation (5). Recently, safe and effective tyrosinase inhibitors became important for their potential applications in improving food quality and melanin-related health problems in human beings (2,6). Furthermore, tyrosinase inhibitors are also important in cosmetics for skin-whitening effects because lighter skin colour is preferred by many ethnic groups (7). Since plants are rich sources of bioactive chemicals, which are mostly free from harmful side-effects, there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as kaempferol (8), quercetin (9) and gallic acid derivatives (10) have been isolated from various plants. In addition, a fungal metabolite, kojic acid [5-hydroxy-2-(hydroxymethyl)-*r*-pyrone], has been demonstrated to be a potent tyrosinase inhibitor and is extensively used as a cosmetic agent with an excellent whitening effect (11,12).

Active oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) have been implicated both in the aging process and in degenerative diseases such as cancer (13). In particular, it is now recognized that the extremely reactive hydroxyl radical (·OH) derived from O_2^- and H_2O_2 causes DNA strand scission resulting in cellular damage. The importance of removing active oxygen species from living organisms is becoming increasingly recognized (15).

Korean mistletoe (*Viscum album* L. var. *coloratum*), a subspecies of European mistletoe (*Viscum album*), has long been recognized as a therapeutic herb. Mistletoe contains high molecular weight components such as lectins (MW > 60 kDa), viscotoxins (MW=5 kDa) and polysaccharides, and low molecular weight components such as phenypropanes and lignans (16), triterpenoids (17) and flavonoids (18,19). Mistletoes are traditionally used as sedative, analgesic and cardiac traditional medicines in Korea and other East-Asian countries (16,17,20).

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The herbs are also used to strengthen tendons and bones, expel pathogens associated with rheumatism and, even more important, as anticancer agents (20). Among several active components in mistletoe, the anticancer properties are considered to be related to glycoproteins and lectins (21-23). Recently we reported the inhibition of porcine pancreatic elastase by mistletoe extract (24). In this study, we investigated the tyrosinase inhibitory and antioxidant activity of mistletoe extract and its fractions.

MATERIALS AND METHODS

Plant material

Korean mistletoe (*Viscum album* L. var. *coloratum*) growing on oak trees were collected in winter at Kangwon province, Korea. The botanical identity was established by Prof. Jong Suk Lee, College of Natural Sciences, Seoul Women's University. Leaves, berries and 1 to 5 year old stems of the plants were sorted and sliced.

Preparation of water extract and fractionation by ultrafiltration

To prepare the mistletoe extract, dried plant slices were extracted with 5 volumes of distilled water for 3 h at 100°C in a flask equipped with a reflux condenser and then concentrated to dryness *in vacuo*. To fractionate the different molecular weight components of mistletoe by ultrafiltration, 10 mg/mL of water extract was filtered through a 10 kDa cut-off membrane with nitrogen gassing, and the filtrate was refiltered through a 1 kDa cut-off membrane (Diaflo, Amicon Co., Danvers, MA, USA) using Amicon Standard UF-Cell 8050 (Amicon Co., Danvers, MA, USA). To eliminate the lower molecular weight components of each step, the remainder of filtration was washed by filtering twice with saline (Fig. 1).

Preparation of methanol extract and partial fractionation by solvents

For the preparation of methanol (MeOH) extract, dried samples were extracted with 4 volumes of methanol for 3 h at 70°C three times in a flask equipped with a reflux condenser and the extracts were concentrated to dryness *in vacuo*. The MeOH extract was suspended with two volumes of hot water (90~95°C) and the sufficiently

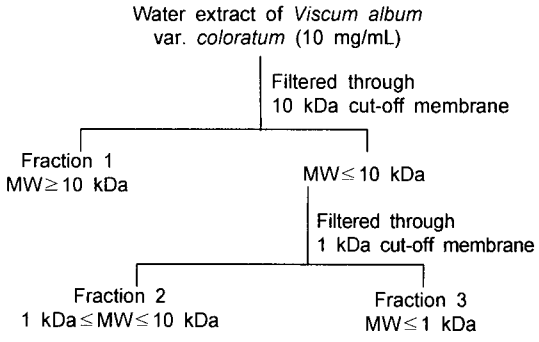

Fig. 1. Fractionation process of water extract from *Viscum album* L. var. *coloratum*. To eliminate the lower molecular weight components of each step, the remainder of the filtrate was washed with saline by filtering twice and the volume of each fraction was adjusted to 100 mL with saline. The volume of the filtrate through 1 kDa cut-off membrane was also adjusted to 100 mL with saline.

