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EFFECT OF 1,2,3,4,6-PENTA-O-GALLOYL-β-D-GLUCOSE ON ELASTASE AND HYALURONIDASE ACTIVITIES AND ITS TYPE II COLLAGEN EXPRESSION

SONG-JA KIM[1#], SANDESH A. SANCHETI[1#], SHRUTI S. SANCHETI[1#], BYUNG-HUN UM[2], SEON-MI YU[1] and SUNG-YUM SEO[1,3]*

[1]Department of Biology, Kongju National University, Kongju 314-701, Korea
[2]Natural Products Research Center, KIST Gangneung Institute, Gangneung 210-340, Korea
[3]Korean Collection of Herbal Extract, Inc., Kongju 314-701, Korea

Abstract: In the current era, natural products are gaining prime attention in the fields of cosmeceuticals and pharmaceuticals due to higher safety margins and biological functions, as they have a considerable amount of potential in treating different ailments. Thus, to find effective elastase and hyaluronidase inhibitors from natural resources, fifty Korean plants were screened, and the fruit of *Terminalia chebula* RETZIUS (Combretaceae) was selected for further structural isolation due to its potent efficacy. The methanol crude extract of the fruits showed 80% elastase and 87% hyaluronidase enzyme inhibition activities at 1 mg/mL. The crude extract, upon bioassay-directed fractionation, led to the isolation of compound **1**, whose structure was found by spectral analysis to be 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG). PGG displayed significant elastase and hyaluronidase inhibitory activities with IC_{50} values of 57 µg/mL and 0.86 mg/mL, respectively; also, treatment of PGG on rabbit articular chondrocytes significantly induced the type II collagen expression. Based on elastase and hyaluronidase inhibitions, and type II collagen expression, it could be suggested that PGG might have an influence on skin conditions when used cosmetically as an active anti-aging ingredient with no cytotoxicity; also, it might be beneficial in relieving painful joint conditions, and thus have relevance for treating arthritis. Therefore, it can be concluded that PGG may prove to be an active ingredient in cosmeceutical and pharmaceutical formulations, and that it definitely merits further *in vivo* investigations.

Keywords: 1,2,3,4,6-penta-O-galloyl-β-D-glucose, elastase, hyaluronidase, *Terminalia chebula*, type II collagen

Elastin is the main component of the elastic fibers of the connective tissue and tendons, while collagen is one of the important components in bone and joint composition; both elastin and collagen in the skin decrease naturally with age. The elastic fibers in the skin, together with the collagenous fibers, form a network under the epidermis (1-3). Elastase is the only enzyme that is capable of degrading elastin; it also plays a critical role in inflammation. It is capable of hydrolyzing nearly all proteins, including supporting and structural proteins of the connective tissues such as collagen and elastin (2, 4). In UVA-irradiated skin, mild inflammation occurs repeatedly in the dermis, and it is assumed that connective tissue proteins may be attacked by elastase released from polymorphonuclear leukocytes, resulting in damage to elastin and collagen fibers and finally causing sagging (5). Since this elastic fiber is easily decomposed by elastase secretion and activation, caused by exposure to UV light or reactive oxygen species, an approach that inhibits the elastase activity could be applied as a useful method to protect against skin aging (1-3, 6).

Hyaluronidase is involved in many pathophysiological processes such as inflammation, allergy, ovum fertilization, tumor growth, and metastasis. It degrades high molecular weight hyaluronic acid (hyaluronan, HA), which leads to inflammation, angiogenesis, fibrosis, and collagen deposition in wound healing. HA is distributed in the skin's connective tissue and is one of the components of the

* Corresponding author:e-mail: dnalove@kongju.ac.kr, tel: 82-41-850-8503, fax: 82-41-854-8503

