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Total Phenolics Levels and Antioxidant Properties in Methanol Extracts from Several Vietnamese Wild Plants

Hee-Ock Boo, Tae-Soon Kim, Kaihei Koshio[1], Jeoung-Hwa Shin[2] and Sang-Uk Chon[3]*

Department of Biology, Chosun University, Gwangju 501-759, Korea
[1]Department of International Agricultural Development, Tokyo University of Agriculture, Tokyo 156-8502, Japan
[2]Korea Basic Science Institute, Seoul Center, Seoul 136-713, Korea
[3]EFARINET Co. Ltd., BI Center, Chosun University, Gwangju 501-759, Korea

Abstract - The aim of this study was to determine total phenolics (TP) content, total flavonoids (TF) level, and antioxidant activity of methanol extracts from leaf parts of 15 Vietnamese wild plants. TP content showed the highest amount in methanol extracts from *Altenanthera sessilis* (409.7 ± 1.4 ppm), and followed by *Eclipta prostrata* (183.6 ± 3.8 ppm) and *Cassia tora* (159.3 ± 5.7 ppm). The highest TF level also was found in *A. sessilis* (156.3 ± 1.7 ppm), followed by *E. prostrata*, and *C. tora*, showing similar tendency to TP. Methanol extracts of *A. sessilis*, *E. prostrate* and *C. tora* at 1000 ppm exhibited the highest DPPH radical scavenging activity by 94.5, 92.6 and 92.7%, respectively. The methanol extracts from *A. sessilis* showed the highest ABTS and nitrite scavenging activities by 97% and 92%, respectively. The highest correlation coefficient (r^2) was 0.9522 between TP and TF, and followed by 0.8919 between DPPH and ABTS activities. However, polyphenols and antioxidant activities showed low correlation coefficients, ranging from r^2=0.4114 to 0.4826. It was concluded that Vietnamese wild plants contain polyphenol compounds with antioxidant activities depending on plant species.

Key words - Vietnamese wild plants, Methanol extracts, Polyphenols, Flavonoids, Antioxidant activity

Introduction

Some higher plants possess important bioactive properties including antioxidant, anti-inflammatory, anticancer and antidiabetic (Yang *et al*., 2006). Recently, there has been a worldwide trend towards the use of wild plants do to their bioactive phytochemicals and first of all phenolics (Watanabe *et al*., 2007). Phenolic compounds are secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wounding, and UV radiation (Canter *et al*., 2005). These compounds occur ubiquitously in plants and are a diversified group of phytochemicals derived from phenylalanine and tyrosine (Shahidi and Naczk, 2004).

Free radical scavenging is generally the accepted mechanism for antioxidants inhibiting lipid oxidation are important not only for food protection but also for the defense of living cells against oxidative damage (Mastaloudis *et al*., 2004). Therefore, now not only antioxidants of natural products, but also synthesized antioxidants are widely used (Haruenkit *et al*., 2007). The toxic and other unfavorable effects of synthesized food antioxidants have been noted (Aggarwal and Shishodia, 2006). Phenolic compounds, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ), have been widely used as synthetic antioxidants in food. Although those antioxidants are considered as safe as natural antioxidants, they do not always provide effective protection against oxidation *in vitro* (Frankle, 1980). Nevertheless, these phenolic antioxidants are still used extensively as food antioxidants because of their low cost. However, when these phenolic antioxidants were administered in doses of 50 mg/kg/day to rodents and monkeys, certain pathological changes including enzyme and lipid alterations as well as carcinogenic effects have been observed (Branen, 1975). Therefore, research on new natural antioxidants must be considered to pose no health risk to consumers (Wanasundara *et al*., 1997).

