BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 21(1),72-78 (2013)

Anti-Inflammatory, Antioxidant, Anti-Angiogenic and Skin Whitening Activities of *Phryma leptostachya* var. *asiatica* Hara Extract

Hyun-Joo Jung[1], Young-Wook Cho[2], Hye-Won Lim[3], Hojin Choi[4], Dam-Jung Ji[5] and Chang-Jin Lim[5,*]

[1]*Department of Anatomy, Yonsei University College of Medicine, Seoul 120-749,*
[2]*Korea Basic Science Institute Chuncheon Center, Chuncheon 200-701,*
[3]*Shebah Biotech Inc., Chuncheon Biotechnology Foundation, Hi-Tech Venture Town, Chuncheon 200-161,*
[4]*Department of Neurology, Hanyang University College of Medicine, Seoul 133-791,*
[5]*Department of Biochemistry, Kangwon National University, Chuncheon 200-701, Republic of Korea*

Abstract

This work aimed to assess some pharmacological activities of *P. leptostachya* var. *asiatica* Hara. The dried roots of *P. leptostachya* var. *asiatica* Hara were extracted with 70% ethanol to generate the powdered extract, named PLE. Anti-angiogenic activity was detected using chick chorioallantoic membrane (CAM) assay. *In vitro* anti-inflammatory activity was evaluated via analyzing nitric oxide (NO) content, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. Antioxidant activity was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and reactive oxygen species (ROS) level in the stimulated macrophage cells. Matrix metalloproteinase-9 (MMP-9) and -2 (MMP-2) activities in the culture media were detected using zymography. PLE exhibits an anti-angiogenic activity in the CAM assay, and displays an inhibitory action on the generation of NO in the LPS-stimulated macrophage cells. In the stimulated macrophage cells, it is able to diminish the enhanced ROS level. It can potently scavenge the stable DPPH free radical. It suppresses the induction of iNOS and COX-2 and the enhanced MMP-9 activity in the stimulated macrophage cells. Both monooxygenase and oxidase activities of tyrosinase were strongly inhibited by PLE. Taken together, the dried roots of *P. leptostachya* var. *asiatica* Hara possess anti-angiogenic, anti-inflammatory, antioxidant and skin whitening activities, which might partly provide its therapeutic efficacy in traditional medicine.

Key Words: Anti-angiogenic, Anti-inflammatory, Antioxidant, Matrix metalloproteinase-9, Skin whitening, *Phryma leptostachya* var. *asiatica* Hara

INTRODUCTION

Phryma leptostachya var. *asiatica* Hara (Phrymaceae), a perennial, erect, and herbal species that usually grows in moist forests, is distributed across Japan, Korea, China, Himalayas, east Siberia and eastern North America. It is used to treat inflammatory diseases, such as allergic dermatitis, gout and itch, and for the treatment and chemoprevention of cancers in traditional medicine. It is also used as larvicide, insecticide, parasiticide and detoxifying agent. Its therapeutic efficacy has not been clearly backed up in the aspect of pharmacology. Nonetheless, a very limited set of experimental findings on its constituents and pharmacological properties were documented.

As natural insecticidal components, several lignans, such as phrymarolins I and II, and haedoxan A were isolated from *P. leptostachya* L. (Taniguchi and Oshima, 1972a; Taniguchi and Oshima, 1972b; Taniguchi *et al.*, 1989). The additional two lignans, such as leptostachyol acetate and 8'-acetoxy-2,2',6-trimethoxy-3,4,4',5'-dimethylenedioxyphenyl-7,7'-dioxabicyclo-[3.3.0]octane, were isolated from the roots of *P. leptostachya* var. *asiatica* Hara, which contained effective larvicidal activity against three types of mosquito and was suggested as potential mosquito larval control agents or lead compounds (Park *et al.*, 2005). Ursolic acid, isolated from the ethyl acetate soluble fraction of *P. leptostachya* L., was identified to contain

Open Access **http://dx.doi.org/10.4062/biomolther.2012.059**

Received Aug 21, 2012 **Revised** Dec 21, 2012 **Accepted** Dec 24, 2012

***Corresponding Author**
E-mail: cjlim@kangwon.ac.kr
Tel: +82-33-250-8514, Fax: +82-33-242-0459
The first two authors contributed equally to this work.

cytotoxic activity against several murine and human cancer cell lines (Lee *et al.*, 2002). However, no other scientific reports on pharmacological activities and effective components of *P. leptostachya* var. *asiatica* Hara have been available. In this work, we demonstrate that the dried roots of *P. leptostachya* var. *asiatica* Hara possess anti-angiogenic, anti-inflammatory, antioxidant and tyrosinase inhibitory activities, which might in part support its folkloric medicinal use in traditional medicine.

