Journal of Medicinal Plants Research Vol. 4(8), pp. 674-679, 18 April, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR09.464
ISSN 1996-0875© 2010 Academic Journals

Full Length Research Paper

In vitro anti-oxidant activity of *Lespedeza cuneata* methanolic extracts

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Accepted 19 March, 2010

In this study, the anti-oxidant activities of *Lespedeza cuneata* extract and fractions were evaluated by various *in vitro* assays, including electron donation ability (EDA) assay, reducing power, ferric thiocyanate (FTC) and thiobarbituric acid (TBA) tests. The electron donation ability of the ethylacetate fraction (95%) demonstrated stronger activity than butylated hydroxyanisole (16%). The ethylacetate fraction (OD_{700} = 0.68) also exhibited the strongest reducing power. Total phenol and flavonoid content were highest in the ethylacetate fraction (359.54 mg GAE/g and 209.63 mg QE/g, respectively). Methanolic extracts had greater anti-lipid peroxidative effects than that of an identical concentration of butylated hydroxyanisole. This result suggests the potential of development of useful natural anti-oxidants.

Key words: Electron donation ability, ferric thiocyanate method, *Lespedeza cuneata*, reducing power, thiobarbituric acid test.

INTRODUCTION

Lespedeza cuneata is an aggressive, warm-season perennial legume that was introduced from Asia for use in hay production and foraging of poor soils, and for controlling erosion along roadsides (Wang et al., 2008). In its native Asia, *L. cuneata* grows on exposed ground and grassy lowlands (Ohiwi, 1965), blooming from July through October. The plant grows up to 2 m in loamy soils and has an extensive taproot that extends up to 120 cm or more. Leaves of *L. cuneata* are trifoliate and have a short petiole.

β-Sitosterol, succinic acid, triacontan-1-ol, quercetin, kaempferol, pinitol, avicuralin, juglanin and trifolin were isolated from the leaves of *L. cuneata* and their structures determined (Shin et al., 1978). This allowed a number of other chemical constituents such as 6,8-di-C-pentosylapigenin, 6,8,3',4'-tetrahydroxy-2'-methoxy-7-methylisoflavanone, 6,8,3',4'-tetrahydroxy-2'-methoxy-6'-(1,1-dimethylallyl)-isoflavone, betulinic acid, hexacosanoic acid 2,3-dihydroxy-propyl ester, hyperin, hirsutrin, desmodin, homoadonivernite, isovitexin, isoorientin, vicenin-2 and lucenin-2 to be isolated and analyzed from the leaves, roots and aerial parts of *L. cuneata* (Deng et al., 2007; Kwon et al., 2007; Kwon and Bae, 2009; Numata et al., 1979, 1980). *L. cuneata* has been used as folk remedy for premature ejaculation, impotence, involuntary emission of semen, coughing and asthma (Kee, 1993). Current pharmaceutical studies confirmed the effects of *L. cuneata* on sexual function (Chung and Lim, 2005). In fact, *L. cuneata* was shown by several reports to possess anti-oxidant activity when investigated by DPPH assay and HPLC analysis (Kim and Kim, 2007; Kim and Ryu, 2008). Therefore, further analysis using various assay methods will be required to better understand the anti-oxidant properties of *L. cuneata*.

In this study, we reported the anti-oxidant activities of *L. cuneata* crude extract and fractions. In order to better understand the anti-oxidant activity, we determined the amount of phenols and flavonoids. Furthermore, careful analysis of electron donation ability, reducing power and

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Abbreviations: **BHA**, Butylated hydroxyanisole; **BHT**, butylated hydroxytoluene; **DPPH**, 1,1-diphenyl-2-picrylhydrazyl; **EDA**, electron donation ability; **FTC**, ferric thiocyanate; **TBA**, thiobarbituric acid; **ROS**, reactive oxygen species.

anti-lipid peroxidative effects were performed.

MATERIALS AND METHODS

Chemicals

α-Tocopherol, butylated hydroxyanisole (BHA), butylated hydroxy-toluene (BHT), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade or higher.

Plant material and extraction

L. cuneata G. Don was purchased from Segyero co. (Yeongju, Korea). The voucher specimen (KNUA-O-1) was established botanically and deposited in the Division of Bio-resources Technology, Kangwon National University. Dried leaves of *L. cuneata* (1 kg) were extracted with methanol at room temperature. The solution was filtered, evaporated under reduced pressure and lyophilized. The combined methanolic extract was partitioned with organic solvents to yield *n*-hexane, ethyl-acetate (EtOAc), *n*-butanol (BuOH; water saturated) and aqueous fractions.

