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Evaluation of Total Antioxidant Activity of Several Popular Vegetables and Chinese Herbs: A Fast Approach with ABTS/H_2O_2/HRP System in Microplates

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ABSTRACT

Total antioxidant activities for several popular vegetables and traditional Chinese herbs were evaluated in an ABTS/H_2O_2/HRP system with a microplate reader. This improved method offers a rapid and sensitive way to measure total antioxidant activity of testing samples. Most samples had antioxidative capacities. Among the tested vegetables, sugar beet and red cabbage had the highest total antioxidant activity, whereas tropical Almond Terminalia and Moutan Radicis Cortex had exceptionally high total antioxidant activity in the tested Chinese herbs.

Key words: total antioxidant activity, vegetables, Chinese herb

INTRODUCTION

Reactive oxygen species (ROS) are involved in the development of various diseases and also accelerate aging. ROS scavengers such as superoxide dismutase and antioxidants can effectively reduce damage from oxidation. Experimental evidence suggests that free radicals and ROS can take a part in many diseases[1,2]. As plants produce antioxidants to control the oxidative stress caused by sunlight and oxygen, they became a source of useful new compounds with antioxidant activity for human consumption. Traditional Chinese medicinal plants possessing natural antioxidants such as anthraquinones, flavonoids, aromatic acids, and tannins have recently been shown to have ROS scavenging and lipid peroxidation prevention effects[3-7]. Evidence is mounting for a role of dietary phytochemicals, including flavonoids, ascorbic acid, α-tocopherol, and carotenoids, in the maintenance of health and protection from disease[8-14]. The epidemiological evidence for a protective effect of natural food and herb antioxidants against cancer, coronary heart disease, age-related macular degeneration, respiratory disease, cystic fibrosis, immuno disease, and Alzheimer's disease is impressive[15,16]. Numerous surveys have shown an inverse relationship between the intake of vegetables and the incidence of those disorders[17,18].

The relationship between these discoveries and the mechanisms of traditional herbal therapies remains open to investigation. Chinese have used herbal medicine for over four thousand years. Most herbs are boiled with water and the water extracts are then used for treatment. For example, the boiled soup of fallen Tropical Almond Terminalia Leaves is beneficial in treating hepatitis[19]. Moutan Radicis Cortex and Rehmanniae Radix et Rhizoma are claimed to remove heat from blood and activate blood circulation. Ligustri Lucidi Fructus, Asari Herba Radice, and Angelicae Dahuricae relieve pain by dispelling wind, coldness and dampness. Leonuni Herba is said to regulate menstruation by activating blood circulation and inducing diuresis[20]. The proportions of phytochemicals with antioxidative activity vary among foods. In order to investigate possible action mechanisms for such herbal remedies, a method to measure the total antioxidant activity in food and herb should be a substantial asset, regardless of the individual antioxidants which contribute toward this activity[21].

Established methods for the measurement of total antioxidant activity are all essentially inhibition methods: a free radical species is generated, there is an end point by which the presence of the radical is detected, and the antioxidant activity of the added sample inhibits the end point by scavenging the free radical. The ABTS/H_2O_2/HRP system presented here is reliable and practical for measuring total antioxidant activity in fluid samples[22]. When ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) is incubated with peroxidase and hydrogen peroxide, the relatively long-lived radical cation, $ABTS^{\cdot+}$, is formed[23]. In the presence of antioxidant reductants and hydrogen donors, the radical cation is quenched and green color formation delayed. If the maximum $ABTS^{\cdot+}$ absorp-

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tion at 414 nm is traced, the lag time is proportional to the total antioxidant activity in the sample[21]. A microplate assay was reported[24] for determining superoxide dismutase. We had studied total antioxidant activity of plants for years, and in this report we intended to study that of several popular vegetables and traditional Chinese herbs in the ABTS/H_2O_2/HRP system via microplates reader.

MATERIALS AND METHODS

I. *Reagents and Materials*

All fresh vegetables were purchased from a local supermarket in Taipei, Taiwan. All Chinese herbs were obtained from local supermarket or drug store in Taipei or Taichung. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), HRP (horseradish peroxidase, type I) and L-ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide as a 35% solution was purchased from Merck (Darmstadt, Germany). All other reagents were analytical grade and were used without further purification.

