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Antioxidant and Anti-diabetes Activities of Methanolic Extract and Fractions of *Astragalus membranaceus* Roots

Jae Hyo Park[1*], Yu Yin[2*], and Myeong-Hyeon Wang[2†]

[1]*Department of Neurosurgery, Kangwon National University Hospital, School of Medicine, Kangwon National University, Gangwon-do 200-701, Korea*
[2]*College of Biomedical Science, Kangwon National University, Gangwon 200-701, Korea*

Abstract

The potential biological activities of methanol extract and 5 fractions (hexane, CH_2Cl_2, EtOAc, BuOH and water) from roots of *Astragalus membranaceus* were examined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical (·OH) scavenging activity, reducing power assays, lipid peroxidation inhibitory activity, α-amylase and α-glucosidase inhibition assays. The EtOAc fraction showed high DPPH free radical scavenging activity (EC_{50}=170.34 μg/mL), hydroxyl radical scavenging activity (EC_{50}=32.14 μg/mL), lipid peroxidation inhibitory activity (EC_{50}=52.46 μg/mL) and a concentration dependence, with OD value ranging from 0.234 to 0.345 (0.1 to 0.5 mg/mL), for reducing power. The EtOAc fraction has the highest total phenolic content (142.13 Gal μg/mg) and the CH_2Cl_2 fraction has the highest flavonoid content (71.63 Que μg/mg). Meanwhile, hexane and EtOAc showed certain α-amylase and α-glucosidase inhibition activities. These results suggest that the methanol extract and fractions from *Astragalus membranaceus* root have significant antioxidant and anti-diabetes activities, which could be used as a potential source of pharmaceutical materials.

Key words: antioxidant activity, *Astragalus membranaceus*, extract, fractions

INTRODUCTION

Free radicals are atoms, molecules or ions with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive, so radicals are likely to take part in biochemical reactions. For example, superoxide and nitric oxide can control vascular tone; hydroxyl radical can make a very dangerous compound to the organism (1). Free radicals belong to reactive oxygen species (ROS), which are highly reactive due to the presence of unpaired valence shell electrons. ROS can induce oxidative damage, such as DNA damage, RNA damage, and protein damage, which theoretically contributes to the physiology of aging (2). An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules, and can scavenge free radicals to prevent damage (3).

The diseases of diabetes, obesity, or hyperlipidemia in the population are high and still increasing. In these metabolic disorders, the levels of blood glucose, serum insulin, and lipoproteins frequently differ from normal, especially after food intake (4). A reasonable way to control these carbohydrate-dependent diseases would be to limit intestinal carbohydrate digestion. α-Amylase and α-glucosidase are the key enzymes involved in starch breakdown and intestinal absorption. The inhibition of these enzymes significantly decreases the digestion and uptake of carbohydrate (5).

Astragalus membranaceus is a well-known traditional medicinal plant that is used as adjunctive therapy in the treatment of colds and influenza, chronic diarrhea, edema, abnormal uterine bleeding and diabetes mellitus, and as a cardiotonic agent (6). Both pharmacology and clinical practices have demonstrated that *A. membranaceus* exhibited hepatoprotective, immunostimulating, cardiotonic and antiaging activities (7). The main constituents of the root of *A. membranaceus* include flavonoids, polysaccharides, saponins, amino acids, and trace elements. Since the isoflavones are known to be one of the major beneficial components with suitable chromophores for UV detection, they have been chosen as “marker compounds” for the chemical evaluation or standardization of *A. membranaceus* and its products. Isoflavones derived from many edible plants have been reported to possess various biological activities (8).

Therefore, the aims of this study were to evaluate antioxidant and antidiabetes activities of extract and fractions (MeOH extract, hexane, CH_2Cl_2, EtOAc, BuOH

[*]These authors are equally contributed to this work.
[†]Corresponding author. E-mail: mhwang@kangwon.ac.kr
Phone: +82-33-250-6486, Fax: +82-33-241-6480

and water fra.) of *A. membranaceus.*

MATERIALS AND METHODS

Preparation of extract and fractions

Astragalus membranaceus was obtained from Chuncheon, Gangwon, Korea. Dried *A. membranaceus* root powder (256.22 g) was refluxed with methanol for 3 hr and 3 times at 70°C, and 61.1 g MeOH extract was obtained. Then MeOH extract (42.72 g) was suspended in distilled water (1 L) and partitioned with hexane, CH_2Cl_2, EtOAc and BuOH in sequence to afford the fractions of hexane (0.56 g), CH_2Cl_2 (1.74 g), EtOAc (1.03 g), BuOH (6.59 g) and water (30.32 g) (Table 1).