Electron donation ability (EDA) assay

The EDA of sample was determined by the method of Kim et al. (2010). This assay is based on the capacity of a substance to scavenge stable DPPH free radicals. The EDA of *L. cuneata* was measured as follows. The reaction mixture contained 1 ml of 0.15 mM DPPH-methanol solution, 3.98 ml of methanol, and 20 µl of different concentration samples, or α-tocopherol, BHT, and methanol (control). The mixture was allowed to react for 30 min at room temperature and the absorbance values were measured at 517 nm using a spectrophotometer (V-530, Jasco Co., Tokyo, Japan). The experiment was conducted in triplicate. The EDA was expressed as the reduction rate of absorbance in accordance with the following equation:

EDA (%) = [1-(absorbance value of sample/absorbance value of control)] × 100.

Determination of reducing power activity

The reducing power of samples was determined via the method of Oyaizu (1986), with some modifications. Reducing power activity is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants (14). Samples with different concentrations were mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 ml of 1% potassium ferricyanide (w/v). The mixture was incubated for 20 min at 50°C. After incubation, 2 ml of 10% TCA (w/v) was added to the mixture, followed by 10 min of centrifugation at 650 rpm. The upper layer (0.5 ml) was mixed with 0.5 ml of deionized water and 0.1 ml of 0.1% ferric chloride (w/v), and the absorbance of the resultant solution was measured at 700 nm. α-Tocopherol and BHT were used as reference compounds.

Total phenol and flavonoid analysis

The total phenolic content was determined using Folin-Ciocalteu reagent in accordance with the method described by Singleton and Rossi (1965). In brief, 0.1 ml of sample and 50 µl of 2 N Folin-Ciocalteu reagents were added to a 5 ml volumetric flask. The solutions were mixed and allowed to stand for 3 - 5 min at room temperature. Next, 0.3 ml of a 20% sodium carbonate solution (w/v) was added. The solutions were mixed and kept aside for 15 min. Finally, 1 ml of distilled water was added. The blue color was measured against a reagent blank at 725 nm. The total phenolic content was expressed in milligrams of gallic acid equivalents (GAE) per gram of samples.

The total flavonoid content of the extracts was determined via the colorimetric method as described by Park et al. (1997). An aliquot of 0.2 ml was added to test tubes containing 0.1 ml of 10% aluminum nitrate (w/v), 0.1 ml of 1 M potassium acetate, and 4.6 ml of 80% ethanol. After 40 min at room temperature, the absorbance was determined at a wavelength of 415 nm. The total flavonoid contents of the sample were determined by comparison with the optical density values of different concentrations of a standard flavonoid compound, quercetin. This analysis for each sample was analyzed in triplicate, and a calibration curve of quercetin was plotted by plotting the absorbance vs. the concentration of quercetin.

Ferric thiocyanate test (FTC)

The test was conducted via the FTC method in a linoleic acid emulsion with some modifications (Haraguchi et al., 1992). The reaction medium contained 0.02 ml of sample (10 mg/ml), 0.2 ml of 2.51% linoleic acid in ethanol, 0.4 ml of 0.04 M potassium phosphate buffer (pH 7.0), and 0.38 ml of distilled water. The solution (1 ml) was mixed and incubated for 20 min at 70°C in darkness. The same reaction medium without any additive was used as a control. Synthetic antioxidants (BHA) were used for comparison at an identical concentration.

At regular intervals during incubation, a 0.05 ml aliquot of the mixture was diluted with 2.85 ml of 75% ethanol, followed by the addition of 0.05 ml of 30% ammonium thiocyanate (w/v) and 0.05 ml of 20 mM of ferrous chloride in 3.5% HCl. The absorbance of the red-colored portion of the sample was measured at 500 nm. These steps were repeated every 3 h until the control achieved its maximum absorbance value.

Thiobarbituric acid (TBA) method

The sample solution was prepared and incubated as described above. The assay was based upon the reaction of TBA with malonaldehyde, an aldehyde product produced by lipid peroxidation. One ml of 20% TCA and 1 ml of 0.67% TBA solution were added to 0.5 ml of the sample mixture prepared by FTC method. This mixture was placed in a heating block (95°C) for 10 min and was centrifuged at 3000 rpm for 20 min after cooling to room temperature. Anti-oxidant activity was based on the absorbance of the supernatant at 532 nm at the final time of the FTC method.