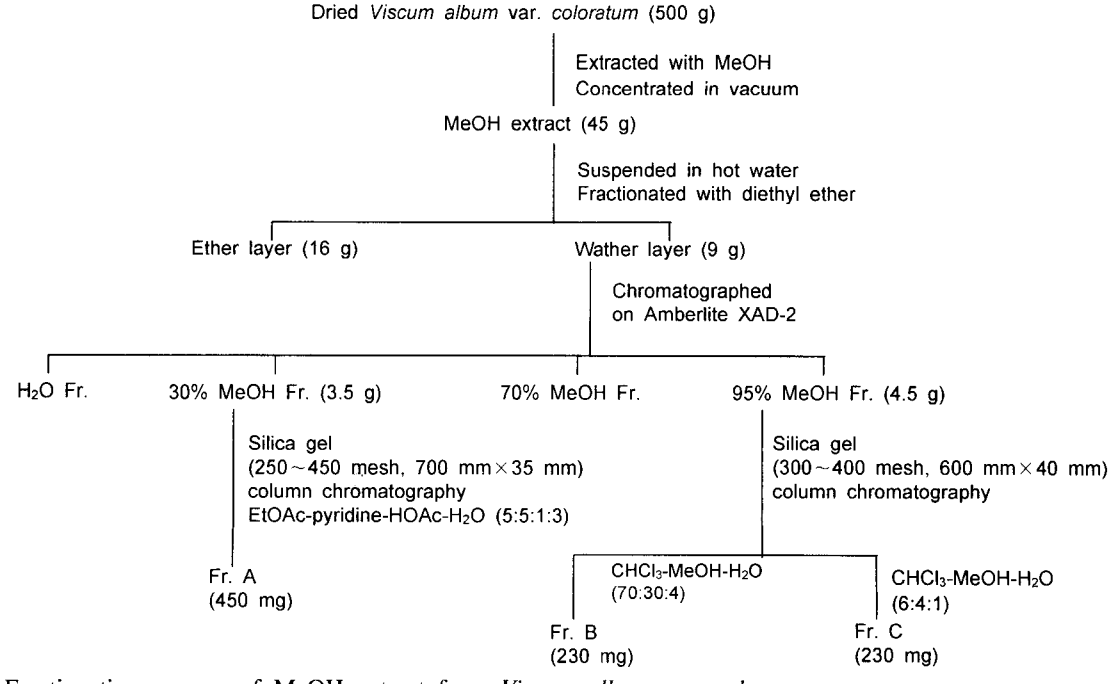

Fig. 2. Fractionation process of MeOH extract from *Viscum album* var. *coloratum*.

cooled suspension was fractionated with diethyl ether. The water layer was chromatographed on highly porous polymer (Amberlite XAD-2, Sigma) using H_2O, 30% MeOH, 70% MeOH and 95% MeOH. Fraction A was obtained from the 30% MeOH fraction by SiO_2 column chromatography with the solvent system, EtOAc-pyridine HOAc-H_2O (5:5:1:3, v/v). Two fractions B and C were obtained from 95% MeOH fraction by SiO_2 column chromatography using the solvent system, $CHCl_3$-MeOH-H_2O (70:30:4) for fraction B and $CHCl_3$-MeOH-H_2O (6:4:1) for fraction C (Fig. 2).

