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Type I pro-collagen promoting and anti-collagenase activities of *Phyllanthus emblica* extract in mouse fibroblasts

PITHI CHANVORACHOTE, VARISA PONGRAKHANANON, SUDJIT LUANPITPONG, BOONTARIKA CHANVORACHOTE, SUMALEE WANNACHAIYASIT, and UBONTHIP NIMMANNIT,
Department of Physiology (P.C.) and Pharmaceutical Technology (International) Program (V.P., S.L., B.C., S.W., U.N), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

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Synopsis

As part of an ongoing search for the novel pharmacological activities of *Phyllanthus emblica,* the present study has shown its type I collagen promoting and anti-collagenase effects on primary mouse fibroblast cells. At a concentration of 0.1 mg/ml, emblica extract significantly increased the type I pro-collagen level up to 1.65-fold, and 6.78-fold greater than that of an untreated control, determined by immunocytochemistry and Western blot analysis, respectively. Emblica extract caused an approximately 7.75-fold greater type I pro-collagen induction compared to the known herbal collagen enhancer asiaticoside at the same treatment concentration (0.1 mg/ml). Moreover, emblica extract inhibited collagenase activity in a dose-dependent manner. Maximal inhibition was observed (78.67 ± 3.51%) at a concentration of 1 mg/ml. In summary, emblica extract has a promising pharmacological effect that benefits collagen synthesis and protects against its degradation and could be used as a natural anti-aging ingredient.

INTRODUCTION

Anti-aging active compounds from herbal extracts are currently in demand in the cosmetics industry. They not only help to prevent skin damage, but also to regenerate the new skin components. Among a great number of potent biologically active herbal extracts, *Phyllanthus emblica* or emblica fruit extracts represent several pharmacological properties attributable to their high vitamin C content (1). In addition, emblica fruit contains several biologically active tannins proposed to have a potent antioxidant activity (2).

Address all correspondence to Ubonthip Nimmannit and Pithi Chanvorachote.

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Skin aging is a process involving an alteration of type I collagen, the major component of dermis. An increase in type I collagen degradation and a decrease in its regeneration are considered major causes of wrinkle formation (3). Type I collagen is originally synthesized from intracellular type I pro-collagen containing propeptide extensions at both ends of the molecule (4,5). The pro-collagen is secreted to the extracellular matrix where the propeptides are removed and the collagen molecules aggregate to form the fibril (5,6). The matrix metalloproteinases (MMPs) are a large family of zinc-dependent endoproteases degrading all extracellular matrix proteins (ECMs) including collagen. MMP-1, interstitial collagenase, mediates type I and type III collagen degradation (7). Several factors promoting this degradation process have been identified, including ultraviolet (UV) radiation and reactive oxygen species (ROS) (8).

Due to their potent antioxidant activity, emblica extracts appear to have promise as effectors for anti-aging actives. The objective of the present study is to investigate the novel pharmacological activities of emblica extract, namely type I collagen promoting and anti-collagenase activities.

MATERIALS AND METHODS

MATERIALS

Dulbecco's Modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin/streptomycin, and phosphate-buffered saline were obtained from Gibco-BRL (Gaithersburg, MD). Formaldehyde, triton X-100, glycerol, sodium formate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoside (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Pro-collagen type I rabbit polyclonal antibody, horseradish peroxidase-coupled isotype-specific secondary antibodies, and FITC-coupled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A protease inhibitior mixture was obtained from Roche Molecular Biochemicals (Switzerland). A chemiluminescence detection system was obtained from Amersham Biosciences (Piscataway, NJ), and a Western blot system was obtained from Bio-Rad (Hercules, CA). An EnzChek® gelatinase/collagenase assay kit was purchased from Molecular Probes®, Invitrogen (Carlsbad, CA).

ISOLATION OF MOUSE EMBRYONIC FIBROBLASTS

Isolation of fibroblast cells from mouse embryo was carried out according to a previously described method (Bradley, Baylor College of Medicine, Waco, TX). Briefly, mouse uterus was dissected from 13- or 14-*post coitum* pregnancy female mice. Each embryo was separated and washed with phosphate buffer solution. Then the embryo was forced through a syringe that was fitted with an 18G 11/2" needle into Dulbecco's Modified Eagle's medium (DMEM). Primary fibroblasts were the only cells that attached and proliferated after five days incubation at 37°C. Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mmol/l L-glutamine, and 100 units/ml of penicillin/streptomycin in a 5% CO_2 environment at 37°C. The cells from passages 2–5 were used for the experiments.

