

Inhibitory effect of burdock leaves on elastase and tyrosinase activity

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Abstract. Burdock (*Arctium lappa* L.) leaves generate a considerable amount of waste following burdock root harvest in Taiwan. To increase the use of burdock leaves, the present study investigated the optimal methods for producing burdock leaf extract (BLE) with high antioxidant polyphenolic content, including drying methods and solvent extraction concentration. In addition, the elastase and tyrosinase inhibitory activity of BLE was examined. Burdock leaves were dried by four methods: Shadow drying, oven drying, sun drying and freeze-drying. The extract solution was then subjected to total polyphenol content analysis and the method that produced BLE with the highest amount of total antioxidant components was taken forward for further analysis. The 1,1-diphenyl-2-picrylhydrazyl scavenging, antielastase and antityrosinase activity of the BLE were measured to enable the evaluation of the antioxidant and skin aging-associated enzyme inhibitory activities of BLE. The results indicated

that the total polyphenolic content following extraction with ethanol (EtOH) was highest using the freeze-drying method, followed by the oven drying, shadow drying and sun drying methods. BLE yielded a higher polyphenol content and stronger antioxidant activity as the ratio of the aqueous content of the extraction solvent used increased. BLE possesses marked tyrosinase and elastase inhibitory activities, with its antielastase activity notably stronger compared with its antityrosinase activity. These results indicate that the concentration of the extraction solvent was associated with the antioxidant and skin aging-associated enzyme inhibitory activity of BLE. The reactive oxygen species scavenging theory of skin aging may explain the tyrosinase and elastase inhibitory activity of BLE. In conclusion, the optimal method for obtaining BLE with a high antioxidant polyphenolic content was freeze-drying followed by 30-50% EtOH extraction. In addition, the antielastase and antityrosinase activities of the BLE produced may be aid in the development of skincare products with antiwrinkle and skin-evening properties.

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Abbreviations: BLE, burdock leaf extract; EtOH, ethanol; ROS, reactive oxygen species; CHA, chlorogenic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; SANA, N-Suc-(Ala)3-nitroanilide

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Introduction

The skin is the largest organ of the human body and has essential functions, including protecting internal organs and regulating body temperature. Collagen and elastin, which are naturally occurring structural proteins in the dermis of the skin, maintain the plumpness, flexibility and elasticity of the skin. Skin aging presents as wrinkling, roughness, dryness, decreased elasticity and uneven pigmentation (1,2). In addition to intrinsic aging, extrinsic aging caused by exposure of the skin to solar radiation is an important concern. Reactive oxygen species (ROS) caused by ultraviolet (UV) radiation are clinically associated with specific markers of photoaging, including wrinkling, and increased elastin and collagen damage (2,3). Elastase digests elastin (4,5). Pigmentary changes due to the accumulation of melanin are another major characteristic of photoaging. Tyrosinase is a key enzyme that catalyzes melanin biosynthesis in melanocytes (4,6). Previous studies have identified a significant association between the activities of topical treatments, including anti-aging, skin

evening, and anti-wrinkling, and decreased elastase and tyrosinase activity (4,5,7).

Burdock (*Arctium lappa* L.), a biennial plant belonging to the *Compositae* family, has been cultivated in Eastern Asian countries, particularly in Taiwan, China and Japan for use in cooking and in folk medicine as a diuretic, antipyretic and blood purifying agent (8-10). Pharmacologically, burdock has been identified to possess hepatoprotective (11,12), desmutagenic (13), antibacterial (14,15), gastroprotective (16,17), anti-hypoglycemic (18,19), anti-hypolipidemic (20), anti-inflammatory (21), antifatigue (22), antioxidant (10,21,23), body weight management (24) and aphrodisiac (25) activities. Therefore, burdock-containing health drinks and foods are widely consumed in Taiwan. However, burdock leaves are discarded following burdock root harvest, contributing to environmental waste.

