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Electron Donating Ability of MeOH Extracts from Three Korean Mistletoes*1

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ABSTRACT

This experiment was accomplished to investigate antioxidative activity of Korean mistletoe by organic solvents partitioning of methanol extact of 3 Korean mistletoes, fractionationing on column chromatography, and evaluation the reduction of a free radical α,α-diphenyl-β-picrylhydrazyl. Butanol partition of *Loranthus yadoriki* MeOH extract showed higher electron donating ability than α-tocopherol. It was thought phenolic compounds including gallic acid account for antioxidative activity, on execution sub-fractionation, electron donating ability evaluation, and GC/MS analysis, but further studies on what are major actives must be investigated exactly.

Keywords : Mistletoe, antioxidative activity, electron donating ability, gallic acid

1. INTRODUCTION

Oxidation by-products or products that produced by radiation are known to cause extensive damage to DNA, proteins, and lipids (Hong, 1992). Frei (1994) appointed that this damage appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction. Pryor (1994) reported that free radicals play a important role in lipid peroxidation, and showed how the free radical acts in oxidation process to produce oxidation by-products or other radicals. In the earlier time, for example, Davies and Goldberg (1987) reported that exposure of red blood cells to oxygen radical can induce hemoglobin damage and stimulate protein degradation, lipid peroxidation, and hemolysis. Free radicals are chemical fragments that cause body damage that can lead to food deterioration and diseases conditions including accelerated aging, cancer, heart disease and arthritis (Streejayan and Rao, 1996). Antioxidants act as radical scavengers. Larson (1998) reviewed the antioxidants of higher plants, and he reported that many enzymes and secondary compounds of higher plants have been demonstrated to protect against oxidative damage by inhibiting or quenching free radical and reactive oxygen species. He also suggested that the roles of many other compounds as potential antioxidants can be inferred by their similarity to synthetic antioxidants of related structure.

DPPH (α,α-diphenyl-β-picrylhydrazyl) has

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been known as a free radical which is a purple compound having characteristic absorption at 517 nm in MeOH, and very stable in even water or organic solvents (Takao *et al.*, 1994). This compound can be directly detected quantitatively without any detecting aid, as the purple color rapidly faded when DPPH encountered any radical scavenger. Because of this properties, DPPH is very useful as a free radical source to evaluate electron donating ability (EDA) of samples. Change in absorbance value at 517 nm was investigated to evaluate antioxidation ability of sample. Decrease of the absorbance in test solution represents decrease of free radical type DPPH, that is DPPH reduction, by radical scavenger. In this experiment, absorption peaks of DPPH were observed at 327 and 515 nm in MeOH, and the latter represents purple color. Therefore the absorbance value at 515 nm was used to measure the decrease of DPPH concentration in testing solution. The purple color defaded when compound donating electron was added to DPPH solution.

2. MATERIALS and METHODS

2.1 Materials

Mistletoe species and sampling parts used in this experiment were represented in Table 1. This experiment did not consider variable characteristics of the mistletoe sample depending on sampling season and host species. Fresh tissue was charged in frozen state, and drying it in shade gave air-dried tissue.

2.2 Extraction and fractionation

Samples were homogenized with 80% aq. MeOH for 30 min. Freeze-drying of filtrated solutions gave brownish powder. MeOH extract in small volume of 10% aq. MeOH was partitioned into $CHCl_3$(C), EtOAc(E), *n*-BuOH(B), residual water(W) layers successively. Further fractionation of *n*-BuOH partition gave three fractions of X1(X1), X2(X2) and X3(X3) by elution with water, MeOH-water(1:1) and MeOH on Amberlite XAD-7 column. Eluate was separately charged in refrigerator, freeze-dried and weighed. However, LTB, the *n*-BuOH fraction of *L. yadoriki* twig, could not eluted out of Amberlite XAD-7. It was tried to fractionate LTB on silica gel column.

2.3 Electron donating ability

DPPH (α,α-diphenyl-β-picryl-hydrazyl) (Constantin *et al.*, 1990) was adopted as a free radical source to evaluate proton-free radical scavenging effect, that is electron donating ability (EDA) of

Table 1. Characteristics of the Korean mistletoe samples.

