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Prenylated Flavonoids from *Epimedium koreanum* Nakai and their Human Neutrophil Elastase Inhibitory Effects

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Abstract: Human neutrophil elastase (HNE) has been linked to immune system functions, including inflammation, as well as the development of skin wrinkles, suggesting that HNE inhibitors have many practical applications. In this study, the methanol extract of *Epimedium koreanum* Nakai contained potent HNE inhibitory flavonoids (**1–4**) with IC_{50} values of 6.06–125.80 μM. Prenyl groups within these flavonoids were critical for the inhibitory effects. For example, epimedokoreanin B (**1**) (IC_{50} = 6.06 μM) showed 6-fold higher efficacy than that of its parent luteolin (IC_{50} = 36.01 μM). A similar trend was observed for compound **2** (IC_{50} = 6.28 μM) and its parent, apigenin (IC_{50} = 37.94 μM). Lineweaver–Burk plots showed that the compounds exhibit mixed-type inhibition. In a detailed kinetic study, compound **1** was assigned to mixed type I and exhibited the greatest binding to the free enzyme (K_I = 11.7 μM, K_{IS} = 40.9 μM).

Keywords: *Epimedium koreanum* Nakai; prenylated flavonoids; epimedokoreanin B; human neutrophil elastase.

1. Introduction

Human neutrophil elastase (HNE, EC 3. 4. 21. 37) belongs to serine protease family of enzyme, that are able to hydrolyze the extracellular matrix. HNE is a key enzyme for the degradation of foreign microorganisms phagocytized by neutrophils [1]. It also modulates the expression of cytokines involved in immune responses, e.g., it upregulates interleukin-8 (IL-8) and transforming growth factor β (TGF-β) and downregulates IL-1, IL-2, IL-6, and TNF-α [2]. Tissue infections by extracellular bacteria as well as various noninfectious diseases are characterized by the extravascular recruitment of neutrophils, which secrete HNE. The balance between HNE and its inhibitors is essential to sustain normal physiological conditions [3]. HNE is inhibited and controlled by serpins, such as α_1-antitrypsin, elafin, and secretory leukocyte proteinase inhibitor (SLPI) [4−6]. These inhibitors are inactivated by large quantities of oxidants and proteinases released by leukocytes that are recruited to sites of inflammation. Excess HNE is also an important pathological factor in the development of

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several chronic inflammatory lung conditions [7,8]. HNE inhibitors have become therapeutic targets for the control of tissue injury in neutrophilic inflammation in humans. In addition to their roles in immune responses and inflammation, HNEs with a wide range of substrate specificities destroy connective tissue proteins, such as elastin, fibronectin, collagen, and cartilage tissue, and are involved in the formation of wrinkles during aging or UV stress [9]. Therefore, skin aging can be prevented by inhibiting HNE activity [10].

Epimedium koreanum Nakai is a deciduous, herbaceous flower bearing plant belonging to the family Berberidaceae. It is widely distributed in Japan, Korea and China. In traditional Korean and Chinese herbal medicine, the aerial parts of this plant have been used for thousands of years to treat infertility, cardiovascular disease, amnesia, lumbago, impotence neurasthenia, arthritis, tonic, and several immune-modulatory disorders [11]. The primary bioactive constituents of this species are prenylated flavonoids and icariin. These bioactive constituents exhibit anti-inflammatory, ant-osteoporosis anti-oxidant, antidepressant, and neuroprotective effects [12,13].

In this study, we isolated HNE inhibitory flavonoids from *E. koreanum* Nakai, and evaluated each with respect to HNE inhibitory activity. The role of prenyl groups in HNE inhibition was also established by comparisons with parent compounds. The mechanisms by which the compounds target proteases were ascertained by kinetic plots. To the best of our knowledge, this report is the first to examine the inhibition of HNE by flavonoids isolated from *E. koreanum* Nakai.