# These three authors contributed equally to this work.

water retaining matrix; it has drawn great interest as a substance responsible for the retention of moisture in the skin and, thus, has been primarily used in cosmetics and pharmaceutical formulations. It has also been reported that the level of hyaluronic acid is low in the joint fluids of patients suffering from osteoarthritis or chronic rheumatism. Therefore, hyaluronidase inhibitors play a key role in maintaining the HA level occurring in the skin by nature; these inhibitors also act in the moisture retention of the skin, enhancing it. Also, arthritis and other diseases due to the loss of HA can be treated (5, 7, 8). Keeping this in view, in order to investigate a potential herbal medicine with anti-elastase and anti-hyaluronidase activities, we screened fifty Korean plants and found *Terminalia chebula* RETZIUS (*T. chebula*) (Combretaceae) as one promising source with 80% anti-elastase and 87% anti-hyaluronidase inhibitory activities in crude extract.

The ripe fruit of *T. chebula*, a native plant in India and Southeast Asia, has traditionally been used as a popular folk medicine, as carminative, deobstruent, astringent, homeostatic, antitussive, laxative, diuretic, and cardiotonic agent. *T. chebula* exhibits *in vitro* and *in vivo* antioxidant and free radical-scavenging activities. Its antimicrobial, antimutagenic, antifungal, antiviral, anticancer, antianaphylactic, and antidiabetic activities have been reported (9, 10). Also, a patent has been published on the anti-tyrosinase and anti-inflammatory activity of *T.* chebula (11). However, no anti-elastase activity has been reported for this plant. In the present study, we isolated compound **1** (Fig. 1) from the *T. chebula* fruit extract in order to investigate the inhibitory effects of compound **1** on elastase and hyaluronidase, which are involved in the turnover of the main components of the skin matrix; also, the collagen expression of compound **1** was studied on rabbit articular chondrocytes.

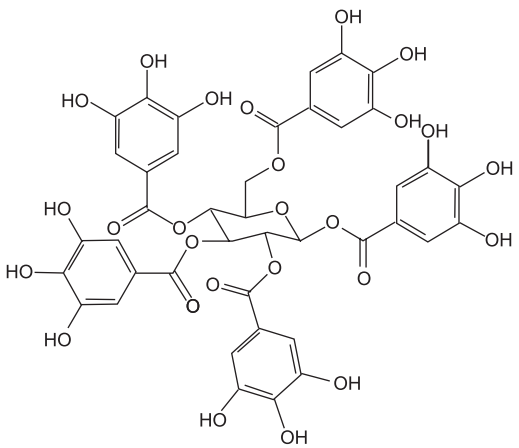

Figure 1. Structure of compound 1

MATERIALS AND METHODS

Plant material

The dried and matured fruits of *T. chebula* were obtained from the "Korean Collection of Herbal Extracts" a Biotech company in Korea. A collection of voucher specimens is available from the company (Korea Collection of Herbal Extracts, 2000).

Reagents

All necessary chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other commercially available reagents and solvents were used as received.

Extraction and isolation

The dried and ripe fruits (1 kg, dry weight) were extensively extracted with 80% aqueous MeOH until residue became colorless. The extract was evaporated to dryness using rotary vacuum evaporator. The vacuum dried crude extract (17.5 g) was then dissolved in water and separated using C-18 column (height: 35 cm, diameter: 2.5 cm) (mobile phase: 100% H_2O to 100% MeOH). The active fraction (2.4 g) (in 60% MeOH) was further separated using LH-20 column (height: 50 cm, diameter: 2 cm) (mobile phase: 100% MeOH), in which 100 fractions were collected and evaluated for the elastase and hyaluronidase inhibitory activities. The 14^{th} active fraction was re-passed through LH-20 column (height: 50 cm, diameter: 2 cm) (mobile phase: 100% MeOH) and from the active fraction (fraction 10), purified compound **1** (dry weight: 115 mg) was obtained, whose structure determination was done by NMR and mass spectral analysis.