Enzyme assay

The tyrosinase activity, using L-Dopa as a substrate, was measured according to the method of Kubo and Kinst-Hori (8) with slight modifications. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Fifty microliters of 1.5 mM L-DOPA solution, 100 μL of 50 mM phosphate buffer (pH 6.8) and 40 μL of the test sample solution were mixed, and 10 μL of mushroom tyrosinase (1,500 U/mL) was added. The enzyme-reacted solution was incubated for 30 min at indicated doses and the solution was immediately monitored for the formation of dopachrome by measuring the linear increase in absorbance at 490 nm. Tyrosinase inhibition was also measured time-dependently and the enzyme inhibition was measured by treating the whole extract and fractions at each time point. The percentage of inhibition of enzyme activity was expressed as $\{1-(C-D)/(A-B)\} \times 100$ (A, absorbance at 490 nm without test sample; B, absorbance at 490 nm without test sample and enzyme; C, absorbance at 490 nm with test sample, enzyme and substrate; D, absorbance at 490 nm without enzyme). Means of triplicates were determined and the 50% inhibition (IC_{50}) of tyrosinase activity was calculated as the concentrations of test samples that inhibited tyrosinase activity by 50% under the experimental conditions.

DPPH radical scavenging effects

The antioxidant activity of mistletoe extract and each solvent fraction was assessed on the basis of the scavenging activity of the stable DPPH free radical (25). MeOH solutions (1 mL) of samples at various concentrations were added to a solution of DPPH in MeOH (4 mL) and the reaction mixture was shaken vigorously. After storing these mixtures for 30 min at room temperature, the remaining amounts of DPPH were determined by colourimetry at 520 nm. In addition, the radical scavenging activity of each sample was expressed as the ratio of the lowering of the DPPH solution in the absence of compounds. The mean values were obtained from triplicate experiments.

Statistical analyses

All laboratory experiments were performed in triplicates. Data points and error bars on the figures represent mean±standard error of the mean (SEM) for each experiment.

RESULTS AND DISCUSSION

Effects of water extract and its fractions on the activity of mushroom tyrosinase

To determine whether mistletoe extract inhibits the activity of mushroom tyrosinase, we first investigated the inhibitory effect of water extract (WE) and WE fractions classified by molecular weight prepared from dried plants and fractionated by ultrafiltration. The enzyme-reacted solution was incubated for 30 min at indicated doses. Fig. 3A shows the dose-response curve for the tyrosinase inhibitory effect of WE and its fractions. WE and its fractions inhibited DOPA oxidase activity when mushroom tyrosinase was added, and the inhibitory effect increased when using higher extract concentrations. Fraction 3 showed the highest tyrosinase inhibitory activity among the fractions, followed by fraction 1, and fraction 2 showed the lowest. Next, the inhibition of tyrosinase by WE and each fraction was measured time-dependently. The enzyme inhibition was measured by treating 10 mg/mL of whole extract and fractions at each time point. The degree of tyrosinase inhibition by WE reached maximum at 30 min (Fig. 3B).

Effect of methanol extract and its fractions on the activity of mushroom tyrosinase

The inhibition of enzyme activity by fraction 3 indicated that the inhibitory activity is due to low molecular weight compounds. Hence, we prepared a methanol extract to evaluate the inhibition of tyrosinase activity by low molecular weight components (< 1 kDa). The methanol extract was suspended in hot water and fractionated with diethyl ether. The enzyme-reacted solution was incubated for 30 min and the degree of tyrosinase-catalyzed formation of DOPAchrome was measured. Fig. 4 shows the inhibition of tyrosinase by the MeOH extract and diethylether fraction. The MeOH extract inhibited the tyrosinase activity significantly in a dose-dependent manner (IC_{50}=0.58 mg/mL). On the other hand, the inhibition by the highly hydrophobic ether fraction reached a maximum (51%) at 0.3 mg/mL and afterwards decreased gradually (Fig. 4A). Also, the inhibition by the MeOH extract and diethylether layer reached a maximum at 30 min and afterwards decreased rapidly (Fig. 4B).

