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Bioguided Fractionation and Isolation of Free Radical Scavenging Components from in Vitro Propagated Chinese Medicinal Plants *Dendrobium tosaense* Makino and *Dendrobium moniliforme* SW

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This study was performed to investigate the free radical scavenging active components from in vitro propagated medicinal herbs of the genus *Dendrobium*, namely, *Dendrobium tosaense* Makino and *Dendrobium moniliforme* SW, using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical antioxidative assay. Seeds of the capsules derived after 12 weeks of hand-pollination germinated asymbiotically (50−74%) on half-strength Murashige and Skoog's (MS) basal medium with 3% sucrose and solidified with 0.9% Difco agar. Active growth in the germinated seedlings was achieved by reculturing on full-strength MS basal medium supplemented with 8% banana homogenate, 8% potato homogenate, 8% coconut water, 1.5% sucrose, and 0.9% Difco agar. Healthy plantlets transferred to plastic trays containing moss or moss and tree fern successfully acclimatized (84−100%) in the greenhouse. Extracts were prepared from plants grown in the greenhouse for a period of 6 months. Methanolic extracts of *D. tosaense* and *D. moniliforme* scavenged DPPH at 95.9 and 83.4%, respectively, at a concentration of 0.4 mg/mL. Therefore, methanolic solubles of *D. tosaense* and *D. moniliforme* were subjected to bioguided fractionation and separation by column chromatographic methods individually. After chromatographic separation of these crude extracts, the obtained fractions (Dm 1, Dm 2, Dm 3, Dt 1, Dt 2, and Dt 3) were tested for their activity. Among them, fractions Dm 2 and Dt 1 showed significant antioxidant activity by DPPH radical antioxidative assay. Active fractions were purified further by column chromatography and resulted in identification of the antioxidant components alkyl ferulates from *D. moniliforme* and quercetin from *D. tosaense*.

KEYWORDS: ***Dendrobium*; radical scavenging activity; mass propagation; alkyl ferulates**

INTRODUCTION

Activated oxygen species such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen may cause various disease states such as carcinogenesis, drug-associated toxicity, inflammation, atherogenesis, and aging in aerobic organisms, as well as food deterioration (*1*−*4*), for which the naturally occurring antioxidants are known to be effective. However, many naturally occurring antioxidants have been limited in their practical usage due to their low effectiveness, even though they are considered to be active in eliminating reactive oxygen and controlling toxic effects (*5*). These economic, health, and environmental concerns have highlighted the need to develop new types of antioxidants. The potential value of antioxidants has prompted investigators to search for natural compounds with potent antioxidant activity but low cytotoxicity. Recent studies showed that a number of plant products including polyphenols, flavanoids, coumarins, curcuminoids, and terpenes and various plant extracts exerted an antioxidant action (*6*−*8*). In recent years, therefore, the search for natural antioxidants and other preparations of plant origin to achieve this objective has been intensified.

Dendrobium orchids are one of the leading cut and potted floricultural crops grown in the tropics. The genus *Dendrobium* (Orchidaceae) includes about 1600 species in the world (*9*), 15 of which are found in Taiwan (*10*). The stems of several *Dendrobium* species are used in traditional Chinese medicine

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as a yin tonic to nourish the stomach, promote the production of body fluid, and reduce fever (*11*). *Dendrobium* species are known to produce a variety of secondary metabolites such as phenanthrens (*12*−*15*), bibenzyls (*16*−*18*), fluorenones and sesquiterpenes (*19*−*21*), and alkaloids (*22*) and are responsible for their wide variety of medicinal properties. With a view to the medicinal value, the present study was undertaken in an effort to conserve the wild population of *Dendrobium moniliforme* using in vitro techniques. We have established an efficient protocol for micropropagation of this species. In the laboratory studies described herein, we assessed the antioxidant activities of the methanolic extract and the components isolated from *Dendrobium tosaense* and *D. moniliforme* through bioguided fractionation and column chromatography to search for plant-derived materials for potentially useful products as commercial antioxidants. To our knowledge, no previous investigations have been done on the radical scavenging effects of pure components obtained from these plants.

MATERIALS AND METHODS

Chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical and α-tocopherol were purchased from Sigma Chemical Co. All solvents and other chemicals used were of the highest analytical grade.