RESULTS AND DISCUSSION

For determination of the EDA, *L. cuneata* extract and fractions were prepared according to the DPPH method. The EDA of the EtOAc and BuOH fractions of *L. cuneata* was stronger than the BHT, but did not exhibit higher anti-oxidant activity than α-tocopherol. Each sample showed different activities. In particular, the EtOAc fraction (94.5%) exhibited the highest activity at 20 µg/ml, which was approximately 5.9 fold higher than that of the positive control BHT (16.1%) (Figure 1).

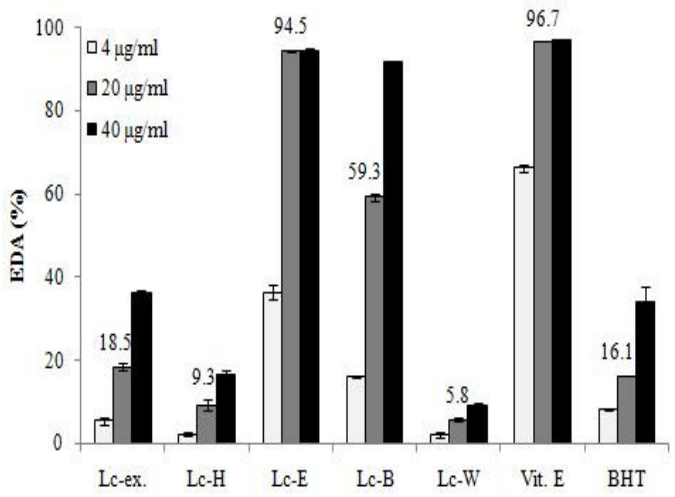

Figure 1. Electron donation ability (EDA) of extract and fractions from *Lespedeza cuneata*. Lc-ex., methanolic extract; Lc-H, *n*-hexane fraction; Lc-E, EtOAc fraction; Lc-B, BuOH fraction; Lc-W, aqueous fraction; Vit. E, α-tocopherol; BHT, butylated hydroxytoluene.

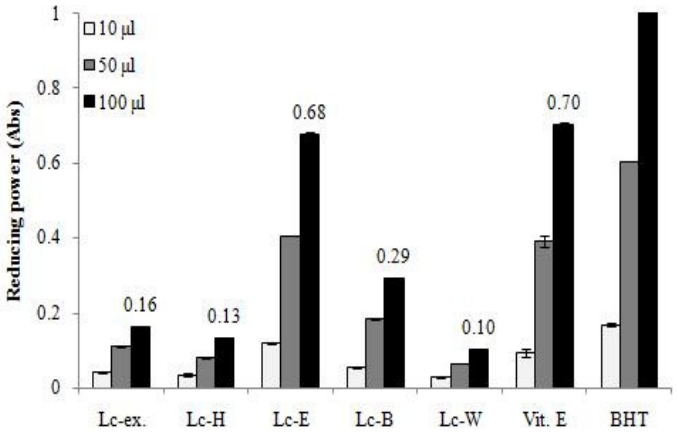

Figure 2. Reducing power of extract and fractions from *Lespedeza cuneata*. Lc-ex., methanolic extract; Lc-H, *n*-hexane fraction; Lc-E, EtOAc fraction; Lc-B, BuOH fraction; Lc-W, aqueous fraction; Vit. E, α-tocopherol; BHT, butylated hydroxytoluene.

The reducing power was determined using a reaction solution that changed color from yellow to green and blue at 700 nm, depending on the reducing power of the sample concentration. The high absorbance of the reaction mixture indicates high reducing power. Figure 2 shows the dose-dependent reducing power of *L. cuneata* extract and fractions, which along with the positive control, increased steadily with increasing sample concentration. EtOAc fractions (OD_{700}=0.68) had the highest levels of activity, and the other extracts exhibited OD_{700} values ranging from 0.10 - 0.29.

The total phenolic content ranged from 21.24 mg GAE/g to 359.54 mg GAE/g. The EtOAc fraction had higher total phenolic content. The results were highly consistent with the EDA and reducing power activity results, and demonstrated that the EtOAc fraction had a higher total flavonoid content equal to 209.63 mg of QE/g. Total flavonoid levels were determined to be in following order: EtOAc fraction, BuOH fraction, hexane fraction, methanolic extract, aqueous fraction (Table 1).

