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

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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 choorioallantoic 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 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...
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**

Retinoid 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, phenylmethylsulfonyl 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, MO, USA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin VA, USA), and cultured in DMEM containing 10% heat-inactivated FBS, 25 mM HEPES (pH 7.5), 100 units/ml penicillin and 0.04 mM DPPH in a 96-well microtiter plate were incubated 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 absorbance at 540 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 5×10⁴ 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 Stettler-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 confirmed 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-angiogenic 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 disc 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.

**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 erythematosus (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...
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 monoxygenase 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 monoxygenase and oxidase activities of tyrosinase were significantly inhibited by PLE. At a concentration of 30 μg/ml, PLE was able to diminish the monoxygenase 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 monoxygenase and oxidase activities of tyrosinase were 9.0 μg and 11.6 μg, respectively. β-Arbutin, used as a positive control, exhibited rather high IC_{50} values, 0.9 mg/ml for the monoxygenase 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.

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 glyceraldehyde-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. 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 monoxygenase 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.

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