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SHORT COMMUNICATION

α-Chymotrypsin inhibition studies on the lignans from *Vitex negundo* Linn

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◆*Dedicated to the memory of Dr. Mohammad Hussain Panjwani (1940-1992), a renowned philanthropist and scholar*

Abstract
The lignans (**1–8**) isolated from the roots of *Vitex negundo* Linn. were screened against the serine proteases α-chymotrypsin, thrombin and prolyl endopeptidase. Compounds **3** and **4** were found to be active only against α-chymotrypsin and were noncompetitive and competitive inhibitors of the enzyme, respectively. *Ki* values were found to be in the range 31.75–47.11 μM.

Keywords: Vitex negundo *Linn, Lignans, α-Chymotrypsin, inhibition*

Introduction

Vitex negundo Linn. belongs to the family Verbenaceae, a large family of herbs, lianas, shrubs, or trees comprising about 36 genera and 1035 species and is indigenous to the Mediterranean countries and central Asia, and also found in parts of Pakistan, India and Burma [1,2].

In many clinical conditions like emphysema, gingivitis, tumour invasion and inflammatory infections, it has been suggested that tissue destruction is caused by proteases [3]. Among the enzymes involved in extracellular matrix degradation, a few serine proteases (elastase, collagenase, cathepsin G, chymotrypsin) are able to solubilize fibrous proteins such as elastin and collagen [4,5]. Given the specific recognition by proteases of defined amino acid sequences, it may be possible to inhibit these enzymes when they are involved in pathological processes. Potent inhibitors have the potential to be developed as new therapeutic agents. which offers huge opportunities for medicine. Thus, the development of non-toxic protease inhibitors extracted from natural sources for *in vivo* application may be quite important. Since the current serine protease (like α-chymotrypsin) inhibitors are still far from perfection, the interests and efforts in the discovery of novel serine protease inhibitors are expected to continue in the future. As part of our ongoing investigations on serine proteases inhibition by natural products, we here describe the isolation, characterization, α-chymotrypsin inhibitory potential and inhibition kinetics of the lignans isolated from the roots of *Vitex negundo* (Figure 1).

Materials and methods

Plant material

The roots of *Vitex negundo* Linn. were collected in November 2001 from Bannu district and identified by Prof. Abdur Rehman (Plant Taxonomist), Department of Botany, Govt. Post Graduate College Bannu, Pakistan. A voucher specimen (no. 318b) has been deposited at the herbarium of the Botany Department of Post Graduate College, Bannu, Pakistan.