In order to determine how anti-oxidant activity is related to the phenol or flavonoid levels of *L. cuneata* extract and fractions, we established a positive linear correlation between phenol content and EDA (linear correlation coefficient r^2 = 0.989) (Figure 3A). A similar result was observed in our examination of total phenol content and reducing power (r^2 = 0.997) (Figure 3C).

This positive linear correlation shows that the sample with the highest total phenol content likewise has the highest EDA values, whereas the sample with lowest total phenol content produces the lowest reducing power values. Correlations were also established between flavonoid content and EDA (r^2 = 0.891) (Figure 3B), as well as between total flavonoid content and reducing power (r^2 = 0. 944) (Figure 3D). Polyphenols are the principal plant compounds that have anti-oxidant activity (Moure et al., 2001; Pitta, 2000; Rice-Evens et al., 1997). Since the EtOAc and BuOH fractions contained the highest amounts of phenolic compounds (Table 1), both fractions exhibited higher anti-oxidant activities. The anti-oxidant activities of phenolic compounds are due primarily to their redox properties, which allow them to function as reducing agents, hydrogen donors and singlet-oxygen quenchers (Kang et al., 2006). This suggests that the polyphenolic constituents of *L. cuneata* may be responsible for its high anti-oxidant activity during the DPPH assay.

These observations clearly suggest a close linkage between total phenolic content and anti-oxidant activity, such as reducing power and radical scavenging effect on DPPH radicals (Do et al., 2005). Similarly, linear relationships between these parameters have been found for extracts of several types of honey, indicating that the correlation coefficient can be used as an indicator of anti-oxidant capacity (Bertoncelj et al., 2007; Blasa et al., 2006). Taken together, the high correlation between the phenolic and flavonoid levels of *L. cuneata* extract and anti-oxidant activities suggest that phenolic compounds act as anti-oxidants in this plants.

The anti-oxidant activities of the extracts were compared with the commercial anti-oxidant BHA by the ferric thiocyanate method, which measures the amount of peroxide generated at the initial stage of linoleic acid emulsion during incubation.

Here, peroxide reacts with ferrous chloride to form ferric chloride, which in turn reacts with ammonium thiocyanate to produce ferric thiocyanate, a reddish pigment. Low absorbance values measured via the FTC method indicate high anti-oxidant activity. Figure 4 shows the changes in absorbance for each sample during 30-h of

Table 1. Total phenolic content, and total flavonoid content of the extract and fractions from *Lespedeza cuneata*.

Sample	Lc-ex[c]	Lc-H[c]	Lc-E[c]	Lc-B[c]	Lc-W[c]
TPC[a] (mg GAE/g)	46.33 ± 4.97	39.62 ± 0.32	359.54 ± 5.83	137.52 ± 12.24	21.24 ± 2.72
TFC[b] (mg QE/g)	32.73 ± 0.31	67.97 ± 1.85	209.63 ± 0.63	73.81 ± 2.51	1.94 ± 0.03

[a]Total phenol content analyzed as gallic acid equivalent (GAE) mg/g of extract, values are the average of triplicates.
[b]Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract, values are the average of triplicates.
[c]Lc-ex, methanolic extract; Lc-H, *n*-hexane fraction; Lc-E, EtOAc fraction; Lc-B, BuOH fraction; Lc-W, aqueous fraction.