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EXTRACTION AND SAMPLE PREPARATION

Emblica fresh fruits were freed from foreign matter like dust or other organic matter. The cleaned raw material was chopped up to reduce its size. The raw material was then agitated in water. The liquid part was separated and then converted to powder form by spray drying. Various samples of emblica extract were prepared by dissolving the dry emblica extract powder in deionized water to the indicated concentrations.

CELL VIABILITY ASSAYS

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells in 96-well plates were incubated with 500 µg/ml of MTT for four hours at 37°C. The intensity of the MTT product was measured at 550 nm using a microplate reader. The relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

WESTERN BLOT ANALYSIS

After specific treatments, cells were incubated in lysis buffer containing 20 mmol/l Tris-HCl (pH 7.5), 1% Triton X-100, 150 mmol/l NaCl, 10% glycerol, 1 mmol/l Na_3VO_4, 50 mmol/l NaF, 100 mmol/l phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Molecular Biochemicals) for 20 minutes on ice. After insoluble debris was pelleted by centrifugation at 14,000*g* for 15 minutes at 4°C, the supernatants were collected and the protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Proteins (40 µg) were resolved under denaturing conditions by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for one hour in 5% nonfat dry milk in TBST [25 mmol/l Tris-HCl (pH 7.4), 125 mmol/l NaCl, 0.05% Tween 20] and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBST for ten minutes and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for one hour at room temperature. The immune complexes were detected by an enhanced chemiluminescence detection system (Amersham Biosciences) and quantified using analyst/PC densitometry software (Bio-Rad). Mean densitometry data from independent experiments were normalized to control results. The data were presented as the mean ± SD and analyzed by the Student's *t*-test.

IMMUNOCYTOCHEMISTRY

After specific treatment, cells in six-well plates were rinsed once with cold PBS and fixed for three minutes with a fixing reagent containing 4% formaldehyde in phosphate-buffered saline (PBS) solution, pH 7.4. After removal of the fixing reagent, cells were washed twice with TBS buffer (pH 7.4) and incubated with permeabilizing solution (1% Triton X-100 in PBS) at room temperature for five minutes. After washing with TBS buffer, the cells were blocked with blocking buffer (2.5% FBS in TBS) for 30 minutes at room

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temperature and further incubated with pro-collagen type I rabbit polyclonal antibody (Santa Cruz) for one hour at room temperature. After washing with TBST buffer for ten minutes, the cells were incubated with secondary antibody (FITC-coupled anti-rabbit, Santa Cruz) for one hour with gentle rocking at room temperature. They were then washed, trypsinized, resuspended in PBS (1×10^6/ml), and immediately analyzed by flow cytometry using an excitation wavelength at 488 nm and an emission wavelength at 520 ± 20 nm (FACSort, Becton Dickinson, Rutherford, NJ) with CellQuest software (Becton Dickinson).

ANTI-COLLAGENASE ASSAY

Collagenase inhibitory activity was performed using the EnzChek® gelatinase/collagenase assay kit (E-12055) (Molecular Probes) that was used in the previous study (9). The emblica extract was diluted in 1X reaction buffer. The diluted collagenase inhibitor was added to each well of a 96-well plate, and 1,10-phenanthroline served as a control inhibitor. DQ gelatin solution was added. Then 100 µl of the diluted enzyme or 100 µl of 1X reaction buffer (blank) was added to the sample wells preloaded with substrate and inhibitor. The samples were incubated at room temperature and protected from light for two hours. The fluorescence intensity was measured by a fluorescence microplate reader set for excitation at 485 nm and emission detection at 535 nm (Molecular Probes, product information, 2001). The increase in fluorescence is proportional to its proteolytic activity. Therefore, the decrease in fluorescence compared with the enzyme activity alone was observed to assay for a potential gelatinase/collagenase inhibitor. The percent inhibition of collagenase reaction was calculated as follows:

$$\% \text{ Collagenase inhibition} = \frac{[(A - B) - (C - D)] * 100}{A - B}$$

where A is the fluorescence after incubation without the test sample (control); B is the fluorescence after incubation without the test sample and enzyme (blank of A); C is the fluorescence after incubation with the test sample; and D is the fluorescence after incubation with the test sample, but without the enzyme (blank of C).