Measurement of phenolic and flavonoid compounds

Samples of different concentrations (1 mL) of *A. membranaceus* root extract were mixed with 95% EtOH (1 mL), 50% Folin-Ciocalteu reagent (0.5 mL) and 5 mL of distilled water. After reaction of 5 min, 5% sodium carbonate (1 mL) was added. The mixtures were shaken thoroughly. The absorbance at 725 nm was determined after incubation at room temperature for 1 hr in dark. Phenolic content was determined from a standard curve obtained with μg equivalents of gallic acid (μg Eq Gal) (9).

Flavonoid contents were determined as follows: one mL of plant extract in methanol (1 mg/mL) was mixed with 2.8 mL distilled water, 1 mL methanol, 0.1 mL aluminium trichloride in ethanol (10%) and 1 M potassium acetate. The absorption at 415 nm was read after 40 min. Flavonoid content was determined from a standard curve obtained with μg equivalents of quercetin (μg Eq Que) (10).

DPPH free radical scavenging assay

The scavenging activity the DPPH free radical was determined by the method of Yoshida (11) with a slight modification. Two milliliters of various concentrations of sample or water (control) were added to 2 mL DPPH solution (0.2 M in MeOH). Blanks contained 2 mL each of distilled water and sample solution. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark, and the decrease in absorbance at 517 nm was measured after 30 min and continued until the reaction reached at steady state. All the experiments were run in triplicate. DPPH radical scavenging activity was calculated as follows:

$$\text{Scavenging effect (\%)} = \{1-(A_i - A_j)/A_0\} \times 100$$

where A_0 is the A_{517} of DPPH without sample (control), A_i is the A_{517} of sample and DPPH, and A_j is the A_{517} of sample without DPPH (blank). The effective concentration required for 50% plaque reduction (EC_{50}) was determined from a curve relating plaque number to the concentration of a sample.

Hydroxyl radical scavenging assay

The Fenton reaction mixture consisted of 200 μL of $FeSO_4 \cdot 7H_2O$ (10 mM), EDTA (10 mM) and 2-deoxyribose (10 mM). 200 μL sample and 1 mL of 0.1 M phosphate buffer (pH 7.4) were added to make up a total volume of 1.8 mL. Thereafter, 200 μL of 10 mM H_2O_2 was added and the reaction mixture was incubated at 37°C for 4 hr. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and the mixture was placed in boiling water for 10 min. The sample was centrifuged (5 min, 300 rpm) and the absorbance was measured at 532 nm. Each assay was performed in triplicate. The hydroxyl radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = \{1-(A_1 - A_2)/A_0\} \times 100$$

where A_0 was the absorbance of the control (blank), A_1 was the absorbance in the presence of sample, and A_2 was the absorbance without 2-deoxyribose (12).

Measurement of lipid peroxidation inhibitory activity

Liposome sample (egg lecithin 6 mg/mL phosphate buffer, pH 7.4) was sonicated using an Ultrasonicator for 1 hr. Then 0.1 mL of sample was dissolved in methanol at different concentrations and added to 0.5 mL of the liposome mixture. The control was made without the test samples. Lipid peroxidation was induced by adding 10 μL of $FeCl_3$ (200 mM) and 10 μL of ascorbic acid (200 mM). After incubating for 1 hr at 37°C, the reaction was stopped by adding 2 mL of 0.25 N HCl containing 15% trichloroacetic acid (TCA) and 0.375% TBA (thiobarbituric acid). The reaction mixture was boiled for 15 min, cooled and centrifuged, and the absorbance of the supernatant was measured at 532 nm. The blank consisted of all the reagents without the lipid. The lipid peroxidation inhibitory activity was calculated as

$$\text{Inhibitory activity (\%)} = (1 - \text{absorbance of sample}/\text{absorbance of control}) \times 100$$

The lipid peroxidation inhibitory activity of BHT was also assayed for comparison. The EC_{50} (50% of the radicals scavenged by the test sample) values were also determined, with the result that the lower the EC_{50} value, the higher the antioxidant activity.

Reducing power assay

Sample solution (1 mL) was mixed with 2.5 mL of 0.2 M (pH 6.6) phosphate buffer and 2.5 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$), then incubated at 50°C for 20 min. Then 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture, followed by cen-

trifugation at 3000 rpm for 15 min. The upper layer of solution (2.5 mL) was mixed with an equal volume of water and 0.5 mL of ferric chloride ($FeCl_3$) and the absorbance was measured photometrically at 700 nm. The reducing power tests were run in triplicate. Increase in absorbance of the reaction indicated the reducing power of samples. α-Tocopherol was used as a positive control.