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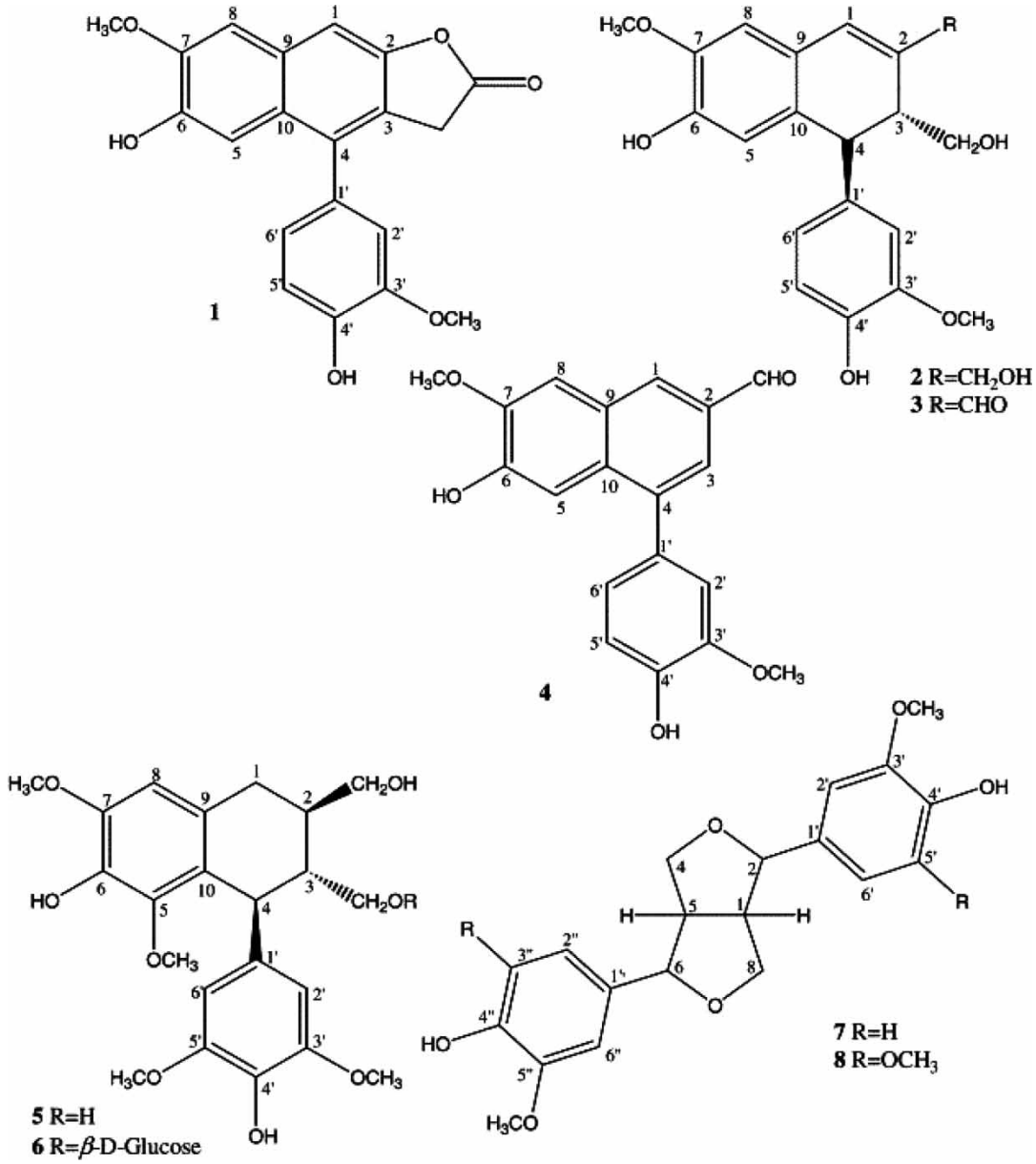

Figure 1. Structures of the lignans isolated from the roots of *V. negundo* Linn.

General experimental procedures

The ^{1}H- and ^{13}C-NMR, HMQC and HMBC spectra were recorded on Bruker spectrometers operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C-NMR respectively. MS and HR-MS were obtained on a JMS-HX-110 with a data system and on JMS-DA 500 mass spectrometers. The UV spectra were recorded on a Hitachi UV-3200 spectrophotometer (λ_{max} nm). Silica gel (230–400 mesh) was used in flash chromatography. TLC plates and pre-coated silica gel G-25-UV$_{254}$ plates were used to check the purity of the compounds under UV at 254 and 366 nm and by spraying with ceric sulphate reagent with heating. The IR spectra were recorded on a 460 Shimadzu spectrometer.

Extraction and isolation

The shade dried roots (40 kg) of *Vitex negundo* were extracted three times, seven days each, with methanol. The combined methanolic extract was evaporated *in vacuo*. The resulting residue (1.5 kg) was suspended in water and extracted successively with *n*-hexane, chloroform, ethyl acetate and *n*-butanol.

