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Full Length Research Paper

Antioxidant and tyrosinase inhibitor from *Leucaena leucocephala*

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The experimental design is divided into two parts: chemical analysis and bioactive assay. One antioxidant lupeol (4) and one inhibition of tyrosinase pheophorbide a methyl ester (7) were identified in *Leucaena leucocephala*. Both showed effective 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity compared with vitamin C, and mushroom tyrosinase compared with kojic acid. These results suggest that these constituents of *L. leucocephala* act as natural antioxidants and play a potential role in prevention of pigmentation.

Key words: *Leucaenana leucocephala*, lupeol, pheophorbide a methyl ester, antioxidant, mushroom tyrosinase.

INTRODUCTION

Designing safe and effective tyrosinase inhibitors for pharmacological preparations was important aims of many research works during the last decade. They have shown potential applications in preventing pigmentation disorders and other melanin-related health problems in human (Dooley, 1997). Research in this area includes both synthetic (Lee et al., 2009; Liu et al., 2009) and natural (Lo et al., 2009; Dorman et al., 2003) compounds acting as tyrosinase inhibitors as well as antioxidants (Willcox et al., 2004; Naik et al., 2004). Therefore, search of natural antioxidant and tyrosinase inhibitors from plant sources would have many industrial outcomes. The bioactivities of these compounds, including anti-tyrosinase and antioxidant were thus examined.

Leucaena leucocephala is a leguminous plant which is full of vitality and fertility. When the number of the *L. leucocephala* ethnic reaches a specific limit, this plant would secrete Mimosine and thus inhibit the growth of other plants (Chou and Kuo, 1986). It was classified as one of the 100 invasive species which brings serious damage to the biological environment by International Union for Conservation of Nature and Natural Resources (IUCN). Additionally, if we could gain its additive value such as the dominant species, we could also develop and use this value to inhibit the expansion and then reach the purpose of ecological balance. Previous studies showed that gallocatechin, epigallocatechin, catechin and epicatechin, extracted from the roots of *L. leucocephala* was found to exhibit the nitrification inhibition bioassay against the bacterium *Nitrosomon aseuropaea* (Erickson et al., 2000). Previously, we isolated 21 compounds, including one polyprenol: ficaprenol-11; two terpenoids: squaleneand lupeol; four steroids: β-sitostenone,5α,8α-epidioxy-(24ξ)-ergosta-6,22-dien-3β-ol,β-sitosterol, β-sitostenone and stigmastenone; one glyceride: 1, 3-dipalmitoyl-2-oleoylglycerol; one alkanoid: linoleic acid; four benzenoids: *trans*-coumaric acid,*cis*-coumaric acid, methylparabene and isovanillic acid and four chlorophylls: pheophytin-a,pheophorbide a methyl ester, methyl-13^2-hydroxy-(13^2-S)-pheophorbide-b,13^2- ydroxy-(13^2-S)-pheophytin-a and aristophyll-C from this species (Chen and Wang, 2010).

In this study, lupeol (4) and pheophorbide a methyl ester (7) of pure compounds isolated from *L. leucocephala* were screened for their antioxidative activities. Previous studies have shown lupeol as effective in the inhibition of human cancer cells. Hence,

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we evaluated their (4 and 7) protective effects of antioxidation which has been reported to be linked with human disease, including cardiopathy and cancer.

MATERIALS AND METHODS

The specimen of *L. leucocephala* was collected from Pingtung County, Taiwan in May, 2009. A voucher specimen was characterized by Dr. Jin-Cherng Huang of the Department of Forest Products Science and Furniture Engineering, NationalChiayi University, Chiayi, Taiwan and deposited in the School of Medical and Health Sciences, Fooyin University, Kaohsiung County, Taiwan.

Vitamin C, dimethyl sulfoxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-tyrosine, mushroom tyrosinase, were purchased from Sigma Chemical (St. Louis, MO). All buffers and other reagents were of the highest purity commercially available.

