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Phenolic compositions and antioxidant attributes of leaves and stems from three inbred varieties of *Lycium chinense* Miller harvested at various times

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ABSTRACT

Antioxidant components and properties (assayed by scavenging DPPH radicals, TEAC, reducing power, and inhibiting Cu^{2+}-induced human LDL oxidation) of leaves and stems from three inbred varieties of *Lycium chinense* Miller, namely ML01, ML02 and ML02-TY, harvested from January to April were studied. Their flavonoid and phenolic acid compositions were also analyzed by HPLC. For each variety, the leaves and stems collected in higher temperature month had higher contents of total phenol, total flavonoid and condensed tannin. Contents of these components in the samples collected in different months were in the order: April (22.3 °C) > March (18.0 °C) > January (15.6 °C) > February (15.4 °C). Antioxidant activities of the leaves and stems for all assays also showed similar trends. The samples from different varieties collected in the same month also possessed different phenolic compositions and contents and antioxidant activities. Their antioxidant activities were significantly correlated with flavonoid and phenolic contents.

1. Introduction

Lycium chinense named wolfberry or Goji, belonging to *Solanaceae* family, mainly distributes in East Asia and is grown in particular in South China. Its fruits are a very popular ingredient in Chinese cuisine and have been widely used for more than 2000 years in traditional Chinese medicine for immunoregulation, longevity, vision, and wellness (Potterat, 2010). Reports indicated that the fruits containing flavonoids (Qian, Liu, & Huang, 2004), phenolic acids (Noculak-Palczewska et al., 2004), carotenoids (Kim, Kim, Huh, & Kim, 1997), polysaccharides (Qin, Yamauchi, Aizawa, Inakuma, & Kato, 2001) and pyrrole derivatives (Chin et al., 2003) displayed many functional effects such as antioxidant (Ionică, Nour, & Trandafir, 2012), anti-inflammatory (Lin, Chuang, Lin, & Yang, 1997) and hepatoprotective (Chin et al., 2003; Kim, Choi et al., 1997).

The agricultural research organizations of Taiwan have remarkable results in cultivation, harvesting technology and post-harvest treatment of *L. chinense* Miller. Its leaf and stem are used as tea and vegetables, which are now popular in Taiwan due to beneficial effects. Studies revealed that the leaf of *L. chinense* containing plentiful amount of polyphenols including flavonoids and phenolic acids had antioxidant and antimicrobial activities (Haensel & Huang, 1977; Mocan et al., 2014; Terauchi, Kanamori, Nobuse, Yahara, & Nohara, 1997).

In general, the harvest time for the leaves and stems of *L. chinense* Miller cultivated in Taiwan is from January to April. Many literatures showed that harvest time affected chemical composition and yield of crops (Amaducci & Pritoni, 1998; Dong et al., 2003; Lin, Chen, Liu, & Yang, 2009). Pęksa, Golubowska, Rytel, Lisińska, and Aniolowski (2002) illustrated that greater influence of harvest time on bioactive compounds was due to different environmental and weather conditions; environmental temperature might play an important role. There is, however, no thorough investigation regarding the influence of harvest time on phenolic compositions and antioxidant effects of leaves and stems of *L. chinense* Miller.

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In this study, we gathered the leaves and stems from three inbred varieties of *L. chinense* Miller, namely ML01, ML02 and ML02-TY, cultivated in Miaoli, Taiwan from January to April, and compared their phenolic compositions and contents as well as antioxidant activities. The influence of air temperature on the phenolic components and antioxidant effects was also traced. Our results could provide as a reference for exploitation of leaves and stems of *L. chinense* Miller.

2. Materials and methods

2.1. Samples

The leaves and stems were gathered from three inbred varieties of *L. chinense* Miller (named M-L01, ML-02 and ML-02TY, respectively) cultivated in Miaoli District Agricultural Research and Extension Station, Miaoli County, Taiwan from January to April, 2014. Samples were lyophilized through a freeze-drying system (Vastech Scientific Co. Ltd., Taipei, Taiwan), ground to flour with a grinder (model RT08, Rong-Tsong Precision Technology Co., Taichung, Taiwan) and passed through 40 mesh standard sieve before experiment.

2.2. Chemicals

Acetonitrile (ACN), acetic acid (CH_3COOH), ethanol (EtOH) (95%), hydrogen chloride (HCl) and methanol (MeOH) were purchased from Merck Co. (Darmstadt, Germany). Distilled deionized water (dd H_2O) is prepared through Ultrapure™ water purification system (Lotun Co., Ltd. Taipei, Taiwan). Horseradish peroxidase was obtained from Sigma Co. (St. Louis, MO, USA). Standards for phenolic acids including gallic acid, gentisic acid, chlorogenic acid, *p*-hydroxybezoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, syringic acid, *p*-anisic acid and rosmarinic acid, and flavonoids including catechin, epicatechin, rutin, naringin, myricetin, hesperidin, quercitrin, neohesperidin, eriodictyol, diosmin, morin, daidzein, quercetin, glycitein, narigenin, luteolin, genistein, hesperetin, kaempferol, apigenin and isorhamnetin were purchased from Sigma Co. (St. Louis, MO, USA). Chemicals used to assay the antioxidant effects and determine antioxidant components such as cupric sulfate ($CuSO_4$), Folin-Ciocalteu's phenol reagent, potassium ferricyanide ($K_3Fe(CN)_6$), 2-2′-azino-bis-(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), vanillin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), and ferric chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$) and aluminum chloride ($AlCl_3$) were purchased from Sigma Co. (St. Louis, MO, USA). Sodium hydroxide (NaOH), disodium hydrogen phosphate (Na_2HPO_4) and hydrogen peroxide (H_2O_2) were obtained from Wako Co. (Osaka, Japan).

