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Evaluation of antioxidant activity of aqueous extract of some selected nutraceutical herbs

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Abstract

The objectives of this study were to examine the antioxidant activities and free radical scavenging effects of extracts of aqueous leaves of *Psidium guajava* L. (PE), *Camellia sinensis* (GABA tea; CE), *Toona sinensis* Roem. (TE) and *Rosemarinus officinalis* L. (RE). Among the four extracts, PE exhibited the strongest efficiency and showed over 50% scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals at the concentration of 100 μg/mL. The reducing power of four nutraceutical herbs was in the order of PE > RE > CE > TE. The antioxidant activities of nutraceutical herbs were evaluated in a liposomes oxidation system promoted by Fe^{3+}/ascorbic acid/H_2O_2. PE still showed the strongest antioxidant activity and exhibited over 95% inhibition at concentration of 50 μg/mL. The antioxidant activity of TE was still lower than that of other herbal plants; however, it also displayed 89% inhibition at concentration of 250 μg/mL. RE exhibited well inhibitory effects on the UVB-induced oxidation of erythrocyte ghosts at lower concentration (100 μg/mL). However, the protection of PE on the UVB-induced oxidation was significantly raised with increasing the concentrations and reached 95.4% inhibitory effects at concentration of 500 μg/mL. These results show that the tested herbal tea, especially PE could be considered as a natural antioxidant source.

Keywords: *Psidium guajava* L.; *Camellia sinensis*; *Toona sinensis* Roem.; *Rosemarinus officinalis* L.; Antioxidant activity

1. Introduction

Reactive oxygen species (ROS) and free radicals such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are constantly formed in the human body by normal metabolic action, and have been implicated in the pathogenesis of certain human diseases, including cancer, aging, diabetes and atherosclerosis (Moskovitz, Yim, & Chock, 2002). Their action is opposed by a balanced system of antioxidant defenses including antioxidant compounds and enzymes. Upsetting this balance causes oxidative stress, which can lead to cell injury and death (Halliwell & Gutteridge, 1999). Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancers (Kris-Etherton et al., 2002) and neurodegenerative diseases (Di Matteo & Esposito, 2003). Therefore, much attention has been focused on the use of natural antioxidants to inhibit lipid peroxidation, or to protect the damage of free radicals.

Abbreviations: *PE,* aqueous extract from *Psidium guajava* L.; TE, aqueous extract from *Toona sinensis* Roem.; CE, aqueous extract from *Camellia sinensis* (GABA tea); RE, aqueous extract from *Rosemarinus officinalis* L; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GAE, gallic acid equivalents; CAE, catechin equivalents; MDA, malondialdehyde; GABA, γ-aminobutyric acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

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Recently, research on phytochemicals and their effects on human health has been intensified. In particular, research has focused on the search for antioxidants, hypoglycemic agents, and anticancer agents from vegetable, fruit, tea, spice and medical herbs. The objectives of this study were to measure the antioxidant properties of several plant extracts that are very popular as herbal teas or vegetables in Taiwan. The development of γ-aminobutyric acid (GABA) tea (aqueous extract from *Camellia sinensis*) in Taiwan has been over 10 years. GABA tea is one kind of functional tea that is made by fermenting fresh tea leaves under nitrogen gas. Because of richness in γ-aminobutyric acid, naming as GABA based on its initials of the special amino acid contained. In Japan, GABA tea also calls GABARON tea, due to the taste of GABA tea is quite similar to oolong tea. γ-Aminobutyric acid is known to be involved in the regulation of blood pressure by modulating the neurotransmitter release in the central and peripheral sympathetic nervous systems. Abe et al. (1995) indicated that GABA tea seemed not only to decrease the established high blood pressure, but to prevent the development of hypertension in rats fed a high salt diet. Guava tea, the infusion of dried guava leaves, is another popular herbal tea in Taiwan. Guava (*Psidium guajava* L.) is widely cultivated and its fruit is popular. Guava was also used as a hypoglycemic in folk medicine. The leaves and skin of the fruit have greater effects. The anti-diarrhoeal (Lutterodt, 1989) antipyretic (Olajide, Awe, & Makinde, 1999), antimicrobial (Jaiarj et al., 1999) and bio-antimutagenic (Matsuo, Hanamure, Shimoi, Nakamura, & Tomita, 1994) properties of guava leaf extract have been demonstrated. *Toona sinensis* Roem. (Meliaceae) is distributed in Taiwan and is also widely cultivated nowadays in Asia. The leaves of *T. sinensis* have been used as an effective nutritious vegetable and herbal tea for the treatments of enteritis, dysentery and itch in the practice of oriental medicine in Chinese society for a long time. It has been reported that extracts from *T. sinensis* leaves can induce apoptosis of cancer cells (Chang, Hung, Huang, & Hsu, 2002) and ovarian cancer cells (Chang et al., 2006). Moreover, the extracts from *T. sinensis* leaves could improve the secretion of insulin in diabetic rats (Yu, 2002). Hsieh et al. (2004) reported that methyl gallate from root of *T. sinensis* can protect against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells. Rosemary extract has been widely used as antioxidant in food industries. The main constituents that contributed to the antioxidant activity of rosemary were carnosol and carnosic acid (Frankel, Huang, & Aeschbach, 1996).