α-Amylase inhibition assay

Twenty μL of α-amylase (0.05 U/μL) was premixed with 20 μL of sample and 250 μL of 2% starch solution in 0.1 M sodium phosphate buffer (pH 6.9) was added as a substrate to start the reaction. The reaction was carried out at 37°C for 10 min and terminated by the addition of 200 μL of DNS reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The reaction mixture was heated for 15 min at 100°C and then diluted with 5 mL of distilled (13). α-Amylase activity was determined by measuring absorbance at 540 nm. $IR = (1 - (A_i - A_{iB})/(A_0 - A_{0B})) \times 100\%$; A_i is the A_{540} of sample reactive solution, A_0 is the A_{540} of control reactive solution, A_{iB} is the blank of sample and A_{0B} is the blank of control.

α-Glucosidase inhibition assay

One hundred microliters of 3 mM p-nitrophenyl α-D-glucopyranoside in 0.1 M sodium phosphate buffer (pH 6.9) was added as a substrate to the mixture of 50 μL of α-glucosidase (0.3 U/mL) and 50 μL of sample to start the reaction. The reaction was conducted at 37°C for 15 min and stopped by the addition of 750 μL of 0.1 M Na_2CO_3. α-Glucosidase activity was assessed by measuring the release of p-nitrophenol from p-nitrophenyl α-D-glucopyranoside at 400 nm (14).

Statistical analysis

Means were calculated from three replications for each experiment. Data were analyzed employing SPSS v13.0 (Statistical Package for the Social Sciences, Chicago, IL, USA). Tukey's test was used to determine the significance of difference ($p<0.05$) (15).

RESULTS AND DISCUSSION

The contents of total phenolic and flavonoid

As phenolic compounds have been shown to possess strong antioxidant activity (Rice-Evans), we decided to measure the phenolic and flavonoid contents of fractions as it probably contributes to the antioxidant activity of *A. membranaceus* (16).

The Folin-Ciocalteu reagent determines total phenols, producing a blue color by reducing yellow heteropolyphosphomolybdate-tungstate anions. The phenolic contents of the MeOH extract, hexane fraction, CH_2Cl_2 fraction, EtOAc fraction, BuOH fraction and water fraction were 31.43, 47.72, 131.05, 142.13, 50.35 and 16.59 μg Eq Gal/mg, respectively (Table 1).

Flavonoids are one of the most diverse and widespread group of natural compounds, and compounds such as flavones, isoflavones, flavonones, anthocyanins and catechins, are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging and strong antioxidant capacity. Therefore, the content of flavonoid in the fractions was determined. The flavonoid contents of the MeOH extract, hexane fraction, CH_2Cl_2 fraction, EtOAc fraction, BuOH fraction and water fraction were 15.31, 63.74, 71.63, 46.52, 17.28 and 12.63 μg Eq Que/mg (Table 1).

DPPH free radical scavenging activity

Table 2 shows the DPPH radical scavenging ability of the extract and the fractions as well as the commercial standards of vitamin C, BHA and BHT. The EtOAc fraction showed the highest DPPH radical scavenging activity (EC_{50}=170.34±2.34 μg/mL). The CH_2Cl_2 fraction also showed DPPH radical scavenging activity with good EC_{50} values of 180.32±3.25 μg/mL. While, the EC_{50} values of vitamin C, BHA and BHT were 8.12±1.23, 8.23±1.67 and 3.32±1.15 μg/mL, respectively. The results indicate that the EtOAc fraction has the highest, and water fraction has the lowest, phenolic contents among the fractions. For the antiradical assay (DPPH·),

Table 1. Total phenolic and flavonoid content in methanol extract and fractions from *A. membranaceus* roots

Samples	Production ratio (%)	Total phenolic contents (μg Eq Gal[1)]/mg)	Total flavonoid contents (μg Eq Que[2)]/mg)
MeOH ext.	–	31.43±0.92[b]	15.31±0.43[a]
Hexane fra.	1.31	47.72±0.99[c]	63.74±1.76[c]
CH_2Cl_2 fra.	4.07	131.05±9.95[d]	71.63±0.59[d]
EtOAc fra.	2.41	142.13±11.44[d]	46.52±1.72[b]
BuOH fra.	15.43	50.35±1.34[c]	17.28±0.10[a]
Water fra.	70.97	16.59±0.73[a]	12.63±0.21[a]

[1)]Gal: gallic acid. [2)]Que: quercetin.
Values not preceded by the same letter are significantly different ($p<0.05$).