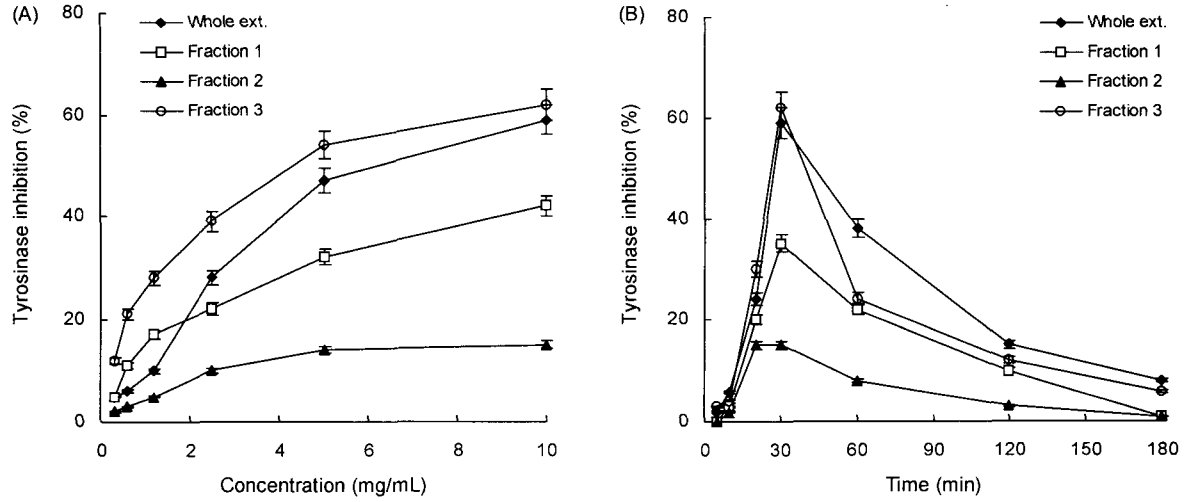

Fig. 3. Dose- (A) and time-dependent (B) inhibition of tyrosinase by whole extract and fractions with different molecules of water extract from *Viscum album* L. var. *coloratum*. Fraction 1, MW $\geq$ 10 kDa; fraction 2, MW=1 ~ 10 kDa; fraction 3, MW $\leq$ 1 kDa. The enzymatic reaction mixture was incubated for 30 min (A) and the concentration of whole extract and fractions for each test was 10 mg/mL (B). The absorbance was measured at 490 nm.

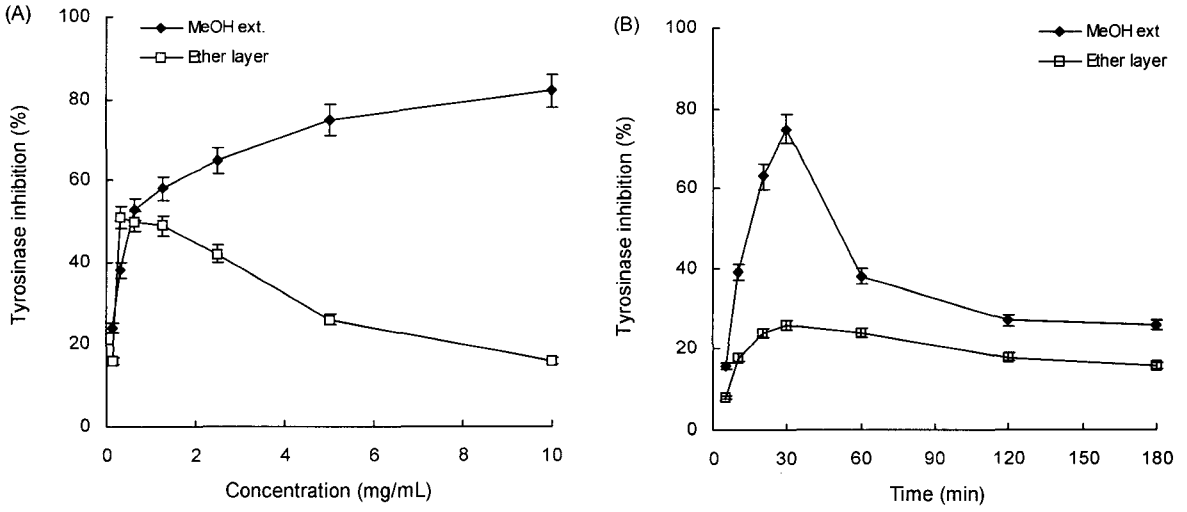

Fig. 4. Dose- (A) and time-dependent (B) inhibition of tyrosinase by 95% MeOH and diethylether extracts. The enzyme-reacted solution was incubated for 30 min (A) and the concentration of extract for each test was 5 mg/mL. The absorbance was measured at 490 nm.

