Screening and compound isolation from selected Thai herbal medicine for anti-hyaluronidase and anti-elastase activities

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Abstract

Ingredients form natural products have been used for centuries for skincare purposes. Nowadays, they are becoming more widespread in cosmetic formulations, due to consumers are becoming increasingly concerned about synthetic ingredients substances. The main advantage reported for plant extracts, used in skincare, include antioxidants, and anti-aging. In this study, ten herbal medicines used in Thailand were investigated for anti-hyaluronidase and anti-elastase activities, and their activities were investigated using the enzyme-substrate assay. Ethanol extract of *Senna garrettiana* at the concentration of 0.25 mg/ml had the highest hyaluronidase and elastase inhibitory activity. The inhibitory percentage of *S. garrettiana* heartwood extract against hyaluronidase and elastase was 60.6 ± 0.8 and 62.5 ± 0.6 %, respectively. The result indicated that *S. garrettiana* heartwood extract has a potential use on anti-aging skincare products due to their aging-enzyme inhibition properties. The ethanol extract of *S. garrettiana* heartwood was therefore selected and subjected to isolate of the active anti-hyaluronidase and anti-elastase compounds. On the basis of bioassay-guided isolation, betulinic acid and piceatannol were obtained as an active compound from *S. garrettiana* heartwood extract, that exhibited satisfactory for anti-elastase activities with IC_{50} values of 20.5 and 15.2 µg/ml, respectively. With regard to anti-hyaluronidase studies, no activity was observed with isolated compounds at the concentration of 100 µg/ml.

Keywords**:** *Anti-elastase, Anti-hyaluronidase, Plant extract, Skin aging, Senna garrettiana*

1. Introduction

Skin aging, a complex process, is associated with the wrinkle, loss of moisture and elasticity, and pigmentation of the skin. Skin aging can result from two ways including intrinsic and extrinsic factors. The intrinsic pathway results from the loss of elasticity and moisture of the skin over time and the external factors can be from nutrition, smoking, alcohol, ultraviolet exposure and contaminated environment (Jenkins, 2002; Sharma & Sharma, 2012)

The degradation of an extracellular matrix is caused by an increase of dermal enzymes including hyaluronidase, elastase, and MMP-1, and this process results in the aging of the skin (Losso et al., 2004). The extracellular matrix comprises proteoglycan interwoven with matrix metalloproteins such as collagen, elastin, fibronectin and hyaluronic acid. Collagen provides the supportive framework to the cells. Elastin maintains the elasticity of the skin, and hyaluronic acid plays a role in the water retention property of the cells. Hyaluronic acid and elastin play a role in the organization, structure, and elasticity of the extracellular matrix of connective tissue. They decrease sharply during maturation as well as in premature aging (Sahasrabudhe & Deodhar, 2004; Nema et al., 2011)

The proteins maintaining youthful skin are decreased with age as the degradation is dominant than the production of proteins; thus, the effective way is to lower the degradation of the proteins. Elastase is a protease for the breakdown of elastin, and it can cleave collagen, fibronectin and other extracellular matrix proteins (Melzig et al., 2001). Hyaluronidase degrades hyaluronic acid by lowering its viscosity and increasing the permeability (Losso et al., 2004).

Thus, skin care products that decrease or relieve skin aging become a great interest especially the ingredients from natural products. Plants are a major resource for skincare, both as antioxidants and inhibitors of enzymes involved in the aging process. The objectives of this study were therefore to explore the extracts

of commonly use herbal plants in Thailand providing the effective inhibitory activity against dermal enzymes including hyaluronidase and elastase. Furthermore, the phytochemical study of the most potent plant extract was also carried out. This could be beneficial to use as the ingredients in anti-skin aging formulations.

2. Objectives

1. To determine the *in vitro* anti-hyaluronidase and anti-elastase activity of ten ethanolic plant extracts.
2. To isolate the active compound from the most potent of plant extract.

3. Materials and Methods

3.1 Chemicals

Hyaluronidase (from bovine testes), hyaluronic acid potassium salt (from the human umbilical cord), Porcine pancreatic elastase (Type IV), N-succinyl-(Ala)-3-*p*-nitroanilide, oleanolic acid, iron (III) chloride, ferrous sulfate, Tris (hydroxymethyl) aminomethane, sodium acetate, sodium chloride, sodium citrate, dibasic sodium phosphate, monobasic sodium phosphate and TPTZ (2,4,6-Tris(2-pyridyl)-1,3,5-triazine) were from Sigma (New Zealand). All other chemicals were of analytical grades.

3.2 Plant material preparation

Ten plants according to Table 1 were collected in Thailand, washed and then dried in a hot air oven at 50 °C for 24 h. The dried materials were ground and passed through a sieve No. 45. The powders were kept in air-tight containers and protected from light.

