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Human Neutrophil Elastase Inhibitory Alkaloids from *Chelidonium majus* L.

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Abstract Human neutrophil elastase (HNE) represents a good therapeutic target for the treatment of inflammatory diseases as well as invasion of microorganism. The methanol extract of a aerial part of *Chelidonium majus* L. showed high activity against the neutrophil elastase with an IC_{50} value of 100 μg/mL. Due to its potency, subsequent bioactivity-guided fractionation of methanol extract led to six alkaloids (**1**-**6**), which were identified as dihydrosanguinarine (**1**), (s)-stylopine (**2**), arnottianamide (**3**), (+)-chelidonine (**4**), spallidamine (**5**), and *N-trans*-feruloyltyramine (**6**). Among of them, three alkaloids (**2**, **5,** and **6**) inhibited HNE in a dose-dependent manner with IC_{50} ranging between 11.6 and 51.0 μM. Lineweaver-Burk and Dixon plots, and their secondary replots showed that alkaloids (**2**, **5**, and **6**) were mixed inhibitors of HNE. The analysis of K_I and K_{IS} value proved that all inhibitors (**2**, **5**, and **6**) had reversible mixed type I mechanism.

Keywords *Chelidonium majus* L. · human neutrophil elastase · inflammation · isoquinoline alkaloid

Introduction

The regulation of the enzymatic activity of human neutrophil elastase (HNE) has been the attractive field because neutrophils are the first cells recruited to inflammatory sites and from the earliest line of defense against the invasion of microorganism (Brice et al., 2010). The human neutrophil elastase (EC 3. 4. 21. 37) is the family of serine proteases that possess the ability to hydrolyze the extracellular matrix protein (Siedle et al., 2007). It is present in azurophil granules in the neutrophil cytoplasm. HNE is a proteolytic enzyme involved in pathogenesis of emphysema, adult respiratory distress syndrome and rheumatoid arthritis (Crocetti et al., 2013). The catalytic site of HNE molecule is composed of the triad His41-Asp99-Ser173, of which the oxygen of serine attacks carbonyl group on the target substrate (Bode et al., 1989). The activity of HNE is controlled by inhibitor named α1-antitrypsin produced in the liver. However, their HNE affinity is strongly decreased by oxidative stress and by proteases released from leukocytes that are recruited to inflammation sites (Brice et al., 2010). Thus, the imbalance between HNE and its inhibitors is implicated in many inflammation diseases as like pulmonary emphysema.

Chelidonium majus L. is a perennial herbaceous plant of the family Papaveraceae, which is widely distributed around the world. This plant has been traditionally used as an herbal medicine for treatment of gastric ulcer, oral infection and liver disease (Lenfield et al., 1981). These effects are mainly due to the alkaloids present in milk sap. The major bioactive components of this plant are isoquinoline alkaloids such as chelidonine, chelerythrine, sanguianarine, bebeerine and coptisine (Barreto et al., 2003). It included flavonoids and various acids such as ferulic, cumaric, caffeic and chelidonic acids (Barens J et al., 2007). The constituents of *C. majus* L. have been found to exhibit antitumor, antiviral, and anti-inflammatory activities (Colombo and Bosisio, 1996). Additionally, the potent and selective acetylcholinesterase inhibition was observed on 8-hydroxydihydrochelerythrine in *C. majus* L (Cho et al., 2006).

During a screening procedure on higher plant to find neutrophil elastase inhibitors, methanol extract of *C. majus* L. was shown to exhibit considerable inhibitory activity. In this study, we isolated six alkaloids targeting to HNE from the methanol extract of aerial

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portion of *C. majus* L. The isolated compounds were evaluated separately for their inhibitory activities against HNE. Their inhibition mechanism was ascertained using Lineweaver-Burk and Dixon plots. The literature revealed that no such work has been reported on the *C. majus* L.

Materials and Methods

Chemicals and Instruments. 1D and 2D NMR spectra were obtained using a Bruker AM-500 spectrometer (300, 500 MHz for ^{1}H, 75, 125 MHz for ^{13}C) in $CDCl_3$ or DMSO-d_6 with TMS as internal standard. ESIMS data and HRESIMS data were recorded on a JEOL JMS-700 mass spectrometer (JEOL, Japan). Enzymatic assays were carried out on a SpectraMaxM_3 Multi-Mode Microplate Reader (Molecular Devise, USA). Column chromatographies were performed using silica gel (230-400 mesh, Merck Co., Germany), Octadecylsilanized (ODS) silica gel (50 μm, YMC Ltd, Japan) and Sephadex LH-20 (50 μm, Amersham Pharmacia Biotech, Sweden). Fractions were monitored by precoated silica gel 60 F_{254} plates (0.25 mm, Merck), and spots were visualized by spraying with 10% sulfuric acid solution followed by heating. Ursolic acid was purchased from Sigma-Aldrich (USA). All solvents used for extraction and isolation were of analytical grade. *C. majus* was purchased from the local market and stored at a freezer before use.