The chloroform soluble fraction was subjected to column chromatography over silica eluting with hexane-ethyl acetate and then ethyl acetate-methanol in increasing order of polarity (fractions 1–30). The fractions showing similar profiles on TLC were combined to afford fractions A-D. Repeated column chromatography of fraction B using hexane-ethyl

acetate (3:1) resulted is the isolation of compounds **1**, **3**, **4**, **7**. Repeated column chromatography of fraction C using hexane – ethylacetate (7:3) solvent system resulted in the isolation of compounds **2**, **5** and **8**. The *n*-butanol soluble fraction was subjected to column chromatography over silica gel, eluting with $CHCl_3$-MeOH in increasing order of polarity. The fraction which eluted with $CHCl_3$-MeOH (8.7:1.3) was subjected to preparative TLC plates using $CHCl_3$-MeOH (85:15) to afford compound **6**.

*Compound (**1**):* Amorphous white solid (18 mg), Mp 125°C. UV λ_{max} (MeOH) nm (log ε): 256.2 (4.64), 288.6 (4.07), 313.6 (4.10). IR (KBr) cm^{-1}: 3529–3266, 2925, 1679, 1621, 1512, 1461, 1266, 1157, 1024. HREIMS: *m/z* 352.0911 $[M]^+$, $C_{20}H_{16}O_6$ requires 352.0942. ^{1}H-NMR (400 MHz, Pyridine-d_5) δ: 8.66 (1H, s, H-4), 7.73 (1H, s, H-8), 7.62 (1H, s, H-5), 7.36 (1H, d, $\mathcal{J} = 7.9$ Hz, H-5′), 7.10 (1H, d, $\mathcal{J} = 1.5$ Hz, H-2′), 7.06 (1H, dd, $\mathcal{J} = 7.9$, 1.5 Hz, H-6′), 4.44 (2H, br s, H_2-9), 3.93 (3H, s, MeO-6), 3.72 (3H, s, MeO-3′); ^{13}C-NMR (100 MHz, pyridine-d_5): δ 133.3 (C-1), 128.9 (C-2), 136.9 (C-3), 121.7 (C-4), 129.6 (C-4a), 108.5(C-5), 150.2 (C-6), 150.3 (C-7), 109.3 (C-8), 131.8 (C-8a), 45.3 (C-9), 171.9 (C-10), 129.1 (C-1′), 113.9 (C-2′), 148.9 (C-3′)147.9 (C-4′),116.9 (C-5′), 123.0 (C-6′), 55.8 (6-OMe), 55.9 (3′ -OMe) [8].

*Compound (**2**):* Amorphous white solid, (15 mg), $[\alpha]_D^{26}$ $-56°$ ($c = 0.11$, MeOH), UV λ_{max} MeOH) nm (log ε): 283 (4.34), 222 (4.70). IR (KBr) cm^{-1}: 3340, 2924, 1599, 1512, 1452, 1225, 1126, 1024. HREIMS: *m/z* 358.1421 requires 358.1416. ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.75 (1H, s, H-8), 6.65 (1H, d, $\mathcal{J} = 1.8$ Hz H-2′), 6.61 (1H, d, $\mathcal{J} = 8.5$ Hz, H-5′), 6.56 (1H, s, H-1), 6.44 (1H, s, H-5), 6.41 (1H, dd, $\mathcal{J} = 8.5$, 1.8 Hz, H-6′), 4.11 (br s, H-4), 4.01 (2H, dd, $\mathcal{J} = 5.5$, 1 Hz, H-2a), 3.85 (3H, s, 3′ -OMe), 3.71 (3H, s, 7-OMe), 3.57 (1H, m, H-3a), 3.30 (1H, m, H-3a), 2.51 (1H, m, H-3).^{13}C-NMR (100 MHz, CD_3OD): δ121.3 (C-1), 138.1 (C-2), 65.9 (C-2a), 48.5 (C-3), 63.5 (C-3a), 45.1 (C-4), 115.8 (C-5), 147.0 (C-6), 148.6 (C-7), 111.5 (C-8), 137.1 (C-9), 127.0 (C-10), 130.1 (C-1′), 112.7 (C-2′), 147.8 (C-3′),145.6 (C-4′), 118.0 (C-5′), 124.2 (C-6′), 56.6 (7-OMe), 56.3 (3′-OMe) [8].