*Corresponding author. E-mail : chonsu4100@yahoo.co.kr

Vegetables, fruits and whole grains contain a wide variety of phytochemicals that have the potential to interfere with the development of cancer (Yang *et al*., 2006). These phytochemicals are isothiocyanates (cruciferous vegetables), carotenoids including alpha-carotene, gamma carotene, beta-cryptoxanthin, zeatxanthin, rutin, lycopene (tomatoes), resveratrol (grapes and wine), ellagic acid (various berries), glutathione-S-transferase (garlic), diallyl sulphide (garlic), genistein (soybean), curcumin (turmeric), indole-3-carbinol, inositol, organosulfur compounds, sulforaphane, squalene and terpenes (Wattenberg, 1998). Also successive intake of tea decreased the risk of various types of cancer (Suzuki *et al*., 2004; Sasazuki *et al*., 2004).

Therefore, according to the above cited studies, extracts of various Vietnamese wild plants, grown in cropland or orchard, could act as preventative or therapeutic agents similar to prescription drugs. This research was conducted to develop health supplements and new medicines offering new possibilities using the Vietnamese wild plants. The objective of this research was to determine total phenolics content, total flavonoids level, and their antioxidant activity of the methanol extracts from leaf part of the 15 Vietnamese wild plants. In order to receive reliable data, the Folin-Ciocalteu assay for determination of total phenolics content and DPPH, ABTS, and nitrite assays for assessment of the antioxidant activity were chosen.

Materials and Methods

Chemicals

Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid and 2,2-azino-bis-(3-ethylbenzothiazoline-6-dulfonis acids) (ABTS) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All reagents were of analytical grade. Deionized and distilled water were used throughout.

Plant material

Methanol extracts from leaves of 15 Vietnamese wild plants (*Altenanthera sessilis*, *Anthocephalus cadamba*, *Bauhinia ornata*, *Cassia tora*, *Centella asiatica*, *Chenopodium ficifolium*, *Eclipta prostrata*, *Gymnanthera oblonga*, *Lactuca indica*, *Leonurus japonicus*, *Matricaria chamomilla*, *Polygonum lapothifolium*, *Rhus chinensis*, *Senna siamea* and *Sphaeranthus africanus*) were provided by Korean Plant Component Bank, Daejeon, Korea, 2006. The methanol extracts from each plant were used for determination of total phenolics level, total flavonoids content, and antioxidant activity including DPPH radical, ABTS free radical, and nitrite scavenging activities.

Total phenolics content

The content of total phenolics (TP) was measured using the classical Folin-Ciocalteu assay (Singleton and Rossi, 1965). 5 mL of Nanopure water, 0.5 - 1.0 mL of sample and 1.0 mL of Folin-Ciocalteu reagent were added to a 25 mL volumetric flask. The contents were mixed and allowed to stand for 5-8 min at room temperature. Next, 10 mL of a 7% sodium carbonate solution was added, and followed by the addition of Nanopure water filled to volume. Solutions were mixed and allowed to stand at room temperature for 2 h. Sample aliquots were filtered through a Whatman 0.45 μm polytetrafluoroethylene filter prior to the determination of TP concentration using a UV-1650 spectrophotometer (Shimadzu, Japan) monitoring 640 nm. TP content was standardized against ferulic acid and expressed as ppm of ferulic acid equivalents (FAE). The linearity range for this assay was determined as 0.5-5.0 mg/L FAE ($R^2 = 0.9990$).

Total flavonoids level

The total flavonoid content (TF) of methanol extracts from the medicinal plants was determined according to colorimetric method as described by Bao *et al*. (2005). In brief, 0.5 ml of sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min of incubation, 0.15 ml of 10% $AlCl_3$ solution was added and then allowed to stand for 6 min, followed by adding 2 ml of 4% NaOH solution to the mixture. Immediately, after water was added to the sample to bring the final volume to 5 mL, the mixture was thoroughly mixed and allowed to stand for another 15 min. The mixture absorbance was determined at wavelength 510 nm. The total flavonoid content was expressed in milligrams of rutin equivalents per gram of plant extracts.

DPPH radical-scavenging activity

Free radical scavenging activity of the methanol extracts was determined using the classical 1, 1-diphenyl-2-picrylhydrazyl method (DPPH), because of his ability in a relatively short time compared to other methods to evaluate the antioxidative activities (Brand-Williams *et al*., 1995). Each methanol extract at 1,000 ppm was added to a 1.5 x 10^{-4} M solution of DPPH in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 520 nm, and the radical scavenging activity was obtained from the following equation: Radical scavenging activity (%) = {($OD_{control}$ - OD_{sample}) / $OD_{control}$} × 100.