Mistletoe Scientific Name*1	Part	Sampling Date	Host Scientific Name	Symbol	Tissue
Pseudixus japonicus HAYATA	Leaf & Twig	July '95	*Camellia japonica* Linnaeus	KJ	Fresh
Viscum album var. *coloratum* OHWI	Leaf	October '95	*Quercus acutissima* Carruthers	VL	Fresh
	Twig			VT	Air-dried
Loranthus yadoriki SIEB.	Leaf	October '94	*Camellia japonica* Linnaeus	LL	Air-dried
	Twig			LT	Air-dried

*1 : named according to Lee (1985).

sample. Initial testing solution concentraction was adapated to 240 μg/4 ml. Testing solutions of the concentrations, 120 μg/4 ml, 60 μg/4 ml, 30 μg/4 ml, 15 μg/4 ml, 7.5 μg/4 ml, 5 μg/4 ml, 2.5 μg/4 ml and 0 μg/4 ml (control), were made from this initial testing solution by dilution stepwise with MeOH. 2 ml Testing solution vortex-mixed with 1 ml DPPH solution was stood for 10 minutes at room temperature, and absorbance value of testing solution at 515 nm was measured by UV spectrophotometer.

EDA of sample, which implies the reduction of DPPH by sample in this experiment, was calculated by Equation 1. Absorbance value of DPPH solution without sample, that is control solution, represents initial DPPH concentration. Absorbance value with sample was compared with that of control. Absorbance value of control solution was set to represent zero EDA. α-Tocopherol, a typical natural antioxidant, was used as a reference compound. However, BHT, a synthetic antioxidant, was not included due to its very low EDA of less than 150 μg/ml. All data were average values of triplicate.

$$EDA\ (\%) = 100 \times (ABS_C - ABS_S) / ABS_C \quad \text{Equation 1}$$

where ABS_C is absorbance value of control solution and ABS_S is absorbance value of testing solution at 515 nm.

Absorbance value of control solution decreased depending on time when DPPH solution was exposed to room temperature. It was needed to prepare standpoint for comparing EDA values of sample each other. Therefore, EDA_{50} value, which means the sample concentration in testing solution showing 50% EDA value, was introduced to prepare the standpoint. The EDA_{50} value was calculated from the linear regression analysis by plotting logarithmic values of sample concentrations in testing solution with EDA values of sample (See Equation 2).

$$EDA = a \times \ln(C) + b \quad \text{Equation 2}$$

where a and b are constant, and C is the sample concentration in testing solution, μg/ml.

In the Equation 2, constant a and b were calculated by linear regression, and the calculated values were substituted for a and b in Equation 3. EDA_{50} value was obtained from Equation 3.

$$EDA_{50} = EXP\left(\frac{EDA-b}{a}\right) \quad \text{Equation 3}$$

The EDA_{50} values of sample calculated from Equation 3 were shown in Table 2, and they were compared to that of α-tocopherol (See Equation 4). Linear regression was conducted at 95% confidence level.

$$EDAR_{50}\ (\%) = \left(\frac{EDA_{50,T}}{EDA_{50,S}}\right) \times 100 \quad \text{Equation 4}$$

where $EDA_{50,T}$ is EDA_{50} of α-tocopherol, and $EDA_{50,S}$ is EDA_{50} of tested sample.

2.4 TBA assay

1 g Mouse liver was homogenized with 9 mL saline solution at cool state to give liver homogenate solution. To 300 μl liver homogenate was added 100 μl test solution following addition with 3.7 μl TBA solution(0.3% 2-thiobarbituic acid + 0.4% sodium dodecyl sulfate/7.5% acetate buffer, pH 4). Mixed solution was boiled at 100°C for 1.0 hour and then cooled to room temperature. Cooled mixed solution was added 4 ml *n*-BuOH and vortexed, and then centrifugated at 3,000 rpm for 10 minutes. The absorbance at 532 nm of *n*-BuOH layer was measured.