*Compound (**3**):* Yellow crystals (30 mg), Mp. 126–127°, $[\alpha]_D^{26}$ $-176.0°$ (MeOH; 0.005), UV λ_{max} (MeOH) nm (log ε) 225 (4.31), 359 (4.24); (+NaOMe) 279 (4.20), 427 (4.43),IR (KBr) cm^{-1} 3390 (O-H), 2840 (aldehydic C-H), 1650 (α, β-unsaturated C = O), 1620, 1565, 1515 (C = C), EIMS *m/z* [rel. int]: 356 $[M]^+$(62), 338 $[M\text{-}HOH]^+$(23), 325 $[M\text{-}CH_2OH]^+$(75), HREIMS: *m/z* 356.1270 calcd. for $C_{20}H_{20}O_6$, 356.1259. ^{1}H-NMR (100 MHz, CD_3OD) δ 9.56 (1H, s, CHO), 7.32 (1H, s,H-10), 6.96 (1H, s, H-8), 6.70 (1H, s, H-5), 6.59 (1H, br s, H-2′), 6.54 (1H, d, $\mathcal{J} = 8.0$ Hz, H-5′), 6.23 (1H, dd, $\mathcal{J} = 8.0$, 2.7 Hz, H-6′) 4.32 (1H, s, H-4), 3.82 (3H, s, OMe), 3.64 (3H, s, OMe), 3.45 (2H, d, $\mathcal{J} = 5.0$ Hz, OCH_2), 3.28 (1H, s, O-H), 3.21 (1H, s, O-H), 3.12 (s, 1H, O-H). ^{13}C NMR (400 MHz, CD_3OD) δ 141.7 (C-1), 141.4 (C-2), 41.3 (C-3), 47.0 (C-4), 115.0 (C-5), 149.1 (C-6), 147.2 (C-7), 112.6 (C-8), 122.2 (C-9), 131.6 (C-10) 133.7 (C-1′), 115.0 (C-2′) 150.6 (C-3′), 148.0 (C-4′), 119.7 (C-5′), 122.8 (C-6′), 193.9 (CHO), 56.1 (7-OMe) 56.1 (5′-OMe) [2].

*Compound (**4**):* Yellowish amorphous solid (10 mg), UV λ_{max} (log ε) (dioxane) nm 320 (4.1), 267 (4.5) nm, IR (KBr) cm^{-1} 3600–3000, 1684, 1580, 1270, 1211, HREIMS *m/z* 324.0981 $[M]^+$ (calcd for $C_{19}H_{16}O_5$, 324.0998), ^{1}H-NMR (100 MHz, CD_3OD) δ 10.12 (1H, s, 3-CHO), 8.18 (1H, br s,H-4), 7.75 (1H, br s, H-2), 7.42 (1H, s, H-8), 7.33 (1H, s, H-5), 7.04 (1H, d, $\mathcal{J} = 7.9$ Hz, H-5′), 6.16 (1H, s, 7-OH) 6.98 (1H, d, $\mathcal{J} = 7.9$, H-6′), 6.97 (1H, s, H-2′), 5.74 (1H, s, 4′-OH), 4.09 (3H, s, 6-OMe), 3.93 (3H, s, 3′-OMe), ^{13}C NMR (400 MHz, CD_3OD) δ139.9 (C-1), 122.8 (C-2), 132.1 (C-3), 131.7 (C-4) 128.8 (C-4a), 107.5 (C-5), 147.8 (C-6), 148.5 (C-7) 108.7 (C-8), 132.1 (C-8a), 132.1 (C-1′) 112.4 (C-2′) 146.5 (C-3′), 145.3 (C-4′), 114.4 (C-5′), 122.9 (C-6′) 192.3 (3-CHO), 56.1 (6-OMe), 56.1 (3′ -OMe) [9].