MATERIALS AND METHODS

Chemicals and fertilized eggs

Retinoic acid (RA), vitamin C, *E. coli* lipopolysaccharide (LPS, natural, purity≥97%), L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), tyrosinase (EC 1.14.18.1) from mushroom, β-arbutin, gelatin and Griess reagent were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), leupeptin, pepstatin, phenylmethanosulfonyl fluoride (PMSF), 1,1-diphenyl-2-picrylhydrazyl (DPPH), HEPES, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were also obtained from Sigma-Aldrich Chemical Co. (St. Louis, MI, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were from Gibco-BRL (Gaithersburg, MD, USA). Fertilized brown Leghorn eggs were purchased from Pulmuone Food Co., Seoul, Korea. All other chemicals used were of reagent grade or better. All experiments, done in this work, were independently repeated at least three times.

Plant material

The dried roots of *P. leptostachya* var. *asiatica* Hara were obtained from a local market, Jeju Island, Korea, in August, 2011. The botanical identity was authenticated by Prof. Ki-Oug Yoo, Department of Biological Sciences, Kangwon National University, Chuncheon, Korea. Its voucher specimen was deposited in the herbarium of Department of Biological Sciences, College of Natural Sciences, Kangwon National University under the acquisition number KWNU56570.

Preparation of the ethanol extract (PLE)

The dried roots of *P. leptostachya* var. *asiatica* Hara were ground and extracted with 70% ethanol for one week at room temperature. The extract in a liquid state was *in vacuo* evaporated to generate its dried powder (PLE) with a yield of 13.1% on a dry weight basis. For the experiments, PLE was dissolved in 70% ethanol.

Cell culture

The RAW264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM containing 10% heat-inactivated FBS, 25 mM HEPES (pH 7.5), 100 units/ml penicillin and 100 μg/ml streptomycin. The RAW264.7 cells were plated at a density of 5×10^5 cells and pre-incubated for 24 h at 37ºC, and maintained in a humidified atmosphere containing 5% CO_2.

Determination of NO concentration

The RAW264.7 macrophage cells were incubated with LPS (1 μg/ml) in the presence or absence of pre-treated PLE for 24 h. Accumulated nitrite (NO_2^-), as an index of nitric oxide (NO), in the media was determined using a colorimetric assay based on the Griess reaction (Sherman *et al.*, 1993). The media (100 μl) were reacted with a 100 μl Griess reagent (6 mg/ml) at room temperature for 10 min, and then NO2- concentration was determined by measuring the absorbance at 540 nm. The standard curve was constructed using the known concentrations of sodium nitrite.

Chorioallantoic membrane (CAM) assay

Anti-angiogenic activity of PLE was determined employing CAM assay as previously described (Song *et al.*, 2004). The fertilized chicken eggs were kept in a humidified egg incubator at 37°C. After 3.5-day incubation, about 2 ml of albumen was aspirated from the eggs through the small hole drilled at the narrow end of the eggs, allowing the small chorioallantoic membrane and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. In the 4.5-day-old chick embryo, a PLE-loaded Thermanox® coverslip was applied onto the CAM surface. Two days after returning the chick embryo to the incubator, an appropriate volume of 10% fat emulsion (Intralipose®, 10%) was injected into a 6.5-day-old embryo chorioallantois. The eggs were then observed under a microscope. The branching pattern of each egg was graded as 0, 1+ or 2+. Convergence of a few vessels toward the CAM surface was denoted as 1+, and 2+ reflected an increased density and length of vessels toward the CAM face.

Assay for DPPH radical scavenging activity

The DPPH radical scavenging activity of PLE was determined as previously described (Song *et al.*, 2004). In brief, the reaction mixtures containing varying concentrations of PLE and 0.04 mM DPPH in a 96-well microtiter plate were incubated at 37°C for 30 min, and the absorbance was measured at 490 nm. Vitamin C was used as a positive control.

Determination of intracellular ROS

To determine intracellular ROS concentrations, the redox-sensitive fluorescent probe DCFH-DA was used as previously described (Royall and Ischiropoulos, 1993). The 5×10^5 RAW264.7 cells were pre-incubated with various concentrations of PLE for 1 h, and treated with LPS for 24 h. Then, they were incubated with 5 μM DCFH-DA for 30 min at 37°C. Right after the cells were harvested, the DCF (dichlorofluorescein) fluorescence, as indexes of the intracellular ROS levels, was immediately analyzed by a flow cytometry (BD FACSCalibur flow cytometer with BD CellQuest Pro software, BD Biosciences, San Jose, CA, USA). The ROS levels were represented as arbitrary units.