2.5 Apparatus and chemical analysis

Column packing materials for separating systems were Sephadex® LH-20 (Lot No. 227621, Pharmacia Biotech, Sweden), silica gel (Kieselgel 60, 0.063~0.200 mm, Merck Co., Germany), Amberlite XAD-7 (Nonionic polymeric adsorbent, Lot 45H1153, Sigma Co., USA), cellulose powder (DS-0, Fluka, Switzerland), and RP 18 (Silica gel $100C_{18}$-reversed phase, Fluka, Switerland). TLC plates were precoated cellulose F (0.1 mm thickness, Art, 5718, Merck, Germany) and pre-coated silica gel (Kieselgel 60 F_{254}, Merck, Germany). Detecting agent and UV light were used for identifcation of spots. Instruments are follows: homogenizer (ULTRA-TURRAX T50, JANKE & KUNKEL, IKA®-LABORTECHNIK, Germany), rotary evaporator (N-N Series, EYELA, Japan), centrifuge (UNION 5KR, HANIL, Korea), freeze dryer (BONDIRO FD8512, ILSHIN, Korea), incubator (CAN & AM Deluxe, Great Britain), UV-VIS spectrophotometer (UV-1601PC, Shidmazu, Japan), FT-IR spectrophotometer (Spectrum BX Series, Perkin Elmer, UK), automated Bioreactor (Bioscreen; Labsystems Oy), capillary GC (Hewlett packard), GC/MSD (JEOL JMS600, USA) and HPLC (THERMO SEPARATION PRODUCTS, P2000 system, SP, USA).

2.6 Preparation of trimethylsilyl derivative

About 1 mg sample was dissolved with 0.25 ml pyridine in vial, and the resultant clear solution was added to 0.15 ml hexamethyldisilazane and 0.05 ml trimethylchlorosilane. The vial was sealed, set aside at room temperature overnight, centrifugated, and the supernatant was used directly for injection (Dalgliesh *et al.*, 1996; Monachino, 1956). In addition, 100 μg sample was dissolved with 50 μl pyridine, and after adding 100 μl bis (trimethylsilyl)trifluoroacetamide (BSTFA), including 1% trimethylchlorosilane (TMCS), the tube was sealed and heated for 30 minutes at 100°C (Greenaway *et al.*, 1988). The cooled solution was used directly for injection.

3. RESULTS and DISCUSSION

3.1 Primary screening on DPPH reduction

All $CHCl_3$ partitions showed low EDA_{50} values less than 50% of EDA_{50}, 11.8 μg/ml, of α-tocopherol (See Table 2). The result was deviated from the expectation that non-polar fractions including lipids and chlorophyll would be most prominent or major actives in scavenging proton free radical, because those compounds are involved in photosynthetic electron or proton transfer process and they prevent tissue oxidation by auto-oxidation or anti-oxidation. (Larson, 1988). *L. yadoriki* extracts showed the highest EDA among extracts of the Korean mistletoes. All extracts from *L. yadoriki* leaf and twig showed EDA_{50} of less than 31.8 μg/ml. Other samples of *P. japonica* and *V. album* var. *coloratum* showed lower EDA_{50} values than those of *L. yadoriki*. EtOAc and *n*-BuOH partitions of *L. yadoriki* gave high activities to DPPH reduction, comparable to α-tocopherol (Table 2). These partitions lost sharply their EDAs at 25-50ppm dose. All the more, *n*-BuOH partitions had higher activities than EtOAc partitions. LTB showed the highest EDA_{50} value, 11.5 μg/ml, among all samples tested against DPPH reduction. Next to LTB, LLB and LTE showed EDA_{50} values of 14.1 and 14.5 μg/ml, respectively.

LLB and LTB were chosen as candidate parti-

Table 2 . Electron donating ability of extracts from three Korean mistletoes.