*Compound (**5**):* Colorless needles (20 mg), M.p. 117–118°C, (Yield 20 mg), $[\alpha]_D^{23}$ $+68°$ (0.1, CH_3OH), UV λ_{max} (MeOH) nm (log ε) 219 (2.32), 278 (0.40), IR (KBr) cm^{-1} 3380, 1598, 1490, 1463, 1295, 1195, EIMS *m/z* 420 [M^+, 100%], 389, 371, 301, 247, 205, 167, HREIMS: *m/z* 420.1773 calcd. for $C_{22}H_{28}O_8$, 420.1784, ^{1}H-NMR (400 MHz, Acetone-d_6) δ 1.44 (1H, m, H-8′), 1.86 (1H, m, H-8), 2.38 (1H, dd, $\mathcal{J} = 14.8$, 11.8 Hz), 2.58 (1H, dd, $\mathcal{J} = 14.8$, 4.6 Hz, H-7′), 3.26 (2H, m, H-9), 3.35 (3H, s, OMe-5′), 3.45 (1H, m), 3.85 (1H, m) (H-9′), 3.64 (6H, s, OMe-3, 5), 3.77 (3H, s, OMe-3′), 4.23 (1H, d, $\mathcal{J} = 5.8$ Hz, H-7), 6.29 (2H, s, H-2, 6), 6.54 (1H, s, H-2′), ^{13}C NMR (100 MHz, Acetone-d_6) δ 147.7 (C-3 and 5) 147.0 (C-3′) 146.6(C-5′), 137.8 (C-1), 137.3 (C-4′), 134.8 (C-4), 128.8 (C-1′), 125.1 (C-6′), 64.9 (C-9′), 62.8 (C-9), 46.8 (C-8), 40.4 (C-7), 39.3 (C-8′), 59.2 (OMe-5′), 56.4 (OMe-3, 5), 55.9 (OMe-3′) [10].

*Compound (**6**):* amorphous powder (80 mg). $[\alpha]_D^{22}$ + 22.4 (MeOH; c = 1.01). IR (KBr) cm^{-1}: 3600–3100, 1570,1515,1460. ^{1}H and ^{13}C NMR HRFABMS $[M\text{-}H]^+$: *m/z* 581.2240 calcd. for $C_{28}H_{37}O_{13}$, 582.2234, ^{1}H-NMR (400 MHz, CD_3OD) δ 2.60 (2H, m, H-1),

1.68 (1H, m, H-2), 3.64 (2H, m, H-2a), 2.0 (1H, m, H-3), 3.82 (2H, m, H-3a), 4.37 (1H, d, $\mathcal{J} = 6.0$, H-4), 6.52 (1H, s, H-8), 6.37 (2H, s, H-2′), 3.78 (6H, s, 3′, 5′ -OMe), 3.64 (3H, s, 7-OMe), ^{13}C NMR (100 MHz, CD_3OD) δ 66.2(C-2a) 46.7 (C-3), 71.6(C-3a), 42.7 (C-4), 148.6(C-5), 139.3 (C-6), 147.5 (C-7), 107.1 (C-2′, 6′), 148.9 (C-3′, 5′), 138.9 (C-4′), 104.8 (C-1″), 75.2 (C-2″), 77.9(C-3″), 71.7 (C-4″), 78.2 (C-5″), 62.8 (C-6″), 56.8 (OMe-3′,5′), 56.6 (OMe- 7), 60.1 (OMe- 5) [11].

