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Identification of potential and selective collagenase, gelatinase inhibitors from *Crataegus pinnatifida*

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ABSTRACT

Four oligomeric procyanidins were isolated from the MeOH extracts of the leaves of *Crataegus pinnatifida* (Rosaceae). Investigation of collagenase and gelatinase inhibitory natural components afforded four oligomeric procyanidins. Of these, **2** and **3** exhibited collagenase inhibitory activity (IC_{50}) at a concentration of less than 1 μM, and **3** showed gelatinases A and B inhibitory activity (IC_{50}) at 0.4 and 2.3 μM, respectively.

Collagenase and gelatinase are a family of zinc-dependent endoproteinases that play pivotal roles in the dynamic remodeling of extracellular matrix. The enzyme that specifically cleaves collagen is collagenase. Collagen is the major protein of connective tissue with a unique triple helical structure.[1] Based on substrate preference and structural homology, matrix metalloproteinases (MMPs) are sub-classified into functional groups: collagenases, gelatinases, stromelysins, matrilysins, membrane type-MMPs (MT-MMPs) and other non-classified MMPs.[2] This enzymes are frequently over-expressed by the various extracellular stimuli including growth factors, cytokines and tumor promoters which have a crucial role in normal physiological processes such as embryogenesis and the aberrant expression of MMPs is associated with many pathological abnormalities such as tumor invasions.[3] *Crataegus pinnatifida* (Hawthorn) is widely distributed in the northeast part of China, Japan and South Korea. It is used as a medicinal plant to improve digestion, remove retention of food, promote blood circulation and resolve blood stasis both in traditional and folk medicine. The species most often used are *Crataegus monogyna* and *Crataegus laevigata*.[4] Dried flowers, leaves and fruits are used as crude drugs. Several studies have been shown to increase myocardial contractility, reduce reperfusion arrhythmias, dilate peripheral arteries, and mildly decrease blood pressure.[5] Currently, hawthorn leaves, flowers, and both green (unripe) and red (ripe) berries are used to make herbal preparations to treat patients with severe heart disease. Oligomeric procyanidins and (−)-epicatechin are considered to be the main active constituents, in addition to flavone- and flavonol-type flavonoids.[6] This paper reports that the isolation of components from the extract of the leaves of *C. pinnatifida* based on collagenase inhibitory activity afforded four oligomeric procyanidins and gelatinases A and B inhibitory activities of **3** were also evaluated. TLC and preparative HPLC methods of the EtOAc-soluble fraction of the MeOH extract of *C. pinnatifida* (leaves) led to the isolation of four oligomeric procyanidins. **1** was the main trimer, epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin. **2** was identified as epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin. **3** was identified as epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, and **4** was identified as epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8) epicatechin. The **1** and **3** have previously been reported from Crataegus leaves and flowers by Rohr.[7] The compounds **2** and **4** have previously been reported from Crataegus leaves and flowers by Svedstroma et al.[8] (see Fig. 1).The *n*-$CHCl_3$- and H_2O-soluble fraction from methanol extract exhibited weak collagenase (IC_{50} >50 μg/ml). However, the EtOAc fraction exhibited higher collagenase activity (IC_{50} = 34.2 μg/ml). So, we investigated carefully the inhibitory activity of collagenase by the EtOAc fraction from *C. pinnatifida.* The collagenase inhibitory activity of isolated four compounds was tested using a literature method.[9] As shown in Table 1, the four compounds were active with IC_{50} values ranging from 0.3 μM to 22 μM. In particular, **3** was found to have the most effective activity of other compounds and the positive control. Positive control (phosphramidon) inhibitory effect on collagenase