The purified compound **1** (amorphous powder) was identified by one- and two-dimensional NMR spectroscopy. ^{1}H NMR, ^{13}C NMR, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bonded coherence (HMBC), and correlated spectroscopy (COSY) were recorded using a Varian NMR system 500 MHz (Varian, Palo Alto, CA, USA). The system was equipped with a carbon enhanced cold probe with reference to tetramethylsilane (TMS). Molecular weights were verified by electrospray ionization mass spectrometry (ESI-MS). Chemical shifts in ppm were reported with reference to the respective solvent peaks and residual

solvent peaks (δ_H 3.31 and δ_C 49.15 for CD_3OD). ESI-MS data were obtained on an ABI Mariner mass spectrometer.

Elastase assay

The elastase activity was evaluated according to the method previously reported by Kraunsoe et al. with minor modifications (2, 12).

In order to evaluate the inhibition of elastase activity, the amount of released *p*-nitroaniline, which was hydrolyzed from the substrate, N-succinyl-Ala-Ala-Ala-*p*-nitroanilide, by elastase, was assayed by measuring absorbance at 410 nm. In brief, a 1.015 mM solution of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide was prepared in a 0.1 M Tris-Cl buffer (pH 8.0) and this solution (130 µL) was added to the test sample (10 µL) in a 96 well microplate. The microplate was pre-incubated for 5 min at 25°C before an elastase (0.0375 Unit/mL) stock solution (10 µL) was added. After enzyme addition, the microplate was kept at 25°C for 30 min, and the absorbance was measured at 410 nm using microplate reader. All experiments were carried out in triplicate. The extent of inhibition, by the addition of samples, is expressed as the concentration required for 50% inhibition (IC_{50}).

HYALURONIDASE ASSAY

Hyaluronidase activity was determined according to the method previously described by Tung et al. (13) with minor modifications.

This method monitors undigested hyaluronic acid that is precipitated in agarose gel with cetylpyridinium chloride. Agarose was dissolved in 0.3 M phosphate buffer (pH 7.0) by heating and was maintained at 55°C in a water bath before use. Hyaluronic acid was dissolved in distilled water at a concentration of 8 mg/mL. The hyaluronic acid solution was preheated to 55°C and mixed with the agarose (1 volume hyaluronic acid : 9 volumes agarose) to give a final concentration of 0.8 mg/mL of hyaluronic acid and 0.8% (w/v) of agarose. The warm hyaluronic acid-agarose mixture (100 µL) was dispensed into each well of the microplate in a temperature-controlled room (37°C), and then the microplate was returned to room temperature (RT) to allow the gel to set. Each well was then filled with 20 µL of hyaluronidase and 20 µL of sample solution and incubated at 37°C for 5 h. At the end of incubation, the enzyme samples were removed and the absorbance was measured at 595 nm; samples were then filled with 100 µL of 10% (w/v) aqueous cetylpyridinium chloride. After reaction at 37°C for 30 min, the absorbance at 595 nm was measured by the microplate reader. All experiments were carried out in triplicate. The extent of inhibition, by the addition of samples, is expressed as the concentration required for 50% inhibition (IC_{50}).

Ethical clearance

Protocol used in this study for the use of animals was approved by the University Animal Ethical Committee.

Culture of rabbit articular chondrocytes

Rabbit articular chondrocytes were isolated from knee joint cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion with collagenase type II in Dulbecco's Modified Eagle's Medium (DMEM). After collecting individual cells by brief centrifugation, the cells were resuspended in a culture medium supplemented with 10% (v/v) fetal bovine calf serum, 50 µg/mL streptomycin, and 50 units/mL penicillin; samples were then plated on culture dishes at a density of 2×10^4 cells/well and cells were confluent after approximately five days. At 3.5 days into the culture, the cells were treated with the different concentrations of compound **1** (14).