Compound (7): Courless needles (11 mg), mp 168–170°C. $[\alpha]_D^{26} + 0$ (MeOH; c = 0.50).IR (KBr) cm^{-1}: 3600–3200, 1606, 1510, 1460. HREIMS: *m/z* 358.1410 calcd. for $C_{20}H_{22}O_6$, 358.1416. Colorless needles, ^{1}H-NMR (400 MHz, CD_3OD) δ 2.71 (2H, m, H-1, 5), 3.83, 4.22 (4H, m, H-4, 8), 4.60 (2H, d, $\mathcal{J} = 5.0$ Hz, H-2, 6), 6.98 (2H, d, $\mathcal{J} = 1.7$ Hz, H-2′, 2″), 6.80 (2H, d, $\mathcal{J} = 8.2$ Hz, H-5′, 5″), 6.83 (2H, dd, $\mathcal{J} = 8.2$, 1.7 Hz, H-6′, 6″), 3.85 (6H, s, OMe-3′, 3″) ^{13}C NMR (100 MHz, CD_3OD) δ 53.7 (C-1, 5), 85.7 (C-2, 6), 71.3 (C-4, 8), 132.0 (C-1′, 1″), 108.8 (C-2′, 2″), 146.8 (C-3′, 3″), 145.8 (C-4′, 4″), 114.4 (C-5′, 5″), 118.5 (C-6′, 6″), 55.8 (3′, 3″-OMe) [12].

Compound (8): amorphous powder (16 mg). mp 170–172°C, $[\alpha]_D^{25} + 110$ (c = 0.1, $CHCl_3$), UV λ_{max} (MeOH) nm (log ε):212 (4.11), 240 (4.16), 278 (4.09). IR (KBr) cm^{-1}: 3400, 1600, 1500 cm^{-1}. HREIMS: 418.1634 calcd. for $C_{22}H_{26}O_8$, 418.1627, ^{1}H-NMR (100 MHz, CD_3OD) δ 3.18 (2H, m, H-1 and 5), 3.57 (2H, dd, $\mathcal{J} = 9.6$, 3.6 Hz, H-4b and 8b) and 3.73 (2H, dd, $\mathcal{J} = 9.6$, 6.8 Hz, H-4a and 8a), 4.90 (2H, d, $\mathcal{J} = 4.3$ Hz, H-2 and 6), 3.91 (12 H, s, 4-OMe) 5.49 (2H, s, OH) and 6.61 (4H, s, H-2′, 6′, 2″ and 6″) ^{13}C NMR (400 MHz, CD_3OD) δ 49.5 (C-1 and 5), 56.3 (4-OMe) 68.7 (C-4 and 8), 84.1 (C-2 and 6), 103.0 (C-2′, 6′, 2″, 6″), 130.0 (C-1′ and 1″), 133.8 (C-4′ and 4″), and 147.0 (C-3′, 5′, 3″, and 5″) [13].

Enzyme inhibition assays

Chymotrypsin inhibitory activity of compounds was performed by the method of Cannel et al (1988) [14]. Chymotrypsin (9 units/ mL of 50 mM Tris-HCl buffer pH 7.6; Sigma Chemical Co. USA) was preincubated with the compounds for 20 min at 25°C. 100 μL of substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide 0.01–0.06 mM in 50 mM tris-HCl buffer pH 7.6) were added to start the enzyme reaction. The absorbance of released *p*-nitroaniline was continuously monitored at 410 nm until a significant color change had occured. The final DMSO concentration in the reaction mixture was 7% which had no effect on enzyme activity. Assays of prolyl endopeptidase and thrombin were performed by the method of Diderot et al. (2005) [18].

Estimation of kinetic parameter

Two different methods were applied to monitor the effect of the test compound on both K_m and V_{max} values. This was done first by a Lineweaver–Burk plot, and secondly by a Dixon plot where the reciprocal of the rate of the reaction was plotted against the inhibitor concentration [15]. The secondary replot of the Dixon plot was constructed as the slope of each line of substrate concentration in the original Dixon plot against the reciprocals of the substrate concentrations. K_i values were determined from the Dixon plot, Lineweaver–Burk plot, and secondary replots using initial velocities.

