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Biologically Active *C*-Alkylated Flavonoids from *Dodonaea viscosa*

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A new *C*-alkylated flavonoid (5,7-dihydroxy-3'-(4"-acetoxy-3"-methylbutyl)-3,6,4'-trimethoxyflavone (**1**), along with two known *C*-alkylated flavonoids (5,7-dihydroxy-3'-(3-hydroxymethylbutyl)-3,6,4'-trimethoxyflavone (**2**), 5,7,4'-trihydroxy-3'-(3-hyroxymethylbutyl)-3,6-dimethoxyflavone (**3**) and two new source *C*-alkylated flavonoids (5,7-dihydroxy-3'-(2-hydroxy-3-methyl-3-butenyl)-3,6,4'-trimethoxyflavone (**4**), 5,7,4'-trihydroxy-3,6-dimethoxy-3'-isoprenyl-flavone (**5**) were isolated from the aerial parts of *Dodonaea viscosa*. The structures of all compounds were established on the basis of 1D and 2D NMR spectroscopy and mass spectrometry. The isolated compounds were evaluated for their inhibitory effect on urease and α-chymotrypsin enzyme. All the compounds (**1-5**) exhibited mild inhibition against urease but remained recessive in case of α-chymotrypsin.

Key words: *Dodonaea viscosa,* Spindaceae, Flavonoid, Urease and α-chymotrypsin enzymes

INTRODUCTION

Dodonaea, a genus of 60 species, is found in the warmer parts of the world. *Dodonaea viscosa* (L.) Jacq. (syn-*Ptelea viscosa* Linn.) is a flowering ever-green shrub belonging to the family Spindaceae, which consists of about 150 genera and approximately 2000 species (Abdullah, 1973). The major secondary metabolites isolated from this plant consist of diterpenoids (Mohammad et al., 2010a) organic acids, flavonoids (Mohammad et al., 2010b), tannins, and sterols (Wagner et al., 1987). The plant is widely used to cure skin diseases in folk medicines (Pirzada et al., 2010). The crude extracts of the plant have shown antimalarial activity (Clarkson et al., 2004), antidiabetic activity (Veerapur et al., 2010), gastroprotective effect (Arun and Asha, 2008), and antibacterial activity (Khurram et al., 2009). Taking into account of our interest in the medicinal plants of Pakistan (Riaz et al., 2002; Azhar-ul-Haq et al., 2004; Khan et al., 2005a; Ferheen et al., 2005; Sharif et al., 2005; Hussain et al., 2008, 2009a; Jan et al., 2009) and pharmacological significance of *D. viscosa*, the present phytochemical investigation was undertaken to identify compounds that may be used as potent inhibitor against urease and α-chymotrypsin.

We describe here the isolation and structure elucidation (Khan et al., 2005b; Nisar et al., 2009; Hussain et al., 2009b) of one new and four known *C*-alkylated flavonoids (Fig. 1), exhibiting urease (Table I) and α-chymotrypsin (Table II) inhibitory activity. Urease is responsible for the hydrolysis of urea to ammonia and carbon dioxide. It is a metalloenzyme (urea amidohydrolase EC 3.5.1.5) having nickel in its backbone and functions in nitrogen fixation in plants. It is reported to be one of main causes of pathologies induced by *Helicobacter pylori*, thus allowing the bacteria to survive at the low pH of the stomach and, therefore, play an important role in the pathogenesis of gastric and peptic ulcers. Furthermore, urease plays a very important role in the formation of infection stones and contributes to the pathogensis of urolithiasis, pyelnephritis, ammonia and hepatic encephalopathy, hepatic coma, and urinary catheter encrustation (Arfan et al., 2010). Owing to the therapeutical and pharmacological significance of the inhibitory activity of urease, inhibition associated with this enzyme by secondary

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metabolites could provide an invaluable addition for treatment of chronic infections.

