Separation of the Antioxidant Compound Quercitrin from Lindera obtusiloba Blume and Its Antimelanogenic Effect on B16F10 Melanoma Cells

Chung-Oui Hong,¹ Hyun Ah Lee,¹,² Chae Hong Rhee,¹,³ Sc-Young Chung,⁴ and Kwang-Won Lee¹,†

¹Division of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, South Korea
²Food Professional Engineering R&D Center, Seoul Dairy Co., Ltd., Ansan-si, Gyeonggi 425-839, South Korea
³Department of Quality Assurance, Sangha Plant, Mael Dairies Co., Ltd., Gochang-gun, Jeonbuk 585-912, South Korea
⁴Department of Hygienic Chemistry, College of Pharmacy, Kyung Hee University, Seoul 130-701, South Korea

Received July 18, 2012; Accepted October 9, 2012; Online Publication, January 7, 2013
[doi:10.1271/bbb.120562]

Considering the growing evidence of the presence of antioxidant compounds in plant extracts, the objectives of this study were to identify antioxidant compounds in Lindera obtusiloba Blume (Lauraceae) and to evaluate their antimelanogenic activities on B16F10 melanoma cells. Organic solvent fractions were separated from L. obtusiloba extracts (LOE). The ethyl acetate fraction (LOE-E) was significantly active against oxidative damage induced by tert-butyl hydroperoxide in primary rat hepatocytes. Two single purified compounds, quercitrin (quercetin-3-O-α-L-rhamnopyranoside) and afzelin (kaempferol-3-O-α-L-rhamnoside), were identified by HPLC and NMR. These compounds were evaluated for antioxidant activities by 1,1-diphenyl 2-picrylhydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay, and for their antimelanogenic activities by tyrosinase inhibitory assay melanin formation inhibition assay and Western blot analysis for the signaling pathway. The significant effects of quercitrin on antioxidant and antimelanogenic activities, and signal modulation of ERK and MITF in B16F10 melanoma cells were observed. This is the first report to identify quercitrin in L. obtusiloba and its whitening effect.

Key words: Lindera obtusiloba; antioxidant activity; antimelanogenic activity; quercetin-3-O-α-L-rhamnopyranoside (quercitrin); kaempferol-3-O-α-L-rhamnoside (afzelin)

Recently, much attention has been directed towards antioxidants, as they have been implicated in the reduction of oxidative damage which is a principle factor in many diseases.¹ Natural antioxidants occurring in medicinal plants may prove useful in the development of agents for the prevention of oxidative damage.² Their pharmacological and therapeutic properties have been attributed to various chemical constituents isolated from crude extracts.³ Constituents with antioxidant activity can be found at high concentrations in plants, and are responsible for their preventive effects in various diseases related to oxidative stress.³ Hence the potential antioxidant activities of purified plant compounds have attracted the attention of researchers who intend to determine whether these compounds are effective antioxidant agents.

Lindera obtusiloba Blume, a flowering plant species of the Lauraceae family and a ubiquitous tree distributed mainly in Southeast Asia, is used in traditional medicine for the treatment of inflammation and the improvement of blood circulation.⁴ Many researchers have reported strong antioxidant properties of L. obtusiloba extracts (LOE).⁵-⁷ Despite these antioxidant properties, little is known about purified antioxidant compounds. Furthermore, even though antioxidant activity is pathophysiological related to anti-aging processes in the skin⁵ and many studies have supported the whitening effects of phenolic compounds,⁶ the antimelanogenic compounds in LOE have not been investigated.

The objectives of the present study were to separate antioxidative compounds, and to determine their antimelanogenic effects by investigating the inhibitory activity of tyrosinase and MITF protein molecules.

Materials and Methods

Materials. Fetal bovine serum (FBS) and penicillin-streptomycin (100 U/mL) medium were purchased from Gibco Life Technologies (Paisley, UK). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, tert-butyl hydroperoxide (t-BHP), 1,1-diphenyl 2-picrylhydrazyl (DPPH), mushroom tyrosinase, and synthetic melanin were from Sigma (St. Louis, MO). The following antibodies were used: ERK1 (C-16) and α-tubulin (B-7) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell signaling Technology, Beverly, MA); MITF antibody (Thermo Fisher Scientific, Fremont, CA); tyrosinase rabbit monoclonal antibody (Epitomics, Burlingame, CA); and horse-radish peroxidase-conjugated anti-rabbit antibody and anti-mouse antibody (Sigma). All other chemicals used were of the highest grade available.