Table 1 Plant materials used in the study

Scientific names	Family	Part used
Senna garrettiana (Craib.) Inwin & Basneby	Araliaceae	heartwood
Pinus merkusii Jungh et de Vriese	Leguminosae	heartwood
Dalbergia parviflora Roxb	Leguminosae	heartwood
Tarenna hoaensis Pitard	Rubiaceae	heartwood
Dracaena loureiri Gagnep	Dracaenaceae	heartwood
Thysostachy siamensis Gamble	Gramineae	root
Mimusops elengi L.	Sapotaceae	heartwood
Euphorbia antiquorum L.	Euphorbiaceae	heartwood
Piper ribesoides Wall	Piperraceae	stem
Oroxylum indicum (L.) kurz	Bignoniaceae	bark

Plant powder 50 g was extracted with 200 ml of ethanol for 1 h in a bath sonicator. The liquid extract was filtered with filter paper (Whatman No. 1). The solvent was removed with a rotary evaporator. The obtained crude extract was kept in a vial and protected from light.

3.4 Anti-hyaluronidase activity studies

The plant extract was dissolved in ethanol to obtain the extract at a concentration of 6.25 mg/ml, and the solution was then diluted to produce the extract a concentration of 0.5 mg/ml in water. The hyaluronidase enzyme of 0.005 mg/ml was prepared in 20 mM sodium phosphate solution, 77 mM sodium chloride, and 0.01 % bovine serum albumin, pH 7.0. Hyaluronic acid was prepared in 300 mM sodium phosphate buffer, pH 5.35. The extracted sample of 50 µl was incubated with 50 µl of hyaluronidase enzyme (0.1 U/reaction) at room temperature for 30 min. The hyaluronic acid as a substrate of 100 µl was then added, and the mixture was further incubated for 45 min at 37 °C. The undigested hyaluronic acid was precipitated with acid albumin solution of 1 ml (bovine serum albumin 0.1 %w/v in 24 mM sodium acetate, 79 mM acetic acid, pH 3.75) at room temperature for 30 min. The absorbance was measured at 600 nm. Oleanolic acid was used as a positive control. The hyaluronidase inhibitory activity of the extract was calculated by comparing it with oleanolic acid as a standard under exactly the same experimental conditions. The % inhibition was determined by the following equation.

% Enzyme activity = (100%) - [(A_{600nm} sample + hyaluronidase)/A_{600nm} hyaluronic acid] × 100

Solution A was prepared by mixing a Tris-buffer solution (0.2 M, pH 8.0) of 800 µl with elastase enzyme (0.0375 unit/ml) of 100 µl. The extract solution of 100 µl at different concentrations prepared in 0.2 M Tris-buffer was added to the solution A to yield the concentration of the extract ranging from 2-50 µg/ml. The samples were then incubated at 25 °C for 20 min. Then, N-succinyl-(Ala)-3-*p*-nitroanilide (0.8 mM), the substrate of elastase, of 100 µl was added, and the incubation was further carried out at 25 °C for 10 min. The absorbance at 410 nm was recorded. Oleanolic acid and Tris-buffer was used as a positive control and negative control, respectively. The inhibition percentage was calculated as follows.

Inhibition (%) = [($OD_{control}$ – OD_{sample})/OD_{sample}] × 100

3.6 Extraction and isolation of active compound

Dried heartwood powder of *S. garrettiana* (500 g) was extracted with ethanol (2 L) using the sonication method for 1 h, to obtain a reddish-brown extract (150 g). The ethanol extract was subjected to silica gel column vacuum chromatography. Hexane, dichloromethane, ethyl acetate and methanol were used as mobile phases, using a step gradient elution. Four fractions (1-4) were obtained after fractionation. All of the fractions were then subjected to anti-hyaluronidase and anti-elastase assay. The strongest inhibitory activity fraction 3 (ethyl acetate 53 g) was further purified by silica gel column eluted with a mixture of hexane and ethyl acetate (7:3 v/v) to give fifteen pooled fractions (fractions I-XV). The fractions were then subjected to anti-hyaluronidase and anti-elastase assay. The anti-hyaluronidase and anti-elastase active fractions (fractions VI and XII) were subjected to further purification processes as follows. Fraction VI (10 g) was further purified by a silica gel column using hexane and ethyl acetate (9:1 v/v) as eluent. A white crystal (compound 1; 25 mg) was obtained from the subsequent silica gel column of fraction VI (VI-7). Fraction XII was purified by silica gel column eluted with a mixture of dichloromethane and methanol using a step-gradient elution starting from 10% methanol to 50% methanol, to give ten pooled fractions (fractions XII-1 - XII-10). Compound 2 (32 mg) was obtained from fraction XII-4 after being purified using a Sephadex LH‑20 column using methanol as eluent.