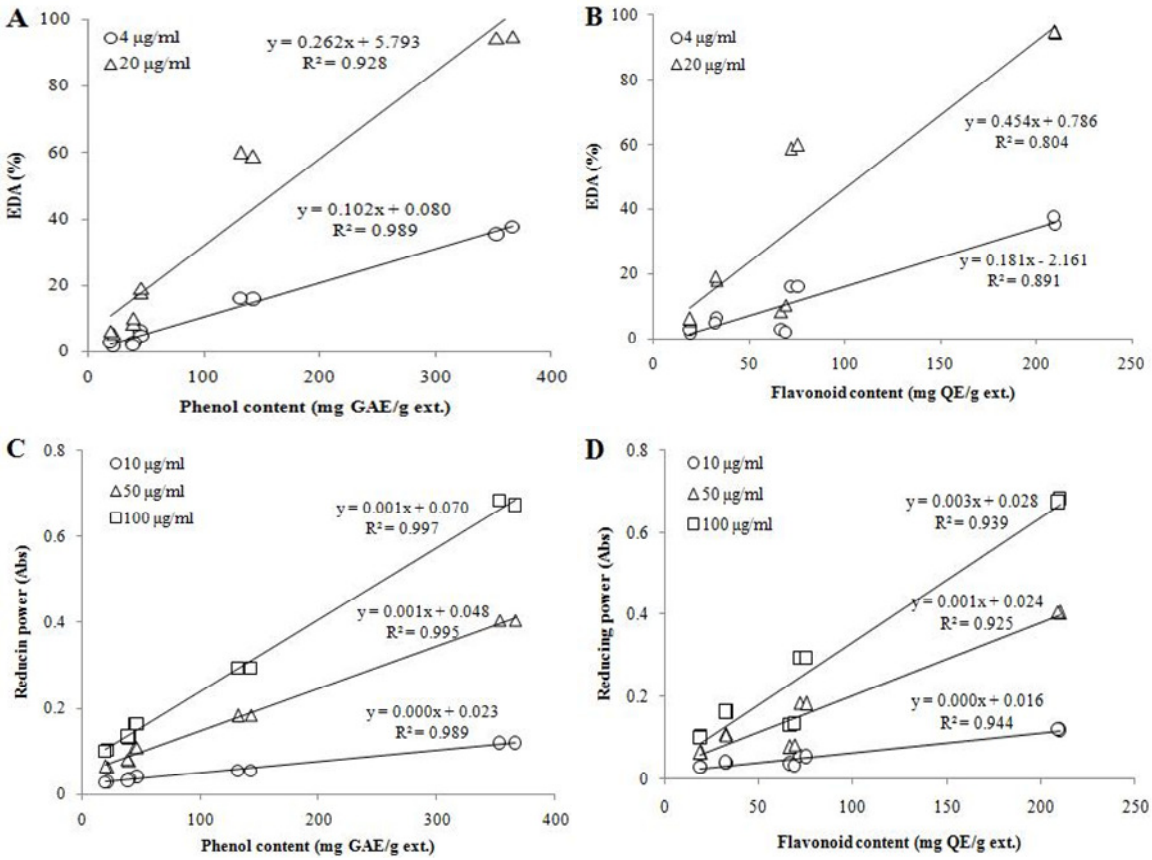

Figure 3. Correlation established between the anti-oxidant activity and total phenolic content of *Lespedeza cuneata*. A: correlation between EDA and total phenol content, B: correlation between EDA and total flavonoid content, C: correlation between reducing power and total phenol content, and D: correlation between reducing power and total flavonoid content.

incubation at 70°C. It was observed that absorbance increases over time, and that the autoxidation of linoleic acid emulsion in the control or aqueous fraction was accompanied by a rapid increase in the peroxide formation. Significantly lower absorbances were observed for the extract and BHA, which indicates the extract have greater anti-oxidant activities. Anti-oxidant activities increased in the following order: methanolic extract>hexane fraction>EtOAc fraction>BuOH fraction> aqueous fraction.

FTC is used to measure the production of peroxide at the initial stages of linoleic acid oxidation, whereas the TBA test is used to estimate the production of secondary products such as aldehydes, ketones, etc (Farag et al., 1989). During the oxidation of linoleic acid emulsion, peroxides are gradually decomposed to lower molecular weight compounds such as malonaldehyde, which can be measured by the TBA method. The results obtained were similar to that of the FTC method. The percentage of methanolic extract, hexane fraction, EtOAc fraction and BuOH fraction with anti-oxidative activity were 93, 88, 57 and 92%, respectively (Figure 5). These results were not