These velocities were obtained over a range of substrate concentrations (0.01 and 0.06 mM). The assay conditions for measurement of the residual activities in the presence of the inhibitors was identical except that fixed concentrations of inhibiting compounds were used in the assay. The types of inhibition were determined from the appearance of the Dixon and Lineweaver–Burk plots.

Statistical analysis

All assays were conducted in triplicate. Graphs were plotted using GraFit program [16].Values of the correlation coefficient, slope, intercept and their standard errors were obtained by the linear regression analysis using the same software. The correlation coefficient for all the lines of all graphs was >0.99, each point in the constructed graphs represents the mean of three experiments.

Results and discussion

Chymotrypsin inhibition studies on the lignans (**1–8**) isolated from the roots of *Vitex negundo* Linn. have been carried out. Chymotrypsin catalyses the hydrolysis of peptide bonds of proteins in the small intestine and is selective for peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met) on the carboxyl side of this bond [17]. K_i values were calculated in three ways; first, the slopes of each line in the Lineweaver-Burk plot were plotted against different concentrations of compounds, secondly the $1/V_{maxapp}$ was calculated by plotting different fixed concentration of substrate versus ΔV in presence of different fixed concentration compounds in the respective assays. Then K_i was calculated by plotting different concentrations of compounds versus $1/V_{maxapp}$. K_i was the intercept on the x-axis. In the third method, K_i was directly measured from the Dixon plot as the intercept on the x-axis. Determination of the inhibition type is critical for the

Table I. In vitro inhibition of α-chymotrypsin by compounds **3–4**.

Serial No	Type of Inhibition	K_i[b] (μM) ± SEM[a]
3	Non- Competitive	31.75 ± 0.85
4	Competitive	47.11 ± 0.72
Chymostatin[c] (Std)		8.24 ± 0.11

[a]Standard mean error of 3–5 assays; [b]K_i is the mean of three values calculated by using the Dixon plot and Lineweaver-Burk secondary plots; [c]Chymostatin source wheat germ (*Triticum vulgaris*)

pendently at the different sites of chymotrypsin but compound **4** binds in the active site where the substrate is bound. The K_i and the type of inhibition are listed in Table I. The graphical analysis of steady state inhibition data for both compounds against α-chymotrypsin has been shown in Figure 2. Close inspections of the structurally similar lignans **1–8** revealed that the inhibition by compounds **3** and **4** may apparently be due to the presence of aldehydic