The water fraction of the MeOH extract was further fractionated by column chromatography on Amberlite XAD-2 using H_2O, 30% MeOH, 70% MeOH and 95% MeOH, respectively. As shown in Fig. 5, the fractions eluted with 95% MeOH showed the strongest tyrosinase inhibition, but the fractions eluted with water and 70% MeOH showed low inhibitory effects. Thirty and 95% Methanol fractions dose-dependently inhibited tyrosinase activity. On the other hand, water and 70% MeOH fractions showed inhibitory effects at lower doses but these effects gradually declined at higher doses. On the half-inhibition concentration (IC_{50}) for the fractions, the 95% MeOH fraction had the lowest IC_{50} value (1.3 mg/mL) indicating that it has the highest tyrosinase inhibitory effect, while the 70% MeOH fraction showed the lowest effect (Table 1). At higher concentrations of the 95% MeOH fraction, up to 5 mg/mL, the fraction inhibited the tyrosinase activity by 83%.

It has been reported that phenypropane and lignan such as syringin (16), triterpenoids such as oleanolic acid and β-amyirin (17) and flavonoids such as viscumneoside and viscoside (18,17) were isolated from mistletoe. In the present study, fraction A further purified from the 30% MeOH fraction reacted positively to vanillin-phosphate, which indicated that it may contain lignan or phenylpropane. Both fraction B and C further purified from the 95% MeOH fraction showed a positive reaction in Liebermann-Burchard and Molish tests, which indicates

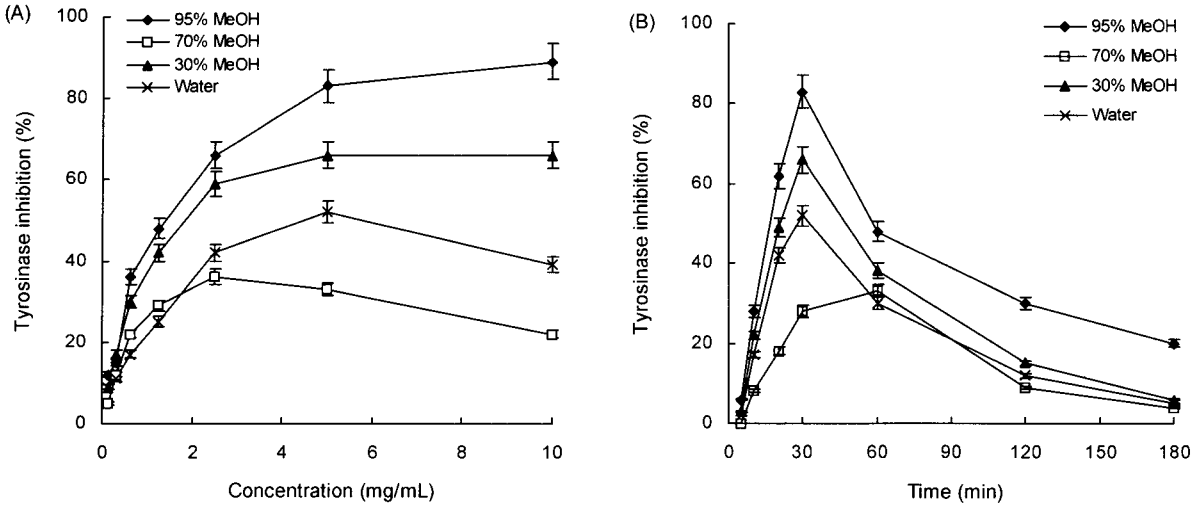

Fig. 5. Dose- (A) and time-dependent (B) inhibition of tyrosinase by fractions eluted with different concentrations of MeOH on Amberlite XAD-2. The enzyme-reacted solution was incubated for 30 min (A) and the concentration of fractions for each test was 5 mg/mL (B). The absorbance was measured at 490 nm.