¹ To whom correspondence should be addressed. Tel: +82-2-3290-3027; Fax: +82-2-3290-4984; E-mail: kwangwon@korea.ac.kr

Abbreviations: DPPH, 1,1-diphenyl 2-picrylhydrazyl; FBS, fetal bovine serum; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; afzelin, kaempferol-3-O-α-L-rhamnopyranoside; LOE, Lindera obtusiloba extract; LOE-E, ethyl acetate fraction of LOE; MDA, malondialdehyde; MTT, microculture tetrazolium technique; PBS, phosphate buffered saline; QE, quercetin equivalents; quercetin, quercetin-3-O-α-L-rhamnopyranoside; t-BHP, tert-butyl hydroperoxide; TF, total flavonoid; TP, total polyphenol.
Plant material and sample preparation. Leaves of Lindera obtusiloba were collected from Cheonan, Chungcheongnam Province, Korea, and were identified by Dr. Kwon-Woo Park (College of Life Science, Korea University). A voucher specimen has been deposited in the herbarium of the College of Life Sciences and Biotechnology of Korea University, under register no. KUST-2006. All the plant leaves were cleaned, and small cut pieces were freeze-dried. The freeze-dried samples were ground into a fine powder with a dry grinder. The ground samples were sieved to get uniformly sized particles.

Extraction and isolation. The extraction and isolation procedure for the purified single compounds are described in Fig. 1. The dried materials were extracted with methanol (MeOH; 511 g/4 L), followed by refluxing for 3 h and cooling. The undissolved materials were removed by passing them though Whatman no. 41 filter paper (Clifton, NJ, USA). The solvent was removed under reduced pressure, and the crude methanol extract (LOE; 103 g) was then subjected to serial extraction with four organic solvents of increasing polarity: n-hexane (30 g), chloroform (10 g), ethyl acetate (17 g), and water (28 g). The ethyl acetate fraction (LOE-E) was separated by silica gel column chromatography (40–63 μm; elution at 5 mL/min and collection of 3 L/fraction) eluted with a stepwise gradient solvent mixture: EtOAc–MeOH (from EtOAc 100% to MeOH 50%) and EtOAc–MeOH (9:1). Two fractions were collected.

Spectral analysis. Analytical high-performance liquid chromatography (HPLC) profiles were obtained using a Varian ProStar (Model 210) HPLC apparatus (Walnut Creek, CA) and a Waters Spherisorb ODS-2 column (particle size 5 μm, 4.6 mm × 250 mm, Milford, MA). The mobile phase was operated as a linear gradient that consisted of MeOH (20% to 100%). The flow rate was 1.0 mL/min, and UV detection was done at 340 nm. Nuclear magnetic resonance spectroscopy (NMR) spectra were obtained using a Varian NMR system 500 MHz (Varian, Palo Alto) with tetramethylsilane (TMS) as internal standard, and chemical shifts were expressed as δ values.

Primary cell and cell-line culture. Male Sprague-Dawley rats (body weight 200 ± 10 g) were purchased from Suntao Bio Korea (Gaeyonggi, Korea), and rat hepatocytes were prepared by collagenase perfusion in our laboratory, as previously described.10 The cells were seeded into 24-well plates at 1.8 × 10^4 cells/well for the microculture tetrazolium technique (MTT) assay, or in 6-well plates at 1.5 × 10^5 cells/well for malondialdehyde (MDA) measurement. In addition, a mouse melanoma cell line, B16F10, was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (HyClone, Logan, UT) supplemented with 10% (v/v) FBS and 100 U/mL of penicillin-streptomycin at 37°C in an incubator under a humidified atmosphere of 5% CO₂. The B16F10 melanoma cells were treated with quercitrin for measurement of antimelanogenic activity.

Antioxidant activity. Cell viability. Cell viability was determined by MTT as previously described.10 Total polyphenol (TP) and total flavonoid (TF) contents. The total polyphenol content was measured using Folin-Ciocalteu reagent, and the total flavonoid content was determined as previously described in our laboratory.10 DPPH free-radical scavenging and ferric reducing/antioxidant power (FRAP) assay. Scavenging activity was estimated using DPPH as a free-radical model and the reducing power method adapted from Lee et al.10 The FeSO₄·7H₂O concentration was used for calibration of the FRAP assay.