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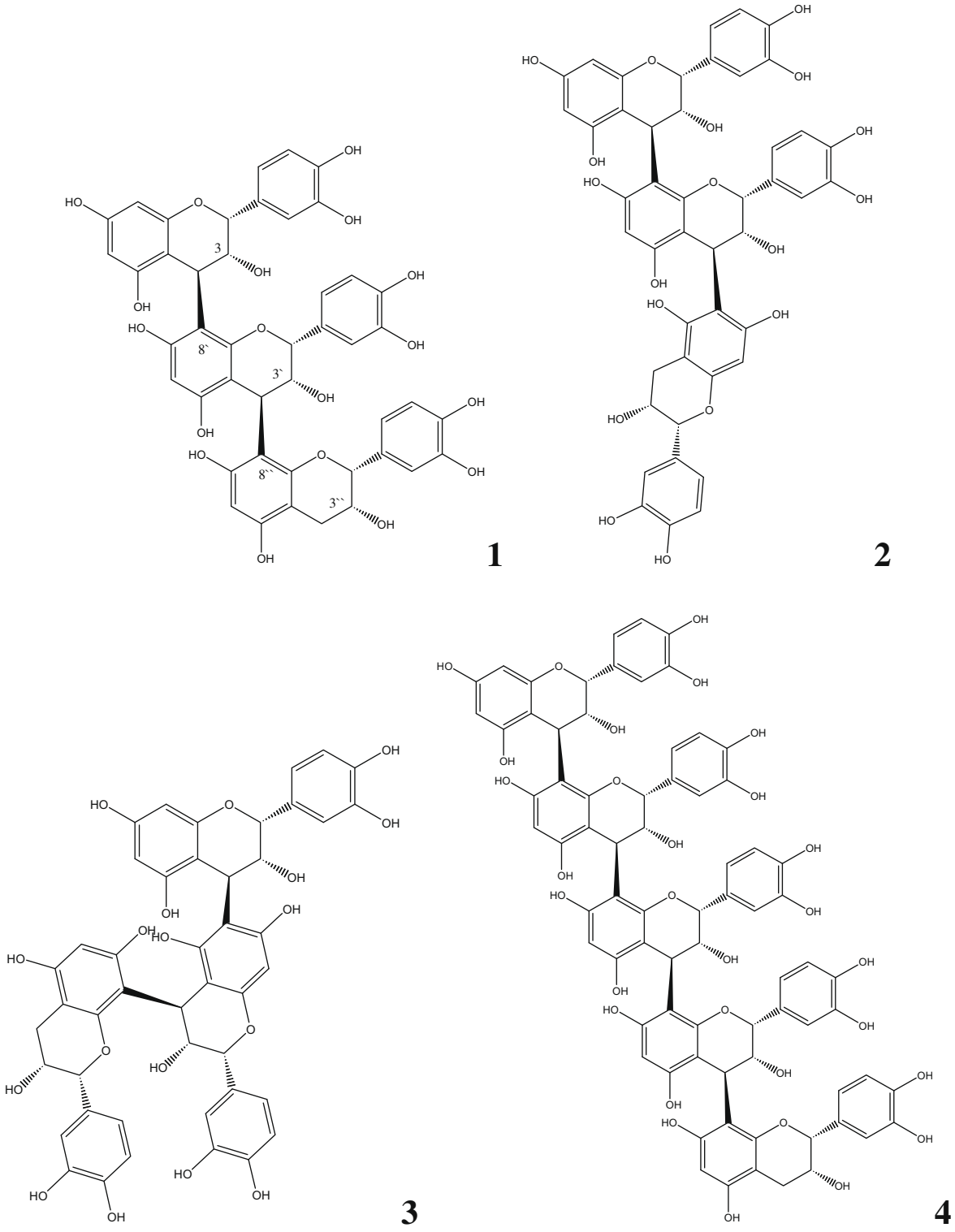

Figure 1. Structure of compounds **1**–**4**.

with IC_{50} values of 7.63 μM respectively. The fact that **3** was more active than **2**, suggested that the connecting position and/or the linkage of epicatechin-(4β→6) in the epicatechin-(4β→6) moiety might be important for inducing collagenase inhibition. Next the gelatinases A and B inhibitory activity of **2** and **3** was evaluated. As shown in Table 2, the IC_{50} of gelatinases A and B inhibitory activities of **3** were 0.4 and 2.3 μM, respectively. As the activities of **3** were stronger than that of **2**, it is likely that the connecting

Table 1
Collagenase inhibitory activity of compounds **1**–**4**

Compounds	IC_{50} (μM)
1	11.3 ± 1.3
2	0.98 ± 0.08
3	0.34 ± 0.05
4	21.4 ± 1.9
Phosphramidon	7.63 ± 1.4

The values are mean ± SD (n = 5).

Table 2
Gelatinases A and B inhibitory activity of compounds **2** and **3**

Compounds	IC_{50} (μM)	
	Gelatinase A	Gelatinase B
1	31.4 ± 3.4	53.2 ± 4.8
2	12.4 ± 1.3	27.3 ± 1.5
3	0.4 ± 0.1	2.3 ± 0.9
4	43.2 ± 6.3	39.2 ± 5.1
Chlorhexidine	7.63 ± 1.4	7.63 ± 1.4

The values are mean ± SD (n = 5).

position and/or the linkage of epicatechin-(4β→6) in the epicatechin-(4β→6) moiety also had a key role in their inhibitory activities. Some synthetic MMP inhibitors are currently in clinical trials for cancer treatment but carry undesirable side effects.