Studies investigating the biological activities and application of burdock leaves are warranted. Although burdock leaves have been reported to possess antioxidant and antiproliferative activities (26-28), little is known about their inhibitory effect on skin aging-associated enzymes. Therefore, the present study investigated the potential inhibitory effect of burdock leaves on elastase and tyrosinase activity. In addition, to increase the use of burdock leaves the present study investigated processes for optimizing their polyphenolic content.

Materials and methods

Materials. Five kilograms Taiwan Good Agriculture Practice (TGAP) certified burdock leaves were obtained from Kang-Li burdock farm in the Gueilai region of Pingtung in Taiwan. Gallic acid, chlorogenic acid (CHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-3,4-dihydroxyphenylalanine (L-DOPA), N-Suc-(Ala)₃-nitroanilide (SANA), mushroom tyrosinase, porcine pancreatic elastase and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Liquid chromatography grade methanol and glacial acetic acid were purchased from Macron Fine Chemicals (Avantor Performance Materials, Center Valley, PA, USA). A Cosmosil 5C₁₈-MS-II reverse-phase high performance liquid chromatography (HPLC) column (4.6x250 mm internal diameter; Nakalai Tesque, Inc., Kyoto, Japan) was used for HPLC. Ethanol and phosphoric acid were of analytical reagent grade.

Preparation of the burdock leaf extract (BLE). Fresh burdock leaves were dried through four methods: Sun drying, shadow drying, oven drying and freeze-drying. For sun drying, fresh leaves were exposed directly to sunlight for 16 h. In shadow drying, fresh leaves were dried at an ambient temperature of 27°C and a relative humidity of 39% for 72 h. For oven drying, fresh leaves were dried in an oven at 60°C for 16 h. In freeze-drying, a lyophilizer (cat no. 8530023; FD-series, Panchun, Taipei, Taiwan; http://www.panchun.com.tw/product_show.asp?id=1038) was used to dry the burdock leaves for 72 h at -80°C. The four types of BLE (30 g of each) were separately added to 600 ml of ethanolic aqueous solution [30, 50 or 95% ethanol (EtOH)] and refluxed for 3 h in a reflux extraction apparatus (cat no. 203-330; Angu, Kaohsiung, Taiwan). Subsequently, the extract solution was filtered and the total polyphenol content was prescreened,

as described below. The solution from each drying method with the highest amount of total antioxidant components was lyophilized to obtain the BLE at -80°C for 24 h, which was stored in an Eureka Auto Dry Box at 25°C and 40% relative humidity (AD-88S; Taiwan Dry Tech Corp., Taipei, Taiwan) until required for analysis.

Determination of total polyphenol content. The total polyphenol content was estimated spectrophotometrically using Folin-Ciocalteu reagent based on a colorimetric oxidation/reduction reaction, as described previously (22,29) with minor modifications. Briefly, BLE solution (20 µl) obtained through the different drying methods, 100 µl 0.2 N Folin-Ciocalteu reagent and 80 µl 7.5% Na₂CO₃ were added to each well of a 96-well microplate and mixed thoroughly. The solution was left to stand for 30 min at room temperature and the absorbance was measured at 765 nm using a microplate reader. The total polyphenol content was calculated as the gallic acid equivalent (GAE) on the basis of a calibration curve of gallic acid standard and expressed in mg/ml, as previously described (22).

DPPH scavenging activity. BLE antioxidant activity was evaluated on the basis of the scavenging capacity toward a stable DPPH free radical as described previously (22,30,31). A stock solution of BLE (1 mg/ml) was prepared and diluted with methanol to a suitable concentration at 0.5 mg/ml. An aliquot of 50 µl of each dilution was transferred into the wells of a 96-well microplate. A working solution of DPPH (250 µM) in methanol was freshly prepared and an aliquot of 150 µl was added to each well. After incubation for 30 min at 25°C, the remaining DPPH was measured at 490 nm using an ELISA reader. The antioxidant activity was expressed as the DPPH scavenging percentage, as previously described (22,31).