Table 2. Antioxidant activity of methanolic extract and fractions from *A. membranaceus* roots

Samples	DPPH radical scavenging activity (EC_{50}: μg/mL)	Hydroxyl radical scavenging activity (EC_{50}: μg/mL)	Lipid peroxidation inhibition activity (EC_{50}: μg/mL)
MeOH ext.	1030.16 ± 10.25^{f}	231.23 ± 4.25^{d}	273.54 ± 5.19^{e}
Hexane fra.	670.35 ± 9.98^{d}	123.24 ± 9.12^{c}	172.84 ± 3.64^{d}
CH_2Cl_2 fra.	180.32 ± 3.25^{c}	57.13 ± 1.32^{b}	91.23 ± 2.79^{c}
EtOAc fra.	170.34 ± 2.34^{c}	32.14 ± 5.34^{b}	52.46 ± 4.13^{b}
BuOH fra.	665.12 ± 14.23^{d}	102.58 ± 5.87^{c}	69.71 ± 3.42^{b}
Water fra.	3717.89 ± 32.12^{e}	341.43 ± 12.12^{de}	316.89 ± 4.15^{f}
Positive Control			
Vitamin C	8.12 ± 1.23^{b}	ND	ND
BHA	8.23 ± 1.67^{b}	ND	ND
BHT	3.32 ± 1.15^{a}	ND	15.58 ± 3.68^{a}
α-Tocopherol	ND[1)]	6.66 ± 1.45^{a}	ND

[1)]ND: Not detected.
Values not preceded by the same letter are significantly different ($p<0.05$).

the antioxidant activity of fractions was likely induced by phenols.

Hydroxyl radical scavenging activity

Methanol extract and fractions of *A. membranaceus* showed considerable hydroxyl radical scavenging activity (Table 2). The EtOAc fraction showed the highest scavenging activity among 5 fractions, its EC_{50} is 32.14 ±5.34 μg/mL. The EC_{50} of MeOH extract, hexane fraction, CH_2Cl_2 fraction, BuOH fraction and water fraction were 231.23±4.25, 123.24±9.12, 57.13±1.32, 102.58 ±5.87, 341.43±12.12 μg/mL, respectively. α-Tocopherol was used as a positive control (EC_{50}=6.66±1.45 μg/mL). These results suggest that hydroxyl radical scavenging activity is related to the levels of phenolic compounds present in the scavenger. Phenolic compounds are found in both edible and inedible plants, and they have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triple oxygen, or decomposing peroxides (17).

Lipid peroxidation inhibitory activity

To evaluate the antioxidant activity of fractions and BHT (to inhibit lipid peroxidation in biological systems), a liposome model system was used. Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. Malondialdehyde is the major product of lipid peroxidation and has been studied widely as an index of lipid peroxidation and as a marker for oxidative stress (18). The lipid peroxidation inhibitory activity of fractions and BHT is given in Table 2. The inhibitory activity of EtOAc fraction was higher compared with other fractions. EC_{50} values for MeOH extraction, hexane fraction, CH_2Cl_2 fraction, EtOAc fraction, BuOH fraction and water fraction were 273.54 ±5.19, 172.84±3.64, 91.23±2.79, 52.46±4.13, 69.71 ±3.42, and 316.89±4.15 μg/mL, respectively. The lower the EC_{50} value, the higher the antioxidant activity. Generally, phenolics and flavonoids inhibit the lipid peroxidation by chain termination through scavenging the peroxyl radicals (19). Thus, the EtOAc fraction has the highest lipid peroxidation inhibitory activity owing to contain abundant phenolic and flavonoid compounds. The mechanism of lipid peroxidation in this study might be through electron donation or by scavenging the peroxyl radicals.

Reducing activity

The reducing activity of the *A. membranaceus* increased with increasing sample concentration (Fig. 1). EtOAc also has the strongest reducing power activity in dose-dependent manner among 5 fractions. Reducing

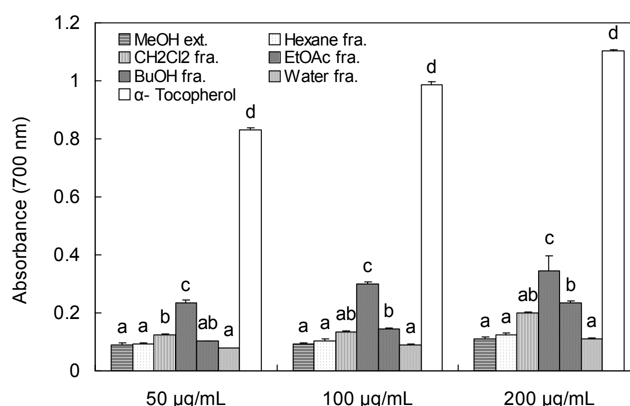

Fig. 1. Reducing power of methanolic extract and fractions from *A. membranaceus* roots. α-Tocopherol was used as a positive control. Vertical bars represent means of three replications±SD. Bars labeled with different letters indicate significant difference at $p<0.05$.