Table 1. The IC_{50} values and degree of inhibition at 3 mg of water- and MeOH-extract of mistletoe and those fractions against mushroom tyrosinase

Fraction	IC_{50} (mg/mL)	Inhibition (%)
Water ext.	6.2	35
Fr. 1	4.1	42
Fr. 2	-	10
Fr. 3	-	25
MeOH ext.	0.6	67
H_2O Fr	4.8	47
30% MeOH Fr.	1.8	62
70% MeOH Fr.	-	36
95% MeOH Fr.	1.3	73
Fr. A	1.5	68
Fr. B	0.8	85
Fr. C	0.5	92

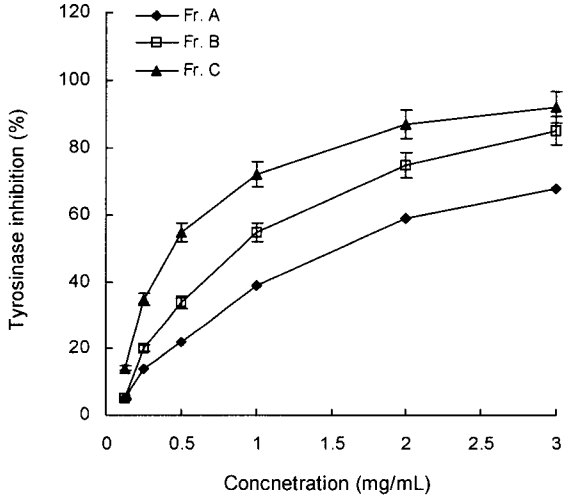

Fig. 6. Dose-dependent inhibition of tyrosinase by fractions purified from 30% MeOH and 95% MeOH fractions on silica gel column chromatography. Fraction A was obtained from the fraction of 30% MeOH on the repeated SiO_2 column chromatography with the solvent system, EtOAc-pyridine-HOAc-H_2O (5:5:1:3, v/v). Fractions B and C were obtained from the 95% MeOH fraction by repeated SiO_2 column chromatography with the solvent system, $CHCl_3$-MeOH-H_2O (70:30:4) for fraction B and $CHCl_3$-MeOH-H_2O (6:4:1) for fraction C. The enzyme-reacted solution was incubated for 30 min and the absorbance was measured at 490 nm.

that they may contain triterpenoid saponin. To study the effects on tyrosinase inhibition by fraction A, B and C, we first assumed from the previous experiments that 30 min is the optimal time to incubate the enzyme-reacted solutions. As a result, compared with those of 30% and 95% MeOH fraction, the fraction C at 3 mg/mL significantly inhibited the enzyme activity up to 92% while the inhibitory effects of fraction A and B were moderately increased (Fig. 6).

DPPH radical scavenging effect

Some potentially active agents, such as kojic acid and arbutin, have not yet been demonstrated to have clinical efficacy (26). A possible way to overcome some of these limitations could be the use of a combination of compounds in a mixture, which may act as whitening agents through different mechanisms and in a complementary way, such as combining tyrosinase inhibitors with efficient antioxidants. In addition, the browning processes are mediated through enzymatic and non-enzymatic oxidation in most foods. The enzymatic oxidation can be prevented by tyrosinase inhibitors and the non-enzymatic oxidation can be prevented by antioxidants (14,27).

To determine the DPPH radical scavenging effects of mistletoe, we first investigated the inhibitory effect of the water extract (WE) and different molecular size fractions of WE. It was found that the WE and all fractions of WE exerted radical scavenging effects (Fig.