RESULTS

EFFECT OF EMBLICA EXTRACT ON PRIMARY MOUSE FIBROBLAST VIABILITY

Collagen fiber is primarily synthesized by fibroblasts as a pro-collagen protein, which is secreted and further processed to be a collagen fiber in the extracellular matrix (10,11). Among collagens, type I is the most abundant. It comprises between 85% and 90% of the total collagen in skin (4). To investigate the effect of emblica extract on collagen synthesis, we first characterized cell viability response to emblica treatment in primary mouse fibroblast cells. Cells were treated with various concentrations of emblica extract (0, 0.01, 0.1, 0.5, 1, and 2 mg/ml). Cell viability was determined after 24 hours incubation by MTT assay. The viability of cells was determined by measuring the optical density (OD) of formazan formation at wavelength 570–620 nm. Treatment of the cells with emblica

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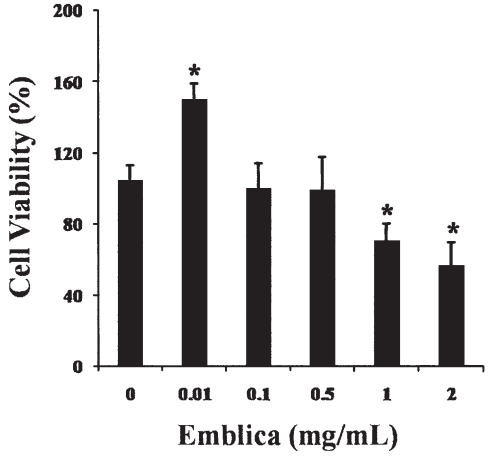

Figure 1. Effect of emblica extracts on cell viability. Various concentrations (0, 0.01, 0.1, 0.5, 1, and 2 mg/ml) of emblica extract were incubated with mouse fibroblast cells for 24 hours. Cell viability was analyzed by MTT assay. The data are presented as percentage of cell viability compared with untreated control. The experiments were performed independently in triplicate, and the number of cells was 20,000 cells in each sample. The data are represented as mean ± S.D. and were analyzed by the Student's *t*-test. *Significant difference, $p < 0.05$, compared to untreated control.

extract at the concentration of 0.01 mg/ml caused a significant increase in cell viability over the control level, (Figure 1). At the treatment concentration of 0.1–0.5 mg/ml, emblica extract did not have a significant effect on cell viability. Emblica extract at high concentrations caused a toxic effect on the cells, as indicated by cell viability that approached 71% and 51% at the treated doses of 1 mg/ml and 2 mg/ml, respectively.

EFFECTS OF EMBLICA EXTRACT ON TYPE I PRO-COLLAGEN EXPRESSION

To determine the effect of emblica extract on pro-collagen expression, mouse fibroblast cells were treated with various concentrations for 24 hours at 37°C. The type I pro-collagen protein level was determined using immunocytochemistry based on the principle of specific protein–antibody complex. After treatment, cells were fixed, permeabilized, and incubated with specific anti-type I pro-collagen antibody followed by chemiluminescence FITC secondary antibody. The fluorescence intensity correlating to the type I pro-collagen level was detected by flow cytometry.

Treatment with emblica extract significantly increased the type I pro-collagen level in a dose-dependent manner (at a concentration ranging from 0.01 to 1 mg/ml), with the maximum response at a concentration of 0.1 mg/ml (1.65 ± 0.085-fold). However, further increasing emblica concentrations (0.5–1 mg/ml) slightly decreased pro-collagen levels compared to the maximal response, but levels were significantly increased compared to the control (Figure 2). Due to its cytotoxic effect, treatment of emblica extract at 2 mg/ml significantly decreased the type I pro-collagen level.