II. *Instruments and Conditions*

In this report, a centrifuge, Sigma 3K30, Sigma Laborzentrifugen (Harz, Germany) and a Microplate Spectrophotometer, SPECTRAmax® PLUS, Molecular Devices (Sunnyvale, CA) were used instead of a conventional spectrophotometer. The microplate reader was set up in kinetic mode with absorbance read at 414 nm every 10 sec. The microplate reader provided rapid and sensitive UV/VIS measurements of up to 96 analytes at the same time.

III. *Preparation of Sample Extracts*

The extraction buffer was 6% metaphosphoric acid with 1 μM EDTA in double deionized water. For fresh tissues, each sample was diluted with 3 equivalents of cold extraction buffer, whereas dry matter samples were diluted with a 10-times volume of extraction buffer. The samples were homogenized in a pestle and mortar, the homogenate centrifuged at 4°C, 12000×g and the supernatant taken for antioxidant activity assay[25,26].

IV. *Total Antioxidant Activity Assay*

The ABTS/H_2O_2/HRP assay system for antioxidant activity was reported by Arnao *et al.*[21]. The working solution contained 2.0 mM ABTS and 0.86 nM horseradish peroxidase in 50 mM phosphate buffer, pH 7.0. The reaction was initiated with 1 mM hydrogen peroxide in 50 mM phosphate buffer.

In a 96-well microplate, each well was loaded with 240 μL working solution, 10 μL sample and finally 25 μL of H_2O_2 to initiate the reaction. The reading was taken immediately and the time for green $ABTS^{\cdot+}$ radical formation was calculated. A standard curve was plotted with different concentrations of L-ascorbic acid. Each measurement was expressed with mg L-ascorbic acid equivalent per gram fresh plant tissue.

RESULTS AND DISCUSSION

To save time and reagents, a microplate reader was used to measure total antioxidant activity of fluid samples in this experiment. Ninety-six samples could be measured simultaneously, and it required only one-third the amount of reagents as compared to the conventional spectrophotometric method. Unlike fixed-point method, ABTS/H_2O_2/HRP system uses method and has absorbance readings taken every 10 sec. The plant pigments will make background of absorbance higher without influencing the lag time. This is the advantage of kinetic method.

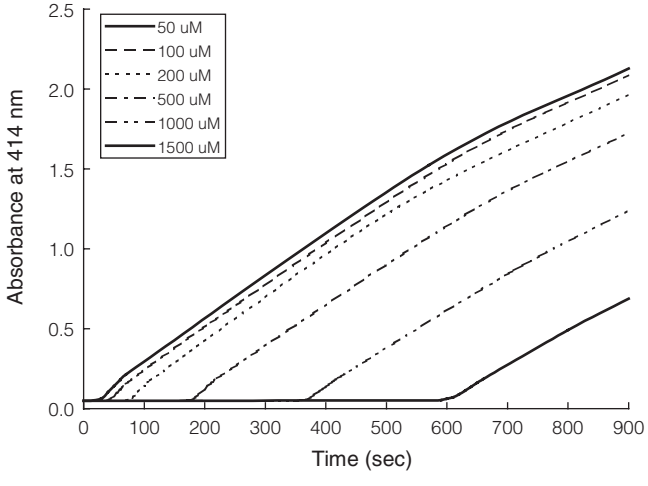

Figure 1. Time course of $ABTS^{\cdot+}$ radical formation in the ABTS/H_2O_2/HRP system using different concentrations of L-ascorbic acid as standard.

Table 1. Total antioxidant activities of numerous vegetables

Common name	Total antioxidant activity (μg/g fresh weight)[a]
Sugar beet root	2029 ± 77
Red cabbage leaf	1217 ± 231
Edible rape leaf	710 ± 101
Pak-choi leaf	475 ± 72
Lettuce leaf	446 ± 67
Spinach leaf	434 ± 55
Pea sprout	296 ± 44
Mungbean sprout	243 ± 56
Bitter melon fruit	228 ± 33
Carrot root	169 ± 20
Tomato fruit	143 ± 12
Head lettuce leaf	123 ± 15
Cucumber fruit	104 ± 15
Celery leaf	83 ± 4.1
Alfalfa sprout	82 ± 6.0
Soybean sprout	79 ± 5.0

[a]All values in this table represent the mean ± SD (n = 3).

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Figure 1 shows the characteristic time course of $ABTS^+$ radical absorption at wavelength 414 nm in $ABTS/H_2O_2/HRP$ system using different concentrations of L-ascorbic acid as standard. A linear correlation was observed between lag time of green $ABTS^+$ product formation and L-ascorbic acid concentration of 50 to 1500 μM L-ascorbic acid (Figure 2).