In a previous study, PE and TE showed more inhibitory effects on glycation of low density lipoprotein (LDL) induced by glucose and glyoxal than that of other selected herbs (Hsieh et al., 2005). In this study, the antioxidant properties and phenolic compound contents of PE, CE and TE were compared with the extracts from rosemary (RE), a known potent plant antioxidant.

2. Materials and methods

2.1. Materials chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, (+)catechin 1,1,3,3,-tetramethoxypropane and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteau reagent and thiobarbituric acid (TBA) were purchased from E. Merck Co. (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Herbal plants and extraction

The dried leaves of *P. guajava* L., *C. sinensis* (GABA tea), *T. sinensis* Roem., and *Rosemarinus officinalis* L. were purchased from a local market in Taichung, Taiwan, their origins were identified and proved by the Institute of Medical Herb (Taichung, Taiwan). Aqueous extracts were obtained as follows. In brief, 50 g of dried leaves were suspended and extracted by refluxing with 10 volumes of boiling water for 30 min. The extracts were filtered through a filter paper and the filtrates were freeze-dried.

2.3. Determination of total phenolic compounds

Total phenolic compounds in the nutraceutical herbs were determined, using Folin–Ciocalteau reagent, by the method of Taga, Miller, and Pratt (1984) and calculated using gallic acid as a standard. Extracts (100 μL) were added to 2 mL of 2% Na_2CO_3. After 2 min, 50% Folin–Ciocalteau reagent (100 μL) was added to the mixture which was then left to stand for 30 min. Absorbance was read at 750 nm using a spectrophotometer and compared to gallic acid calibration curves. The content of total phenolics was expressed as gallic acid equivalents (GAE). All analyses were run in three replicates and mean values recorded.

2.4. Determination of total flavonoids

The spectrophotometer assay for the quantitative determination of flavonoid content was carried out as described by Zhishen, Mengcheng, and Jianming (1999). Briefly, the extract (1 mL, 1 mg/mL) was diluted with 1.25 mL distilled water. At zero time, 75 μL 5% $NaNO_2$ were added to the mixture. After 6 min, 150 μL 10% $AlCl_3$ were added. After another 5 min, 1 mL 1 M NaOH were added to the mixture. Immediately, the absorbance of the mixture, pink in colour, was determined at 510 nm versus prepared water blank. Total flavonoids of fruits were expressed on a fresh weight basis as mg/100 g catechin equivalents (CAE).

2.5. Scavenging effects of nutraceutical herbs on DPPH radical

The scavenging effects of nutraceutical herbs on DPPH radicals were estimated according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992). Plant extracts in 4 mL water were added to a 1 mL solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. The final concentration of DPPH was 0.2 mM. The mixture was shaken vigorously and was allowed to stand for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (U-3000, Hitachi).

2.6. Reducing power of herbal plant extracts

The reducing power of nutraceutical herbs was determined according to the method of Oyaizu (1986). Extracts in 1 mL distilled water were mixed with phosphate buffer (2.5 mL, 2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%); the mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (TCA, 10%) was added to the mixture which was then centrifuged at 1500*g* for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.7. Effect of nutraceutical herbs on the oxidation of liposome induced by $FeCl_3/H_2O_2$/ascorbic acid

The antioxidative effects of nutraceutical herbs on liposome-induced oxidation with $FeCl_3/H_2O_2$/ascorbic acid and lipid peroxidation were quantified, based on thiobarbituric acid reactive substances (TBARS) production, by the method described by Yen, Chen, and Lee (1999). Lecithin (300 mg) was sonicated in an ultrasonic cleaner in 30 mL, 10 mM phosphate buffer (pH 7.4) for 2 h at ice bath. The sonicated solution (10 mg lecithin/mL), $FeCl_3$, H_2O_2, ascorbic acid and nutraceutical herbs were mixed to produce a final concentration of 2.5 mg lecithin/mL, 125 μM $FeCl_3$, 125 μM H_2O_2 and 125 μM ascorbic acid. The mixture was incubated for 4 h at 37 °C. The oxidation of liposome was measured by the TBA method (Tamura & Shibamoto, 1991). The absorbance of the sample was read at 532 nm against a blank which contained all reagents except lecithin.