2. Materials and Methods

2.1. Instruments

^{1}H and ^{13}C NMR as well as 2D NMR data were measured on the Bruker AM-500 Spectrometer (300, 500 MHz; Bruker, Karlsruhe, Germany) in methanol-d_4 or acetone-d_6 with trimethylsilane as an internal standard. HREIMS and HRFABMS analyses were performed using the JMS-700 Mass Spectrometer (JEOL, Tokyo, Japan). Enzymatic assays were performed using a SpectraMax M_3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.2. Plant Materials and Chemicals

E. koreanum Nakai [as permitted by Korea Food and Drug Administration (KFDA)] (approximately 1 kg) was purchased from a local medicinal market. Human neutrophil elastase (EC 3.4.21.37) and methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide were purchased from ENZO Life Sciences, Inc. (Farmingdale, NY, USA). Luteolin and apigenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Separation was performed using various resins, such as an octadecylsilanized (ODS) silica gel (50 μm; YMC Ltd., Kyoto, Japan), silica gel (230–400 mesh; Merck Co., Darmstadt, Germany), and Sephadex LH-20 (50 μm; Amersham Pharmacia Biotech, Uppsala, Sweden). TLC was performed using precoated Silica Gel 60 F_{254} Plates (0.25 mm; Merck) and spots were visualized by UV illumination or with 10% sulfuric acid solution spray followed by heating.

2.3. Extraction, Fractionation, and Isolation of E. koreanum Nakai

Dried and powdered *E. koreanum* Nakai (1 kg) was extracted using MeOH (4 × 10 L) at room temperature for 7 days with frequent shaking. The methanol extract (113 g) was concentrated, filtered, and partitioned sequentially with H_2O and *n*-hexane. The water layer was partitioned with EtOAc, *n*-BuOH, and H_2O. The EtOAc fraction (24 g) was subjected to chromatography on a silica gel column with a stepwise gradient flow of an *n*-hexane/EtOAc mixture (from 20:1 to 0:100), to obtain 50 fractions (F1–F50, each 100 mL). Fractions F18–28 were concentrated to obtain a mixture (7.5 g) with good potential to include HNE. This crude mixture (5 g) was subjected to MPLC using a C18 column

(300 g) using a gradient elution with methanol (0% to 100%) in H_2O and a 20 mL/min flow rate to obtain 80 subfractions (S1–S80) [14]. Subfractions S23–29, enriched for compound **4,** were further purified using the Sephadex LH-20 (MeOH/H_2O mixture, 85:15) to obtain compound **4** (30.3 mg). Subfractions S35–38, enriched for compounds **1** and **3,** were purified using the Sephadex LH-20 (MeOH) to obtain compounds **1** (46.1 mg) and **3** (30.7 mg). Subfractions S57–61, enriched for compound **2,** were purified using the Sephadex LH-20 (MeOH/H_2O mixture, 90:10) to obtain compound **2** (21.3 mg). The purities of all compounds were determined as more than 95% based on ^{1}H-NMR and HPLC.

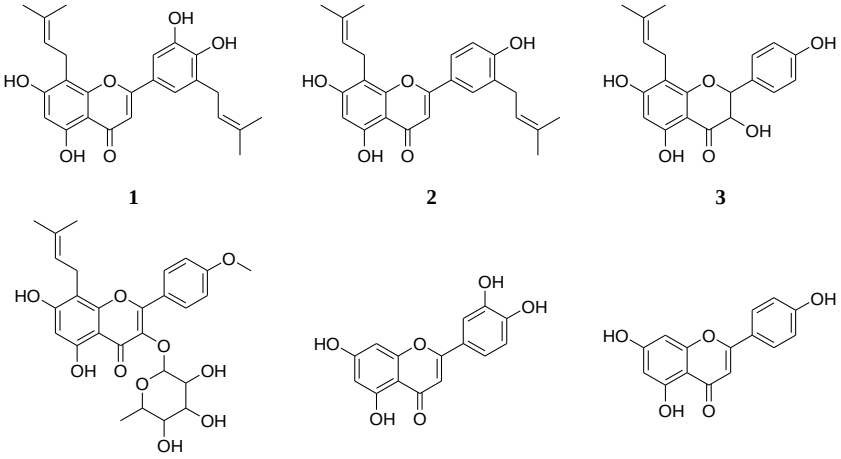

Figure 1. Chemical structures of flavonoids (**1-4**) from *E. koreanum* Nakai and parent compounds.

2.4. Human Neutrophil Elastase Inhibitory Activity

Human neutrophil elastase (HNE, EC 3. 4. 21 37) inhibitory activity was examined using a previously described method, with modifications [15,16]. HNE activity was measured using the substrate methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide in 96-well plates. The initial rate (enzyme activity) was determined by immediately detecting the degree of formation of *p*-nitroaniline at 405 nm for 6 min at 13-s intervals. We dissolved the samples in 100% dimethylsulfoxide (DMSO) at a stock concentration of 1000 ppm and diluted as needed. The mixture containing 130 mL of 0.02 mM Tris-HCl buffer solution (pH 8.0) and 20 μL of enzymes (0.01 Unit/mL) was preincubated at 25 °C for 2 min in the presence of 10 μL of inhibitors or control (DMSO). Then, 40 μL of substrate was added to initiate the reaction. Each analysis was separately repeated three times. The inhibition caused by compounds were expressed as the concentration (IC_{50}) that decreased 50% of the enzyme activity. IC_{50} values were calculated using non-linear regression to fit the data according to the following equation: inhibition (%) = [(rate of control reaction - rate of sample reaction)/rate of control reaction] × 100.