With the exception of red cabbage and sugar beet, total antioxidant activity of the tested vegetables ranged from 79 to 710 μg L-ascorbic acid equivalent per gram plant tissue. Sugar beet and red cabbage had total antioxidant activity of 2029 and 1217 μg L-ascorbic acid equivalent per gram fresh plant tissue respectively (Table 1). The high antioxidant activity of these 2 vegetables is probably due to their red pigments, flavonoids, which are very powerful antioxidants[27,28].

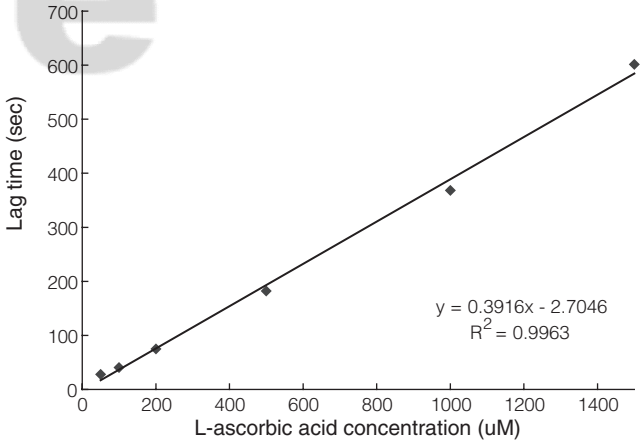

Figure 2. Plot of $ABTS^+$ radical formation lag time versus L-ascorbic acid concentration in the $ABTS/H_2O_2/HRP$ system.

Table 2. Total antioxidant activities of Chinese herbs and folk medicines

Common name	Latin name	Total antioxidant activity[a] (μg/g dry weight)
Tropical Almond Teriminalia Leaves	*Terminalia catappa* L.	28800 ± 9600
Moutan Radicis Cortex	*Paeonia suffruticosa* Andr. (Ranunculaceae)	17100 ± 400
Ligustri Lucidi Fructus	*Ligustrum lucidum* Ait. (Oleaceae)	6220 ± 40
Mori Folium	*Morus alb*a L.(Moraceae)	5070 ± 10
Paeoniae Radix Alba	*Paeonia lactiflora* Pall. (Ranunculaceae)	4910 ± 20
Anemarrhenae Rhizoma	*Anemarrhena asphodeloides* Bge. (Liliaceae)	3990 ± 90
Rehmanniae Radix et Rhizoma	*Rehmannia glutinosa* (Gaertn.) Libosch. (Scrophulariaceae)	2420 ± 150
Ashitaba	*Angelica keiskei*	1890 ± 130
Leonuni Herba	*Leonurus heterophyllus* Sweet (Labiatae)	1630 ± 50
Asari Herba Radice	*Asarum heterotropoides* Fr.Schmidt var. mandshuricum (Maxim.) Kitag. (Aristolochiaceae)	1260 ± 2
Niu-chang-chih	*Antrodia Camphorata* powder	1200 ± 17
Praeparatum Mungo	*Phaseolus radiatus* L. (Leguminosae)	926 ± 62
Ligustici Rhizoma et Radix	*Ligusticum sinense* Oliv. (Umbelliferae)	901 ± 27
Mori Radicis Cortex	*Morus alba* L. (Moraceae)	850 ± 10
Angelicae Dahuricae Radix	*Angelica anomala* Lallem. (Umbelliferae)	817 ± 22
Alpiniae Oxyphyllae Fructus	*Alpinia oxyphylla* Miq. (Zingiberaceae)	568 ± 5.1
Angelicae Sinensis Radix	*Angelica sinensis* (Oliv.) Diels (Umbelliferae)	567 ± 6.0
Eucommiae Cortex	*Eucommia ulmoides* Oliv. (Eucommiaceae)	561 ± 14
Spirodelae Herba	*Spirodela polyrrhiza* Schleid (Lemnaceae)	559 ± 7.0
Albizziae Cortex	*Albizzia julibrissin* Durazz. (Leguminosae)	522 ± 4.1
Tribuli Fructus	*Tribulus terrestris* L. (Zygophyllaceae)	516 ± 1.0
Atractylodis Ovatae Rhizoma	*Atractylodes macrocephala* Koidz (Compositaceae)	471 ± 2.1
Cuscutae Semen	*Cuscuta chinensis* Lam. (Convolvulaceae)	466 ± 1.1
Sileris Radix	*Saposhnikovia divaricata* (Turcz.) Schischk. (Umbelliferae)	460 ± 2.1
Ophipogonis Radix	*Ophiopogon japonicus* (L.f.) Ker-Gawl. (Liliaceae)	411 ± 7.3
Clematidis Radix	*Clematis chinensis* Osbeck (Ranunculaceae)	372 ± 3.2
Trichosanthis Frutus	*Trichosanthes kirilowii* Maxim (Cucurbitaceae)	352 ± 8.1
Codonopsis Pilosulae Radix	*Codonopsis pilosula* (Franch.) Nannf. (Compositaceae)	291 ± 10
Platycodi Radix	*Platycodon grandiflorum* (Jacq.) A. DC.(Campanulaceae)	289 ± 1.1
Persicae Semen	*Prunus persica* (L.) Batsch (Rosaceae)	233 ± 4.0
Magnoliae Liliflorae Flos	*Magnolia liliflora* Desr. (Orchidaeae)	181 ± 13
Ampelopsitis Radix	*Ampelopsis japonica* (Thunb.) Mak. (Vitaceae)	163 ± 7.1
Taiwan Jewel Orchid	*Anoectochilus formosanus* Hayata	146 ± 31
Asparagi Radix	*Asparagus cochinchinensis* (Lour.) Merr.(Liliaceae)	145 ± 3.1
Ginseng Radix	*Panax ginseng* C.A.Mey (Araliaceae)	131 ± 5.0
Pruni Semen	*Prunus japonica* Thunb. (Rosaceae)	120 ± 14
Ginkgo Semen	*Ginkgo biloba* L. (Ginkgoaceae)	99.1 ± 11.4
Coicis Semen	*Coix lacryma-jobi* L. var.ma-yuen (Roman.) Stapf (Gramineae)	85.1 ± 7.7
Benincasae Semen	*Benincasa hispida* (Thunb.) Cogn.(Cucurbitaceae)	52.0 ± 20.6
Armeniacae Semen	*Prunus armeniaca* L. var. ansu Maxim (Rosaceae)	47.1 ± 8.7
Pinelliae Rhizoma	*Pinellia ternata* (Thunb.) Breit. (Araceae)	29.7 ± 6.1
Poria	*Poria cocos* (Schw.) Wolf (Polyporaceae)	21.8 ± 1.3