Species		Sample	EDA_{50} (μg/ml)	$EDAR_{50}$ (%)
Pseudixus japonica		KC	44.0	27
		KE	37.5	31
		KB	50.0	21
		KW	> 550.0	tr
Viscum album var. *coloratum*	Leaf	VLC	230.0	5
		VLE	64.0	18
		VLB	31.3	38
		VLW	126.0	9
	Twig	VTC	80.0	15
		VTE	63.0	19
		VTB	65.0	18
		VTW	230.0	5
Loranthus yadoriki	Leaf	LLC	31.8	37
		LLE	17.5	67
		LLB	14.1	84
		LLW	20.6	57
	Twig	LTC	26.0	45
		LTE	14.5	81
		LTB	1.5	103
		LTW	17.0	69
α-Tocopherol			11.8	100

* : Significant at 95% confidence level

tions in studying what are active compounds in DPPH reduction. However, LTE having EDA_{50} equivalent to LLB was excluded in following EDA screening. LTE showed lower EDA_{50} and yield than LTB. In addition, LTE showed the same development behaviors as LTB when its white-on-purple spots was detected by spraying DPPH solution (80 μg/ml EtOH) after development on cellulose TLC plate.

3.2 Electron donating ability of LTB

LTB showed dark reddish-brown color. It had phenolic compounds having resorcinol or phluoroglucinol skeleton, identified by sequential process of blotting LTB aqueous MeOH solution on cellulose TLC plate and detecting developed spots. Detecting of spots on plate was accomplished by lighting plate with UV lights, and coloring with spraying ferric chloride, Folin-Ciocaltue reagent following exposure to ammonia gas, and Vanilin-HCl. LTB partition was mostly soluble in aq. MeOH and aq. EtOH of concentration of less than 30%, insoluble in water and $CHCl_3$, and partially soluble in water-MeOH mixture(1:1). On cellulose TLC plate, the developing system of mixture of EtOH and water, ratio 8 of 2, was found as the most adaptable developer. Also TLC systems showed that silica gel was not adequate to separate compounds in partition mode.

Silica gel adsorption column chromatography was firstly adopted to fractionation LTB into subfractions, because partition column chromatography on cellulose and silica gel could not elute efficiently the loaded sample and gave severe tailing and lengthened separation time. Successive

elution with $CHCl_3$, $CHCl_3$-MeOH(8:2, 2:8), MeOH, $CHCl_3$-MeOH-water(65:35:10, 5:4:1, 1:8:1 and 1:9:2) gave LTB1, LTB2, LTB3, LTB4, LTB5, LTB6 and LTB7 subfractions, respectively. Each elution was finished when no absorbance of eluate was observed at 280 nm. Each eluate was concentrated by evaporator, freeze-dried, and tested on DPPH reduction.

The subfraction of the highest EDA_{50} was LTB3 having EDA_{50} value of 6.8 μg/ml, and the next was LTB2. α-Tocopherol, the reference compound, had EDA_{50} of 12.4 μg/ml. However, the EDA_{50} values of the other fractions were lower than α-tocopherol (Table 3).

LTB3 is a fraction gained from silica gel chromatography by elution with $CHCl_3$-MeOH(2:8) mixture. This fact suggested that the active compound in scavenging proton free radical have slightly lower polarity than MeOH. LTB3 was refractionated on silica gel column (25 x 2.5 cm) chromatography, where successive elution with $CHCl_3$-MeOH(6:4, 5:5, 3:7, 1:9) and MeOH gave 5 subfractions of LTB3_S1, LTB3_S2, LTB3_S3, LTB3_S4 and LTB3_S5, respectively. Each subfraction was tested on electron donating abilities in DPPH system mentioned before. There was no subfraction having higher $EDAR_{50}$ value than that of LTB3, 182% as shown in Table 3. On the contrary, all subfractions had EDARs of less than 100%. Chromatography of LTB3 on silica gel column caused severe loss in amount of loaded sample and elongated analysis time of three days. Additionally, chromatography gave no improvement of EDA_{50} value. Therefore, LTB3 was tried to fractionate further by C18 column chromatography.