HPLC assay. The expression of CHA (which is an antioxidant component) in BLE was measured using an HPLC assay as described previously (10). Briefly, HPLC analyses were performed using an HPLC system equipped with an L2130 HPLC pump, an L2450 photodiode detector, an L2200 autosampler (Hitachi, Ltd., Tokyo, Japan) and a Cosmosil 5C₁₈-MS-II reverse-phase HPLC column. A mixture of methanol/water/glacial acetic acid (30:69:1, v/v; Sigma-Aldrich; Merck KGaA) was used as the mobile phase. A wavelength of 320 nm was used for monitoring the phenolic compounds and CHA. The flow rate and injection volume were set to 1 ml/min and 10 µl, respectively.

Elastase inhibition assay. The effect of BLE on porcine pancreatic elastase activity was estimated spectrophotometrically using SANA as the substrate according to the method described by Moon *et al* (4) with slight modifications. The release of *p*-nitroaniline was monitored for 15 min at 25°C by measuring the absorbance at 410 nm on a microplate reader. The reaction was performed in a mixture containing 0.2 M Tris-HCl buffer (pH 8.0), 10 µg/ml elastase, 5 mM SANA and the test sample (5 mg/ml) in a 96-well microplate. Each test sample was preincubated for 10 min at 25°C; the reaction commenced upon adding the substrate. The inhibition of elastase was calculated as follows: Inhibition (%) = [(A-B)/A] x100,

where A and B were the absorbance at 410 nm without and with the test sample, respectively.

Tyrosinase inhibition assay. The tyrosinase inhibition activity of BLE was determined by spectrophotometrically measuring the rate of dopachrome formation using L-DOPA as the substrate according to the method described by Choi *et al* (32) with slight modifications. Briefly, L-DOPA (15 mM; 40 μ l), phosphate buffer (pH 6.8; 67 mM; 80 μ l) and 40 μ l of either the same buffer or the test sample (5 mg/ml) were added to each well of a 96-well microplate. Subsequently, 40 μ l mushroom tyrosinase (125 units) was added to the reaction mixture. After incubation at 37°C for 30 min, this reaction mixture was evaluated for the formation of dopachrome using a microplate reader, by measuring the linear increase in the optical density at 492 nm. The tyrosinase inhibition was calculated as follows: Inhibition (%) = [(A-B)/A] x100, where A and B were the absorbance at 492 nm without and with the test substance, respectively.

Statistical analysis. The results are presented as the mean \pm standard deviation (SD) of triplicate experiments. The statistical difference between the results of different groups was analyzed using one-way analysis of variance and a post hoc Duncan's new multiple range test. SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Polyphenolic content of BLE. Four drying treatments for fresh burdock leaves were assessed; shadow drying, oven drying, sun drying and freeze-drying, and the total polyphenol content of the resulting BLEs was analyzed. As illustrated in Fig. 1, the total polyphenolic content following extraction with 95% EtOH was highest using the freeze-drying method, followed by the oven drying, shadow drying and sun drying methods. The polyphenolic content obtained from the freeze-drying method was significantly (~3 times) increased compared with that obtained through the other methods tested (P<0.05; Fig. 1). The effect of the concentration of ethanolic solvent used for extraction on the polyphenol content of BLE was investigated in the present study. As depicted in Fig. 2, freeze-drying the BLEs following extraction using 95% EtOH yielded a significantly lower polyphenolic content (GAE) compared with solvents extracted using 30 and 50% EtOH (P<0.05), indicating that an increase in the aqueous ratio results in a higher polyphenol content.

BLE has DPPH free radical scavenging activity. A DPPH-scavenging assay was used to measure the antioxidant capacity of the BLE obtained using different concentrations of EtOH as presented in Fig. 3. A similar effect was observed, with BLE extracted using 30 and 50% EtOH yielding a higher DPPH scavaging ability compared with BLE extracted using 95% EtOH.