Plant Collection. The mature plants of the two species were collected from Taiwan: *D. moniliforme* was collected from Chi Lan and *D. tosaense* from Tien Hsiang in March 2000. A voucher specimen of the plants has been deposited at the China Medical College herbarium, Taichung, Taiwan (*Dendrobium tosaense* Makino CMC DT 0303; *Dendrobium moniliforme* SW CMC DM 0302).

Mass Propagation. Seeds from the artificially pollinated 14-week-old capsules of *D. tosaense* Makino and *D. moniliforme* were cultured in 22 × 120 mm glass test tubes each containing 10 mL of half-strength MS basal medium (*23*) with 3% sucrose and solidified with 0.9% Difco agar for 20 weeks. The germinated seedlings were subcultured in 500 mL flasks containing MS basal medium supplemented with 8% potato homogenate and 1.5% sucrose and solidified with 0.9% Difco agar. All of the cultures were incubated at 25 ± 1 °C under cool white fluorescent light at 38 μmol·m^{-2}·s^{-1} (Philips) with a 16-h photoperiod per day. The pH of all media was adjusted to 5.7 ± 0.1 with 1 N NaOH or HCl before autoclaving at 121 °C and 105 kPa for 15 min. After 20 weeks of culture, seed-derived plantlets were removed from flasks and washed thoroughly under tap water for 2−3 min to remove traces of agar-gelled medium sticking to them. These were then planted in plastic trays (diameter = 6 cm × height = 5.5 cm × 20 hole) containing moss and tree fern as a substrate. The plants were acclimatized in the greenhouse conditions with 10−80% relative humidity and 25/20 °C day/night temperature.

Extraction, Bioguided Fractionation, and Isolation. The plants grown for 6 months in the greenhouse were collected and freeze-dried in a lyophilizer (FTS System, New York). The dried plants (200 g) were finely ground with a mortar and pestle and extracted independently with methanol (300 mL × 3) under 20 min of sonication (Branson ultrasonic cleaner, Branson Cleaning Equipment Co., Shelton, CT) to ensure the complete extraction. These extracts were filtered through an Advantec no. 1 filter paper (Toyo Roshi Kaisha Ltd.), and the methanol was evaporated in vacuo to dryness. The dry residue was further extracted with methanol and water. The methanol solubles were dried over anhydrous sodium sulfate and concentrated to get the crude residue under reduced pressure. The crude extracts of *D. moniliforme* (18 g) and *D. tosaense* (22 g) were fractionated individually in an open column packed with silica gel (400−800 mesh). The column was stepwise eluted with 500 mL of each of four different concentrations of 10, 25, and 50% chloroform in methanol, respectively. The fractions obtained from *D. moniliforme* (Dm 1, Dm 2, and Dm 3) and *D. tosaense* (Dt 1, Dt 2, and Dt 3) were tested for their activity. Active fractions (Dt 1 and Dm 2) were purified further using mixtures of hexane and ethyl acetate and chloroform/methanol by silica gel column chromatography. Alkyl ferulates (**1**, 4.8 mg) from the active fraction of *D. moniliforme* and quercetin (**2**, 12 mg; *24*) from the active fraction of *D. tosaense* were isolated. Inactive fractions were also purified by column chromatography to identify chemical components. Purification of Dm 1 resulted in the isolation of β-sitosterol (20 mg; *25*) and *p*-methoxybenzaldehyde (5 mg), whereas chromatography of fraction Dm 3 gave β-sitosterol glucoside (5 mg; *25*). Chromatography of Dt 2 gave scoparone (3 mg; *26*). Purification of Dt 3 gave sucrose (150 mg) and β-sitosterol glucoside (4 mg; *25*). The structures of the known compounds were identified by comparison of NMR and mass spectral data with those reported in the literature.