2.8. Effects of nutraceutical herbs on the oxidation of erythrocyte ghost induced by UVB radiation

Fresh blood was obtained from healthy adult male by venipuncture, and collected in tubes containing citrate phosphate dextrose adenine solution (CAPD-1). Blood was centrifuged at 1500*g* for 10 min at 4 °C. The plasma and the buffy coat which contains the platelets, white blood cells, and some erythrocytes were removed by aspiration and the erythrocytes were washed three times with phosphated buffer saline (PBS). Hemoglobin-free ghost was prepared according to the methods of Tsuda et al. (1994) and Virgili, Battistini, Canali, Vannini, and Tomasi (1996) with some modification. Washed erythrocytes were hemolyzed in 40 volumes of 5 mM phosphate buffer solution (pH 7.4) and centrifuged at 37,000*g* for 20 min at 4 °C. The pellet was washed repeatedly until colourless ghosts were obtained.

The UVB radiation was applied at wavelength 280–350 nm (peak of emission near 302 nm) using a fluorescent tube (UVP CL-1000: UVP, Inc., CA). The radiation intensity was measured with a UVX Radiometer (UVP CL-1000: UVP, Inc., CA). Erythrocyte ghost suspensions at 10% hematocrit were mixed with or without different concentrations of nutraceutical herbs and were treated by UVB radiation (5.2 mW) for 2 h. After the UVB radiation, the TBARS in ghost were measured according to the methods of Budge and Aust (1978) and Yagi (1976). After radiation, 1 mL of 10% erythrocyte suspension solution was added to butylated hydroxytoluene (BHT) (the final concentration was 0.5 mM). Two millilitres of 7.5% TCA was added to the mixture to precipitate protein. Draw the upper layer and add 1 mL of 1% TBA to combine with malondialdehyde (MDA) and determine the fluorescence at excitation 515 nm and emission 555 nm. Using 1,1,3,3-tetramethoxy- propane as a standard to convert the MDA content.

2.9. Statistical analysis

All analyses were run in triplicate and averaged. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($P < 0.05$) between the means were determined using Duncan's multiple range test.

3. Results

3.1. Total phenolics and total flavonoids contents

The amounts of total phenolics and flavonoids in the studied nutraceutical herbs are summarized in Table 1. A high content of total phenolics was observed in RE in com-

Table 1
The contents of total flavonoids of aqueous extracts from selected herbs[a]

Sample	Total phenolic compounds (mg/g)	Total flavonoids (mg/g)
CE	149.27 ± 2.31	33.20 ± 0.51
PE	154.36 ± 2.97	82.85 ± 0.22
RE	185.04 ± 4.99	141.2 ± 2.85
TE	64.95 ± 2.40	12.92 ± 0.95

[a] Total phenolic compounds and total flavonoids were expressed as gallic acid and catechin equivalents, respectively. Data expressed in mean ± standard deviation from triplicate experiments.

parison with other herbal plant extracts, followed by PE, CE and TE extracts which had the lowest. Flavonoids are one of the most diverse and widespread group of natural phenolics and then contents in the herbs studied decreased in the order of RE > PE > CE > TE. The data in Table 1 shows that more than 50% of the extracted phenolic substances in RE and PE were of flavonoid origin. However, only about 20% of the extracted phenolic substances in CE and TE were flavonoids. In our results, CE also contained a lower level of flavonoids which may due to the fermentation under nitrogen gas during the preparation of GABA tea.

3.2. DPPH radical scavenging effects

The scavenging effects of nutraceutical herbs under investigation on DPPH radicals are shown in Table 2. Among the four extracts examined, PE exhibited the strongest efficiency and showed over 50% scavenging effect of DPPH at a concentration of 100 μg/mL. Therefore, PE had the highest hydrogen-donating capacity, followed by RE and CE, while TE rendered the weakest effect.