Western blot analysis

The RAW264.7 cells were incubated with LPS (1 μg/ml) in the presence or absence of PLE for 24 h and then washed twice with ice-cold phosphate-buffered saline (PBS). Cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin. For immunoblotting, anti-inducible nitric oxide synthase (anti-iNOS; Transduction Laboratories, Lexington, KY, USA), anti-cyclooxygenase-2 (anti-COX-2; Transduction Laboratories, Lexington, KY, USA), and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) antibod-

ies were used.

Zymographic determination of MMP-2 and -9 activities

MMP-2 and -9 activities were determined by gelatin zymographic analysis as described by Kleiner and Stetler-Stevenson (1994). The culture media were electrophoresed on 8% (w/v) SDS-PAGE gel impregnated with 1 mg/ml gelatin under a non-reducing condition. After SDS was removed from the gel by 2.5% Triton X-100, the proteins in the gel were renatured by incubation with calcium-containing buffer. The gel was stained with 0.1% Coomassie Brilliant Blue R-250, and gelatin-degrading enzyme activities were convinced as clear zones against a blue background. Identification of MMP-2 and -9 activity bands was in accordance with their appropriate molecular weights estimated using molecular mass markers.

Tyrosinase assay

In tyrosinase assay, L-tyrosine was used as a substrate for monooxygenase (cresolase) activity assay, while L-DOPA was used as a substrate for oxidase (catecholase) activity assay.

The monooxygenase activity of tyrosinase was determined by dopachrome method using a 96-well plate reader as previously described (Moon *et al.*, 2010). The enzymatic reaction was performed in 0.1 M potassium phosphate buffer (pH 6.7) containing 1.5 mM L-tyrosine and 100 units/ml mushroom tyrosinase at 37°C. The mixture was pre-incubated for 15 min before adding the substrate, and the change in the absorbance at 490 nm was measured.

The L-DOPA oxidation activity of tyrosinase was spectrophotometrically determined as previously described (Masuda *et al.*, 2005). Each reaction mixture contained 0.04 mM potassium phosphate buffer (pH 6.8), 0.5 mM L-DOPA and 6 units/ml mushroom tyrosinase, and was incubated at 37°C. The change in the absorbance at 475 nm was measured using a plate reader.

GC/MS analysis

GC/MS analysis was performed on an HP-6890 GC/HP 5973 MSD system using a fused silica capillary column (30 m×0.25 mm i.d., thickness 0.25 μm, DB-5MS 122-5532). The oven temperature, maintained at 100°C, was programmed at 5°C/min to 270°C. Helium was used as a carrier gas. Identifications were performed by comparison of mass spectra based upon the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) 2005 database.

Statistical analysis

The results were expressed as mean ± SD. Statistical comparisons between experimental groups were performed by unpaired Student's *t*-test. *p*-values less than 0.05 were considered to be significant. The half maximal inhibitory concentration (IC_{50}) values were calculated from the dose/response linear regression plots.

RESULTS

Anti-angiogenic activity

The chick chorioallantoic membrane (CAM) is an extra-embryonic membrane commonly used to determine both *in vivo* pro-angiogeneic and anti-angiogenic activities. The CAMs were used for examining the inhibitory activity of PLE on the vascular development. Retinoic acid (RA) was used as a positive control for the assay, since it inhibits angiogenesis by down-regulating the expression and release of pro-angiogenic factors (Iurlaro *et al.*, 1998). The disk itself did not give rise to changes in vascular density, indicating that it was unable to modulate the growth of blood vessels in the CAM assay (data not shown). After the 2-day treatment, the eggs treated with RA at 1 μg/egg showed approximately 85.2% inhibition in the branching patterns of blood vessels (Fig. 1). When 0.3, 1.0, 3.0 and 10.0 μg/egg of PLE was applied onto the CAMs, the inhibition percentages in the CAM angiogenesis were 30.0%, 33.3%, 36.7% and 43.3%, respectively (Fig. 1). This indicates that PLE has an anti-angiogenic activity in a dose-dependent manner. The dose required for half-maximal inhibition (IC_{50}) of PLE was 1.27 mg/egg. Taken together, PLE possesses anti-angiogenic activity.

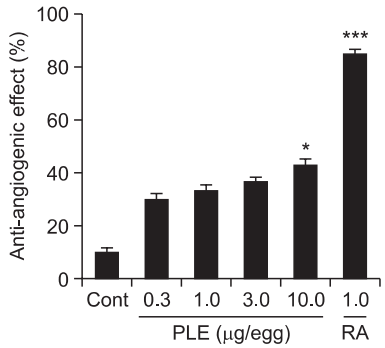

Fig. 1. Dose-dependent anti-angiogenic activity of PLE in the chick embryo chorioallantoic membrane (CAM) assay. Retinoic acid (RA, 1 μg/egg) was used as a positive control. Each group contained at least 20 eggs. Each bar represents the mean ± SD of the three independent experiments. **p*<0.05; ****p*<0.001, compared with the control.