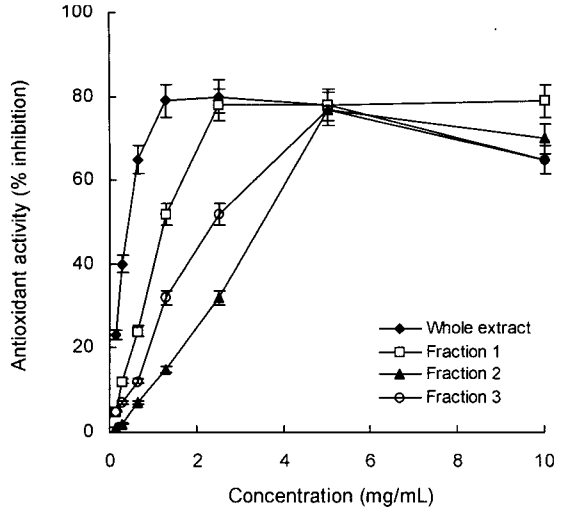

Fig. 7. Dose-dependent DPPH radical scavenging activity by whole extract and fractions with different molecules of water extract from *Viscum album* var. *coloratum*. Fraction 1; MW≥ 10 kDa, fraction 2; MW=1~10 kDa, fraction 3; MW≤1 kDa. The absorbance was measured at 520 nm.

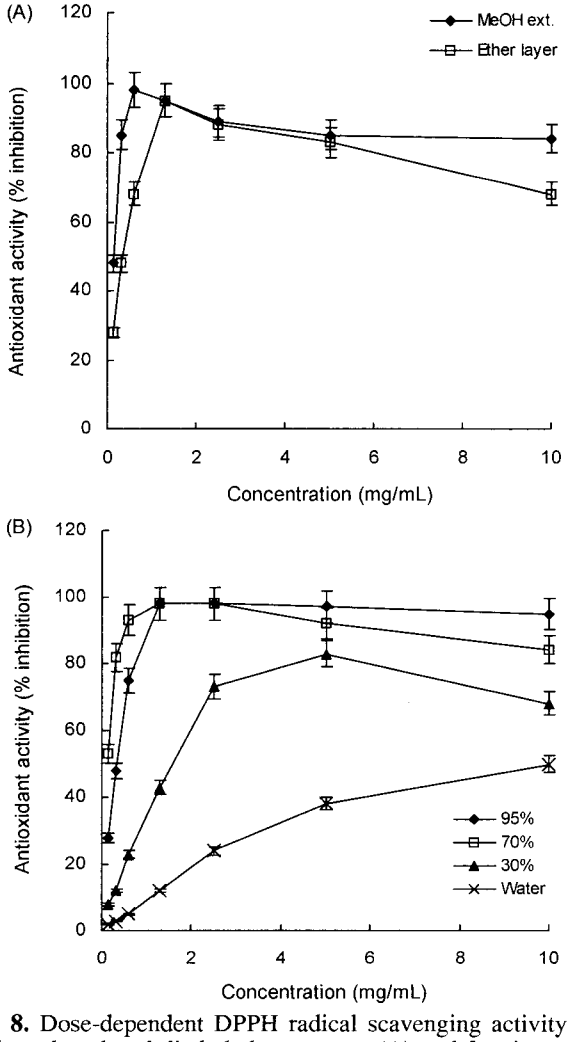

Fig. 8. Dose-dependent DPPH radical scavenging activity by 95% methanol and diethylether extracts (A) and fractions eluted with different concentrations of methanol on Amberlite XAD-The absorbance was measured at 520 nm.

7). Next, we investigated the radical scavenging effects of the MeOH extract and its fractions. Fig. 8A shows the radical scavenging effects of MeOH extract and diethylether layer. MeOH extract and diethylether layer exerted the radical scavenging effects up to 100% and 95%, respectively. The concentration, leading up to a 50% scavenging effect (IC_{50}), was estimated to be 0.16 mg/mL and 0.32 mg/mL, respectively. The fractions eluted with 70% MeOH and 95% MeOH showed high radical scavenging activities while the inhibition by water fractions showed low activities (Fig. 8B).

In conclusion, these results suggest that Korean mistletoe extract may be used for the treatment of pathological processes such as various dermatological disorders and improving food quality. The isolation and the structural elucidation of the active constituents of the extracts will provide useful leads in the development of skin-whitening agents. Therefore, there is a need for further research focusing on isolating and identifying the effective tyrosinase inhibitory components in mistletoe and its inhibitory effect on melanin synthesis.

ACKNOWLEDGEMENTS

This study was supported by research grant of Seoul Women's University in the program year of 2004.

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(Received July 5, 2005; Accepted August 29, 2005)