Plant material, extraction and isolation. The dried aerial part of *C. majus* L. (1 kg) was purchased from local market extracted with methanol (18 L) two times at room temperature. The methanol soluble portion was concentrated to give the crude extract (96 g), which was portioned between H_2O and *n*-hexane. The aqueous layer was further partitioned with EtOAc to give the EtOAc extract (32 g), which was subjected to MPLC (PuriFlash® 450, Interchim, France) on silicagel with a gradient solvent system ($CHCl_3$-MeOH=50:1 to 10:1) to give four fractions (A-D). Potent inhibition targeting to neutrophil elastase was found on faction B that was further chromatographed on a silicagel column with a gradient solvent system (*n*-hexane-EtOAc=100:0 to 1:1) to give fifteen fractions (Fr1-Fr15). The Fr2 was purified by silicagel column with a gradient solvent system (*n*-hexane-acetone=100:1 to 1:1) to yield arnottianamide (37 mg, **3**). Fr4 was chromatographed on a sephadex LH-20 column with methanol as the eluent to give nine main fractions (Fr6-1-Fr6-9). Fr6-7 was purified by ODS column chromatography with 80% MeOH as the eluent to give dihydrosanguinarine (23 mg, **1**) and (s)-stylopine (41 mg, **2**). Fr6-8 was purified on a sephadex LH-20 column with MeOH as eluent to give (+)-chelidonine (19 mg, **4**). Fr7 was purified by ODS column chromatography with 80% MeOH as the eluent to give spallidamine (28 mg, **5**) and *N-trans*-feruloyltyramine (56 mg, **6**). The chemical structures of these compounds were determined by comparison of their spectroscopic data with those previously reported (Ma et al., 1999; Koul et al., 2002; Kanada et al., 2012).

*Dihydrosanguinarine (**1**)*: white powder; mp 185-188°C; EIMS, *m/z* 333 $[M]^+$; HREIMS, *m/z* 333.1004 (calcd for $C_{20}H_{15}NO_4$ 333.3374); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.68 (1H, d, *J*=8.6 Hz), 7.67 (1H, s), 7.47 (1H, d, *J*=8.6 Hz), 7.26 (1H, d, *J*=8.1 Hz), 7.10 (1H, s), 6.85 (1H, d, *J*=8.1 Hz), 6.04 (2H, s), 6.03 (2H, s), 4.19 (2H, s), 2.61 (3H, s).

*(s)-stylopine (**2**)*: yellow powder; $[\alpha]_D$ +82 (C 0.45, $CHCl_3$); mp 198-202°C; EIMS, *m/z* 323 $[M]^+$; HREIMS, *m/z* 323.1158 (calcd for $C_{19}H_{17}NO_4$ 323.34); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.75 (1H, s), 6.71 (1H, d, *J*=7.9 Hz), 6.66 (1H, d, *J*=8.0 Hz), 6.62 (1H, s), 5.98 (1H, s), 5.95 (1H, s), 5.94 (2H, s), 4.12 (1H, d, *J*=15.3 Hz), 3.60 (1H, br s), 3.57 (1H, br s), 3.26 (1H, dd, *J*=16.0, 3.46 Hz), 3.16 (2H, m), 2.83 (1H, t, *J*=28.0, 15.5, 11.6 Hz), 2.67 (2H, m).

*Arnottianamide (**3**)*: ivory crystal; EIMS, *m/z* 381 $[M]^+$; HREIMS, *m/z* 381.1212 (calcd for $C_{21}H_{19}NO_6$ 381.3787); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.09 (1H, s), 7.66 (1H, d, *J*=8.7 Hz), 7.24 (1H, d, *J*=8.3 Hz), 7.13 (1H, s), 7.01 (1H, s), 6.73 (1H, d, *J*=8.6 Hz), 6.47 (1H, d, *J*=8.6 Hz), 6.01 (2H, s), 3.94 (3H, s), 3.83 (3H, s), 2.93 (3H, s).