3.3. Reducing power

Fig. 1 shows the dose–response curves for the reducing powers of herbs studied using the potassium ferricyanide reduction method. The amount of Fe^{2+} complex was then monitored by measuring the formation of Perl's prussian blue at 700 nm. The reducing power of all extracts increased with concentration and these varied in the order of PE > RE > CE > TE.

3.4. Antioxidant effect on the oxidation of liposome

In this study, the antioxidant activities of four herbs in liposomes composed of egg lecithin with Fe^{3+}/ascorbic acid/H_2O_2 were examined, as shown in Fig. 2. Among the four herbs tested, PE showed the strongest antioxidant activity and exhibited over 50% and 95% inhibition at a concentration of 10 and 50 μg/mL, respectively. The antioxidant activity of TE was still lower than that of other herbs tested, however, it also displayed 89% inhibition at concentration of 250 μg/mL.

Table 2
Scavenging effect of aqueous extracts from selected herbs on DPPH radicals

Sample	Concentration (μg/mL)	Scavenging effects (%)[a]
CE	100	32.71 ± 1.50
	500	49.47 ± 0.11
PE	100	51.69 ± 1.29
	500	57.42 ± 0.55
RE	100	39.41 ± 0.51
	500	55.32 ± 0.52
TE	100	33.11 ± 0.95
	500	38.02 ± 0.52

[a] Scavenging effects % (capacity to scavenging the DPPH radicals) = [(absorbance of control at 517 nm) − (absorbance of sample at 517 nm)/(absorbance of control at 517 nm)] × 100. Data expressed in mean ± standard deviation from triplicate experiments.

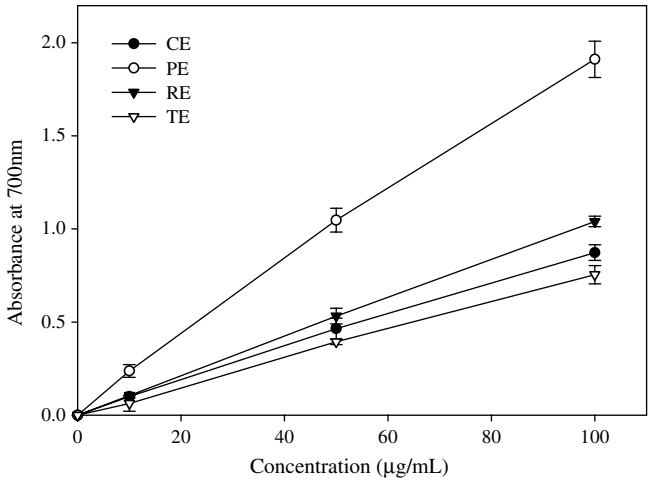

Fig. 1. Reducing power of four nutraceutical herbs. Reducing power: increase in the absorbance at 700 nm indicates the activity of reducing power. Each value is expressed as mean ± standard deviation ($n = 3$).

3.5. Antioxidant effect on the oxidation of erythrocyte ghost

The effects of nutraceutical herbs on the oxidation of erythrocyte ghost are shown in Fig. 3. RE exhibited good inhibitory effects on the oxidation of erythrocyte ghosts at a lower concentration (100 μg/mL). However, the protection of PE on the UVB-induced oxidation was significantly raised with increasing concentration and reached 95.4% inhibitory effects at 500 μg/mL.

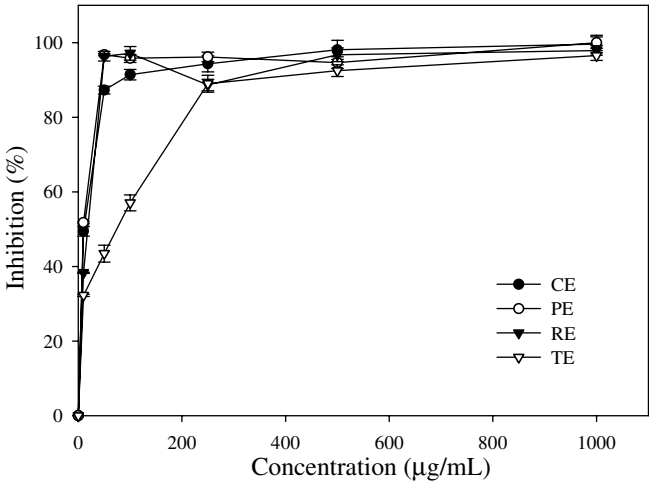

Fig. 2. Inhibition of four nutraceutical herbs on Fe^{3+}/ascorbic acid/H_2O_2-induced lipid peroxidation in a liposome model system. The inhibition of samples on the formation of TBARS was compared with control reaction in the absent of samples. Each value is expressed as mean ± standard deviation ($n = 3$).