Purification of active ingredients from the *Leucaena leucocephala*

The air-dried green beans of *L. leucocephala* (4.0 kg) were extracted with MeOH (80 L × 6) at room temperature and the MeOH extract (152.5 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed over silica gel (800 g, 70~230 mesh) using *n*-hexane/acetone as eluent to produce 4 fractions. Part of fraction 1 (5.04 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (50:1), enriched with acetone to furnish 7 fractions (1-1~1-7). Fraction 1-1 (1.49 g) was re-subjected to Si gel chromatography, eluting with *n*-hexane/acetone (80:1) to obtain linoleic acid (1) and 1, 3-dipalmitoyl-2-oleoylglycerol (2) mixture (49 mg, 0.0321%). Part of fraction 4 (6.36 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (40:1) to obtain pheophorbide a methyl ester (7) (18 mg, 0.0118%). Part of fraction 3 (6.77 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (8:1). Part of fraction 4 (7.00 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (6:1), then enriched with acetone to furnish 7 fractions (4-1~4-6). Fractions 4 to 5 (0.62 g) was further purified by another silica gel column using *n*-hexane/acetone to obtain methyl-13^2-hydroxy-(13^2-*S*)-pheophorbide-b (8) (11 mg, 0.0072%).

The air-dried leaves of *L. leucocephala* (4.0 kg) were extracted with MeOH (80 L × 6) at room temperature and the MeOH extract (107.5 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed over silica gel using *n*-hexane/acetone as eluent to produce 5 fractions. Part of fraction 1 (5.94 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (80:1), enriched with acetone to furnish 6 fractions (1-1~1-6). Fraction 1-1 (1.49 g) was re-subjected to Si gel chromatography, eluting with *n*-hexane/acetone (80:1) to obtain *β*-sitostenone (3) (7 mg, 0.0065%). Part of fraction 2 (6.51 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (70:1), then enriched with acetone to furnish 6 fractions (2-1~2-6). Fraction 2-4 (2.50 g) was re-subjected to Si gel chromatography, eluting with *n*-hexane/Acetone (50:1) to obtain lupeol (4) (51 mg, 0.0474%). Part of fraction 4 (6.33 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (10:1), then enriched with acetone to furnish 7 fractions (4-1~4-7). Fraction 4-3 (0.44 g) was further purified by another silica gel column using *n*-hexane/acetone to obtain isovanillic acid (5) (7 mg, 0.0065%) and methylparabene (6) (15 mg, 0.0140%). Part of fraction 5 (6.22 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (8:1) to obtain pyropheophorbide (9) (15 mg, 0.0140%).

Determination of DPPH· radical scavenging capacity

The antioxidant activity was measured in terms of radical scavenging ability using the DPPH method, as modified by Maric et al. (2007). Briefly, the various concentrations of the sample were added to 0.2 ml of stable DPPH (60 μM) solution. When DPPH· reacts with an antioxidant compound donating hydrogen, it is reduced, resulting in a decrease in absorbance at 490 nm. The absorbance was recorded at 5 min intervals up to 30 min using a UV–vis spectrophotometer. Vitamin C was used as a positive control. The experiment was performed in triplicate and results were reported as the average of three trials by the IC_{50} value, which was evaluated by Kaleida Graph 4.0 statistical software.

Mushroom tyrosinase activity

Tyrosinase activity inhibition was determined spectrophotometrically according to the method described previously (Likhitwitayawuid and Sritularak, 2001; Chen et al., 2010), with minor modifications. Assays were conducted in a 96-well micro-plate, and ELSA plate reader was used to determine the absorbance at 490 nm (Molecular Devices). Kojic acid was used as a positive control on the tyrosinase inhibitory assay. The testing substance was dissolved in aqueous DMSO, and incubated with L-tyrosine (2.5 mg/ml) in 50 mMphosphate buffer (pH 6.8). All samples weredissolved in DMSO which did not affect tyrosinase activity when DMSO was less than 1% of the total volume. Then, 25 U/ml of mushroom tyrosinase in the same buffer was added, and the mixture was incubated at 37°C for 30 min.

RESULTS AND DISCUSSION

The chemical constituents in the plants of *L. leucocephala*were were separated with column chromatography. Investigation on the MeOHextract of the plants led to the isolation of 9 compounds; one alkanoid: linoleic acid (1) (Tsai et al., 2001); one glyceride: 1, 3-dipalmitoyl-2-oleoylglycerol (2); (Arishima et al., 1996); one steroid: *β*-sitostenone (3) (Gaspar and Neves, 1993); one terpenoid: lupeol (4) (Ishii et al., 1982); two benzenoids: isovanillic acid (5) (Bulgakov et al., 1996) and methylparabene (6) (Wang and Lee, 1997); and three chlorophylls: pheophorbide a methyl ester (7) (Nakatani et al., 1981); methyl-13^2-hydroxy-(13^2-*S*)-pheophorbide-b (8) (Buchanane et al., 1996) and pyropheophorbide (9) (Yahara et al., 1991) (Figure 1).