Antioxidant activity

First of all, the *in vitro* antioxidant activity of PLE was determined in terms of the scavenging of the stable free radical DPPH. Since the proton-radical scavenging activity is considered as a crucial mechanism of antioxidation, DPPH, containing a proton free radical, is commonly used to determine the scavenging activity (Lu *et al.*, 2003). As shown in Fig. 2A, the DPPH radical scavenging activity of PLE appeared to be very strong. Its scavenging activity was comparable with that of vitamin C used as a positive control, suggesting that PLE would possess potent antioxidant principle(s).

PLE was able to suppress the reactive oxygen species (ROS) level enhanced in the LPS-stimulated RAW264.7 macrophage cells. As shown in Fig. 2B, when the macrophage cells were treated with LPS only, the intracellular ROS level increased about 8.2-fold. When the RAW264.7 macrophage cells were pretreated with 10, 50 and 200 μg/ml PLE, the ROS levels reduced to 99.6%, 79.1% and 25.6% of that of the cells treated with LPS only (Fig. 2B). However, PLE was unable to significantly modulate the ROS levels in the absence of LPS (Fig. 2B). This finding might suggest that PLE exhibits its decreasing effect chiefly on the process inducing ROS production under stimulation. In other words, PLE is thought to be unable to disturb the pre-existing normal level of ROS in the non-stimulated macrophages. These results imply that

PLE may significantly diminish the ROS level elevated by LPS stimulation in the macrophages. In brief, PLE contains strong antioxidant activity.

Inhibitory activity on NO production

NO, known as an important intracellular pro-inflammatory mediator, reacts with superoxide radical to generate a peroxynitrite ion, which can induce the inflammatory activity and leads to a variety of pathological conditions, such as arthritis, sepsis, ulcerative colitis, and systemic lupus erythematous (Lechner *et al.*, 2005; Bastos *et al.*, 2008). Suppression of NO production is linked with an anti-inflammatory action. As shown in Fig. 3, the effect of PLE was evaluated on the LPS-induced NO production in RAW264.7 macrophages. When the macrophage cells were stimulated with LPS, the nitrite content, as an index of NO, increased about 6.5-fold (Fig. 3). When the macrophage cells were pre-treated with 10, 50 and 200 μg/ml PLE, the nitrite level reduced to 94.9%, 54.8% and 25.7% of that of the cells treated with the LPS only (Fig. 3). This finding implies that PLE is able to suppress the NO production induced by LPS in the RAW264.7 macrophage cells in a concentration-dependent manner.

With the assumption that suppression of NO production by PLE would be caused by a diminishment in the inducible nitric oxide synthase (iNOS) level, the effect of PLE on the iNOS expression was examined in the macrophages cells treated with LPS. As shown in Fig. 4A, PLE concentration-dependently suppressed iNOS induction without changes in the levels of β-actin, an internal control, indicating the specific inhibition of iNOS expression by PLE. At the concentrations of 50 μg/ml and 200 μg/ml, PLE could completely suppress the iNOS induction in the stimulated macrophage cells (Fig. 4A). Taken together, PLE suppresses NO production through inhibiting induction of iNOS in the stimulated macrophage cells.

Suppressive activity on COX-2 induction

Cyclooxygenase (COX), known as a molecular target of

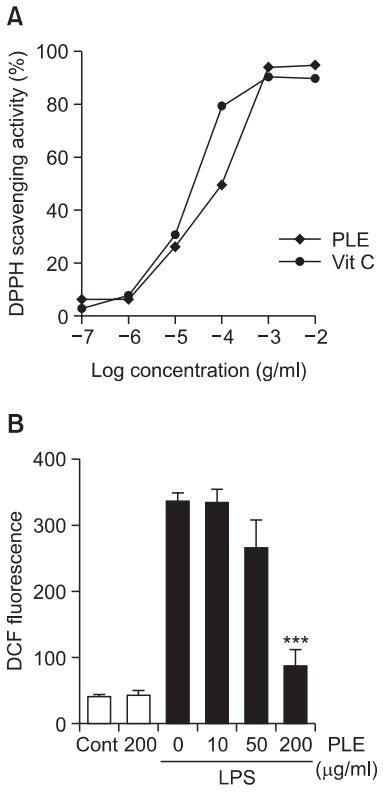

Fig. 2. Antioxidant activity of PLE. The stable free radical DPPH-scavenging activity (A) of PLE and its inhibitory activity (B) on the generation of reactive oxygen species (ROS) in the LPS-stimulated RAW264.7 macrophage cells were determined. The 5.0×10^5 mammalian cells were incubated for 24 h with LPS (1 μg/ml) in the presence or absence of indicated concentrations of PLE. In B, each bar shows the mean ± SD of cultures grown in triplicate, repeated in the three independent experiments, and the ROS level was represented as DCF fluorescence, an arbitrary unit. ***p<0.001 versus the LPS only.