ABTS free radical activity

The scavenging activity of the extracts from mushrooms on 2,2-azino-bis-(3-ethylbenzothiazoline-6-dulfonis acids) (ABTS) radical cation was measured according to method of Roberta *et al*. (1999) with some modifications. Briefly, ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate solution and the mixture was left to stand for overnight in a dark place at room temperature. The ABTS radical cation solution was diluted with distilled water to obtain an absorbance of 1.4-1.5 at 414 nm (molar extinction coefficient $\varepsilon=3.6\times10^4$ mol^{-1} cm^{-1}). Diluted ABTS radical cation solution (1 mL) was added to 50 μL of extract, ascorbic acid standard solution or distilled water. After 90 min, the absorbance was measured at 414 nm using a spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The ABTS radical cation scavenging activity was expressed as ascorbic acid equivalent antioxidant activity (AEAC) and defined as the milligrams of ascorbic acid equivalents per 100 g of sample. AEAC was calculated by the following equation:

$$AEAC = (\Delta A_{sample} / \Delta A_{aa}) \times C_{aa} \times V \times (100/W_{sample})$$

Where ΔA_{sample} is the change of absorbance in the presence of the sample extracts, ΔA_{aa} is the change of absorbance after the addition of ascorbic acid standard solution, C_{aa} is the concentration of absorbance after the addition (mg/mL), V is the volume of sample extracts (mL) and W_{sample} is the weight of sample used for extraction (g). All extracts were analyzed in triplicate.

Nitrite scavenging activity

The nitrite scavenging activity (NSA) was evaluated using a UV-Visible spectrophotometer (UV-1601, Shimadzu) at a wavelength of 520 nm (Gray and Dugan, 1975). One milliliter of 1 mM $NaNO_2$ solution was added to 1 mL of each sample, and pH values of the resulting mixtures were adjusted to 1.2,

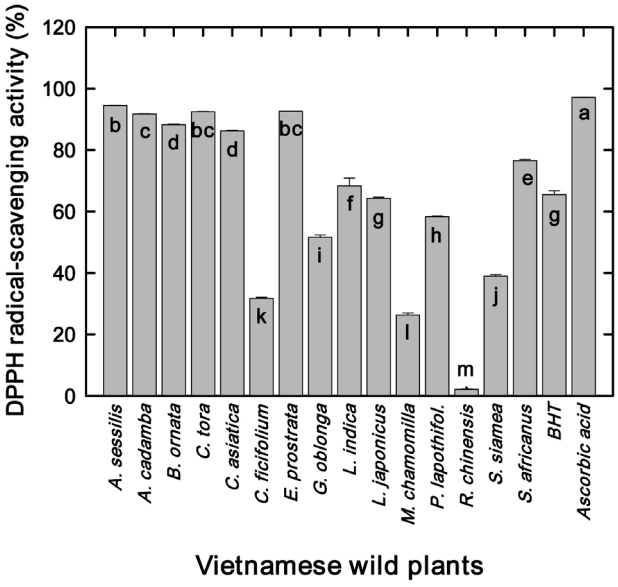

Fig. 1. DPPH. Radical scavenging activities of methanol extracts of 15 Vietnamese wild plants at 1000 ppm. Means with same letter within are not significantly different (p<0.05).