ODS packed column (150 x 25 I.D. mm) was also used as the stationary phase, where outlet part was connected UV/VIS spectrophotometer. The mobile phase was composed of MeOH and water. Elution with MeOH-water (0:10, 1:9, 2:8, 3:7, 2:8, 3:7, 4:6, 5:5 and 6:4) gave LTB3_OD1, LTB3_OD2, LTB3_OD3, LTB3_OD4, LTB3_OD5, LTB3_OD6, and LTB3_OD7, respectively. Final washing was carried out with MeOH. The flow rate was set to 2.0 ml/min. Absorbance at 340 nm was plotted with time and each elution continued to no absorbance at 340 nm. Characteristic heading pattern was observed on chromatograms.

LTB3_OD4 fraction obtained from MeOH-water(3:7) elution had the highest EDA of 202%. Fractionation of LTB3 in direction to give higher EDAR could be carried out successfully on ODS column without severe loss in EDAR and yield.

Absorbance of DPPH at 515 nm changed on the concentration of LTB3_OD4 (Figure 1). The absorbance values showed pattern to reduce stable values independently on sample concentration in 200 seconds. Absorbance value decreased more sharply as LTB3_OD4 concentration became higher. It is suggested that the electron donating ability of LTB3_OD4 is determined in very early time of DPPH reduction.

Table 3. Effect of subfractions of LTB on DPPH reduction.

Silica gel column chromatography				ODS column chromatography	
Sample	EDAR (%)	Sample	EDAR (%)	Fraction	EDAR (%)
LTB1	23	LTB3_S1	<5	LTB3_OD1	162
LTB2	132	LTB3_S2	75.8	LTB3_OD2	134
LTB3	182	LTB3_S3	36.0	LTB3_OD3	106
LTB4	83	LTB3_S4	6.5	LTB3_OD4	202
LTB5	24	LTB3_S5	<5	LTB3_OD5	80
LTB6	31			LTB3_OD6	88
LTB7	28			LTB3_OD7	29

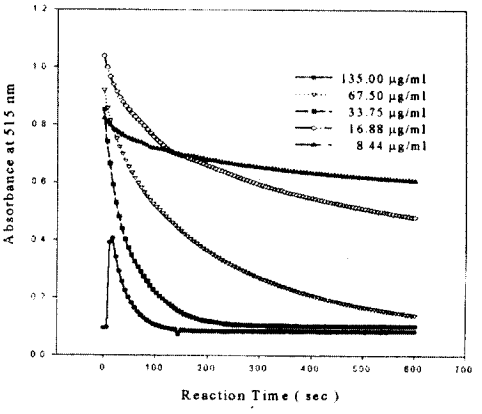

Fig.1. Absorbance change of DPPH solution depending on reaction time and LTB3_OD4 concentration.

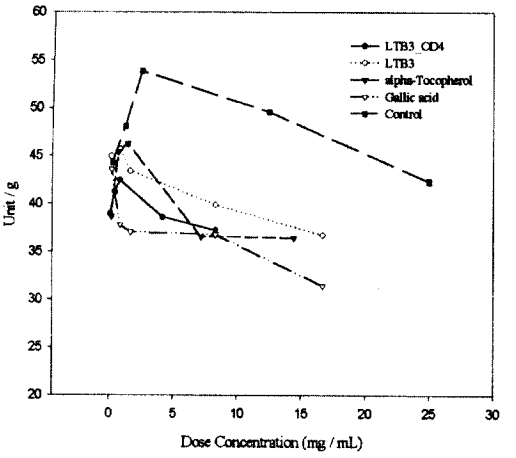

Fig. 2 . TBA activity of LTB, LTB3 and Gallic acid.

This result was similar to Streejayan and Rao's result (Streejayan and Rao, 1996), where curcuminoids reduced DPPH concentration in inverse functionally dependently on reaction time. They also reported that compounds capable of scavenging superoxide are active in reducing DPPH.