3.7 Identification of isolated compounds

Compound 1 and 2 were identified based on their ^{1}H-NMR spectra data. The ^{1}H-NMR spectral data were compared with the data in the literature.

Betulinic acid (compound 1; white crystal) ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 4.66 (1H, d, H-3), 4.54 (1H, s, H-29), 2.95 (1H, m, H-19), 1.62 (3H, s, H-30), 0.95 (3H, s, H-27), 0.90 (3H, s, H-26), 0.84 (3H, s, H-23), 0.74 (3H, s, H-25), 0.62 (3H, s, H-24).

Piceatannol (compound 2; white powder) ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.97 (1H, d, J = 2.0 Hz, H-6'), 6.89 (1H, d, J = 16.0 Hz, H-8), 6.83 (1H, dd, J = 2.0, 8.0 Hz, H-5'), 6.76 (1H, d, J = 16.0 Hz, H-7), 6.74 (1H, d, J = 8.0 Hz, H-2'), 6.43 (2H, d, J = 2.0 Hz, H-2; H-6), 6.16 (1H, t, J = 2.0 Hz, H4).

3.8 Statistical analysis

All data were expressed as mean ± SD of three independent experiments assayed in triplicate. Statistical significance of differences between means was established by the Student *t*-test. P values < 0.05 were examined as statistically significant. IC_{50} values were obtained by interpolation from the linear regression analysis.

4. Results and Discussion

The percent yield data of the crude extract of different plants are shown in Table 2. *D. parviflora* produced the highest crude extract yield of 26.25%, then *E. antiquorum*, *D. loureirin* and *P. merkusii*, respectively while *T. siamensis* resulted in the lowest yield.

The anti-hyaluronidase and elastase activities of plant extracts were evaluated. All plant extract used in this study exhibited hyaluronidase inhibitory activity. While *S. garrettiana* extract showed the most potent activity with percent inhibition of 30.4 ± 0.8 (Table 3).

Table 2: The yield percentage of the extracts of different plants

Plants	% Yield of crude extract ± SD
C. garrettiana	2.63 ± 0.12
P. merkusii	18.4 ± 1.31
D. parviflora	26.25 ± 2.51
T. hoaensis	1.24 ± 0.03
D. loureiri	11.85 ± 0.83
T. siamensis	0.48 ± 0.02
M. elengi	3.32 ± 0.45
E. antiquorum	19.1 ± 1.02
P. ribesoides	3.51 ± 0.31
O. indicum	1.58 ± 0.01

Table 3 Anti-hyaluronidase and anti-elastase activities of the extracts

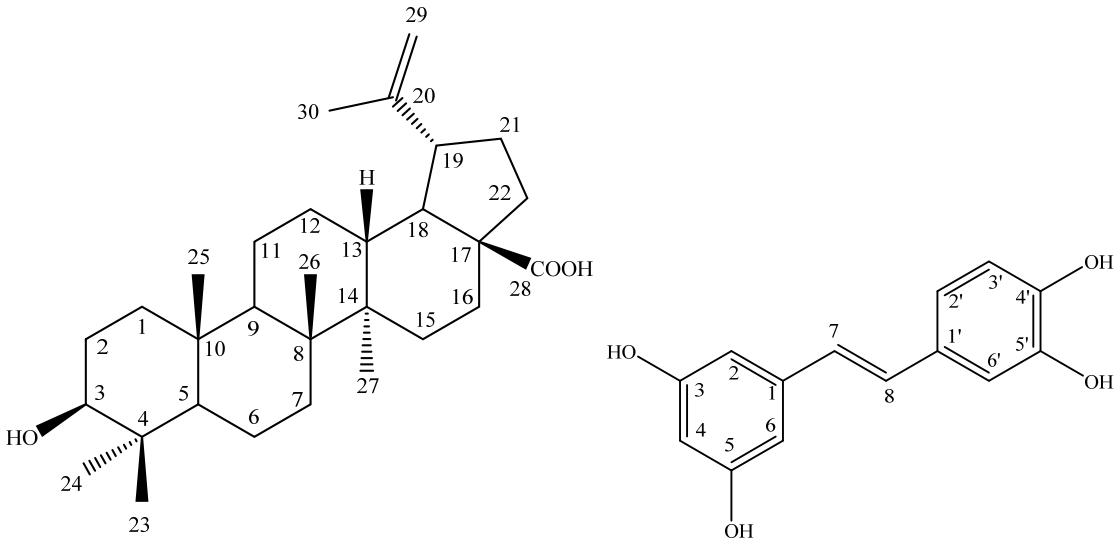
Plants extract	% Inhibition ± SD	
	Hyaluronidase	Elastase
S. garrettiana	30.4 ± 0.8	62.5 ± 0.6
P. merkusii	13.0 ± 0.4	-
D. parviflora	14.6 ± 0.7	32.0 ± 2.9
T. hoaensis	5.2 ± 0.7	-
D. loureiri	9.1 ± 0.3	23.7 ± 1.2
T. siamensis	5.4 ± 0.5	10.7 ± 3.9
M. elengi	6.7 ± 0.3	-
E. antiquorum	12.8 ± 0.1	28.2 ± 0.4
P. ribesoides	10.9 ± 0.8	-
O. indicum	10.0 ± 0.3	-
Oleanolic acid (25µg/ml)	40.6 ± 0.2	73.5 ± 0.6