[a]All values in this table represent the mean ± SD (n = 3).

Antioxidation activity represents the capability of scavenging free radical and offering hydrogen atom. The higher the antioxidation activity, the stronger its capability is[29]. Various methods used to evaluate total antioxidant activity of common vegetables had been reported, including oxygen radical absorption capacity method[30], ferric reducing antioxidant capacity method[30], liposome assay[31], and total oxyradical scavenging capacity assay[32]. The tested samples were not overlapped with the materials used in this study, so the results cannot be compared. However, the method of total antioxidant activity determination with ABTS/H_2O_2/HRP system in microplate offers a fast and reliable approach.

The tissue of some Chinese herbs or folk medicines demonstrated potent antioxidative activity. For example, that of Tropical Almond Terminalia fallen leaves and Moutan Radicis Cortex were overwhelmingly high. They were 28800 and 17100 μg L-ascorbic acid equivalent per gram weight respectively. Their high antioxidative capacity may constitute one of the beneficial factors in these traditional Chinese medication (Table 2). However, the active components that contribute to the strong antioxidant activity of these Chinese herbs is still unidentified.

Herbal drugs such as Moutan Radicis Cortex, Ligustri Lucidi Fructus, Mori Folium, Paeoniae Radix Alba, Anemarrhenae Rhizoma, Rehmanniae Radix et Rhizoma, Ashitaba, Leonuni Herba, and Asari Herba Radice were found to have higher total antioxidative activities. These drugs used to nourish qi (in Chinese, vital energy), blood, lung, and liver[33]. However, other Chinese herbs such as Asparagi Radix, Coicis Semen, Codonopsis Pilosulae Radix, and Ginseng Radix, etc. showed lower total antioxidant activities. This finding indicates that antioxidative effects are not the only beneficial factors of these medicinal herbs.

Oxidation is found to have a strong relationship with melanin formation. Antioxidants and free radical scavengers could contribute to lightening of skin pigment[34,35]. Meanwhile, skin possesses an extremely efficient antioxidant system[36] by two major groups: enzymes (e.g., superoxide dismutase, SOD) and small molecules (e.g., L-ascorbic acid). Both antioxidants could protect skin damage from oxidation. The herbs with higher antioxidant activities have the potential to be used in cosmetics.

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