MATERIALS AND METHODS

General experimental procedures

All reagents, chemicals, and solvents used were of analytical grade. Urease (*Jack bean*), thiourea, chymostatin (bacterial source), α-chymotrypsin (from bovine pancreas), N-succinyl-L-phenylalanine-*p*-nitroanilide (SPpNA) were purchased from Sigma-Aldrich. The UV spectra were recorded on a Shimadzu UV-240 (Shimadzu Corporation) spectrophotometer. The IR spectra were recorded on a Shimadzu IR-460 (Shimadzu Corporation) instrument. The ^{1}H-NMR spectra were scanned on AM 300 FT NMR, AM 400 FT NMR and AM 500 FT NMR spectrometers (Bruker) using tetramethyl silane as the internal standard. The ^{13}C-NMR spectra were recorded at 100 and 125 MHz on the aforementioned spectrometers. The mass spectra were scanned on a Varian-MAT 112S and Finnigan MAT-112 and 312A double focusing mass spectrometers connected to DEC PDP 11/34 and IBM-AT compatible PC based system, respectively. Fast atomic bombardment (FAB) experiments were either performed on a Varian/Finnigan-MAT-312A or on Jeol-JMS HX-110 mass spectrometers. FABMS were recorded in a glycerol-water (1:1) matrix in the presence of KI. High-resolution electron impact mass spectra (HREIMS) were recorded on a Jeol-JMS H X-110 mass spectrometer. Column chromatography was carried out on silica gel (type 60, 70-230 mesh; Merck). Ceric sulphate reagent was also used for the detection of compounds.

Plant material

The aerial parts of *Dodonaea viscosa* were collected from the hills of Kurram Agency, Khyber-Pakhtoonkhwa, Pakistan and identified by Dr. Ijaz Khan, a plant taxonomist at the Department of Botany, Post Graduate College, Kohat, Pakistan. A voucher specimen (DVPGCK-098) has been deposited in the herbarium of Department of Botany, Post Graduate College, Kohat.

Extraction and isolation

The shade-dried plant material (20 kg) was ground into powder and extracted at room temperature with methanol (MeOH; 35 L × 3 × 15 days). The combined extracts were concentrated under reduced pressure to give brown gummy residue (2 kg). The crude extract was suspended in 2 L water and successively extracted with *n*-hexane (5 L × 3), chloroform (5 L × 3), ethyl acetate (5 L × 3), and *n*-butanol (5 L × 3) to yield *n*-hexane-soluble (620 g), chloroform-soluble (890 g), ethyl acetate-soluble (507 g), and *n*-butanol-soluble (750 g) fractions, respectively. The ethyl acetate (EtOAc) soluble fraction was subjected to Medium Pressure Liquid Chromatography (MPLC) on a silica gel and eluted with *n*-hexane-$CHCl_3$, $CHCl_3$-EtOAc, EtOAc-MeOH, and MeOH with gradient increase of polarity to yield 38 fractions (Fr. 1-38). Compound **1** (30 mg) was obtained from fraction 7 (1.2 g) by repeated silica gel column chromatography (CC) using *n*-hexane-EtOAc (8:2), *n*-hexane-$CHCl_3$ (1:1). Fraction 9 (980 mg) was rechromatographed on repeated silica gel column, eluted with hexane-EtOAc (8:2) to yield compound **3** (25 mg), compound **2** (40 mg), and compound **4** (20 mg). Fraction 10 (670 mg) was purified by silica gel CC eluted with n-hexane-EtOAc (8:2) to give compound 5 (18 mg). The purity of all isolated compounds was assessed by TLC, NMR and electron ionization-mass spectrometry (EI-MS), and exceeded 99%.

5,7-Dihydroxy-3'-(4''-acetoxy-3'-methylbutyl)-3,6,4'-trimethoxyflavone (1)

Yellowish gummy solid. UV λ_{max} (MeoH) nm (log ε): 346 (4.42), 274 (4.53). IR max (KBr) cm^{-1}: 3398, 2923, 1655, 1599, 1191. ^{1}H-NMR (CD_6CO, 400 MHz) δ: 12.94 (5-OH), 8.00 (1H, dd, J = 8.4, 2.1 Hz, H-6'), 7.96 (1H, d, J = 2.1 Hz, H-2'), 7.11 (1H, d, J = 8.4 Hz, H-5'), 6.57 (1H, s, H-8), 3.97 (3H, s, MeO-4'), 3.86 (3H, s, MeO-6), 3.85 (3H, s, MeO-3), 3.91 (1H, dd, J = 11.0, 6.6 Hz, H-4''a) and δ 3.89 (1H, dd, J = 11.0, 6.0 Hz, 4''b), 2.78 (2H, t, J = 6.8 Hz, H-1''), 1.48 (2H, m, H-2''), 1.69 (1H, m, H-3'') and 1.01 (3H, d, J = 6.9 Hz, H-5''), 1.99 (3H, s, CH_3-7''). ^{13}C-NMR (CD_6CO, 125 MHz) δ: 179.1 (s, C-4), 171.0 (s, C-6''), 160.6 (s, C-4'), 157.6 (s, C-7), 156.1 (s, C-2), 153.6 (s, C-5), 153.1 (s, C-9), 138.1 (s, C-3), 132.2 (s, C-6), 131.7 (s, C-3'), 130.6 (d, C-2'), 129.0 (d, C-6'), 123.5 (s, C-1'), 111.3 (d, C-5'), 105.7 (s, C-10), 69.4 (t, C-4''), 60.7 (q, OCH_3-6), 60.2 (q, OCH_3-3), 56.0 (q, OCH_3-4'), 33.2 (d, C-3''), 34.2 (t, C-2''), 28.2 (t, C-1''), 20.76 (q, C-7''), 17.1 (q, C-5''). EI-MS m/z: 472 (100), 457 (33.2), 429 (16), 357 (11.4), 341 (19.5), 183 (5.1). FAB-MS m/z: 473 [M+H]$^+$. HR-FAB-MS m/z: 473.1276 [M+H] (calcd for $C_{25}H_{29}O_9$: 473.1380).