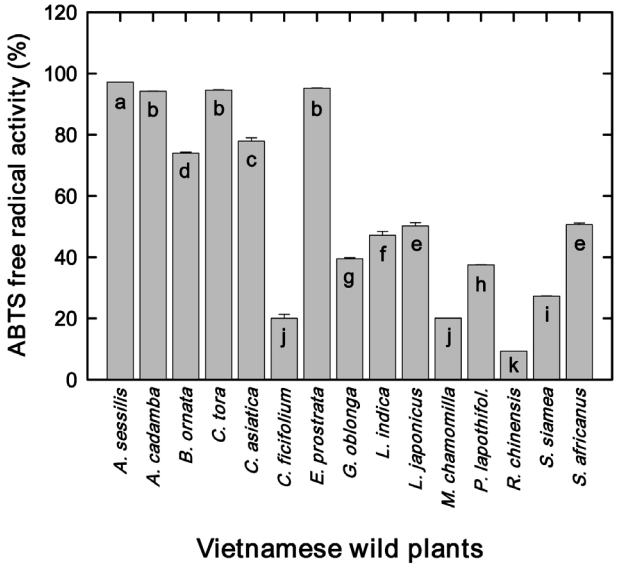

Fig. 2. ABTS radical scavenging activities of methanol extracts of 15 Vietnamese wild plants at 1000 ppm. Means with same letter within are not significantly different (p<0.05).

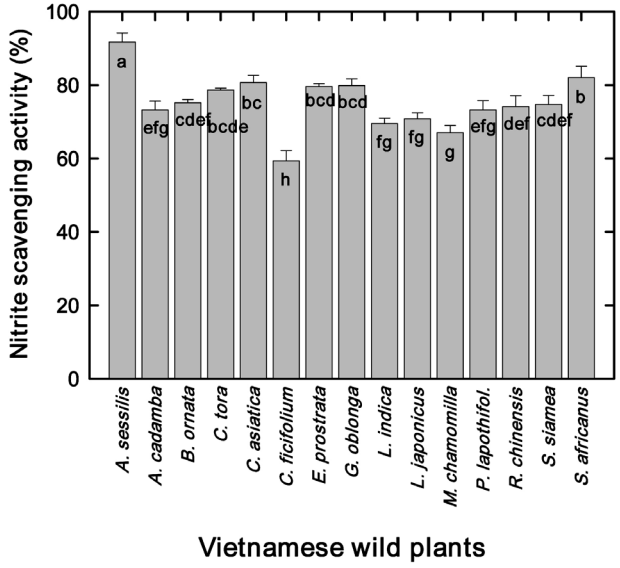

Fig. 3. Nitrite scavenging activities of methanol extracts of 15 Vietnamese wild plants at 1000 ppm. Means with same letter within are not significantly different (p<0.05).

3.0 and 4.2 using 8 mL of buffer solution: 0.1 N HCl for pH 1.2 and 0.2 N citric acid for 3.0 and 4.2. The final volume of each sample wad adjusted to 10 mL. Each sample was allowed to react at 30℃ for 1h, after which 1 mL of each sample was taken from the solutions, mixed thoroughly with 5 mL of 2% acetic acid and 0.4 mL of Griess reagent, and maintained at room temperature for 15 min. Griess reagent was prepared by mixing equal amounts of 1% sulfanilic acid and 1% napthylamine, which were made with 3% acetic acid. A blank was prepared by adding 0.4 mL of distilled water instead of the Griess reagent. The nitrite scavenging activity was determined based on the following formula: Nitrite scavenging activity (%) = ((1-A-C)/B)*100. Where A is the absorbance of the mixture sample during a reaction with 1 mM $NaNO_2$ after a 1 h reaction, B is the absorbance of a mixture of distilled water and 1 mM $NaNO_2$ after a 1 h reaction and C is the absorbance of the sample.

Statistical analysis

To verify the statistical significance, mean ± SD of three independent measurements were calculated. Differences between groups were tested by two-way ANOVA. When the means were significant at F-test, the means were separated by least significant difference (LSD) test. In the assessment of the antioxidant potential, Spearman correlation coefficient (R) was used. Linear regressions were also calculated. The p values of < 0.05 were considered significant (SAS Institute, 2000).

Results

Total phenolics content

Total phenolic content showed the highest amount in methanol extracts from *A. sessilis* (409.7 ± 1.4 ppm) and followed by *E. prostrata* (183.6 ± 3.8 ppm), *C. tora* (159.3 ± 5.7 ppm) and *A. cadamba* (99.1 ± 0.7 ppm) (Table 1). However, the extracts from *L. indica*, *M. chamomilla*, *R. chinensis* and *S. africanus* showed lower levels of total phenolics (Table 1). The result was highly consistent with the finding of DPPH radical scavenging activity (Velioglu *et al*., 1998). Also Zhou and Yu (2006) reported that total phenolic content of the tested vegetable extracts was correlated with the DPPH radical scavenging activity, suggesting that total phenolics can play a major role in the antioxidant activity of plant materials.