2.5. Kinetic Assay of HNE and Progress Linear Determination

The kinetic study of HNE inhibitors (**1** and **2**) were assessed by double reciprocal Lineweaver–Burk plots. Similarly the kinetic parameters associated with the inhibitory mechanism of HNE were determined by progress curves, which were obtained for different inhibitor concentrations and various substrate concentrations. K_I and K_{IS} are the coupling constants for the inhibitors binding to the free enzyme or enzyme-substrate complex. Hereafter, methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide was used as a substrate. These two constants were determined from the second plots of Lineweaver–Burk plots, the slopes of the straight lines or vertical intercept ($1/V_{max}$) versus the concentration of each inhibitor. These parameters are given in Equations **(1)–(3)** [17, 18]:

$$\frac{1}{V} = \frac{K_m}{V_{max}}(1 + \frac{[I]}{K_I}) \times \frac{1}{S} + \frac{1}{V_{max}}(1 + \frac{[I]}{K_{IS}}) \quad (1)$$

$$\text{Slope} = \frac{K_m}{K_I V_{max}}[I] + \frac{K_m}{V_{max}} \quad (2)$$

$$\text{Intercept} = \frac{K_m}{K_{IS} V_{max}}[I] + \frac{K_m}{V_{max}} \quad (3)$$

3. Results and Discussion

During solvent fractionation of the methanol extract of *E. koreanum* Nakai, we found that the ethylacetate fraction had potent inhibitory activity against HNE with an IC_{50} of 35 μg/mL. The significant inhibition by ethylacetate fraction encouraged us to determine the compounds responsible for its HNE inhibition. Subsequent bioactivity-guided isolation of the ethylacetate fraction yielded prenylated flavonoids (**1**–**4**), which were purified by MPLC over a octadecyl-functionalized silica gel and silica gel. The structures of isolated flavonoids (**1**–**4**) were identified using established spectroscopic analysis methods and comparisons with previously reported data. Isolated flavonoids (**1**–**4**) were identified as epimedokoreanin B (**1**), 5, 7, 4′-trihydroxy-8, 3′-prenylflavone (**2**), neophellamuretin (**3**), and icariside II (**4**) [19]. The structure of the most effective compound, i.e., compound **1,** was briefly elucidated as follows. It had the molecular formula $C_{25}H_{26}O_6$ and twelve degrees of unsaturation, established by HREIMS (*m/z* 422.1727 [M^+], calcd 422.1729). An analysis of the unsaturation degree pointed a tricyclic skeleton with two aromatic rings. ^1H and ^{13}C in conjugation with DEPT experiments indicated the presence of 25 carbon atoms, consisting of 2 methylenes (sp^3), 6 methines (sp^2), 4 methyls, and 13 quaternary carbons. HMBC correlation clearly indicated that two 3, 3-dimethylallyl groups were located on C8 and C5′.

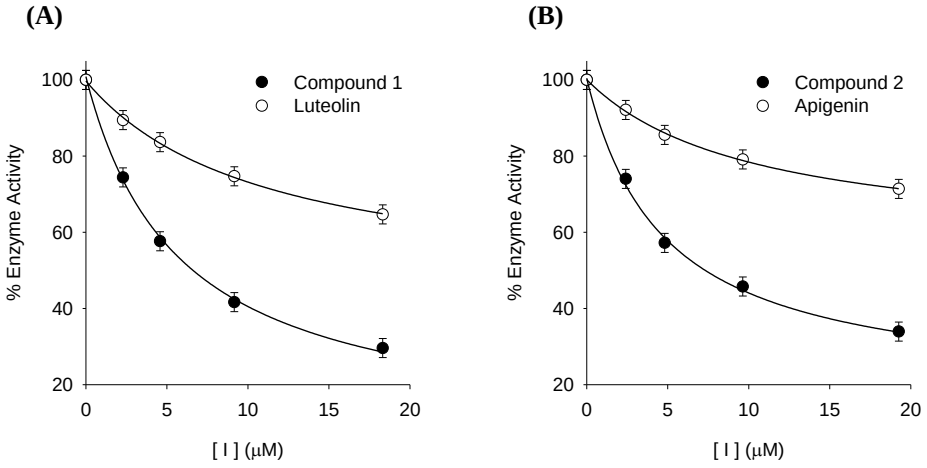

Figure 2. Effects of compound **1** and **2** on HNE compared with parent compounds, luteolin and apigenin.