C. pinnatifida (leaves) was collected at Pohang, Kyungsangbuk-do, South Korea, in July 2004, was identified by Dr. Tae-Jin Kim, KRIBB, Eoeun-dong, Yuseong-gu, Daejeon, South Korea. The air dried powder of the leaves (1.0 kg) of *C. pinnatifida* was extracted by percolation in 95% methanol (4 l) at room temperature for one week and filtered. The residue was re-percolated again. This process was repeated four times. The combined methanol extracts were concentrated under reduced pressure at a temperature not exceeding 35 °C to yield a dry extract (365 g). Water (1 l) was added and the resultant mixture successively extracted with chloroform, ethyl acetate, and *n*-butanol, respectively. The ethyl acetate phase was evaporated to dryness, and the raw extract (5 g) was transferred to a polyamide CC 6 column (30 cm × 20 mm id). Elution was performed with methanol (500 ml), methanol/water (7:3) (500 ml) and acetone/water (7:3), and fractions (20 ml) were collected. The flow rate was 1.5 ml/min. Elution of the compounds was monitored by TLC and HPLC. The eluents were evaporated under reduced pressure (below 35 °C), and the residue was freeze-dried. Compounds **1–3** were isolated in fractions 101–113 from polyamide CC 6 column. The combined fractions were rechromatographed with ethanol on a Sephadex LH-20 column (33 cm × 15 mm i.d.), and fractions (10 ml) were collected. Compound **1** (R_f 0.43, t_R 19.2 min, $[M+H]^+$ at m/z 867) was isolated from fractions 32–41. Compound **2** (R_f 0.48, t_R 24.2 min, $[M-H]^+$ at m/z 865) was isolated from fractions 54–57, and **3** (R_f 0.46, t_R 16.8 min, $[M-H]^+$ at m/z 865) from fractions 63–66 by semi-preparative HPLC. Compound **4** (R_f 0.11, t_R 21.0 min) was isolated in fractions 172–179 from polyamide CC 6 column, +ESI-MS m/z: $[M+H]^+$ 1443. The TLC separations were performed with ethyl acetate/formic acid/acetic acid/water (75:3:2:20) according to Vanhaelen and Vanhaelen-Fastre.[10] The spots were made visible with vanillin (1%)-sulfuric acid. The elution conditions were as described in Rigaud et al.:[11] solvent A 2.5% acetic acid, solvent B acetonitrile/2.5% acetic acid (80:20). Linear gradients: solvent B 5–50% in 35 min, 50–100% in 40 min. Flow rate 1 ml/min, detection at 280 nm detection at 280 nm. Column: YMC-GEL ODS-A (12 nm, S-75 mm, YMC). The elution conditions in semi-prep. HPLC were: solvent A 2.5% acetic acid, solvent B methanol/2.5% acetic acid (80:20), solvent B 0–30% in 20 min. Collagenase, gelatinase A and gelatinase B inhibitory activity were examined using the modified method described by Ohtsuki et al.[9] Collagenase type IV (EC 3.4.24.3) was purchased from Sigma–Aldrich (Seoul, South Korea). The substrate peptide, MOCAc-Pro-Leu-Gly-Leu-A2pr(Dnp)-Ala-Arg-NH2, and phosphramidon were obtained from the Peptide Institute (Takara, Seoul. South Korea). Gelatinase A proenzyme from human rheumatoid synovial fibroblasts (EC 3.4.24.24), Gelatinase B monomer from human neutrophils (EC 3.4.24.35) and chlorhexidine were purchased from Calbiochem (La Jolla, USA). Briefly, the test samples, enzyme solution (final concentrations, collagenase: 10 μg/ml; gelatinase A and gelatinase B 9: 0.5 μg/ml) and 50 mM Tris–HCl buffer (pH 7.3) were added to 96-well microtitre plate, and preincubated for 10 min at 37 °C. Afterwards, the substrate solution ((7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-leucylglycyl-L-leucyl-[*N*β-(2,4-initrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) at a final concentration of 10 μM was added to initiate the reaction. The fluorescence values were measured at an excitation of 320 nm and an emission of 405 nm after 0 min and 30 min incubation at 37 °C using a fluorescence plate reader. These assays were performed in triplicate using phosphramidon or chlorhexidine as a positive control.[12] The inhibition ratio of the samples was calculated by comparing the fluorescence increase produced by the sample with that of the negative control.

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