In order to confirm the effect of emblica extract on the type I pro-collagen level, Western blot analysis for type I pro-collagen was performed. Cells were treated with various

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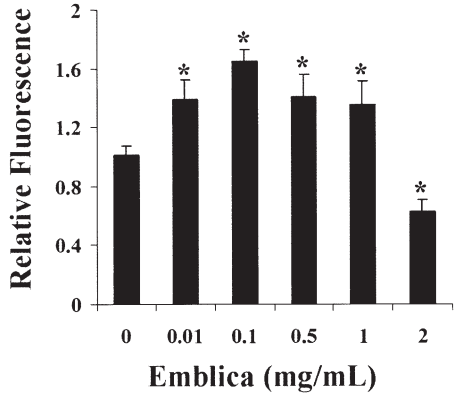

Figure 2. Effect of emblica extracts on type I pro-collagen level in mouse fibroblast cells. Cells were treated with various concentrations of emblica extract (0, 0.01, 0.1, 0.5, 1, and 2 mg/ml) for 24 hours. Type I pro-collagen protein was determined by immunocytochemistry assay and flow cytometry. Data are shown in terms of relative fluorescence intensity to untreated control. The experiments were performed independently in triplicate, and the number of cells was 100,000 cells in each sample. The data are represented as mean ± S.D. and were analyzed by the Student's *t*-test. *Significant difference, $p < 0.05$, compared to untreated control.

concentrations of emblica (0, 0.01, 0.1, 0.5, 1, and 2 mg/ml) for 24 hours. The cell lysates were analyzed by Western blot analysis using a specific antibody for type I pro-collagen protein.

Consistent with the immunocytochemistry assay, our Western blot results showed that emblica extracts dramatically increased intracellular type I pro-collagen in mouse fibroblast cells. Approximately 6.87-fold induction of type I pro-collagen was observed at the treatment concentration of 0.1 mg/ml emblica (Figure 3). As the dose was increased to 0.5 mg/ml and 1 mg/ml, the pro-collagen level appeared to decrease and the level was significantly decreased compared to the untreated control at the concentration of 2 mg/ml.

Since asiaticoside has been recently reported to possess a collagen-enhancing effect (12), we used the same concentrations of asiaticoside (0.1 mg/ml and 0.5 mg/ml) for comparison. Our results indicated that emblica extract dramatically up-regulated the type I pro-collagen level to 7.55-fold compared to the untreated control (Figure 4), whereas only 1.01- and 2.57-fold inductions were found in 0.1 mg/ml and 0.5 mg/ml of asiaticoside treatment, respectively.

EFFECTS OF EMBLICA EXTRACT ON COLLAGENASE ACTIVITY

Collagenase is a metalloproteinase with an active site zinc ion that is important in facilitating interaction with an inhibitor (13). Quantification of the anti-collagenase activity of emblica extracts was determined by using an EnzChek® gelatinase/collagenase assay kit.

As shown in Figure 5, we found that emblica extract significantly inhibited collagenase activity in a dose-dependent manner. At the concentration of 0.5 mg/ml, emblica extract showed collagenase inhibition of 72.11 ± 5.95%, and the inhibition activity slightly increased up to 78.67 ± 3.51% when the concentration was extended to 1 mg/ml.

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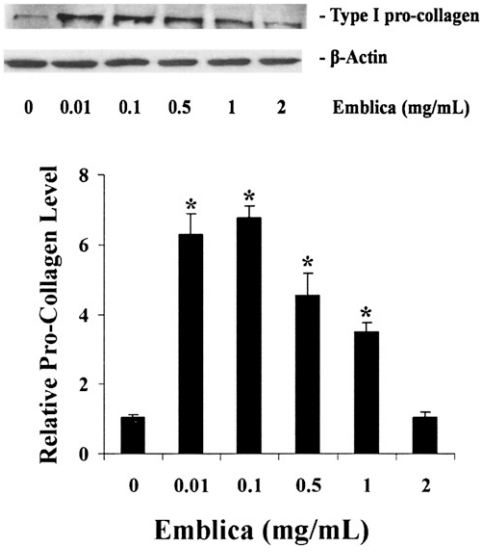

Figure 3. Effect of emblica extract on type I pro-collagen expression level. Cells were treated with various concentrations of emblica extract (0, 0.01, 0.1, 0.5, 1, and 2 mg/ml) for 24 hours, and cell lysates were subjected to Western blot analysis detection with specific antibody for type I pro-collagen protein. All Western blot results were quantified using analyst/PC densitometry software. Mean densitometry data from three independent experiments were normalized to result in cells in the control. The data are represented as mean ± SD and were analyzed by the Student's *t*-test. *Significant difference, $p < 0.05$, compared to untreated control.