*(+)-Chelidonine (**4**)*: ivory crystal; $[\alpha]_D$ –319 (C 0.50, $CHCl_3$); mp 136-138°C; EIMS, *m/z* 353 $[M]^+$; HREIMS, *m/z* 353.1266 (calcd for $C_{20}H_{21}NO_5$ 353.3844); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.77 (1H, s), 6.68 (1H, s), 6.66 (1H, s), 5.97 (1H, d, *J*=4.3 Hz), 5.95 (2H, t, *J*=3.2, 1.5 Hz), 5.01 (1H, d, *J*=1.4 Hz), 4.26 (1H, br s), 4.11 (1H, d, *J*=15.6 Hz), 3.59 (1H, br s), 3.45 (1H, d, *J*=15.6 Hz), 3.25 (1H, d, *J*=17.6 Hz), 3.08 (1H, dd, J=17.5, 4.4 Hz), 2.98 (1H, t, *J*=2.5 Hz), 2.30 (3H, s).

*Spallidamine (**5**)*: yellow powder; $[\alpha]_D$ + 17 (C 0.45, $CHCl_3$); EIMS, *m/z* 391 $[M]^+$; HREIMS, *m/z* 391.1056 (calcd for $C_{18}H_{19}NO_4$ 391.3735); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.70 (1H, d, *J*=8.5 Hz), 7.58 (1H, d, *J*=8.5 Hz), 7.41 (1H, s), 7.35 (1H, d, *J*=8.0 Hz), 7.14 (1H, s), 6.91 (1H, d, *J*=8.0 Hz), 6.08 (2H, s), 6.06 (2H, s), 4.69 (1H, t, *J*=7.5 Hz, H-6), 2.79 (3H, s, N-CH_3), 2.45 (2H, d, *J*=7.5 Hz).

*N-trans-feruloyltyramine (**6**)*: ivory crystal; EIMS, *m/z* 313 $[M]^+$; HREIMS, *m/z* 313.1315 (calcd for $C_{18}H_{19}NO_4$ 313.3478); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.33 (1H, d, *J*=15.1 Hz), 7.01 (1H, d, *J*=1.5 Hz), 6.96 (1H, s), 6.94 (1H, s), 6.92 (1H, dd, *J*=8.7, 18.1 Hz), 6.69 (1H, d, *J*=13.7 Hz), 6.30 (1H, d, J=15.1 Hz), 6.63 (1H, s, *J*=8.5 Hz), 6.61 (1H, s), 3.77 (3H, s), 3.36 (3H, t, *J*=13.7, 28.0 Hz), 2.65 (2H, t, *J*=13.6, 28.0 Hz).

Measurement of neutrophil elastase activity. Human neutrophil elastase (EC 3. 4. 21. 37) (Sigma-Aldrich) was assayed as described previously with slight modification (Johansson et al., 2002), using succinyl-Ala-Ala-Ala-*p*-nitroaniline (Bachem., USA) as substrate. In spectrophotometric experiments, enzyme activity [initial velocity (v_i)] was monitored by observing the hydrolysis of *p*-nitroaniline at 405 nm. All samples were dissolved in 100% DMSO at 200 μM stock concentrations and used for the experiment with dilution. The mixture contained 130 μL 0.02 mM Tris-HCl buffer solutions (pH 8.0) and 20 μL enzymes (0.02 Unit/mL) were preincubated for 10 min in the presence of test compounds or vehicle (DMSO). The final concentration of DMSO was 5% throughout. The reaction was started by the addition of 40 μL substrate (1.5 mM Suc-Ala-Ala-Ala-*p*NA). The percent inhibition ratio (percent) was calculated according to the following equation:

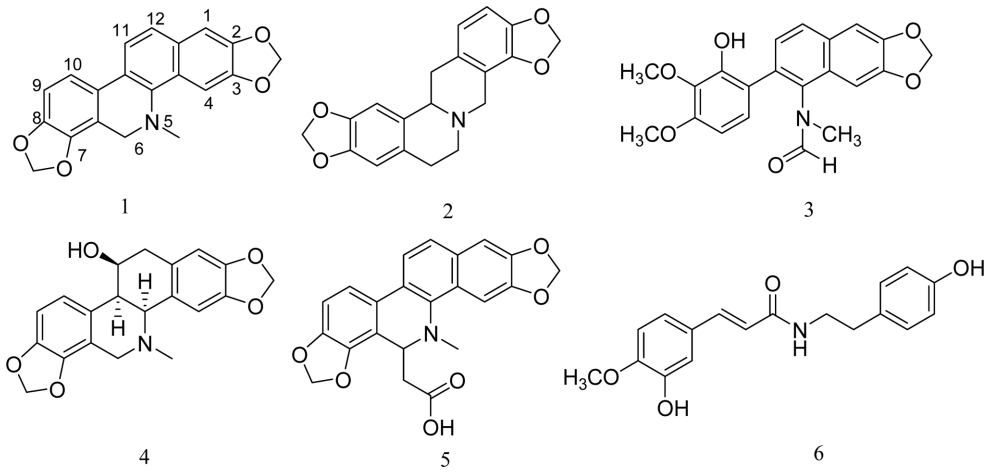

Fig. 1 Chemical structures of alkaloids (**1**−**6**) from *Chelidonium majus* L.

% inhibition =[(rate of control reaction−rate of sample reaction)/ rate of control reaction]×100

Enzyme kinetic assay and progress linear determination. Compounds (**2**, **5**, and **6**) showing the highest inhibitory activities were further characterized by the assay conditions. The inhibition kinetics of human neutrophil elastase by the compounds (**2**, **5**, and **6**) was analyzed by Lineweaver-Burk plots and compared to data obtained in the absence of inhibitors. Kinetic parameters associated with inhibition mechanism of HNE, steady-state rates were obtained at several inhibitor concentrations. The two inhibition constants for inhibitor binding with either free or enzyme-substrate complex, K_I or K_{IS}, were obtained from secondary plots of the slopes of the straight lines or vertical intercept, respectively, versus the concentration of inhibitors. The constants for inhibitor binding with free or enzyme-substrate complex, K_I or K_{IS} with Suc-Ala-Ala-Ala-*p*NA as substrate, were obtained from the second plots of the slopes of the straight lines or vertical intercept ($1/V_{max}^{app}$), versus the concentration of inhibitors. The data were analyzed using the a nonlinear regression program [Sigma Plot (SPCC Inc., USA)] to give the individual parameters for each curve. The parameters are represented by Eqs.: (1)-(3) (Zhang et al., 2006; Chiari et al., 2011):

$$1/V = K_m/V_{max}(1+[I]/K_I)1/S+1/V_{max} \quad (1)$$

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

$$\text{Intercept} = 1/K_{IS}V_{max}[I]+1/V_{max} \quad (3)$$

Results and Discussion

Structural identification of human neutrophil elastase inhibitors. In the preliminary study, we confirmed human neutrophil elastase inhibitory activity of methanol extract of *C. majus*. The methanol extract exhibited 88% inhibition at 100 μg/mL. The high potency of the methanol extract encouraged us to identify the compounds responsible for its human neutrophil elastase inhibition. The six alkaloids (**1**-**6**) in Fig. 1 were isolated from methanol extract of *C. majus* L. by chromatography over silicagel, sephadex LH-20 and octadecyl functionalized silicagel, compounds (**1**-**6**) were identified as dihydrosanguinarine (**1**), (s)-stylopine (**2**), arnottianamide (**3**), (+)-chelidonine (**4**), spallidamine (**5**), and *N-trans*-feruloyltyramine (**6**) by comparing their spectroscopic data of those previously reported (Ma et al., 1999; Kanada et al., 2012). The representative quinolone (spallidamine, **5**) was isolated as a yellow powder with molecular formula $C_{22}H_{17}NO_6$ established by the [M^+] ion at 391.1056 (Calcd 391.3755) in HREIMS. ^{1}H and ^{13}C NMR in conjunction with DEPT experiments indicated the presence of 22 carbons consistency of the following functional groups: 7 methines (sp^2), 3 methylenes (sp^3), 1 methyl and 11 quanternary carbons. The ^{13}C NMR data enabled carbons corresponding to the 8 C-C double bonds and 1 carbonyl group to be identified and thus accounted for 9 of 15 degrees of unsaturation. The extra six degrees of unsaturation were ascribed to six rings, three of which were aromatics. The existence of two methylenedioxy groups was revealed by the signals at δ_H 6.08 (*d*, 4H). Analysis of spectroscopic data and comparison of previous data showed compound **5** to be spallidamine (Ma et al., 1999).