Therefore LTB3_OD4 isolated from the Korean mistletoe, *L. yadoriki*, is expected to have very active in scavenging superoxide. Similar to this result, recently, Wagner *et al.* (1998) reported that they isolated flavonoids, flavonans and proanthocyanidins from Loranthaceae and Viscaceae which grow in Argentine, and following assays for antioxidative activity showed positive activities of especially the proanthocyanidins. However, all LTB fraction and α-tocopherol showed no prominent TBA activity as shown in Figure 2.

3.3 Spectroscopic and chemical observation on LTB3

IR spectrum of LTB3 showed characteristic absorbance pattern in which there are O-H stretch (3,400-3,500 cm^{-1}), aromatic C-H stretch (3,100-3,000 cm^{-1}), C=C ring stretch (1,600 cm^{-1} around, strong), and C-O stretch (1,100 cm^{-1} around) (Silverstein *et al.*, 1991). These data showed that LTB3 might be composed of phenolic compounds. Characteristic absorption peaks of LTB2, LTB3 and LTB4 are shown in 280.6 and 222.2 nm, 280.5 and 212.5 nm, and 280.0 and 216.5 nm, respectively. Absorption peak of LTB3 was similar to LTB2 and LTB4. This fact suggests that three subfractions have similar chemical structure skeleton each other. Phenols are known to have aromatic $\pi \rightarrow \pi^*$ transition absorption at 210 and 270 nm. Aromatic $\pi \rightarrow \pi^*$ pattern are shown at 180, 200, and 255 nm in benzene, 244 and 282 nm in styrene, 208 and 262 nm in toluene, and 240 and 278 nm in acetophenone (Silverstein *et al.*, 1991) Cellulose TLC of LTB3 was accomplished by elution with the developer of EtOH-water mixture(8:2). Irradiating with $UV_{366/254}$ lighting gave 4 tailed spots and 1 original point, and coloring with spraying 1% $FeCl_3$ in EtOH showed blue-grey coloured spots. Additionally, Folin-Ciocaltue reagent following exposure to

ammonia gas revealed blue to grey colors and Vanilin-HCl solution, 1 g/100 ml HCl, gave scarlet spots. Silica gel TLC gave dark brown spots or bands with spraying concentrated sulfuric acid and following heating at 100℃ after developed with solvent systems, although spot resolution was not good.

From the above chemical analyses, it is suggested that the major components of LTB3 are aromatic phenolics including compounds of resorcinol or phluoroglucinol skeleton, and they consist of major 5 kinds components.

GC/MS analysis of LTB3_OD4 by pyridine-catalyzed sillylation showed that gallic acid is most prominent major component. There were also several sugars and other phenolic compounds. Gallic acid showed more less EDAR than that of LTB3_OD4. Howeyer, it must be further researched whether antioxidative activity of *L. yadoriki* extractives may caused by the synergistic effect of gallic acid and its related polyhydroxy phenolic derivatives.

Monachino (1956) reported that *Loranthus yadoriki* S_{IEB}. and Z_{UCC}. had the bark being effective in treatment of hypertension and this species grows in China, Japan, and Korea. Frei (1994) appointed damage by free radicals to degenerative diseases of cardiovascular disease. It is anticipated that later studies would show extract of *L. yadoriki* in Korea has antioxidative activity and this result gave Korean mistletoe potentiality as medicinal source of cardioactive drug or treatment of cardiovascular disease, similar with Wagner *et al.*'s suggestion (1986) in the study on extractive of *Viscum album*.

4. CONCLUSION

In order to find out the biological activity of extracts of the Korean mistletoes, MeOH extracts and their partitions were tested on their electron donating ability against DPPH, a free radical. Species showing most prominent electron donating ability (EDA) was *L. yadoriki* among the Korean mistletoes, and LTB3, a butanol subfraction of *L. yadoriki* twig, had highest EDA_{50} value. Further fractionation of LTB3 on ODS column chromatography caused increase in EDA_{50} while on silica gel did decrease. TLC and GC/MS analyses of LTB3_OD4 showed that gallic acid is major component and that there were other phenolic compounds and sugars. It is suggested that antioxidative activity of *L. yadoriki* extracts may be improved efficiently by purification on reverse-phase chromatography.

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