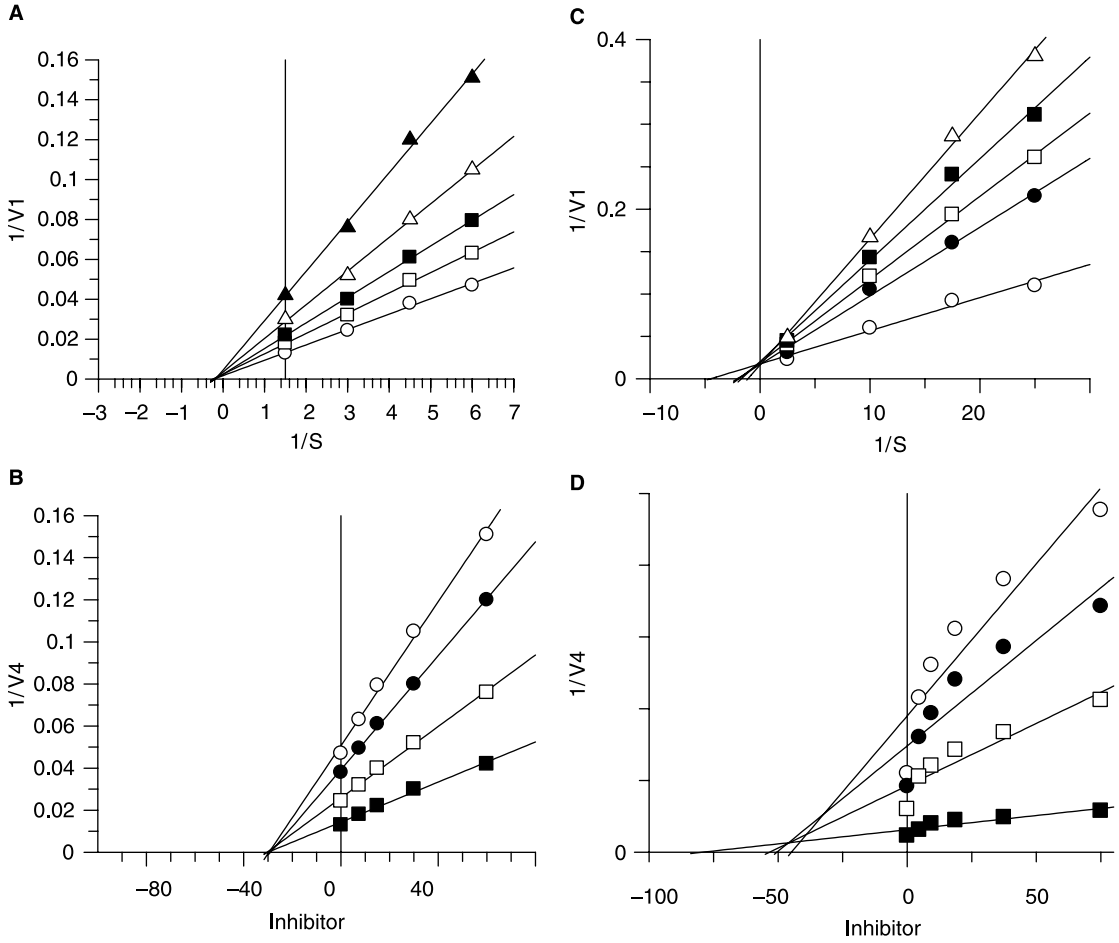

Figure 2. Inhibition of chymotrypsin by compounds **3** and **4;** (A) is the Lineweaver–Burk plot of reciprocal of initial velocities versus reciprocal of four fixed substrate concentrations in absence (o) and presence of 10 μM (□), 20 μM (■), 30 μM (Δ), (▼) 40 μM of compound **3.** (B) is the Dixon plot at fixed substrate concentrations, (■) 0.06 mM, (□) 0.045 mM, (●) 0.033 mM and (o) 0.01 mM (C) is the Lineweaver–Burk plot of reciprocal of initial velocities versus reciprocal of four fixed substrate concentrations in absence (o) and presence of 12.5 μM (●), 25 μM (□), 50 μM (Δ), (■) 75 μM, (Δ) 100 μM of compound **4**. (D) is the Dixon plot at fixed substrate concentrations, (■) 0.06 mM, (□) 0.045 mM, (●) 0.033 mM and (○) 0.01 mM.

identification of mechanism of inhibition and the sites of inhibitor binding. Lineweaver-Burk, Dixon plots and their re-plots indicated pure non-competitive and pure competitive type of inhibition for compounds **3** and **4,** respectively, against chymotrypsin. In the case of compound **3** there was decrease in V_{max} without affecting the affinity (K_m values) while for compound **4** V_{max} was not effected while K_m increased. In other words we can say that compound **3** and N-succinyl-phenylalanine-*p*-nitroanilide bind randomly and independently at the different sites of chymotrypsin but compound **4** binds in the active site where the substrate is bound. The K_i and the type of inhibition are listed in Table I. The graphical analysis of steady state inhibition data for both compounds against α-chymotrypsin has been shown in Figure 2. Close inspections of the structurally similar lignans **1–8** revealed that the inhibition by compounds **3** and **4** may apparently be due to the presence of aldehydic functionalities which might play an important role in ligand-receptor interaction. So we can conclude that compounds **3–4** are specific natural inhibitors of α-chymotrypsin.

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