Total flavonoids content

The highest TF content of the methanol extracts was found in *A. sessilis* (156.3 ± 1.7 ppm), followed by *E. prostrata* (55.5 ± 1.8 ppm), *C. tora* (41.9 ± 1.4 ppm) and *A. cadamba* (22.5 ± 1.8 ppm) (Table 1), showing same tendency to results of TP. The differences were very significant (P<0.05). *C. asiatica*, *G. oblonga*, and *R. chinensis* showed the lowest content of total flavonoids (12.5, 12.5 and 12.1 ppm, respectively) (Table 1).

DPPH radical scavenging activity

Methanol extracts of *A. sessilis* had the highest DPPH radical scavenging activity, followed by *E. prostrata*, *C. tora* and *A. cadamba*, indicating over 90% more than at 1000 ppm. Especially, their values showed higher activity than the synthetic antioxidant BHT, with 66% activity. All samples of plant species proved that DPPH radical scavenging activity is dose-dependent, and their activities depend on plant species.

ABTS free radical activity

The free radical scavenging ability of 15 Vietnamese wild

Table 1. Total phenolic content and total flavonoids level of methanol extracts (1000 ppm) from 15 Vietnamese wild plants.

Vietnam Plant samples	Total phenolics content (ppm)	Total flavonoids level (ppm)
Altenanthera sessilis	409.7 ± 1.4[a†]	99.5 ± 3.3[a†]
Anthocephalus cadamba	99.1 ± 0.7[d]	22.5 ± 1.8[d]
Bauhinia ornata	58.9 ± 1.8[f]	14.4 ± 1.8[e]
Cassia tora	159.3 ± 5.7[c]	41.9 ± 1.4[c]
Centella asiatica	89.0 ± 4.7[e]	12.5 ± 0.4[e]
Chenopodium ficifolium	52.4 ± 2.5[fg]	16.0 ± 1.2[e]
Eclipta prostrata	183.6 ± 3.8[b]	55.5 ± 1.8[b]
Gymnanthera oblonga	51.1 ± 2.6[fg]	12.5 ± 0.2[e]
Lactuca indica	43.7 ± 3.7[gh]	13.2 ± 1.5[e]
Leonurus japonicus	50.1 ± 3.1[fg]	16.5 ± 1.2[e]
Matricaria chamomilla	26.4 ± 1.5[i]	16.0 ± 1.4[e]
Polygonum lapothifolium	46.8 ± 2.2[gh]	14.4 ± 1.2[e]
Rhus chinensis	14.6 ± 1.2[j]	12.1 ± 1.0[e]
Senna siamea	50.8 ± 3.4[fg]	22.4 ± 1.2[d]
Sphaeranthus africanus	38.5 ± 3.2[h]	16.0 ± 1.2[e]

[†]Means with small letters within a column are not significantly different at $p<0.05$ level by LSD test.

Table 2. Correlation among physiological substances and their activities of methanol extracts from Vietnamese wild plants.

	TP[†]	TF[†]	DPPH[†]	ABTS[†]	NSA[†]
TP	1.0000	0.9522	0.3249	0.4826	0.4651
TF		1.0000	0.2243	0.3741	0.4114
DPPH			1.0000	0.8919	0.3172
ABTS				1.0000	0.3669
NSA					1.0000

[†]Total phenolics content (TP), total flavonoid level (TF), DPPH radical scavenging activity (DPPH), ABTS free radical scavenging activity (ABTS), and nitrite scavenging activity (NSA).

plants was also determined using ABTS radical cation. ABTS radical cation has been often used in the evaluation of antioxidant activity of single compounds and complex mixtures of various origins (body fluids, foods, beverages, plant extracts). In this assay ABTS radical cation was generated directly in stable form using potassium persulfate. Generation of radical before the antioxidant was added to prevent interference of compounds, which affect radical formation. Methanol extracts of *A. sessilis* had the highest ABTS radical scavenging activity and followed by *E. prostrate*, *C. tora* and *A. cadamba*, indicating over 94% more than at 1000 ppm extracts.