MTT assay

Proliferating cells were quantified by counting surviving cells using an MTT assay kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. Primary rabbit cells at a concentration of 2×10^4 cells/well in 100 µL culture medium containing different concentrations of compound **1** [final concentration: 16 µg/mL to 5 mg/mL] were seeded into microplates (tissue culture grade, 96 wells, flat bottom). Cell cultures were incubated for four days at 37°C and 5% CO_2. After the incubation period, 10 µL of the MTT labeling reagent was added (final concentration 0.5 mg/mL) to each well. The microplate was incubated for 4 h in a humidified atmosphere (37°C, 5% CO_2). Hundred µL of the solubilization solution was added into each well. The plate was allowed to stand overnight in the incubator in a humidified atmosphere (37°C, 5% CO_2). The purple formazan crystals were checked for complete solubilization and the spectrophotometric absorbance of the samples was measured using a microplate (ELISA) reader at 600 nm. The control in this assay was treated with PBS alone. All assays were performed at least three times with triplicate samples.

Western blot analysis

Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl,

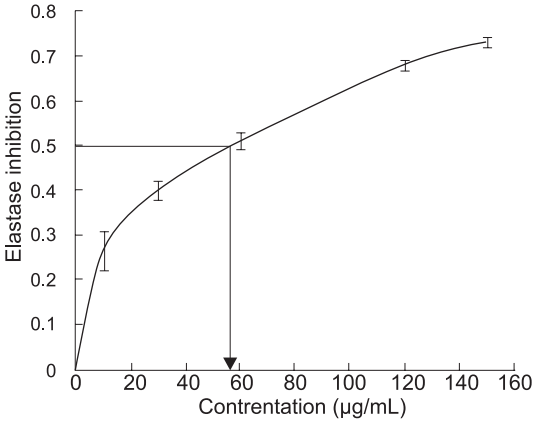

Figure 2. Elastase inhibitory activity of compound 1

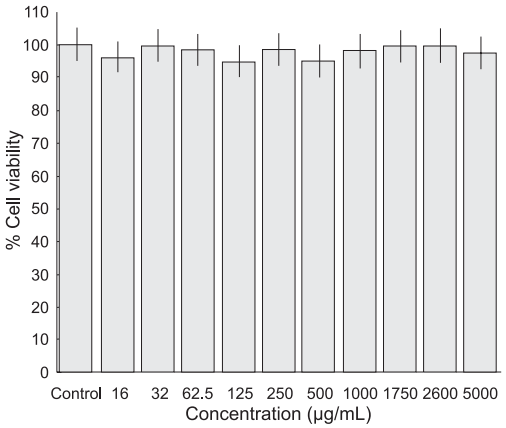

Figure 4. Cytotoxicity (MTT) assay for compound 1 on rabbit articular chondrocytes

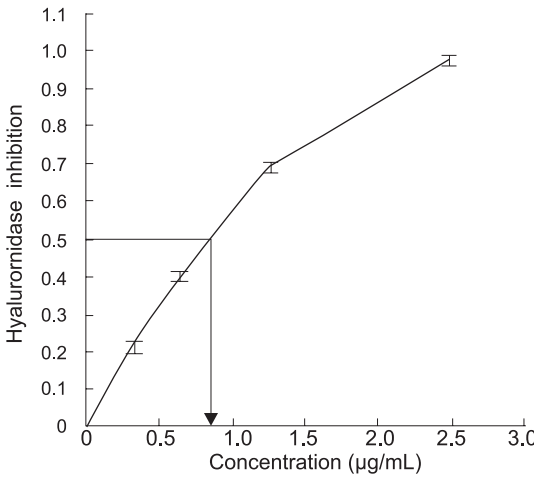

Figure 3. Hyaluronidase inhibitory activity of compound 1

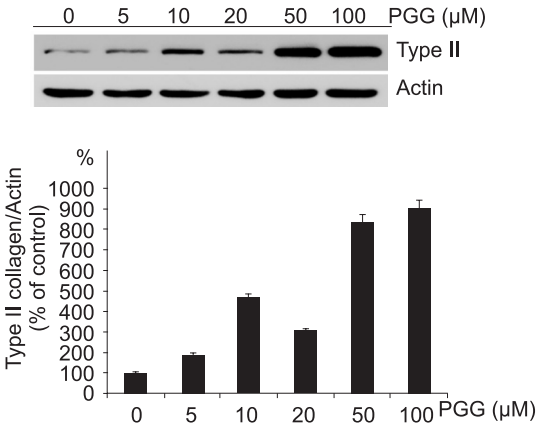

Figure 5. Type II collagen expression in rabbit articular chondrocytes by Western blot analysis for compound 1

pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecylsulfate, supplemented with protease inhibitors [10 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL aprotinin and 1 mM of 4-(2-aminoethyl)benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was then blocked with 3% non-fat dry milk in Tris-buffered saline. Type II collagen and actin were detected using antibody purchased from Chemicon (Temecula, CA). The bands were visualized using peroxidase-conjugated secondary antibodies and chemiluminescence.