The prenylated flavonoids (**1**–**4**) and their parent compounds luteolin and apigenin were screened for in vitro HNE inhibitory activity at various concentrations using methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide as a substrate, whereas caffeic acid was used as positive control [20]. All isolated flavonoids (**1**–**4**) inhibited HNE in a dose-dependent manner with IC_{50} values ranging from 6.06 to 134.34 μM. Prenylated compounds (**1** and **2**) were much more effective than their parent compounds [21]. For example, epimedokoreanin B (**1**) inhibited HNE with an IC_{50} of 6.06 μM, but its parent compound luteolin (IC_{50} = 36.01 μM) was less active. Epimedokoreanin B (**1**) was 6-fold more effective than luteolin. Similar trend were also observed for compound **2** (IC_{50} = 6.28 μM) and apigenin (IC_{50} = 37.94 μM). Given the low efficacies of luteolin and apigenin, the prenyl fractions

within the isolated flavones (**1** and **2**) appear to play critical roles in the inhibition of HNE. In particular, a dramatic difference in dose-dependence curves was observed between prenylated flavonoids and their corresponding parent compounds, as shown in Figure 2.

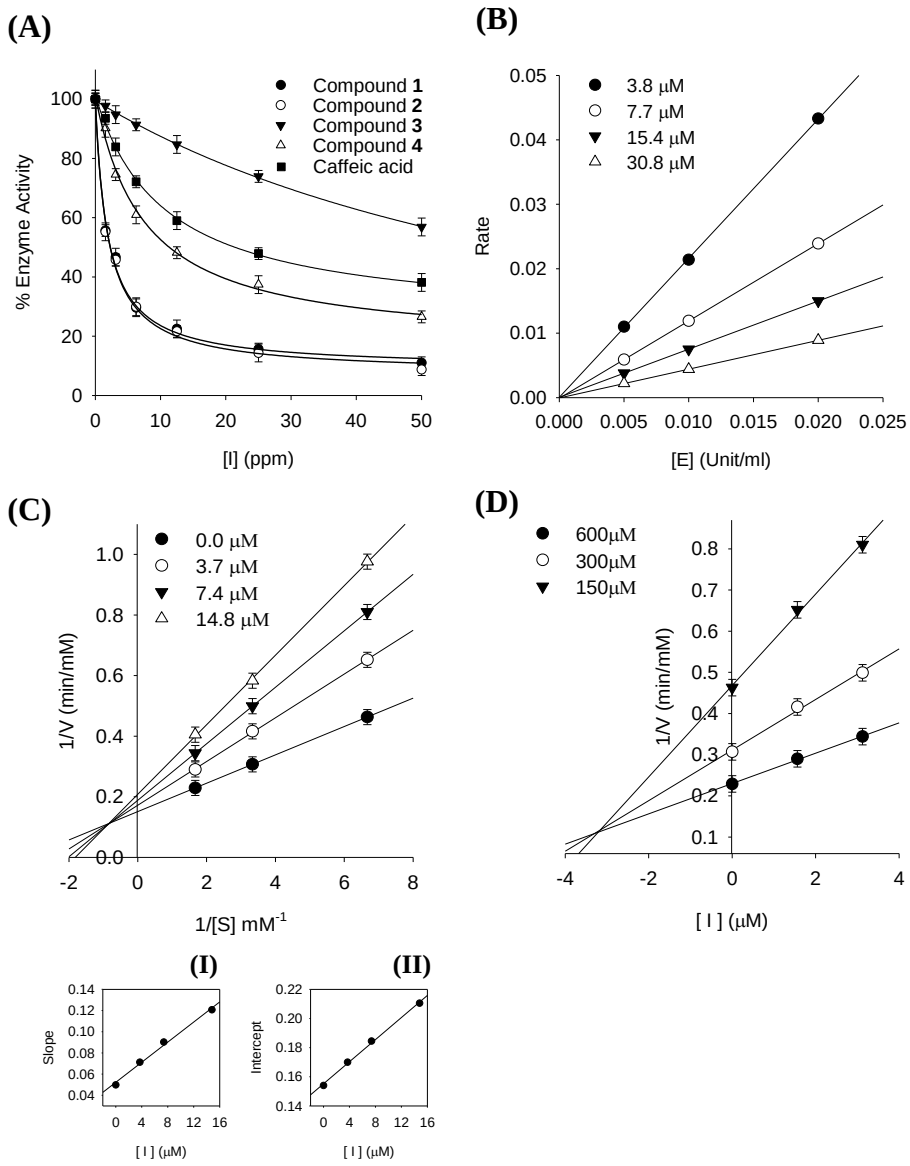

Figure 3. (**A**) Effects of isolated compounds (**1**-**4**) on human neutrophil elastase for the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-*p*NA (● compound **1**; ○ compound **2**; ▼ compound **3**; ∆ compound **4**; ■ caffeic acid), (**B**) catalytic activity of HNE as a function of enzyme concentration at different concentration of compound **1**. (**C**) Lineweaver-Burk plot were constructed for the inhibition of HNE by compounds **1**. Insets (I) and (II) represent secondary plot of slope and intercept of the straight lines versus concentration of compounds **1**, respectively. (**D**) Dixon plots for the inhibition of compound **1** on HNE activity.

For all isolated compounds (**1**–**4**), a similar relationship between enzyme activity and concentration was observed. Increasing the concentration of the inhibitors drastically lowered residual enzyme activity (Figure 3A). The inhibition of HNE by compound **1**, the most potent inhibitor (IC_{50} = 6.06 μM), is illustrated in Figure 3B. A family of straight lines passing through the origin was observed on the plots of the remaining enzyme activity against enzyme concentration for different inhibitor concentrations (Figure 3A). The presence of an inhibitor did not affect the amount of enzyme, but decreased the enzyme activity. An increase in the inhibitor concentration was associated