Antityrosinase and antielastase activities of the BLE. CHA is an antioxidant component of burdock leaves (26,27). As

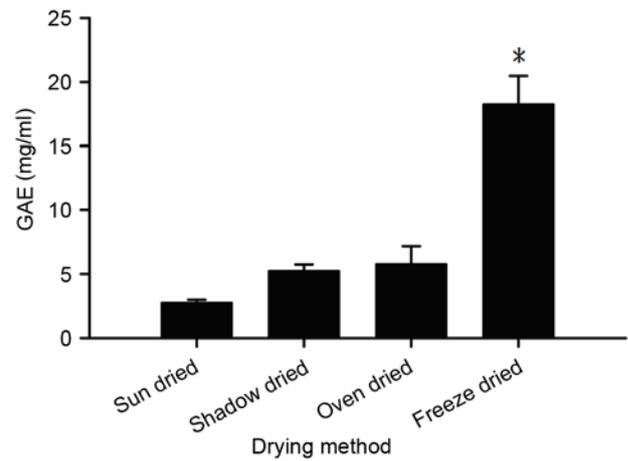


Figure 1. Polyphenolic content of BLE produced from different drying methods. GAE, gallic acid equivalent. *P<0.05 vs. sun dried, shadow dried and oven-dried samples. The results are presented as the mean \pm standard deviation of triplicate experiments.

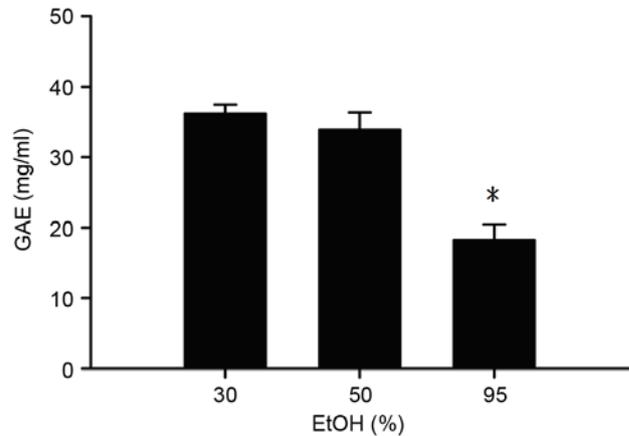


Figure 2. Polyphenolic content of the BLE obtained using different concentrations of EtOH for extraction following freeze-drying. *P<0.05 vs. 30 and 50% EtOH. The results are presented as the mean \pm standard deviation of triplicate experiments. GAE, gallic acid equivalent; EtOH, ethanol.

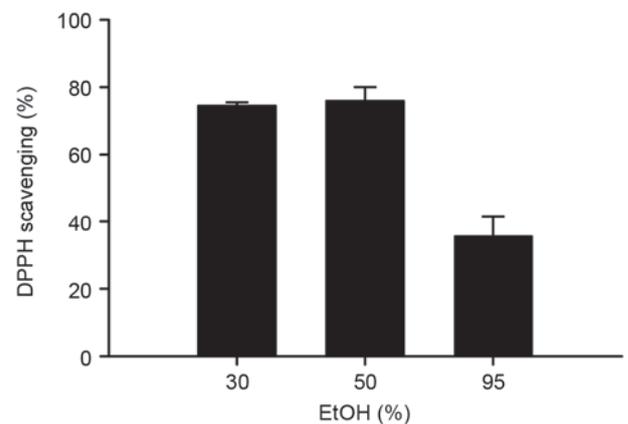


Figure 3. DPPH free radical scavenging activity of the BLE obtained using different concentrations of EtOH for extraction following freeze-drying. The free radical scavenging activity was evaluated as the DPPH scavenging percentage in the presence of BLE for 30 min. The results are presented as the mean \pm standard deviation of triplicate experiments. BLE, burdock leaf extract; EtOH, ethanol; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