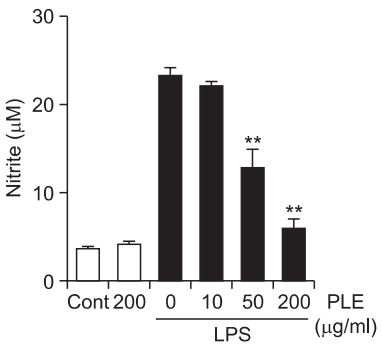

Fig. 3. Inhibitory effect of PLE on LPS-induced NO production in the RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μg/ml) in the presence or absence of indicated concentrations of PLE. Accumulated nitrite in the culture medium was determined by the Griess reaction. Each bar shows the mean ± SD of the three independent experiments each repeated in triplicate. **p<0.01 versus the LPS only.

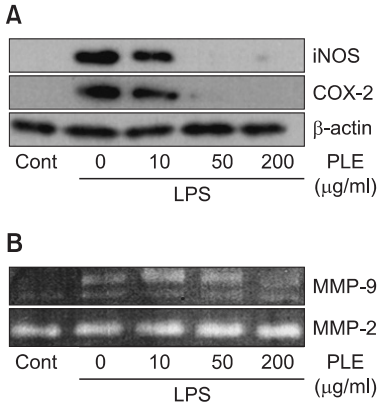

Fig. 4. Suppressive effects of PLE on LPS-induced expression of iNOS and COX-2 (A) and matrix metalloproteinase-9 (MMP-9) (B) in RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μg/ml) in the presence or absence of indicated concentrations of PLE. After 24-h incubation, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with appropriate antibodies. β-Actin was used an internal control. MMP-9 and -2 activities in the culture media were detected using gelatin zymography. Representatives of the three independent experiments are shown.

non-steroidal anti-inflammatory drugs (NSAIDs), which are very beneficial in the treatment of various types of inflammatory disorders, is an enzyme catalyzing a rate-limiting step involved in the conversion of arachidonic acid into inflammatory prostaglandins. In contrast to constitutive cyclooxygenase-1 (COX-1), inducible cyclooxygenase-2 (COX-2) is activated by tissue damage during inflammatory process. Various natural products of plant and marine origin have been shown to contain their anti-inflammatory activities through suppression of COX-2 (Jachak, 2006). As shown in Fig. 4A, PLE was found to suppress the induction of COX-2 in the stimulated macrophage cells at the concentrations capable of reducing the iNOS induction. This finding strongly suggests that anti-inflammatory activity of PLE depends on COX-2 suppression. Taken together, PLE possesses anti-inflammatory activity through suppressing induction of iNOS and COX-2.

Effect on the activities of matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) play an important role in the degradation and remodeling of the extracellular matrix at the sites of inflammation. MMP-9, not MMP-2, was markedly induced in LPS-stimulated RAW264.7 macrophage cells (Fig. 4B). Treatment with PLE at the concentrations of 10, 50 and 200 μg/ml was able to suppress the gelatinolytic activity of MMP-9 induced in the stimulated macrophages (Fig. 4B). However, PLE at the used concentrations couldn't modulate the gelatinolytic activity of MMP-2 in the stimulated macrophage cells. Taken together, PLE is specifically capable of down-regulating MMP-9 in the LPS-stimulated macrophage cells.

Skin whitening activity

Tyrosinase is a bifunctional copper-containing enzyme that is mainly responsible for the formation of the natural pigment melanin. It catalyzes the first two rate-limiting reactions in the biosynthesis of melanin, such as the hydroxylation of L-tyrosine to L-DOPA catalyzed by the monooxygenase activity of tyrosinase and the oxidation of L-DOPA to dopaquinone catalyzed by the oxidase activity of tyrosinase. Since tyrosinase plays a key role in human melanogenesis, tyrosinase inhibitors have been a great concern in the development of skin whitening agents. As shown in Fig. 5, the monooxygenase and oxidase activities of tyrosinase were significantly inhibited by PLE. At a concentration of 30 μg/ml, PLE was able to diminish the monooxygenase activity to 26.9% of the uninhibited value (Fig. 5A), whereas the uninhibited oxidase activity level was reduced to 45.2% by PLE (Fig. 5B). The IC_{50} values of PLE for the monooxygenase and oxidase activities of tyrosinase were 9.0 μg and 11.6 μg, respectively. β-Arbutin, used as positive control, exhibited rather high IC_{50} values, 0.9 mg/ml for the monooxygenase activity and 18.5 mg/ml for the oxidase activity. These results imply that PLE can suppress the biosynthesis of melanin through inhibiting the activity of the rate-limiting melanogenic enzyme. Collectively, PLE possesses potential skin whitening activity.