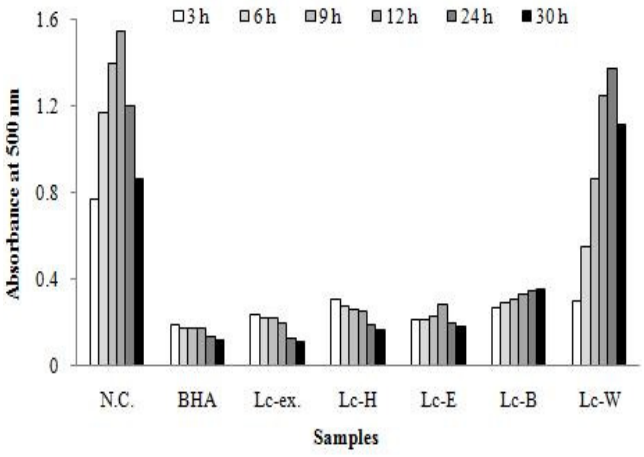

Figure 4. Anti-oxidative activity of extract and fractions from *Lespedeza cuneata* determined by the ferric thiocyanate method. N.C., negative control; BHA, butylated hydroxyanisole; Lc-ex., methanolic extract; Lc-H, *n*-hexane fraction; Lc-E, EtOAc fraction; Lc-B, BuOH fraction; Lc-W, aqueous fraction.

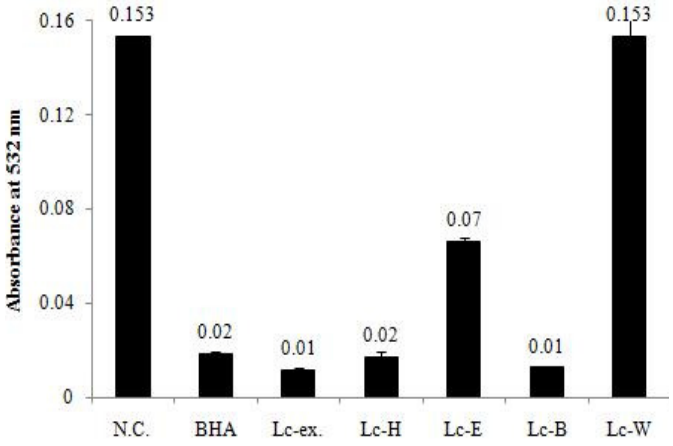

Figure 5. Anti-oxidative activity of extract and fractions from *Lespedeza cuneata* determined by the thiobarbituric acid method. N.C., negative control; BHA, butylated hydroxyanisole; Lc-ex., methanolic extract; Lc-H, *n*-hexane fraction; Lc-E, EtOAc fraction; Lc-B, BuOH fraction; Lc-W, aqueous fraction.

significantly different from BHA, in which the percentage of anti-oxidant activity was 88%. Free radicals are defined as atoms or molecular fragments, easily generated during normal cellular metabolism, that contain a number of unpaired electrons in their respective atomic or molecular orbitals (Valko et al., 2006, 2007). Although reactive oxygen species (ROS) function in cellular signaling systems, an excessive ROS are responsible for other pathologies such as atherosclerosis, hypertension, cardiac disease, diabetic complication, autoimmune rheumatic disease, cancer and aging (Gutteridge, 1995; Halliwell, 1996; Valko et al., 2006, 2007). Therefore, anti-oxidant therapy using free radical scavengers has received increasing attention in a clinical setting, and consequently several anti-oxidants have been developed and used in primary and/or complementary therapies (Delanty and Dichter, 2000).

In conclusion, we analyzed the anti-oxidant activities of *L. cuneata* extract and fractions using *in vitro* anti-oxidant assays that tested for EDA, reducing power and anti-lipid peroxidative effects. We found that the anti-oxidant potential of the EtOAc fraction is higher than that of the extract and other fractions. Our findings strongly support *L. cuneata* as a possible candidate for the production of anti-oxidants. To clarify the anti-oxidant properties of *L. cuneata* and develop natural anti-oxidant agents, the purification and identification of active compounds will be necessary. Our study provides basic information concerning the relationship between phenolic compounds from *L. cuneata* and anti-oxidant properties.

ACKNOWLEDGEMENT

This work was supported by the Oriental Bio-herb Research Institute, Kangwon National University, Korea.

REFERENCES

Bertoncelj J, Dobersek U, Jamnik M, Golob T (2007). Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. Food Chem. 105: 822-828.

Blasa M, Candiracci M, Accorsi A, Piacentini MP, Albertini MC, Piatti E (2006). Raw *Millefiori* honey is packed full of antioxidants. Food Chem. 97: 217-222.

Chung YH, Lim CH (2005). Bioactivity of an extract of *Lespedezea cuneata* G. Don to rabbit corpus cavernosum smooth muscle tone. J. Agri. Sci. Chungnam Nat'l Univ., Korea 32: 63-70.

Delanty N, Dichter MA (2000). Antioxidant therapy in neurologic diseases. Arch. Neurol. 57: 1265-1270.