Structure of alkyl ferulates: mixture of *n*-pentacosyl *trans*-ferulate and *n*-tetracosyl *trans*-ferulate (1:4); amorphous powder, mp 78−80°C; EIMS, *m*/*z* (%) (rel int) 545 $[M + H]^+$ (4) (*n*-pentacosyl *trans*-ferulate), 531 $[M + H]^+$ (21) (*n*-tetracosyl *trans*-ferulate), 502 (100), 194 (55), 177 (44), 57 (12), 43 (10); ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 6.6 Hz, CH_3), 1.24 (br s, $-CH_2$-*n*), 1.67 (2H, t, J = 6.6 Hz, $OCH_2\underline{CH_2}$), 3.92 (3H, s, OCH_3), 4.18 (2H, t, J = 6.6 Hz, $O\underline{CH_2}$), 5.87 (1H, br s, OH), 6.29 (1H, d, J = 16.0 Hz, H-2), 6.88 (1H, d, J = 7.8 Hz, H-5′), 7.03 (1H, d, J = 2.5 Hz, H-2′), 7.07 (1H, dd, J = 8.2, 2.0 Hz, H-6′), and 7.60 (1H, d, J = 15.7 Hz, H-3); ^{13}C NMR ($CDCl_3$) δ 14.1 (CH_3), 22.7−31.9 ($-CH_2$-*n*), 28.7 ($-OCH_2\underline{CH_2}-$), 55.9 (OCH_3), 64.6 ($-O\underline{CH_2}-$), 109.2 (C-2′), 114.6 (C-5′), 115.6 (C-2), 123.0 (C-6′), 127.0 (C-1′), 144.6 (C-3), 146.7 (C-4′), 147.8 (C-3′), and 167.4 (C-1). The proton and carbon NMR data were confirmed further by HMBC, HMQC, and ^{1}H−^{1}H COSY spectral data.

DPPH Radical Scavenging Spectrophotometric Assay. Scavenging activity on DPPH radicals of *Dendrobium* extracts, fractions, and pure components was measured according to the method reported by Blois (*27*) with minor modifications. Each sample stock solution was diluted to final concentrations of 400, 200, 100, 50, and 25 μg/mL, and 0.2 mL of methanol and 0.3 mL of various concentrations of the samples in methanol were mixed in a 10 mL test tube. To this was added 2.5 mL of 75 μM DPPH in methanol to achieve a final volume of 3 mL. The solution was kept at room temperature for 90 min, and the absorbance at 517 nm (A_{517}) was measured. α-Tocopherol was used as a reference compound. The DPPH scavenging effect was calculated as

$$\text{scavenging effect (\%)} = [(A_0 - (A - A_b)/A_0)] \times 100$$

where $A_0 = A_{517}$ of DPPH without sample, $A = A_{517}$ of sample and DPPH, and $A_b = A_{517}$ of sample without DPPH.

RESULTS AND DISCUSSION

In vitro propagation is a viable tool for the conservation and plant propagation of plant species of importance (*28*). Although various plant parts have been used as explants, immature seeds are widely used for the propagation of orchids. Immature seeds from the capsule have been reported to germinate normally under in vitro conditions (*29*). Also, it is more convenient to handle and surface sterilize capsules than seeds (*30*). Maximum seeds from the 14-week-old capsule germinated in 4 months on half-strength MS basal medium with 3% sucrose, solidified with 0.9% Difco agar. The germinated seedlings were subcultured on medium supplemented with 8% banana homogenate, 8% potato homogenate, and 8% coconut milk and cultured for 5 months. The plantlets were transferred on moss and tree fern and hardened under greenhouse conditions. Plants grown in the greenhouse for 6 months were harvested and used to study the DPPH activity.

The use of DPPH radical for screening antioxidants by spectrophotometric assay appears to be well suited for the detection of antioxidant activity in crude plant extracts or pure compounds isolated from plant material. DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. When DPPH encounters a proton-donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance at 517 nm reduced. On the basis of this principle, the antioxidative activity of a substance can be expressed as its