Table 3. α-Glucosidase inhibition rate of extract and fractions of *A. membranaceus* roots

Fractions	α-Glucosidase inhibition rate (IC_{50}: μg/mL)
Hexane fra.	18.65 ± 0.24^{b}
CH_2Cl_2 fra.	71.39 ± 0.53^{c}
EtOAc fra.	113.21 ± 1.39^{d}
BuOH fra.	NE[1)]
Water fra.	28.59 ± 2.14^{b}
Positive control	
Acarbose	0.013 ± 0.0003^{a}

[1)]NE: No effect.
Values not preceded by the same letter are significantly different ($p<0.05$).

power activity is associated with the presence of reductones (20), which have been shown to exert an antioxidant effect by donating a hydrogen atom and breaking the free radical chain. Reductones are reported to react with certain peroxide precursors, which preventing peroxide formation (21). In this study, the EtOAc fraction had high reducing activity, suggesting that the reducing activity of the fraction contributes significantly to its antioxidant effect and that there are likely some kinds of reductones in it.

α-Glucosidase and α-amylase inhibition activities

α-Glucosidase and α-amylase inhibition activities were measured in this study. Table 3 showed that all fractions have certain α-glucosidase inhibition activities except BuOH fraction. The order of IC_{50} value is hexane fraction (18.65 μg/mL)< water fraction (28.59 μg/mL)< CH_2Cl_2 fraction (71.39 μg/mL)< EtOAc fraction (113.21 μg/mL). All fractions exhibited α-amylase inhibition activity at the concentration of 50∼200 μg/mL (Table 4). The EtOAc fraction inhibited α-amylase 25.53% at the concentration of 200 μg/mL, which is the highest among the five fractions. Acarbose was used as a positive control in these two inhibition assays.

Non-insulin-dependent diabetes mellitus is a heterogeneous disease with both environmental and genetic causative factors, and is characterized by hyperglycemia induced by decrease in the secretion of insulin from the pancreatic Langerhans β-cells. Inhibiting α-amylase and α-glucosidase are alternative therapeutic approaches for treating non-insulin diabetes mellitus, which are the key enzymes involved in starch breakdown and intestinal absorption. The digestion and uptake of carbohydrate significantly decreases with the inhibition of these enzymes, which decreases the postprandial blood glucose level in the non-insulin-dependent diabetes mellitus patients (22). Acarbose is presently used as α-amylase and α-glucosidase inhibitors. Most fractions exhibited significant inhibition activity against α-glucosidase except the BuOH fraction (Table 3) and some effect against α-amylase (Table 4). Perhaps α-glucosidase and α-amylase inhibitor can be isolated from *A. membranaceus* roots to treat diabetes disease.

Table 4. α-Amylase inhibition rate of extract and fractions of *A. membranaceus* roots

Fractions	Concentration (μg/mL)	α-Amylase inhibition ratio (%)
Hexane fra.	50	6.06 ± 1.32^{aA}
	100	14.49 ± 2.69^{bA}
	200	14.83 ± 5.30^{bB}
CH_2Cl_2 fra.	50	11.52 ± 2.28^{aB}
	100	16.93 ± 1.84^{bB}
	200	16.06 ± 1.60^{bB}
EtOAc fra.	50	23.09 ± 2.72^{aD}
	100	25.53 ± 2.98^{bC}
	200	25.53 ± 2.12^{bC}
BuOH fra.	50	16.06 ± 3.49^{cC}
	100	14.83 ± 4.26^{bA}
	200	12.57 ± 1.39^{aA}
Water fra.	50	11.34 ± 2.98^{aA}
	100	15.53 ± 2.18^{bAB}
	200	16.23 ± 3.66^{bB}
Positive control		
Acarbose	0.01	76.40 ± 0.80

Within the same fraction, values not preceded by the same small letter are significantly different ($p<0.05$).
Within the same concentration, values not followed by the same capital letter are significantly different ($p<0.05$).

CONCLUSION

In conclusion, *A. membranaceus* roots could be considered as an ingredient of functional foods as well as for pharmaceutical purposes. Further studies on the identification of the compounds in *A. membranaceus* roots containing bioactive properties *in vivo* are necessary to better identify the beneficial effects of this plant.

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