Human neutrophil elastase inhibitory activity. Human neutrophil elastase is considered to be the primary source of tissue damage associated with many inflammatory diseases. The isolated alkaloids (**1**-**6**) were screened for their *in vitro* human neutrophil elastase (EC 3. 4. 21. 37) inhibitory activities at different concentration by using succinyl-Ala-Ala-Ala-*p*-nitroaniline as substrate. As shown in Fig. 2A all compounds exhibited a dose-dependent inhibitory effect on the HNE. But among of them, two isoquinolines (**2** and **5**) and cinnamicamide (**6**) appreciably inhibited HNE activity with IC_{50} ranging between 11.6 and 51.0 μM (Table 1). The inhibition of HNE by compound **5**, the most potent inhibitor (IC_{50} =11.6 μM) is illustrated in Fig. 2B, representatively. Other inhibitors (**2** and **6**) manifested a similar relationship between enzyme activity and enzyme concentration. The equilibrium constant for inhibitor binding, K_I was obtained from the values at the intersection of three lines from Dixon plots as shown in Fig. 2C-E. The K_I values of inhibitors are presented in Table 1. All the measurements were

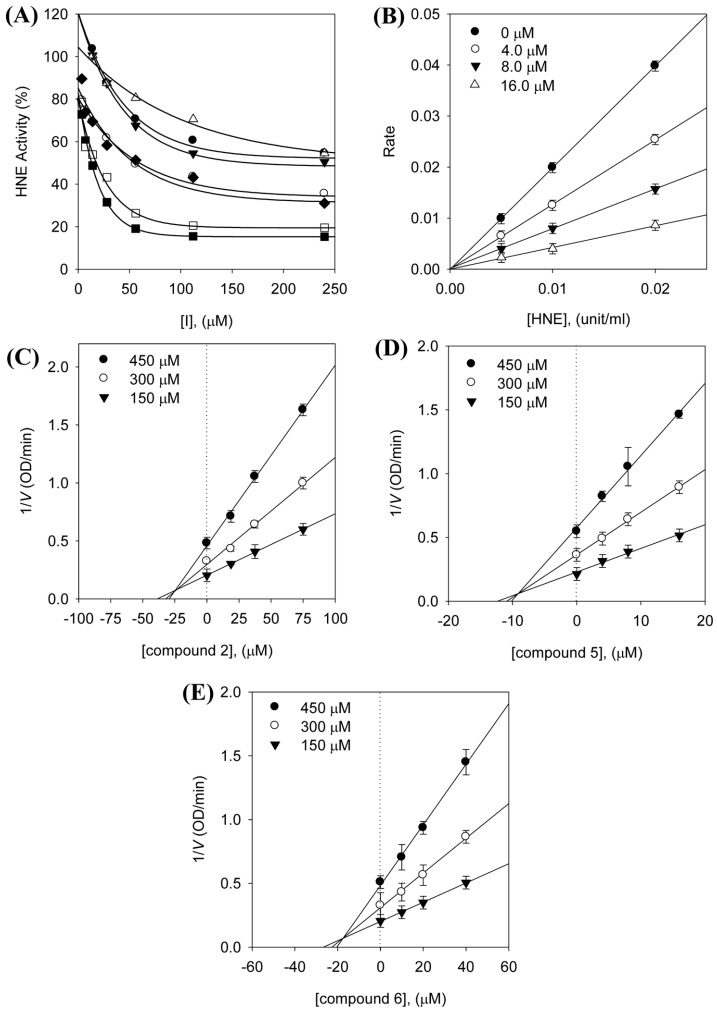

Fig. 2 (A) Effects of isolated compounds **1**–**6** on HNE for the hydrolysis of Suc-Ala-Ala-Ala-*p*NA (● compound **1**; ○ compound **2**; ▼compound **3**; △ compound **4**; ■ compound **5**; □ compound **6**; ◆ ursolic acid), (B) the catalytic activity of HNE as a function of enzyme concentration at different concentrations of compound **5** and (C–E) Dixon plots for the inhibition of compounds (**2**, **5**, **6**), respectively, on the hydrolysis activity of HNE in the presence of different concentrations of substrate.

Table 1 Inhibitory effect of isolated compounds **1-6** on human neutrophil elastase

Compound	IC_{50}[a] value (μM)	Inhibition mode	K_I (μM)	K_{IS} (μM)
1	>200	NT[b]	-	-
2	51.0±0.4	Mixed type I	34.0±0.8	87.5±0.7
3	>200	NT	-	-
4	>200	NT	-	-
5	11.6±1.1	Mixed type I	9.1±0.5	18.8±0.6
6	20.7±0.9	Mixed type I	13.0±0.2	31.0±0.4
Ursolic acid[c]	30.6±1.8	NT	-	-

[a]Sample concentration which lead to 50% enzyme activity loss.
[b]NT is not tested. [c]Ursolic acid was used as a positive control.