5,7-Dihydroxy-3'-(3-hydroxymethylbutyl)-3,6,4'-trimethoxyflavone (2)

Gummy solid. ^{13}C-NMR (CD_6CO, 125 MHz) δ: 178.2 (s, C-4), 160.3 (s, C-4'), 158.2 (s, C-7), 156.4 (s, C-2), 154.2 (s, C-5), 153.7 (s, C-9), 137.8 (s, C-3), 132.0 (s, C-6), 131.4 (s, C-3'), 128.8 (d, C-2'), 130.1 (d, C-6'), 122.8 (s, C-1'), 110.9 (d, C-5'), 106.1 (s, C-10), 65.6 (t, C-4''), 60.3 (q, OCH_3-6), 60.1 (q, OCH_3-3), 56.4 (q, OCH_3-4'), 35.5 (d, C-3''), 33.8 (t, C-2''), 28.4 (t, C-1''), 17.3 (q, C-5''). EI-

MS *m/z*: 430 (100). FAB-MS *m/z*: 430 $[M+H]^+$. HR-FAB-MS *m/z*: 430.1678 [M+H] (calcd for $C_{23}H_{27}O_8$: 430.1619).

5,7,4'-Trihydroxy-3'-(3-hyroxymethylbutyl)-3,6-dimethoxyflavone (3)

Gummy solid. ^{13}C-NMR (CD_6CO, 100 MHz) δ: 179.6 (s, C-4), 161.7 (s, C-4'), 157.8 (s, C-7), 157.2 (s, C-2), 153.9 (s, C-5), 154.5 (s, C-9), 138.3 (s, C-3), 131.7 (s, C-6), 132.2 (s, C-3'), 130.7 (d, C-2'), 129.1 (d, C-6'), 123.6 (s, C-1'), 110.8 (d, C-5'), 105.8 (s, C-10), 65.3 (t, C-4''), 60.5 (q, OCH_3-6), 60.3 (q, OCH_3-3), 34.5 (d, C-3''), 33.3 (t, C-2''), 27.8 (t, C-1''), 16.9 (q, C-5''). EI-MS *m/z*: 416 (100). FAB-MS *m/z*: 416 $[M+H]^+$. HR-FAB-MS *m/z*: 416.1518 [M+H] (calcd for $C_{23}H_{27}O_8$: 430.1520).

Urease inhibition assay

Reaction mixtures comprising one unit of urease enzyme (*Jack bean*) solution and 55 µL of buffers having 100 mM urea were incubated with 5 µL of test compounds (0.5 mM concentration) at 30°C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method. Briefly, 45 µL each of phenol reagent and 70 µL of alkali reagent were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Devices). All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using Soft Max Pro software (Molecular Devices) (Cannell et al., 1988).

Chymotrypsin inhibition assay

Chymotrypsin (12 Units/mL prepared in Tris-HCl buffer, pH 7.6) was pre-incubated with test compounds (prepared to a final concentration of 7% dimethylsulfoxide) for 25 min at 30°C. The substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide, 0.4 mM) was added to initiate the enzymatic reaction. The absorbance of released *p*-nitroaniline was continuously monitored at 410 nm until a significant color change was observed (Coblentz and Coblentz, 1966).

Statistical analysis

Results are presented as means ± S.E.M from three experiments as indicated in each figure legend. IC_{50} values were determined by using EZ-FIT, enzyme kinetics software by Perrella Scientific.