Biochemical assays of lipid peroxidation. Thiobarbituric acid reacting substances (TBARS) are secondary products of lipid peroxidation, the major compound being malondialdehyde (MDA). Determination of the MDA content was done by the thiobarbituric acid (TBA) assay method using freshly diluted 1,1,3,3-tetramethoxypropane as standard.10 This method has been widely adopted as a sensitive assay for lipid peroxidation in animal tissues.11

Antimelanogenic activity. Tyrosinase inhibitory activity. A tyrosinase inhibition assay was performed as reported previously, with a slight modification.12 Twenty μL of an aqueous solution of mushroom tyrosinase (1,000 U/mL, 30 mg) was added to a 100 μL solution of tyrosinase substrate (40 μM L-DOPA, 30 μM L-DOPA). The B16F10 melanoma cells were treated with quercitrin for measurement of antimelanogenic activity.

Figure 1. Fractionation Scheme for the Isolation of Active Antioxidant Compounds from Lindera obtusiloba Blume Leave Extract (LOE).

Values are presented as means ± standard deviations for three replicate experiments. *p < 0.01 compared with cells treated with tert-BHP alone. Cell viability was determined by MTT assay. *Cells were treated with 500 μg/mL of each fractions of LOE and 1.5 mM tert-butyl hydroperoxide (t-BHP) for 30 min. #Cells were treated with 100 μg/mL of each fraction of LOE and 1.5 mM tert-BHP for 30 min.
Results and Discussion

In the present study, we separated the antioxidant compound quercetin from Lindera obtusiloba Blume, and measured its antimelanogenic effect. L. obtusiloba leaves were extracted with crude methanol (LOE), and then the effect of the LOE on the cytotoxicity inflicted by t-BHP was examined. The results obtained for co-incubation of LOE with t-BHP indicated significant reduction in of t-BHP induced cell death (Fig. 2A). The cytoprotective effect occurred in a concentration-dependent manner. t-BHP induces radical stress, and is used as a good experimental tool to mimic the conditions of oxidative stress.13)

Further fractionization of the LOE led to the identification of the most hepatoprotective LOE fraction against t-BHP-induced cytotoxicity (Fig. 1). The cell viabilities of the n-hexane (LOE-H), chloroform (LOE-C), ethyl acetate (LOE-E), n-butanol (LOE-B), and water (LOE-W) fractions along with 1.5 mM t-BHP (co-incubation) were 64.4 ± 1.4%, 66.9 ± 1.5%, 94.4 ± 4.1%, 74.6 ± 2.5%, and 48.3 ± 0.8% respectively. The LOE-E fraction was significantly active against t-BHP induced cell death, and hence was subjected to further investigation for its antioxidant activities. Several analytical assays were proposed to determine the total antioxidant capacity of the LOE-E fraction (Table 1).

Polyphenolic compounds are known to have antioxidant activity, probably due to their redox properties.14) The LOE-E fraction contained comparable levels of phenolic compounds (497.3 ± 12.2 μg GAE/mg DM) and flavonoid compounds (441.0 ± 8.3 μg QE/mg DM). In fact, a strong positive relationship between total phenolic content and antioxidant activity, which appears to hold for many medicinal plants, has been reported.15,16) Abu Bakar et al.17) also reported that phenolic and flavonoid constituents are the phytochemicals responsible for free-radical scavenging activity, and the antioxidant properties of phenolic compounds are contributed by the reactive phenol moiety (the hydroxyl group on aromatic rings) added to a 96-well microplate (Nunc, Roskilde, Denmark), and then mixed with 150 μL of an assay mixture containing 1 mM L-DOPA solution, 50 mM sodium phosphate buffer (pH 6.5), and various concentrations of the samples. After 10 min of incubation at 37 °C, the optical density was recorded at 475 nm using a spectrophotometer (Biotek, Winookski, VT). Inhibitory activity was calculated according to the following equation: % inhibition rate = (A - B)/(C - D) × 100/(A - B), where A represents the absorption value without the test sample, B represents the absorption value without the test sample or enzyme, C represents the absorption value with the test sample, and D represents the absorption value with the test sample but without the enzyme. Kojic acid was used as positive control.