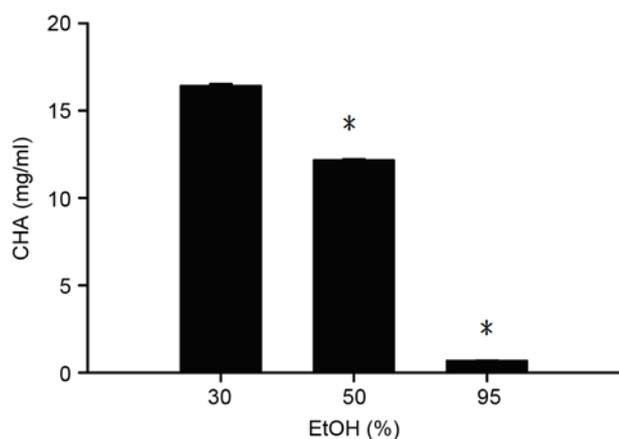


Figure 4. CHA content of the BLE obtained using different concentrations of EtOH for extraction following freeze-drying. * $P < 0.05$ vs. 30% EtOH. The results are presented as the mean \pm standard deviation of triplicate experiments. CHA, chlorogenic acid; EtOH, ethanol.

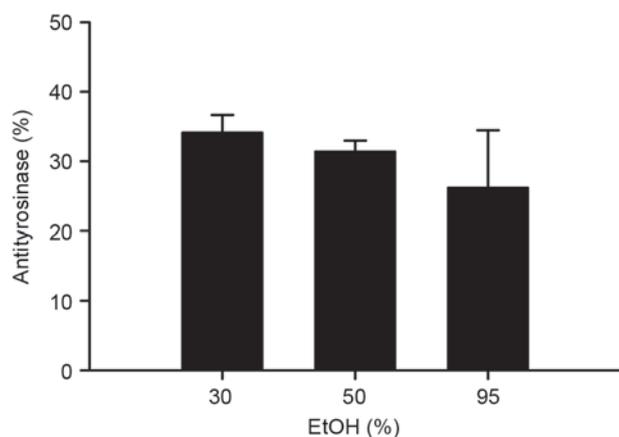


Figure 5. Antityrosinase activity of the BLE obtained using different concentrations of EtOH for extraction following freeze-drying. The results are presented as the mean \pm standard deviation of triplicate experiments. EtOH, ethanol.

observed in Fig. 4, freeze-drying BLE following extraction using 50 and 95% EtOH resulted in a significantly lower CHA content compared with solvents extracted using 30% EtOH ($P < 0.05$). This indicates that an increase in the ratio of ethanol negatively affects CHA extraction. The trend observed in the CHA content in the BLE using different concentrations of EtOH for extraction was only marginally similar to those for polyphenolic content and antioxidant activity. This suggests that polyphenolic components other than CHA contribute to polyphenolic content and antioxidant activity.

As depicted in Fig. 5, the BLEs produced following the use of 30, 50 and 95% EtOH exhibited a marked tyrosinase inhibitory effect ($>25\%$ inhibition). There was no significant difference in tyrosinase inhibition between the groups. In addition, the BLEs obtained using 30 and 50% EtOH notably inhibited elastase activity ($>60\%$), and this inhibition was significantly increased, compared with that if the BLE obtained using 95% EtOH ($P < 0.05$; Fig. 6). These results indicate that the antielastase activity of BLE is higher compared with its antityrosinase activity.