RESULTS AND DISCUSSION

The crude methanolic extract of *Dodonaea viscosa* was suspended in water (2 L) and successively extracted with *n*-hexane (5 L × 3), chloroform (5 L × 3), ethyl acetate (5 L × 3), and *n*-butanol (5 L × 3) to yield *n*-hexane-soluble (620 g), chloroform-soluble (890 g), EtAOc-soluble (507 g), and *n*-butanol-soluble (750 g) fractions. The EtOAc-soluble fraction was subjected to repeated CC to yield C-alkylated flavonoid (**1**-**5**) (Fig. 1). The known flavonoids were identified as 5,7-dihydroxy-3'-(3-hydroxymethylbutyl)-3,6,4'-trimethoxyflavone (**2**) (Sachdev and Kulshreshtha, 1983) 5,7,4'-trihydroxy-3'-(3-hyroxymethylbutyl)-3,6-dimethoxyflavone (**3**). Sachdev and Kulshreshtha (1982) previously reported the isolation of the latter compound from the same plant, but the ^{13}C-NMR data is presently reported for the first time, while flavonoids (5,7-dihydroxy-3'-(2-hydroxy-3-methyl-3-butenyl)-3,6,4'-trimethoxyflavone (**4**) (Anis et al., 2002) and 5,7,4'-trihydroxy-3,6-dimethoxy-3'-isoprenyl-flavone (**5**) (Wollenweber and Roitman, 2007) are isolated for the first time from this plant.

Compound **1** was isolated as a yellowish gummy solid and its molecular formula was established as $C_{25}H_{28}O_9$ by observing $[M+H]^+$ peak at *m/z* 472 in high resolution EI-MS. Characteristic UV absorption maxima in methanol at 274 and 346 nm confirmed that compound **1** is a flavonoid. The IR spectrum showed aromatic stretching at 1599 cm^{-1}, α, β-unsaturated carbonyl group stretching at 1655 cm^{-1}, methoxyl group at 2923 and 1191 cm^{-1}, and hydroxyl group at 3398 cm^{-1}. The ^{1}H-NMR of **1** showed a striking resemblance to the known compound 5,7-dihydroxy-3'-(4''-hydroxy-3''-methylbutyl)-3,6,4'-trimethoxyflavone (**2**) (Sachdev and Kulshreshtha, 1983), indicating an identical substitution pattern in rings A and B, and the side chain, except for the presence of additional acetoxyl moiety (CH_3-7'' protons in acetyl group observed around δ 1.99) at C-4'', which was also confirmed through ^{13}C-NMR by observing signals at δ 171.0 (C-6'') for ester carbonyl and δ 20.76 (C-7'') for methyl group (Iqbal et al., 2004). ^{1}H-NMR also showed proton signals of other three methoxyl groups at δ 3.97 (3H, s, MeO-4'), 3.86 (3H, s, MeO-6) and 3.85 (3H, s, MeO-3), an aromatic signal at δ 6.57 (H-8) of ring A (Sachdev and Kulshreshtha, 1983), and an ABX system in ring B of flavonoid at δ 8.00 (1H, dd, *J* = 8.4, 2.1 Hz, H-6'), 7.96 (1H, d, *J* = 2.1 Hz, H-2') and 7.11 (1H, d, *J* = 8.4 Hz, H-5'). Similarly, the other proton signals of the side chain including benzylic methylene proton attached to ring B resonated at δ 2.78 (2H, t, *J* = 6.8 Hz, H-1''). The proton signal of hydroxyl methylene protons appeared at δ 3.91 (1H, dd, *J* = 11.0, 6.6 Hz, H-4''a) and δ 3.89 (1H, dd, *J* = 11.0, 6.0 Hz, 4''b) and other side chain protons at δ 1.48 (2H, m, H-2''), 1.69 (1H, m, H-3'') and 1.01 (3H, d, *J* = 6.9 Hz, H-5''), which identified the side chain as 4''-acetoxy-3''methylbutyl. The EI-MS spectrum showed