Tyrosinase inhibitory activity in B16F10 melanoma cells. B16F10 cells were plated at a density of 1 × 10^5 cells/mL in 6-well plates and incubated for 24 h in the medium prior to being treated with the samples. After 3 d, the cells were washed twice with cold phosphate buffered saline (PBS), and lysed in 0.1% sodium phosphate buffer (pH 6.5) containing 3% Triton X-100 at 4 °C for 20 min. The supernatants were used in the tyrosinase inhibition assay.

Melanin synthesis inhibition assay in B16F10 melanoma cells. The melanin contents of the cells after sample treatment were determined as follows: After removal of the medium and washing with PBS, the cell pellet was dissolved in 0.1 N NaOH. After heating of the cells at 100 °C for 30 min, the melanin content was estimated at 475 nm. It was determined by calculation from a synthetic melanin standard curve.15)

Effect on the mechanism of melanogenesis inhibition. The mechanism of melanin synthesis was measured in the presence of PD 98059. After 1 h, the cells were treated with various concentrations of LOE along with 1.5 mM t-BHP (co-incubation) for 30 min at 37 °C. MTT assays were carried out to determine cell viability. B, Malondialdehyde (MDA) formation, a measure of lipid peroxidation, was examined by TBARS assay measurements. The results were expressed as mean ± SD (n = 3). *p < 0.01, **p < 0.05, as compared to the cells treated with t-BHP alone.
LH-20 eluted with MeOH–H₂O. Hence this fraction was further fractionated on Sephadex G-25, and it indicated positive reactions to the C-3 position. Based on the spectral data and a comparison with the literature, the chemical structures of compound 1 and 2 were assigned on the basis of 1D and 2D NMR experiments. In the 1H and 13C NMR spectra, it was evident that the aglycone in the molecule was quercetin and that rhamnose was linked to the C-3 position. Based on the spectral data and a comparison with the values, compound 1 was identified as quercetin-3-O-α-L-rhamnopyranoside (quercetin) (Fig. 1). Compound 2 was isolated as a yellow amorphous powder that indicated a dark green color after the FeCl₃ reaction and a pale red color on the Mg–HCl test. It also indicated UV absorption bands (342 and 264 nm) characteristic of flavonol derivatives in the UV/MeOH spectrum. In the 1H and 13C NMR spectra, it was expected that the aglycone in the molecule was kaempferol, and signals of rhamnose were detected. Based on the spectral data and a comparison with the values in compound 2, it was identified as kaempferol-3-O-α-L-rhamnopyranoside (afzelin) (Fig. 1). Quercitin and afzelin share the aglycon quercetin, naturally the most abundant flavonoid, is found in a variety of fruits and vegetables. Table 2 indicates the antioxidant properties of quercitin and afzelin as compared to those of ascorbic acid. The DPPH*EC₅₀ values of quercitin and afzelin were 1.8-fold greater and 2.3-fold less than ascorbic acid respectively. In addition, the FRAP values of quercitin and afzelin were 1.6-fold higher and 2.5-fold lower than ascorbic acid. Quercitin indicated higher antioxidant activities than afzelin. This result is in agreement with Braca et al., who reported that quercitin exhibited stronger DPPH radical scavenging activity than afzelin in Licania licaniaeflora extract. Liu et al. also reported DPPH radical scavenging activity in the order quercitin > ascorbic acid > afzelin, and suggested that the antioxidant activities are related to their structures. Research has shown that quercitin possesses many structural components that contribute to its antioxidant properties, and is a safe antioxidant nutrient. Uppugundla et al., also reported antioxidant activity of quercitin from Panicum virgatum water extracts.