The IC_{50} value of DPPH· for lupeol (4) was 102.3 ± 4.1 μM. In 2009, there was a research that reported that *L. leucocephala* showed positive antioxidant activity (Shieh et al., 2009), which was consistence with our research results. We also investigated the mushroom tyrosinase of these two compounds (Table 1) and the activity was about 704.6 ± 3.4 μM for mushroom tyrosinase. Thus, lupeol (4) and pheophorbide a methyl ester (7) could be used as natural antioxidant to prevent melanin deposition and other diseases.

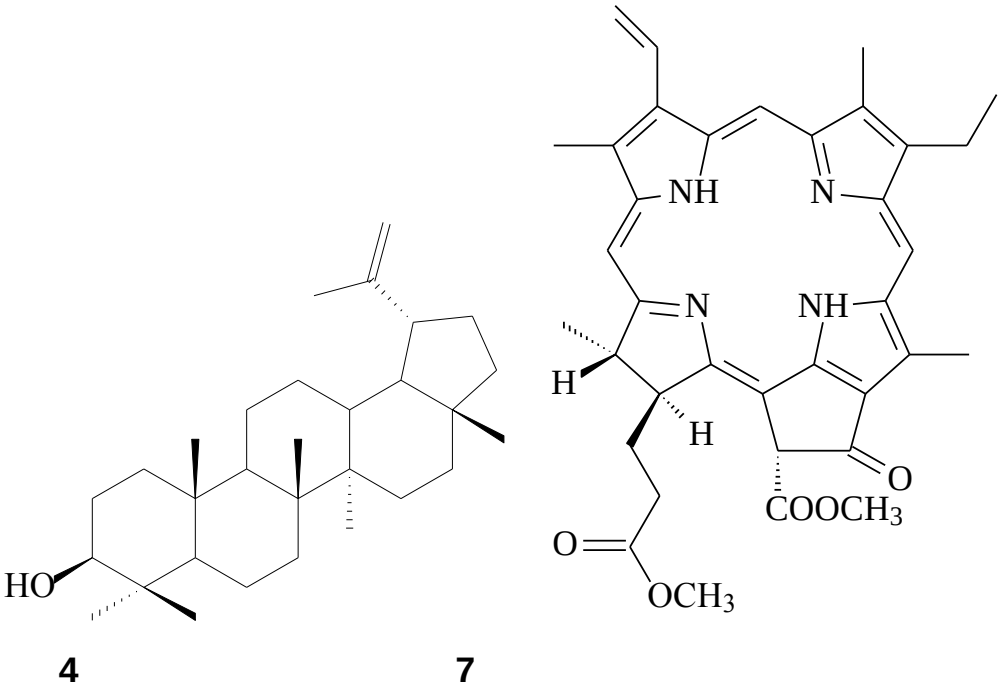
Conclusion

In our research studies, free radical scavenging

Table 1. Antioxidant and tyrosinase inhibition properties of lupeol (**4**) and pheophorbide a methyl ester (**7**).

Sample	DPPH· scavenging IC_{50} (µM)	Mushroom tyrosinaseIC_{50} (µM)
4	102.3 ± 4.1	NS
7	NS	704.6 ± 3.4
Vitamin C	20.7	-
Kojic acid	-	65.6

Data are expressed as a mean value of three independent experiments. Vitamin C was used as a positive control in DPPH assay, and kojic acid in mushroom tyrosinase at 100 µM. (-), no test.



4 **7**

Figure 1. Chemical structures of lupeol (**4**) and pheophorbide a methyl ester (**7**).

capacities and antioxidant activities of lupeol (4) and pheophorbide were assayed effectively by two *in vitro* antioxidant evaluation systems, DPPH / ABTS radical scavenging activity and mushroom tyrosinase activity. The results presented clearly show that the two compounds from *L. leucocephala* efficiently revealed antioxidant and mushroom tyrosinase activities. These two compounds could be used as easily accessible sources of natural antioxidants and are able to act as cosmetics and food supplements against oxidative deterioration to prevent carcinogenesis or other diseases.

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