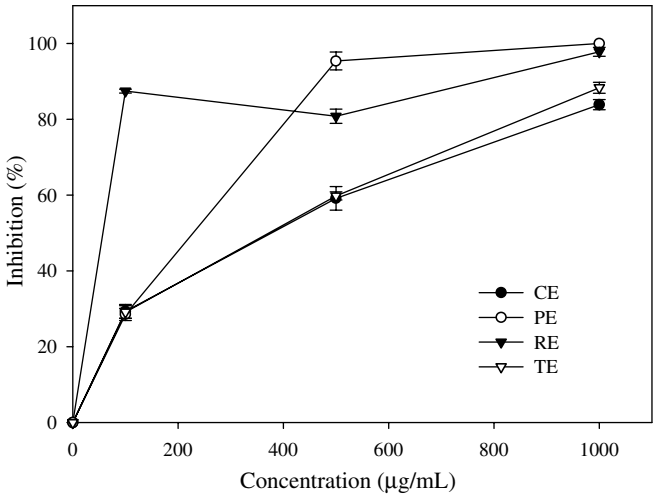

Fig. 3. Effects of four nutraceutical herbs on UV light-induced lipid peroxidation of red blood membrane. The inhibition of samples on the formation of TBARS was compared with control reaction in the absent of samples. Each value is expressed as mean ± standard deviation ($n = 3$).

4. Discussion and conclusions

Herbal plants are known to contain a variety of antioxidants. Numerous substances have been suggested to serve as antioxidants. It has been revealed that various phenolic antioxidants, such as flavonoids, tannins, coumarins, xanthones and more recently procyanidins scavenge radicals dose-dependently, thus they are viewed as promising therapeutic drugs for free radical pathologies (Hollman & Katan, 1998; VanderJagt, Ghattas, VanderJagt, Crossey, & Glew, 2002). In this work, the content of total phenolics in RE was 185.04 mg GAE/g, which was similar to the data of Dorman, Peltoketo, Hiltunen, and Tikkanen (2003) that compared the total phenolics contents in deodorised aqueous extracts of four commonly consumed herbs belonging to the *Lamiaceae* family, and the total phenolics contents of the extracts were oregano 149, rosemary 185, sage 166 and thyme 95.6 mg GAE/g. As shown in Table 1, the contents of total phenolic compounds and total flavonoids were both in the order of RE > PE > CE > TE. Miliauskas, Venskutonis, and van Beek (2004) observed that the amount of flavonoids in 12 medicinal, aromatic plant extracts showed only low correlation with the total amount of phenolics. Duh, Yen, Yen, Wang, and Chang (2004) compared the total phenolics and flavonoids contents of various fermented tea extracts and the results showed that green tea extracts contained the highest total phenolics, 94% of which were flavonoids (catechins) in origin. However, oolong tea and pu-erh tea extracts had middle level of total phenolics and a low level of flavonoids (18.4% and 4.4%, respectively). In our results, GABA tea extracts also had a low level of flavonoids (22.2%) compared with those of RE and PE. The three main categories of tea namely: green, black and oolong, are produced by different processing procedures. Catechins dominate in green tea, and theaflavins and thearubigins predominate in black tea. These kinds of tea flavonoids are thought to have the strongest chemopreventitive effects (Wang, Provan, & Helliwell, 2000). The changes of components in GABA tea that altered in the fermentation process under nitrogen gas are interesting and remain to be studied further.

Phytochemical work on *Toona* species has led to the isolation of triterpenes and phenolic compounds. Known compounds, including gallic acid, methyl gallate, kaempferol, quercitin, quercitrin, rutin, catechin, epicatechin, a mixture of b-sitosterol and stigmasterol, and b-sitosteryl-glucoside, were isolated and identified from this plant (Hsieh, Chang, & Wu, 1999). Although it remains unclear which of the components of *T. sinensis* are active compounds, phenolic compounds such as gallic acid, gallic acid ester, and its catechin derivatives have received increasing attention recently because of some interesting new findings regarding their biological activities (Inoue, Sakaguchi, Isuzugawa, Tani, & Ogihara, 2000; Lopez-Velez, Martínez-Martínez, & Valle-Ribes, 2003; Ow & Stupans, 2003) In our previous report, the main phenolic compounds in guava leaf extracts were quercetin (12.26 mg/g), gallic acid (12.18 mg/g) and ferulic acid (9.42 mg/g) (Hsieh, Lin, Yen, & Chen, 2007). Zhang, Liang, Qian, Yuan, and Yao (2006) also identified gallic acid, chlorogenic acid, kaempferol, procatechuic acid, ferulic acid, caffeic acid, quercetin and rutin in acetone extract from guava leaf by RP-HPLC analysis. From above data indicated that guava extracts contained phenolic acids and flavonoids which appeared to be responsible for its antioxidant activity.