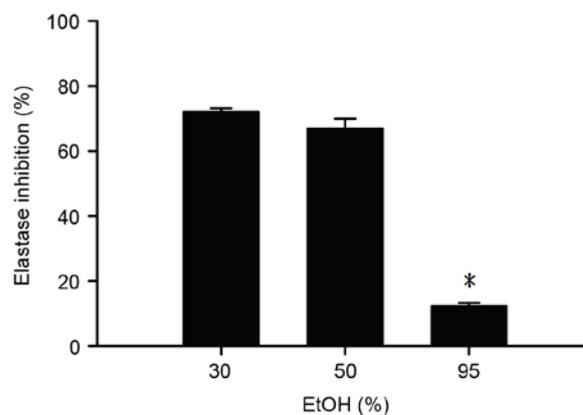


Figure 6. Antielastase activity of the BLE obtained using different concentrations of EtOH for extraction following freeze-drying. * $P < 0.05$ vs. 30 and 50% EtOH. The results are presented as the mean \pm standard deviation of triplicate experiments. EtOH, ethanol.

Discussion

Burdock is cultivated for dietary and traditional medicinal use in Eastern Asian countries (8-10). In Taiwan, burdock leaves are discarded following burdock root harvest, contributing to environmental waste. To increase the number of applications for these leaves, the present study investigated processes for optimizing their polyphenolic content, including certain drying and solvent extraction methods. In addition, the inhibitory activity of skin aging-associated enzymes in burdock leaves was examined in the current study.

Four drying treatments for fresh burdock leaves were determined. The polyphenolic content obtained from the freeze-drying method was significantly increased compared to the other methods assessed. These findings are consistent with previous reports, which identified that temperature strongly influences polyphenolic stability (10,33). Furthermore, these results indicate that freeze-drying is the optimal method of maintaining the polyphenolic content of burdock leaves; therefore, this method was applied to obtain the extracts used in subsequent experiments. The polyphenolic components other than CHA contribute to polyphenolic content and antioxidant activity. This is consistent trend with previous results (33,34), which reported that ethanol and methanol with 50-70% water content improved the extraction of antioxidant compounds from plants.

Skincare products formed the largest proportion (~23%) of cosmetics sales from 1998-2010 worldwide (35). Increased wrinkling, decreased elasticity, uneven pigmentation, and increased roughness and dryness are common signs of skin aging (1,2). Plants with natural antioxidant components that possess antiaging activities may have cosmetic applications. Polyphenols with antioxidant activity are frequently present in plants and are associated with various biological functions, including inhibiting skin aging-associated enzymes (36-38). Burdock leaves have recently been reported to contain polyphenols with antioxidant activity (10,26,27). For burdock leaves to be used effectively the following procedures are required: Measuring their content of antioxidant polyphenols; determining their inhibitory activity on skin aging-associated enzymes; and optimizing BLE preparation methods, including

drying and solvent extraction. The results of the current study indicate that the extraction of burdock leaves with 30-50% EtOH following freeze-drying is the optimal method for extracting antioxidant polyphenols.

ROS formed due to various extrinsic environmental factors and intrinsic endogenous metabolism are the most common causes of skin aging. UV radiation activates elastase secretion via ROS formation, resulting in a decreased elastin content, leading to wrinkles (2,3,33). The inhibition of skin elastase and tyrosinase serves a key role in the antiwrinkle and skin-evening activities (4,7,33). Antioxidant polyphenols possess tyrosinase and elastase inhibitory activity (4,36,39). In the current study, the extract from burdock leaves with antioxidant polyphenols inhibited elastase and tyrosinase activity, with their antielastase activity being stronger compared with their antityrosinase activity, which can be explained using the ROS scavenging theory of skin aging (40). These results indicate that the antiwrinkle activity of burdock leaves may be more effective than its skin-evening activity.

In conclusion, the current study demonstrated that BLE contains polyphenols, including CHA, and exhibits antioxidant activity. In addition, the BLE produced possessed marked tyrosinase and elastase inhibitory activity, which were associated with its polyphenolic content and antioxidant activities. Freeze drying followed by 30-50% EtOH extraction was identified to be the optimal method for obtaining BLE with high levels of antioxidant polyphenols. The antielastase and antityrosinase activity of BLE may be beneficial for developing skincare products with antiwrinkle and skin-evening activities. Further research is required to clarify the individual antioxidant components and the underlying pharmacological mechanisms of these effects of BLE.

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