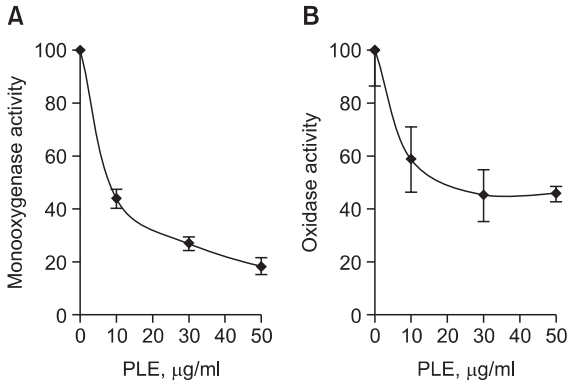

Fig. 5. *In vitro* inhibitory effects of PLE on monooxygenase (A) and oxidase (B) activities of tyrosinase. The monooxygenase and oxidase activities of tyrosinase, represented as relative activities, were spectrophometrically determined as a substrate using L-tyrosine (A) and L-DOPA (B), respectively, in the presence of 0, 10, 30 or 50 μg/ml PLE.

DISCUSSION

Angiogenesis is defined as the growth of new blood vessels from pre-existing capillaries and post-capillary venules, which is crucial in both physiological and pathological aspects. It mainly consists of several distinct phases, such as degradation of the basement membrane of existing blood vessels, migration, proliferation and rearrangement of endothelial cells, and formation of new blood vessels (Risau, 1995). Angiogenesis is closely linked with several kinds of diseases. Especially, it plays a significant role in the growth and metastasis of various tumors and chronic inflammatory diseases, such as rheumatoid arthritis and proliferative diabetic retinopathy (Mu *et al.*, 2008). Since down-regulation of angiogenesis is considered as an effective means for the control of cancer and inflammatory diseases, anti-angiogenic therapy may be a useful and practical trial in the treatment of those diseases. In combination with conventional chemotherapy, anti-angiogenic therapy can have great potential for curing tumors (Quan and Choong, 2006). Anti-angiogenic therapy retains advantages, such as reduced incidence of drug resistance, because angiogenesis inhibitors target generally normal vascular endothelial cells in contrast to conventional cytotoxic chemotherapy that targets genetically unstable tumor cells prone to mutations, and pharmacologic efficacy since endothelial cells are readily accessible to drugs and one endothelial cell supports the growth of a large number of tumor cells (Quan and Choong, 2006). Fibroblast growth factors (FGFs) and their receptors have recently been suggested to be useful targets for the development of anti-angiogenesis strategies (Rusnati and Presta, 2007). Many medicinal herbs traditionally used as anti-tumor agents, such as Chinese wormwood, curcumin, grape seed extract, *Ginkgo biloba*, quercetin, ginger, resveratrol, *Panax ginseng* and Chinese magnolia tree, were shown to possess significant anti-angiogenic activities (Sagar *et al.*, 2006). Screening of anti-angiogenic principles of natural origin is still promising and being continued. In this work, it is demonstrated that PLE contains reasonable anti-angiogenic activity, which was determined using CAM assay, one of typical methods widely used for detecting anti-angiogenic activities. Its anti-angiogenic activity would be partly responsible for its traditional use as anti-tumor and anti-inflammatory agents.

ROS include superoxide anions, hydrogen peroxide, and hydroxyl radicals, which are derived from oxygen. Although

ROS at physiological concentrations are essential for normal cellular function involved in intracellular signaling and redox regulation, excess ROS cause oxidative stress, which threatens the integrity of various biomolecules and is involved in tumor development and aging (Nordberg and Arnér, 2001). ROS initiate various kinds of toxic oxidative reactions, including lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehydes-3-phosphate dehydrogenase, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative modification of proteins (Cuzzocrea, 2006). Since a pathway leading to activation of transcription factor NF-κB, a regulator of inflammation, is under ROS-mediated control, antioxidant compounds, capable of down-regulating the intracellular ROS level, could have anti-inflammatory activities (Bubici *et al.*, 2006). PLE, identified to have strong DPPH-scavenging activity, markedly suppressed LPS-induced ROS generation in macrophages cells. Antioxidant activity of PLE might initiate its biological properties, which lead to its anti-tumor and anti-inflammatory activities. PLE with strong antioxidant activity provokes an indication on the application of *P. leptostachya* var. *asiatica* Hara into various fields other than anti-tumor and anti-inflammatory purposes. PLE would have various kinds of pharmacological activities which are known to be closely linked with antioxidant activity. In *in vitro* experiments, PLE exhibited significant inhibitory activity on tyrosinase utilizing both L-tyrosine and L-DOPA as a substrate, implying that PLE contains skin whitening effect.