The antioxidant activities could be assayed by using several test systems. Recent investigations showed differences between the test systems for the determination of antioxidant activity (Schlesier, Harwat, Böhm, & Bitsch, 2002) are use of at least two methods has been recommended. The hydrogen-radical scavenging action has been known as an important mechanism of antioxidation. DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Shimoji et al., 2002). The reducing power might be due to hydrogen-donating ability (Shimada et al., 1992), and is generally associated with the presence of reductones (Duh, 1998). In our results, the trend of reducing abilities for four plant extracts was similar to the result of the scavenging effect on DPPH radicals. PE exhibited the best effects in both test methods.

The systems of liposome and erythrocyte ghost have been used extensively as biological models for in vitro lipid peroxidation studies. Cellular membranes, which contain abundant phospholipids, such as phosphatidylcholine (lecithin), are major targets of free radicals which induce lipid peroxidation and thereby cause malfunctioning of membranes by altering membrane fluidity and membrane-bound enzyme and receptor functions (Jana, Agarwal, & Chatterjee, 1990).

Oxidation of lipids can also be brought about by other mechanisms such as photooxidation. In recent years,

accumulated evidence has demonstrated that UV induced oxidative damage occurs through the formation of free radicals and ROS which damage cellular components (Zhang, Rosenstein, Wang, Lebwohl, & Wei, 1997). UV light can damage many tissue components including membrane phospholipids, proteins and nucleic acids. Cell membrane is the main target attacked by free radicals. The membranes of mitochondria and erythrocyte are easily oxidation induced by photodynamic and free radicals (Salet & Moreno, 1990). UVB photons could penetrate the capillary plexus in the dermis and interact directly with erythrocytes, resulting photo-hemolysis (Kollias, Baqer, Sadiq, & Sayre, 1992). Acute and chronic exposures to UV light promote premature skin aging, erythema, inflammation, immunodepression and photo-carcinogenesis (Lopez-Torres, Thiele, Shindo, Han, & Packer, 1998). Therefore, antioxidants can be used to protect the cells from UV induced cellular damage by scavenging free radicals and ROS.

In this study, the abilities of the herbs to scavenge free radicals were further confirmed by inhibition of lipid peroxidation indices in a liposome model system and erythrocyte ghost system. Among the four extracts, PE showed the strongest antioxidant activity in liposome model system, and exhibited over 95% inhibition at a concentration of 50 μg/mL. In addition, the antioxidant activities of herbs in a liposome system correlated significantly with the DPPH radical-scavenging activity ($r = 0.973$, $P < 0.05$) and total phenolic compounds ($r = 0.968$, $P < 0.05$). These results reveal that nutraceutical herbs could react with free radicals, converting them to more stable products and terminating the radical chain reaction and supplying antioxidant action; and they may be attributed, in a significant part, at least, to the total phenolic compounds. In erythrocyte ghost system, RE exhibited well inhibitory effects against the UVB-induced oxidation at a lower concentration (100 μg/mL). However, the protections of PE on the UVB-induced oxidation were significantly increased with increasing the concentrations. These results suggested that the percentage of total phenolic compounds in nutraceutical herbs might be a critical factor for their antioxidant activity against UVB radiation when the dosage of extracts used in the reaction system was lower. In contrast, the extent of antioxidant ability provided by specific compounds in nutraceutical herbs seemed more important when the dosage of extracts used in the reaction system was higher.

Although the contents of total phenolic compounds and flavonoids in PE extracts were lower than that of RE, PE showed the strongest antioxidant activity in most of the tested methods. Several phenolic compounds such as gallic acid, chlorogenic acid, kaempferol, procatechuic acid, ferulic acid, caffeic acid, quercetin and rutin have been isolated from PE. Our study shows that the PE is a source of natural antioxidants, which could contribute to the health benefits. However, in vivo studies are needed to confirm the health-promoting potential of PE.

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