Nitrite scavenging activity

The nitrite scavenging activity for the 15 plant extracts at pH 4.0 was ranged from 59 to 92%. Methanol extracts of *A. sessilis* had the highest nitrite radical scavenging activity (91.73%) and followed by *S. africanus* and *C. asiatica*, indicating over 80% more than at 1000 ppm extracts.

Discussion

The research of new natural antioxidants has gained momentum because of their positive role in prevention and treatment of some diseases and pose no health risk to consumers (Wanasundara *et al*., 1997). In order to achieve desirable results in diseases' prevention and treatment only natural products with high content of phenolics and high free radical activity has to be used (Leontowicz *et al*., 2007; Saxena *et al*., 2007). Therefore, content of phenolics and free radical activity of the leaf part of 15 Vietnamese wild plants were determined by classical methods.

It was found that the content of the total phenolics and

flavonoids in methanol extracts of most of the studied plants was high and varied significantly, depending plant species. The highest content of phenolics or flavonoids was in *A. sessilis*, *E. prostrata*, *C. tora* and the lowest *L. indica*, *M. chamomilla*, *R. chinensis* and *S. africanus*. Other studies found that the content of phenolics was highly consistent with the finding of DPPH radical scavenging activity (Velioglu *et al*., 1998). Also Zhou and Yu (2006) reported that total phenolic content of the tested vegetable extracts was correlated with the DPPH, ABTS and nitrite radical scavenging activities, suggesting that total phenolics can play a major role in the antioxidant activity of plant materials.

It was observed that also the radical scavenging activity in methanol extracts of most of the studied plants was high and varied significantly. Especially, methanol extracts of *A. sessilis* had the highest DPPH radical scavenging activity, which was higher than that of a synthetic antioxidant BHT. Also other reported that antioxidant activity of some plants was higher than of synthetic antioxidants (Lee *et al*., 2003). They investigated methanol extracts of nine medicinal plants traditionally used in Chinese medicine *vs* synthetic antioxidant resveratrol and found relatively high levels of DPPH radical scavenging activity in extracts of *Areca catechu* var. *dulcissima*, *Paeonia suffruticosa* and *Cinnamomun cassia* (IC_{50} < 6.0 μg mL^{-1}). The extract of *Areca catechu* var. *dulcissima* showed in all experiments higher antioxidant activity than resveratrol.

Correlations among physiological substances and their biological activities in 15 Vietnamese plants are shown in Table 2. The highest correlation coefficient (r^2) was 0.9522 between TP and TF, and followed by 0.8919 between DPPH and ABTS activites. However, correlation coefficient between TP and ABTS was 0.4826, between TP and NSA 0.4651, and between TF and NSA 0.4114, showing low correlation coefficients. In other paper, the correlation coefficient between polyphenols and antioxidant capacities of Prolipid with 1,1-diphenyl-2-picrylhydrazyl radical assay was about 0.97 (Jastrzebski *et al*., 2007). It was also shown that fifty percent of ethanol extract from mate tea had the greatest antioxidant activity (Jastrzebski *et al*., 2007; Jung *et al*., 2008). The methanol extract of *Ulmus davidiana* (Jung *et al*., 2008) exhibited strong antioxidant activity in the tested model systems. *U. davidiana* extracts may be exploited as biopreservatives in food applications as well as for health supplements of functional food, to alleviate oxidative stress.

In summary, this investigation shows that the content of total phenolics and total flavonoids in some of Vietnamese wild plants is high: the significantly highest in *A. sessilis* and *E. prostrata*, and the same plants have the highest DPPH radical scavenging activity. However, the total phenolics level or total flavonoids content was lowly correlated with the antioxidative activities. The leaf part of the Vietnamese wild plants could be recommended as preventative or/and therapeutic agents in addition to proper prescribed drugs.

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