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Enzyme kinetic analysis. The enzyme inhibition properties of the isolation inhibitors were modeled using double-reciprocal plots of Lineweaver-Burk and Dixon analysis. As shown in Fig. 3A-C, the inhibition kinetics analyzed by Lineweaver-Burk plots show that all compounds (**2**, **5**, and **6**) are mixed-type inhibitors because increasing inhibitor concentration resulted in a family of lines which intersected at a nonzero point on both x- and y-axes. Since the inhibition was mixed, this means that inhibitor has different affinities for the substrate bound and free enzyme. Thus we tried to analyze the respective inhibition constants for the two states. Type I is where inhibitor preferably binds to the free enzyme

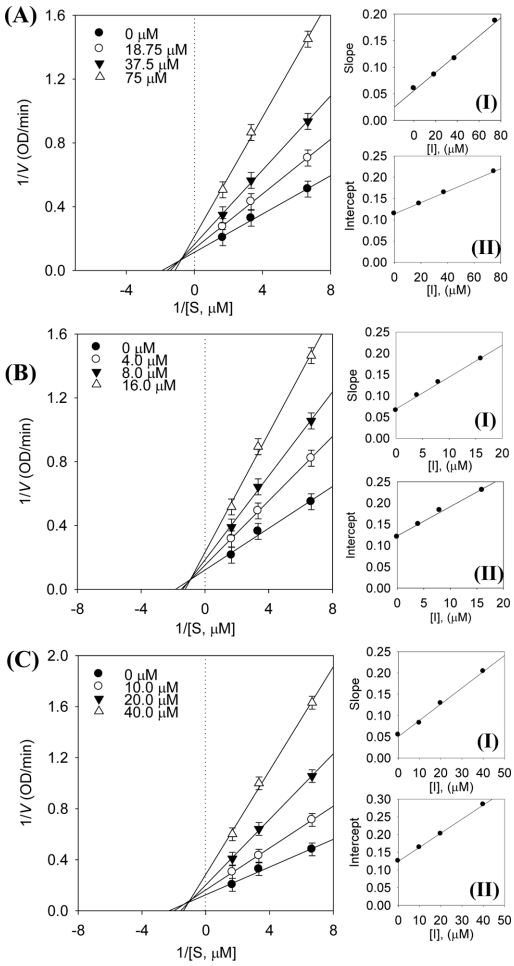

Fig. 3 (A–C) Kinetic assays of HNE inhibition by stylopine (**2**), spallidamine (**5**), and *N-trans*-feruloyltyramine (**6**). Lineweaver–Burk plots were constructed for the inhibition of HNE by compounds (**2**, **5** and **6**). The plot is expressed as 1/velocity versus 1/HNE (Unit/mL) with or without inhibitor. Insets (I) and (II) represent the secondary plot of the slope and the intercept of the straight lines versus concentration of compounds (**2**, **5** and **6**), respectively.

while Type II is where inhibitor preferably binds to the enzyme substrate complex. This analysis is carried out by varying both inhibitor and substrate concentration, as shown by Equations (1)-(3). The equilibrium to free enzyme (K_I) and enzyme-substrate complex (K_{IS}) were obtained from secondary plots of K_m/V_{max} and $1/V_{max}$ versus concentration of compounds **2**, **5**, and **6**, respectively. We thus established the following constants: compound **2**, K_I =34.0 μM, and K_{IS} =87.5 μM; compound **5**, K_I =9.1 μM and K_{IS} =17.8 μM; compound **6**, K_I =13.0 μM and K_{IS} =31.0 μM (Fig. 3 insects). These data show that the affinity of the inhibitor for free enzyme is significantly stronger that the affinity of inhibitor for the enzyme-substrate complex. Thus, compound **2**, **5**, and **6** are mixed type I inhibitors (Roberts, 1977).

This study demonstrates that the methanol extract of *C. majus* L. show potent inhibitory activity toward human neutrophil elastase. Three compounds, (s)-stylopine (**2**), spallidamine (**5**), and *N-trans*-feruloyltyramine (**6**), were the principal contributors to the HNE inhibition. The analysis of K_I and K_{IS} values proved that three inhibitors (**2**, **5**, and **6**) had a reversible mixed type I behavior.

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