RESULTS

Based on the bioassay guided fraction of *T. chebula* for the elastase and hyaluronidase inhibition activities, compound **1** was isolated as an active ingredient; it is a pale yellow solid whose molecular formula was established by ESIMS and ^{13}C NMR spectral data as $C_{41}H_{32}O_{26}$; all the results were equally matched with the authentic standard.

The elastase and hyaluronidase inhibition activities for compound **1** were evaluated in the series of concentrations (Fig. 2 and 3) in order to determine the IC_{50} values, which were calculated for elastase and hyaluronidase as 57 μg/mL and 0.86 mg/mL, respectively. From all of these results, it is

evident that compound **1** showed a marked inhibitory effect on elastase and hyaluronidase inhibitions. Also, cytotoxicity of compound **1** was evaluated *in vitro*: samples of various concentrations (16 µg/mL to 5 mg/mL) were prepared and used to treat rabbit articular chondrocytes for 24 h. The results of this evaluation, as shown in Figure 4, are expressed in percentages (considering control as 100%, with the cells that were treated with PBS alone), which showed that compound **1** has no cytotoxicity in rabbit articular chondrocytes up to 5 mg/mL concentration.

In addition, due to the hyaluronidase inhibitory properties of compound **1**, its effect on rabbit articular chondrocytes was also studied to determine its effect on the type II collagen. This is because type II collagen is mainly found in the articular cartilage and plays a key role in rheumatic arthritis. Therefore, to determine the effect of compound **1** on type II collagen, the chondrocytes were treated with indicated concentrations of compound **1** for 24 h, and cartilage specific matrix molecules were determined. As determined by Western blot analysis, compound **1** caused a dramatic increase in type II collagen (Fig. 5) in a dose dependent manner.

DISCUSSION

As increased elastase and hyaluronidase activities have been associated with wrinkling, sagging, inflammation, and laxity of aged skin (5, 15, 16), compound **1** obtained from a natural source might contribute as a main ingredient in anti-aging and anti-inflammatory formulations, as it is able to inhibit both elastase and hyaluronidase enzymes.

In addition, it is already known that compound **1** is a potent antioxidant agent (17), and that it may have an additive or synergistic effect on anti-aging of the skin; it also has skin-whitening activity (18), which proves that it is a very good cosmetic agent.

As a potential cosmetic agent, the active ingredient should be highly safe without any toxic effects. To prove this, cytotoxicity of compound **1** was evaluated on rabbit articular chondrocytes for 24 h, and results proved it safe with no cytotoxicity up to 5 mg/mL concentration.

As compound **1** is also a very good candidate as an anti-inflammatory, due to its inhibition of hyaluronidase, which is involved in rheumatic arthritis, we also examined the effects of compound **1** on rabbit articular chondrocytes. Based on the data, it could also be used as an active chondroprotective agent for the treatment of rheumatic arthritis, which needs further detailed investigations.

CONCLUSION

The isolated 1,2,3,4,6-penta-O-galloyl-β-D-glucose (compound **1**) from *T. chebula* could be a potentially promising candidate for use in cosmetic products due to its anti-elastase, anti-inflammatory, antioxidant, and antityrosinase activities with no cytotoxicity. Also, based on antihyaluronidase activity and type II collagen expression results, it may prove useful as an active and safe chondroprotective agent.

Therefore, further *in vivo* potential should be investigated in order to strengthen the application of PGG as an active ingredient in antiaging and anti-inflammatory formulations.

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