Deng F, Chang J, Zhang JS (2007). New flavonoids and other constituents from *Lespedeza cuneata*. J. Asian Nat. Prod. Res. 9: 655-658.

Do JR, Kim KJ, Park SY, Lee OH, Kim BS, Kang SN (2005). Antimicribial and antioxidant activities of ethanol extracts of medicinal plants. J. Food Sci. Nutr. 10: 81-87.

Farag RS, Badei AZMA, Hawed FM, El-Baroty GSA (1989). Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. J. Am. Oil. Chem. Soc. 66: 793-799.

Gutteridge JMC (1995). Lipid-peroxidation and antioxidants as biomarkers of tissue-damage. Clin. Chem. 41: 1819-1828.

Halliwell B (1996). Antioxidants in human health and disease. Annu. Rev. Nutr. 16: 33-50.

Haraguchi H, Hashimoto K, Yagi A (1992). Antioxidative substances in leaves of *Polygonum hydropiper*. J. Agric. Food Chem. 40: 1349-1351.

Kang KA, Zhang R, Piao MJ, Park S, Park J, Kim JS, Kang SS, Hyun JW (2006). Screening of antioxidant and anticancer effects from flavonoids. Cancer revention Res. 11: 235-239.

Kee CH (1993). The Pharmacology of Chinese Herbs. CRC Press, Boca Raton, USA.

Kim JS, Kwon YS, Chun WJ, Kim TY, Sun J, Yu CY, Kim MJ (2010). *Rhus verniciflua* Stokes flavonoid extracts have anti-oxidant, anti-microbial and α-glucosidase inhibitory effect. Food Chem. 120: 539-543.

Kim SJ, Kim DW (2007). Antioxidative activity of hot water and ethanol extracts of *Lespedezea cuneata* seeds. Korean J. Food Preserv. 14: 332-335.

Kim YH, Ryu SN (2008). Antioxidant Activity of Methanol Extract from Aerial Parts in *Lespedeza cuneata* G. Don. Korean J. Crop Sci. 53:

121-123.
Kwon DJ, Bae YS (2009). Flavonoids from the aerial parts of *Lespedeza cuneata*. Biochem. Syst. Ecol. 37: 46-48.
Kwon DJ, Kim JK, Ham YH, Bae YS (2007). Flavone glycosides from the aerial parts of *Lespedeza cuneata* G. Don. J. Korean Soc. Appl. Biol. Chem. 50: 344-347.
Moure A, Cruz JM, Franco D, Dominguez JM, Sineiro J, Dominguez H, Nunez MJ, Parajo JC (2001). Natural antioxidants from residual sources. Food Chem. 72: 145-171.
Numata A, Hokimoto K, Shimada A, Yamaguchi H, Takaishi K (1979). Plant constituents biologically active to insects I-feedings stimulants for the larva of the yellow butterfly *Eurema hecabe mandarina*. Chem. Pharm. Bull. 27: 602-608.
Numata A, Hokimoto K, Yamaguchi H (1980). C-glycosylflavones in *Lespedeza cuneata*. Chem. Pharm. Bull. 28: 964-965.
Ohiwi J (1965). Flora of Japan. Smithsonian Institution, Washington DC, USA. pp. 1067.
Oyaizu M (1986). Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr. 44: 307-315.
Park YK, Koo MH, Ikegaki M, Contado JL (1997). Comparison of the flavonoid aglycone contents of *Apis mellifera* propolis from various regions of Brazil. Arquivos de Biologiae Technologia 40: 97-106.
Pitta PG (2000). Flavonoids as antioxidants. J. Nat. Prod. 63: 1035-1042.
Rice-Evens CA, Miller NJ, Paganga G (1997). Antioxidant properties of phenolic compound. Trends Plant Sci. Rev. 2: 152-159.
Shin M, Munekazu L, Emikol, Hiromasa T, Kengo K (1978). Studies on the constituents of the useful plants. VIII. The constituents of *Lespedeza cuneata* G. Don. (1). Yakugaku Zasshi 98: 1542-1544.
Singleton VL, Rossi JA Jr. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticul. 16: 144-158.
Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39: 44-84.
Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biol. Int. 160: 1-40.
Wang C, Zhou B, Palm HL (2008). Detecting invasive sericea lespedeza (*Lespedeza cuneata*) in Mid-Missouri Pastureland using hyperspectral imagery. Environ. Manage. 41: 853-862.