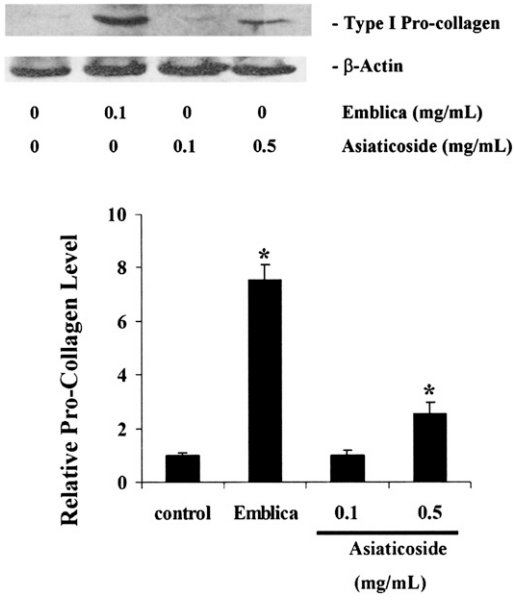

Figure 4. Effect of emblica extract and asiaticoside on type I pro-collagen expression level. Cells were treated with 0.1 mg/ml emblica extract or 0.1 mg/ml and 0.5 mg/ml asiaticoside for 24 hours, and cell lysates were subjected to Western blot analysis detection with specific antibody for type I pro-collagen protein. All Western blot results were quantified using analyst/PC densitometry software. Mean densitometry data from three independent experiments were normalized to result in cells in the control. The data are represented as mean ± SD and were analyzed by the Student's *t*-test. *Significant difference, $p < 0.05$, compared to untreated control.

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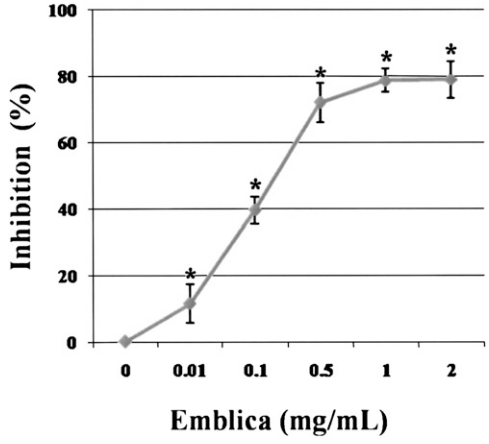

Figure 5. Effect of emblica extract on collagenase activity. Quantification of anti-collagenase activity of emblica extracts was determined by using an EnzChek® gelatinase/collagenase assay kit. The percent collagenase inhibition at indicated concentrations of emblica extract obtained from three independent experiments is presented as mean ± SD. *Significant difference, $p < 0.05$.

DISCUSSION

Skin aging is a process associated with the alteration of collagen and elastin contents in dermis. Among these, type I collagen, the major component of the dermis, has been shown to be degraded in response to several environmental stimuli, including UV-radiation and ROS. An increase in this protein's degradation and a decrease in its regeneration are considered major causes of wrinkle formation (3). Emblica fruit or *Phyllanthus emblica* possesses several pharmacological properties attributable to its high vitamin C content and biologically active tannins. The present study found that emblica extract dramatically increased intracellular type I pro-collagen levels in primary mouse fibroblasts. Moreover, emblica extract showed a strong inhibitory effect against collagenase activity.

Previous studies have reported that ascorbic acid (vitamin C) was able to induce collagen synthesis (14,15). The mechanism by which vitamin C induced collagen synthesis has been shown to involve its pro-oxidant activity. This pro-oxidant effect contributes to the lipid peroxidation that is considered a primary cause of collagen induction (16). Although emblica fruit contain a "high level" of vitamin C, neither lipid peroxidation nor cytotoxicity was detected under the condition of pro-collagen induction in the present study. Our results suggest that the pro-collagen stimulating effect of emblica extract may not be associated with its high vitamin C content. In addition, our results show that emblica has a greater potency than the known collagen enhancer asiaticoside. In summary, the present study indicates the novel effects of emblica extract as a pro-collagen regenerator as well as a collagenase inhibitor.

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