2.3. Sample preparation

The ground sample (50 g) was extracted by 1 L of 80% EtOH with magnetic stirring for 24 h in the dark. After filtration, the solvent was removed under vacuum at 40 °C followed by lyophilization in a freeze-dryer (Vastech Scientific Co., Ltd., Taipei, Taiwan) to obtain the crude ethanolic extract. Each preparative procedure was carried out in triplicate. The extracts were preserved under nitrogen at −20 °C in the dark before assays.

2.4. Antioxidant assays

Each sample extract was dissolved in 95% EtOH (1 mg/mL) and then diluted to prepare the series concentrations for measurement of antioxidant effects. (+)-Catechin was used for comparison in all assays.

2.4.1. DPPH radical scavenging activity assay

The assay referred to the report of Liu, Lin, Wang, Chen, and Yang (2009) was used. An aliquot of each sample extract (200 μL) was mixed with 50 μL of 1 mM DPPH (prepared with MeOH) and reacted for 30 min. The absorbance was measured at 517 nm (Multiskan Spectrum, Thermo Co., Vantaa, Finland). The scavenging activity was estimated based on the percentage of scavenging DPPH radicals. EC_{50} value is the effective concentration that scavenges 50% of the DPPH radicals.

2.4.2. Trolox equivalent antioxidant capacity (TEAC) assay

The assay was executed by the method of Scalzo, Politi, Pellegrini, Mezzetti, and Battino (2005). The preparation of $ABTS^{\cdot+}$ solution ($OD_{734} = 0.70 \pm 0.03$) was done through mixing ABTS, H_2O_2 and peroxidase with the final concentrations of 100 μM, 50 μM and 4.4 unit/mL, respectively. An aliquot of each sample extract (30 μL) reacted with 270 μL of the $ABTS^{\cdot+}$ solution for 3 min. The absorbance was recorded at 734 nm. The scavenging ability of $ABTS^{\cdot+}$ was counted relative to Trolox. The TEAC value was expressed as μmole Trolox equivalent (TE)/g extract.

2.4.3. Reducing power

The assay was estimated according to the report of Oyaizu (1986). An aliquot of each sample extract (125 μL) mixed with 125 μL of sodium phosphate buffer (0.2 M, pH 6.6) and 125 μL of 1% $K_3Fe(CN)_6$ was incubated at 50 °C for 20 min. After adding 125 μL of 10% TCA, the mixture was centrifuged at 3750×*g* for 10 min (Hermle Z300 K centrifuge, Hermle Labortechnik GmbH, Wehingen Württ, Germany). Briefly, the supernatant solution (100 μL) was mixed with 100 μL of dd H_2O and 20 μL of 1% $FeCl_3$ and reacted for 10 min. The absorbance was read at 700 nm. The EC_{50} value corresponds to the sample concentration at which the absorbance is 0.5.

2.4.4. Assay for inhibition of Cu^{2+}-induced human LDL oxidation

LDL (d = 1.019–1.063 g/mL) was separated from the plasma of fasting healthy volunteers with sequential density ultracentrifugation in a Beckman Ultra centrifuge (model: LE-80K; Beckman Instruments Inc., Palo Alto, CA) at 4 °C as the conditions of Hsieh, Shen, Kuo, and Hwang (2008). The isolated LDL was dialyzed against phosphate buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.4) overnight at 4 °C. The protein level of LDL was determined as the report of Lowry, Rosebrough, Farr, and Randall (1951), and further regulated to 300 μg protein/mL with 5 mM PBS. The LDL (70 μL) mixed with 20 μL of each sample extract (dissolved in EtOH and diluted with dd H_2O to 0.02% EtOH) and 10 μL of 250 μM $CuSO_4$ (in 5 mM PBS) was incubated at 37 °C. The formation of conjugated diene was surveyed at 234 nm at 5 min intervals by a Multiskan Spectrum microplate spectrophotometer (Thermo Co., Vantaa, Finland) to conclude the kinetics of LDL oxidation in the initiation, propagation, and termination processes. The increase of lag time (Δtlag) of LDL oxidation compared to control was calculated.

2.5. Determination of antioxidant components

Each sample extract was dissolved in MeOH (50 mg/mL) to determine the antioxidant components.

2.5.1. Total phenolic contents

Total phenolic contents in samples were measured through the method of Julkunen-Titto (1985). An aliquot (50 μL) of each sample extract or standard solution was mixed with 1 mL of dd H_2O and