*The extract concentration used in anti-hyaluronidase and anti-elastase activities studies were 0.5 mg/ml and 25 µg/ml, respectively.

Five plant extracts including *S. garrettiana*, *D. parviflora*, *D. loureiri*, *T. siamensis* and *E. antiquorum* were capable of inhibiting the elastase activity at the concentration of 0.25 mg/ml. However, *S. garrettiana* extract showed the highest anti-elastase activity with the percent inhibition of 62.5 ± 0.6 when compared to other plant extracts while the standard, oleanolic acid showed the percent inhibition of 73.5 ± 0.6.

In the present study, preliminary screening of the extracts exhibiting anti-hyaluronidase and elastase activity from ten plants extract revealed that *S. garrettiana* heartwood extract showed the most effective in inhibition of hyaluronidase and elastase activity. Thus, *S. garrettiana* heartwood extract may have a potential role in skin care cosmetics due to their enzyme inhibition activity.

The active compounds in the extracts possessing effectiveness in anti-aging enzymes will be further characterized. The anti-hyaluronidase and anti-elastase activities of *S. garrettiana* heartwood has not been investigated in any research yet. Thus, the compounds exhibiting anti-enzyme activity in *S. garrettiana* heartwood extracts are needed to be investigated. The ethanol extract of *S. garrettiana* was therefore selected and subjected to isolation of the anti-hyaluronidase and anti-elastase active compounds base on bioassay-guided isolation. Our phytochemical studies on the ethanol extract prepared from the heartwood of *S. garrettiana* resulted in the isolation of two previously known compounds. The chemical structures of these compounds were identified as betulinic acid (1) and piceatannol (2) (Figure 1) by comparing their ^{1}H-NMR spectroscopic data with the relevant works of literature previously published (Kim et al., 2009; Madaka & Charoonratana, 2018).



(1) **(2)**

Figure 1 Structure of isolated compounds from heartwood of *S. garrettiana*; betulinic acid (1) and piceatannol (2)

The isolated compounds were investigated for their anti-hyaluronidase and anti-elastase activities. The test solutions for each substance were prepared at varying concentrations (2-100 µg/ml). The results revealed that betulinic acid and piceatannol showed strong anti-elastase activities with IC_{50} values of 20.5 and 15.2 ug/ml, respectively. However, these isolated compounds did not show anti-hyaluronidase activity at the concentration of 100 µg/ml. The results are summarized in Table 4.

Table 4 Hyaluronidase and elastase inhibitory activities of isolated compounds from heartwood of *S. garrettiana*

Compounds	**IC_{50} (µg/ml)**	
	Hyaluronidase	**Elastase**
Betulinic acid	n.d.	20.5
Piceatannol	n.d.	15.2
Oleanolic acid	95.4	4.3

n.d. = not determined

Our findings indicate *S. garrettiana* heartwood extracts have a potential use on anti-aging skincare products due to their aging-enzyme inhibition properties.

5. Conclusions

The plant extracts showing the potential to be developed as an anti-aging product were determined in this study. The results revealed that the heartwood extracts of *S. garrettiana* showed the most potent anti-hyaluronidase and anti-elastase activity. Therefore, the extracts of *S. garrettiana* showed effective inhibitory activity against hyaluronidase and elastase that might be beneficial for use as an anti-aging agent. Bioassay-guided isolation of *S. garrettiana* heartwood extract led to the isolation of betulinic acid and piceatannol. We determined their hyaluronidase and elastase inhibition for the development of skin anti-aging ingredients in cosmetic formulation. Among the isolated compounds exhibited strong elastase inhibition activity, the investigated activities of the extracts, as well as the isolated compounds from *S. garrettiana* heartwood extract, implied that this plant could be a potential candidate for the development of novel cosmetic additives.

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