ability to scavenge the DPPH free radical. It was used in the present study for the bioguided isolation and identification of active principles from the methanolic extracts of *D. tosaense* Makino and *D. moniliforme* SW by UV spectrophotometric assay. It is generally accepted that the electron-donating ability of chemical substances results in their antioxidant activity toward lipid oxidation. The DPPH radical scavenging test is a short method for investigation of the hydrogen-donating potency (*31*). In vitro plant tissue cultures such as plant cell, callus, and adventitious root could be new sources of antioxidants (*32*). Rakotoarison et al. (*33*) reported the antioxidative activity of proanthocyanidin B2 in cell suspension cultures of *Crataegus monogyna*, and anthocyanins, catechins, and stilbenes isolated from *Vitis vinifera* cell cultures inhibited low-density lipoprotein (LDL) oxidation (*32*). Also, antioxidative activity of extracts and constituents in *Cornus capitata* (*34*) adventitious roots was studied. The plants belonging to the genus *Dendrobium* were chosen for evaluation of their antiradical capacity due to the presence of the secondary metabolites that exhibit some interesting pharmacological properties. Primarily, the influence of methanolic extracts obtained from three in vitro propagated species of *Dendrobium* on DPPH radical activity has been studied. The percentage of DPPH free radical scavenging effect of each methanolic extract at different concentrations (400, 200, 100, 50, and 25 μg/mL) was measured. All of the extracts scavenged DPPH radical significantly in a concentration-dependent manner. Scavenging activity increased from 30 to 95% with increasing concentrations from 0.025 to 0.4 mg/mL as shown in **Figure 1**. The crude extracts of *D. tosaense* and *D. moniliforme* were fractionated individually into three fractions each, Dm 1, Dm 2, and Dm 3 and Dt 1, Dt 2, and Dt 3, with 10, 25, and 50% chloroform in methanol, respectively, by silica gel column chromatography. The fractions obtained from *D. moniliforme* (Dm 1, Dm 2, Dm 3, and Dm 4) and *D. tosaense* (Dt 1, Dt 2, and Dt 3) were tested for their activity. Among them, fractions Dm 2 and Dt 1 exhibited better antioxidant activity. To clarify, the fractions were further purified by silica gel column chromatography and their DPPH scavenging effects were studied. It is evident that the remarkable antioxidative activity of Dm 3 and Dt 2 is due to the alkyl ferulates (**1**, 4.8 mg) and quercetin (**2**, 12 mg), respectively (**Figure 1**). The radical scavenging activity of alkyl ferulates was reported earlier by Kikuzaki et al. (*34*). They explained that the scavenging efficiency of these alkyl ferulates was lower than that of the corresponding ferulic acid. In the present study pure compounds obtained from active fractions were examined for their scavenging activity, and the results obtained were given in **Figure 1**. Inactive fractions were also purified by column chromatography to identify chemical components. Purification of Dm 1 resulted in the isolation of β-sitosterol (20 mg) and *p*-methoxybenzaldehyde (5 mg), and fraction Dm 3 gave β-sitosterol glucoside (5 mg). Chromatography of Dt 2 yielded scoparone (3 mg). Purification of Dt 3 gave sucrose (**7**, 150 mg) and β-sitosterol glucoside (4 mg). All of the structures were identified by NMR and mass spectral data.

The recent study on antioxidative substances in foods and medicinal plants is a comparatively new province. The digestion, absorption, biological activity, and metabolic pathway in each food and medicinal plant are still complicated because a number of substances are included in various proportions in one plant. Meanwhile, their safety has been established to certain extent for empirical and traditional use from ancient times. Therefore, the study of antioxidative substances in foods and medicinal plants will become important, and such antioxidative components might be applied for the treatment and prevention of human diseases. Bioguided fractionation and isolation of the active principles of these plants with radical scavenging action obtained from the present studies may give a rational explanation for the therapeutic use of the herbs of the genus *Dendrobium* in Chinese medicine.

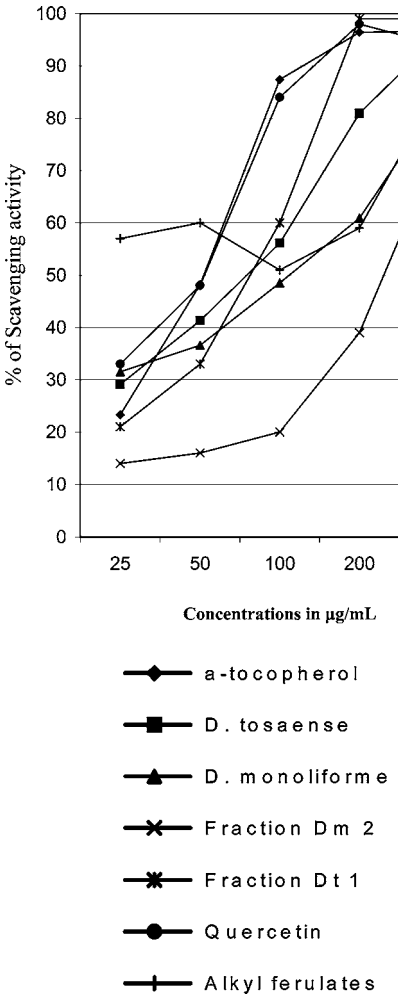

Figure 1. DPPH radical scavenging activity of methanolic extracts, active fractions, and pure compounds isolated from active fractions of *D. moniliforme* and *D. tosaense*, α-tocopherol, at 90 min of reaction. The reaction mixture consisted of DPPH radical (0.1 mM) and the extracts, fractions, and compounds listed at the right different concentrations (25–400 μg/mL).

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