with a decrease in the slope of the regression line. These results indicate that compound **1** is a reversible inhibitor. In Lineweaver–Burk plots, substrate concentration increasing order resulted in a family of lines that shared a common intercept on the left side of the *y*-axis and above the *x*-axis, indicating that compound **1** exhibited mixed inhibition kinetics. The K_i value of compound **1** was 3.22 μM based on the Dixon plot. The K_i values of the other compounds (**2**–**4**) are shown in Table 1. More detailed parameters can be derived by changing the concentration of the substrate and inhibitor and measuring the residual enzyme-substrate complex. Equations (2) and (3) can be used to obtain the parameters K_I and K_{IS}, and these can help identify the two possible mechanisms (mixed type I and II). The equilibrium constants for compound **1** binding to the free enzyme (K_I) and to the enzyme-substrate complex (K_{IS}) were obtained from the second plots of K_m/V_{max} and $1/V_{max}$ against the concentration of compound **1,** respectively. This analysis showed that compound **1** had the following attributes: K_I = 11.6 μM and K_{IS} = 42.2 μM. Thus, the affinity of the inhibitors for the free enzyme was stronger than that of the inhibitor's affinity for the enzyme-substrate complex. Compound **1** was accordingly identified as a mixed type I inhibitor of HNE. Other compounds (**2–4**) showed similar inhibitory behavior to that of compound **1**.

Table 1. Inhibitory effect of isolated compound **1-4** on human neutrophil elastase.

Compound	Human Neutrophil Elastase				
	IC_{50} [a] value (μM)	K_i [b] (μM)	Inhibition Mode	K_I	K_{IS}
1	6.06 ± 0.52	3.22	Mixed type I	11.7	40.9
2	6.28 ± 0.78	2.10	Mixed type I	9.3	20.4
3	125.80 ± 6.68	19.40	Mixed type I	59.6	211.4
4	42.11 ± 2.95	16.43	Mixed type I	44.4	151.8
Luteolin	36.01 ± 1.15		NT[c]		
Apigenin	37.94 ± 2.06		NT		
Caffeic acid[d]	61.34 ± 3.74		NT		

[a] IC_{50} values of compounds represent the concentration that cause 50% enzyme activity loss.
[b] Balues of inhibition constant.
[c] NT is not tested.
[d] Positive control.

In conclusion, we analyzed the flavonoids displaying HNE inhibitory activity within *E koreanum* Nakai. HNE inhibitory flavonoids (**1–4**) had prenyl groups and showed mixed-type inhibition. Based on these findings, *E. koreanum* Nakai may be an excellent source of natural HNE inhibitors for food and medicinal uses.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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