NO, a simple and gaseous molecule, is a mediator and regulator of inflammatory processes, and iNOS plays a regulatory role in the generation of NO upon inflammation. iNOS-derived NO is involved in various pathological conditions such as inflammation and autoimmune diseases and causes cellular injury (Singh *et al.*, 2000). iNOS is notably up-regulated during sustained inflammation such as arthritic disorders (Cuzzocrea, 2006). For the expression of iNOS, the mammalian cells should be triggered by specific stimulants, such as pro-inflammatory cytokines and bacterial LPS (Chesrown *et al.*, 1994). The present study demonstrates that PLE, at relatively low concentration ranges, markedly diminishes NO production and iNOS induction in the LPS-stimulated RAW264.7 macrophages. Taken together, PLE is presumed to play its anti-inflammatory role via suppression of NO production at the inflammation site.

This work additionally demonstrates that PLE is able to suppress induction of COX-2 and to inhibit MMP-9 activity in the LPS-stimulated RAW264.7 macrophage cells. Various compounds of natural origin were shown to exhibit their anti-inflammatory activities through suppression of COX-2 (Jachak, 2006). Based on MMP activity regulating extracellular matrix (ECM) turnover, MMPs modulate the outcome of inflammatory reaction, angiogenesis and tissue remodeling, and cause the release of ECM-bound growth factors and cytokines that regulate many of these processes (Mott and Werb, 2004). MMP-9, a 92 kDa gelatinase B expressed in various cell lines such as keratinocytes, osteoclasts, eosinophils, neutrophils and macrophages, is increased and activated in many kinds of inflammatory and malignant diseases (Sorsa *et al.*, 2006). Suppression of MMP-9 activity by PLE supports its anti-angiogenic and anti-inflammatory activities. However, how PLE inhibits MMP-9 activity remains to be elusive.

GC/MS analysis proved that PLE contains several known compounds, such as 1-N-methyl uracil, 1,7-azulenoquinone, β-D-methylglucopyranoside, D-tagatose, isosorbide and 5-hydroxy-2-methylbenzaldehyde. However, whether these compounds are responsible for PLE's antioxidant and anti-inflammatory activities currently remains unknown. Identification and purification of major antioxidant and anti-inflammatory principle(s) from the dried roots of *P. leptostachya* var. *asiatica* Hara would be firstly needed in future studies.

In conclusion, PLE possesses anti-angiogenic and antioxidant activities in addition to *in vitro* anti-inflammatory activity. PLE decreases the production of NO and ROS in the LPS-stimulated RAW264.7 macrophage cells, and suppresses the induction of iNOS and COX-2 in the same stimulated macrophage cells. PLE also suppresses the gelatinolytic activity of MMP-9 induced in the stimulated macrophage cells. It displays strong DPPH-scavenging activity when compared with vitamin C. PLE shows potent skin whitening activity through inhibiting both monooxygenase and oxidase activities of tyrosinase. Pharmacological activities of PLE, assessed in this work, may offer some knowledge on the traditional efficacies of *P. leptostachya* var. *asiatica* Hara. The current findings, together with previously known information, might suggest that PLE, an ethanol extract of *P. leptostachya* var. *asiatica* Hara, exhibits an anti-inflammatory property through the inhibition of the production of NO, PGE2 and MMP-9 by down-regulating the expression of pro-inflammatory mediators such as iNOS and COX-2, which are induced by inflammatory stimuli including LPS.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No. : A103017). This study was supported in part by Kangwon National University. The authors are grateful to Ms A Rum Park and Ms Yu-Mi Shin for their technical assistances.

REFERENCES

Bastos, G. N., Silveira, A. J., Salgado, C. G., Picanço-Diniz, D. L. and do Nascimento, J. L. (2008) *Physalis angulata* extract exerts anti-inflammatory effects in rats by inhibiting different pathways. *J. Ethnopharmacol.* **118**, 246-251.

Bubici, C., Papa, S., Dean, K. and Franzoso, G. (2006) Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* **25**, 6731-6748.

Chesrown, S. E., Monnier, J., Visner, G. and Nick, H.S. (1994) Regulation of inducible nitric oxide synthase mRNA levels by LPS, INF-gamma, TGF-beta, and IL-10 in murine macrophage cell lines and rat peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **200**, 126-134.