Reactive oxygen species (ROS) are generated by oxidative stress and are thought to play significant roles in the regulation of melanocyte proliferation and melanogenesis, while ROS scavengers and antioxidants...
inhibit hyperpigmentation and melanogenesis.\textsuperscript{27,28} Thus, to determine whether quercitrin and afzelin have antimelanogenic activity associated with antioxidant effects, we evaluated its action as to antimelanogenic activity. With regard to observation of the antimelanogenic effects of quercitrin and afzelin, Tanaka \textit{et al.}\textsuperscript{29} reported the suppressive actions of both compounds against melanin formation in B16F10 melanoma cells. Hence the antimelanogenic activities of the two compounds were observed in the present study. To confirm the cytotoxicity of quercitrin, MTT assay was performed. Compared to the control group without sample, cell viability was 98.1\%, 94.9\%, and 73.2\% when quercitrin was treated at 100\,$\mu\text{M}$, 150\,$\mu\text{M}$, and 300\,$\mu\text{M}$ respectively. Hence we selected concentrations of quercitrin of 100\,$\mu\text{M}$ and 150\,$\mu\text{M}$ to evaluate the antimelanogenic effect. Whereas the treatment with the same concentration of afzelin as quercitrin increased melanin synthesis in the B16F10 melanoma cells to 3.9-fold of control (data not shown). Quercitrin had a suppressive action ($p < 0.05$) against tyrosinase activity in a concentration-dependent manner, indicating that these two flavonol glycosides have opposite effects in terms of their antimelanogenic activities, but its \textit{in vitro} inhibition activity of the tyrosinase activity was much less than that of kojic acid, a well-known tyrosinase inhibitor (Fig. 3A).

On the other hand, in the B16F10 melanoma cells, which are mouse melanoma cells producing melanin, quercitrin indicated inhibitory effects on tyrosinase activity and melanin synthesis in a concentration-dependent manner (Fig. 3B and C), and the inhibitory activities of quercitrin were comparable to those of the same concentration of kojic acid. In our preliminary data, the inhibitory effect of 100\,$\mu\text{g/mL}$ of LOE on melanin synthesis in B16F10 melanoma cells decreased significantly, by 0.8-fold, as compared to control, indicating that a major compound of \textit{L. obtusiloba} in inhibitory melanin synthesis is quercitrin.

In support of the results according to which quercitrin reduced melanin synthesis in cultured B16F10 melanoma cells, we measured phospho-ERK (P-ERK), microphthalmia transcription factor (MITF), and tyrosinase protein levels by Western blot analysis to gain insight on the molecular level into the inhibition of melanogenesis by quercitrin. As shown in Fig. 4A, the P-ERK levels were remarkably induced by quercitrin after 24\,h of treatment, and quercitrin suppressed the amounts of MITF and tyrosinase proteins after 72\,h of treatment. Our results indicate that the melanin content was
significantly reduced even though tyrosinase activity slightly decreased, at no significance. This indicates that the melanin level was highly susceptible to tyrosinase activity in the B16F10 melanoma cells. Moreover, a downregulated protein level in tyrosinase was found by Western blot analysis (Fig. 4A). This was also thought to have caused the decrease in the melanin level.

It has been found that mitogen-activated protein (MAP) kinases are activated during cAMP-induced melanogenesis, and that PD98059 inhibits the activation of MAP kinase kinase (MEK), inhibiting both the phosphorylation and activation of MAP kinases ERK1 and ERK2 in B16 melanoma cells.30 In this study, we confirmed that inhibition of the MAP kinase pathway by PD98058 eliminated the effect of quercitrin on melanin content, reflecting stimulation of melanogenesis (Fig. 4B). As shown in Fig. 4C, quercitrin and kojic acid increased the expression of P-ERK, and hence MITF and tyrosinase levels decreased, but inhibition of the MAP kinase pathway was sufficient to increase MITF and tyrosinase expression. In sum, activation of the MAP kinase pathway by quercitrin in the B16F10 melanoma cells was a key event in melanogenesis. This result is in accordance with the study30 of indicating that activation of the MAP kinase pathway is not required for the induction of melanogenesis. Furthermore, our data correlate well with previous reports that induction of melanogenesis in B16 melanoma cells is characterized by stimulation of MITF and tyrosinase activity and gene expression.31,32

Our study effected the separation and identified the biological activity of quercitrin from L. obtusiloba Blume leaves. To our knowledge, this is the first report on the presence of quercitrin in L. obtusiloba leaves and of the inhibitory effect of melanogenesis. Also, quercitrin possessed potent antioxidant activity. The study further confirms the relevance of L. obtusiloba, containing quercitrin, as a source of effective antioxidants and antimelanogenic activity in developing skin-whitening cosmetic agents.
Acknowledgments

This research was supported by grant nos. 109140-03-SB010 and 111137-03-1-HD120 from the High Value-Added Food Technology Development Program of the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (iPET), and the Ministry for Food, Agriculture, Forestry, and Fisheries of the Republic of Korea. We thank the Korea University-CJ Food Safety Hall and the Institute of Food and Biomedicine Safety for the use of their equipment and facilities.

References