Cuzzocrea, S. (2006) Role of nitric oxide and reactive oxygen species in arthritis. *Curr. Pharm. Des.* **12**, 3551-3570.

Iurlaro, M., Benelli, R., Masiello, L., Rosso, M., Santi, L. and Albini, A. (1998) Beta interferon inhibits HIV-1 Tat-induced angiogenesis: synergism with 13-cis retinoic acid. *Eur. J. Cancer* **34**, 570-576.

Jachak, S. M. (2006) Cyclooxygenase inhibitory natural products: current status. *Curr. Med. Chem.* **13**, 659-678.

Kleiner, D. E. and Stetler-Stevenson, W.G. (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.* **218**, 325-329.

Lechner, M., Lirk, P. and Rieder, J. (2005) Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. *Semin. Cancer Biol.* **15**, 277-289.

Lee, S., Min, B. and Kho, Y. (2002) Brine shrimp lethality of the compounds from *Phryma leptostachya* L. *Arch. Pharm. Res.* **25**, 652-654.

Lu, L.C., Chen, Y. W. and Chou, C. C. (2003) Antibacterial and DPPH free radical-scavenging activities of the ethanol extract of propolis collected in Taiwan. *J. Food Drug Anal* **11**, 277-282.

Masuda, T., Yamashita, D., Takeda, Y. and Yonemori, S. (2005) Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica. Biosci. Biotechnol. Biochem.* **69**, 197-201.

Moon, J. Y., Yim, E. Y., Song, G., Lee, N. H. and Hyun, C. G. (2010) Screening of elastase and tyrosinase inhibitory activity from Jeju Island plants. *EurAsia J. BioSci.* **4**, 41-53.

Mott, J. D. and Werb, Z. (2004) Regulation of matrix biology by matrix metalloproteinases. *Curr. Opin. Cell Biol.* **16**, 558-564.

Mu, P., Gao, X., Jia, Z. J. and Zheng, R. L. (2008) Natural antioxidant pedicularioside G inhibits angiogenesis and tumourigenesis *in vitro* and *in vivo*. *Basic Clin. Pharmacol. Toxicol.* **102**, 30-34.

Nordberg, J. and Arnér, E. S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31**, 1287-1312.

Park, I. K., Shin, S. C., Kim, C. S., Lee, H. J., Choi, W. S. and Ahn, Y. J. (2005) Larvicidal activity of lignans identified in *Phryma leptostachya* var. *asiatica* roots against three mosquito species. *J. Agric. Food Chem.* **53**, 969-972.

Quan, G. M. and Choong, P. F. (2006) Anti-angiogenic therapy for osteosarcoma. *Cancer Metastasis Rev.* **25**, 707-713.

Risau, W. (1995) Differentiation of endothelium. *FASEB J.* **9**, 926-933.

Royall, J. A. and Ischiropoulos, H. (1993) Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302**, 348-355.

Rusnati, M. and Presta, M. (2007) Fibroblast growth factors/fibroblast growth factor receptors as targets for the development of anti-angiogenic strategies. *Curr. Pharm. Des.* **13**, 2025-2044, 2007.

Sagar, S. M., Yance, D. and Wong, R. K. (2006) Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer - Part 1. *Curr. Oncol.* **13**, 14-26.

Sherman, M. P., Aeberhard, E. E., Wong, V. Z., Griscavage, J. M. and Ignarro, L. J. (1993) Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **191**, 1301-1308.

Singh, V. K., Mehrotra, S., Narayan, P., Pandey, C. M. and Agarwal, S.S. (2000) Modulation of autoimmune diseases by nitric oxide. *Immunol. Res.* **22**, 1-19.

Song, Y.S., Kim, S. H., Sa, J. H., Jin, C., Lim, C. J. and Park, E. H. (2004) Anti-angiogenic and inhibitory activity on inducible nitric oxide production of the mushroom *Ganoderma lucidum. J. Ethnopharmacol.* **90**, 17-20.

Sorsa, T., Tjäderhane, L. Konttinen, Y. T., Lauhio, A., Salo, T., Lee, H. M., Golub, L. M., Brown, D. L. and Mäntylä, P. (2006) Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann. Med.* **38**, 306-321.

Taniguchi, E. and Oshima, Y. (1972a) Phrymarolin-I, a novel lignin from *Phryma leptostachya* L. *Agric. Biol. Chem.* **36**, 1013-1025.

Taniguchi, E. and Oshima, Y. (1972b) Structure of phrymarolin-II. *Agric. Biol. Chem.* **36**, 1489-1496.

Taniguchi, E., Imamura, K., Ishibashi, F., Matsui, T. and Nishio. A. (1989) Structure of the novel insecticides sesquilignan, haedoxan A. *Agric. Biol. Chem.* **53**, 631-643.