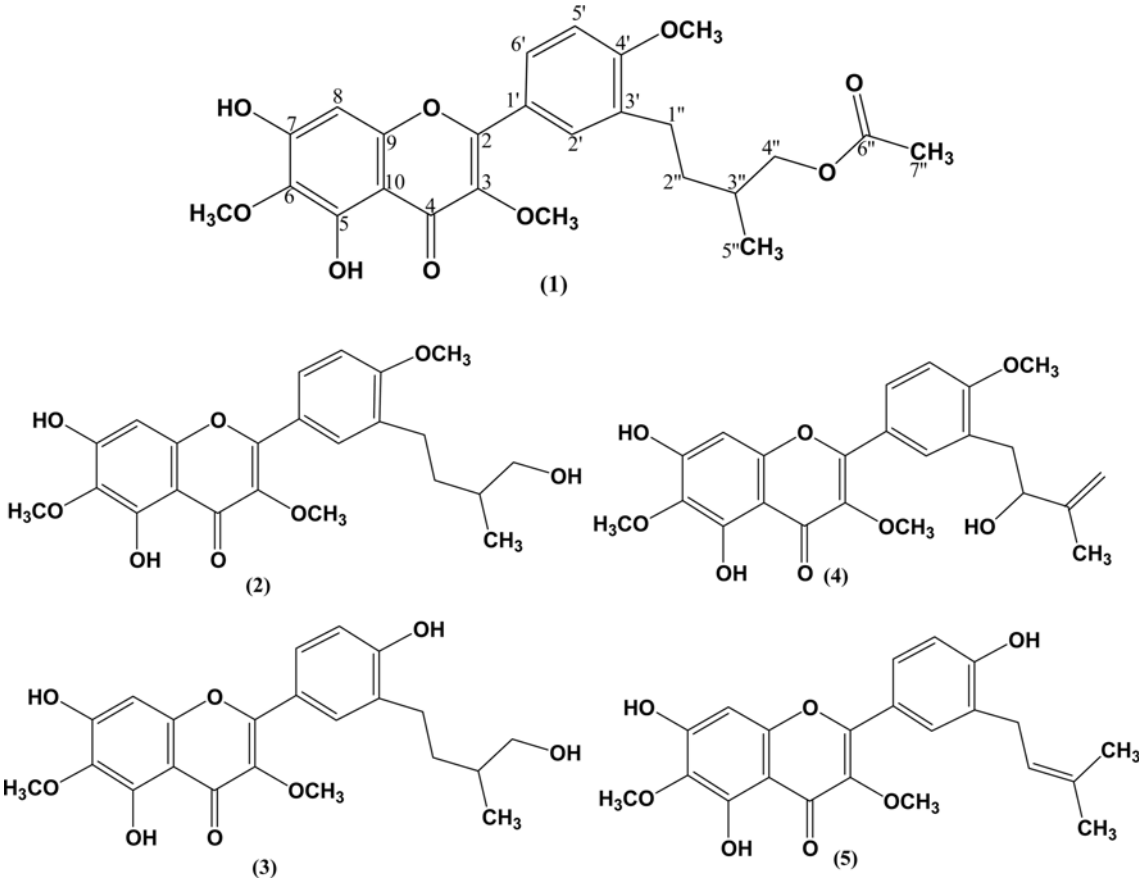

Fig. 1. Chemical structures of compounds **1-5**

M^+ peak at 472 as a base peak. The other fragments at 457, 429 were due to the losses of methyl and acetyl moiety from the molecular ion thus establishing the presence of acetyl moiety in the molecule. Additional characteristic fragment at *m/z* 357 [M-side chain $(C_6H_{11}O_2)]^+$ and 341 [M-side chain $(C_6H_{11}O_2)$-CH_3+H]$^+$ were observed due to the benzylic cleavage of the side chain and methoxyl group in ring B, while fragment at *m/z* 183 [A_1+H, $C_8H_6O_5$+H]$^+$ evidently supported the presence of one methoxyl and two hydroxyl groups in ring A (Sachdev and Kulshreshtha, 1982, 1983). The broad band (BB) and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra of **1** showed a total of 25 carbons. The assignment of all carbons were carried out by hetero atom multiple quantum coherence (HMQC) experiments. Furthermore the heteronuclear multiple bond connectivity (HMBC) of side chain methylenic proton (H-1") at δ 2.78 chain showed ^2J connectivity with C-3' (131.7), while ^3J correlation with C-4' (160.6), C-2' (130.6) and C-3" (33.2). Similarly, the methoxyl protons at δ 3.97 (MeO-4') showed interaction with C-4' (δ 160.6), thus validating the presence of the side chain and one methoxyl in ring B. The location of acetoxyl moiety was also established through a HMBC (Fig. 2) experiment in which the methyl at δ 1.99 (C-7") showed cross peaks with the ester carbonyl at δ 171.0 (C-6"). In addition, hydroxyl methylene protons at δ 3.91 and 3.89 (H-4"a, 4"b) also showed ^3J connectivity with ester carbonyl at δ171.0 (C-6") and methyl at δ17.2 (C-5"). The singlet hydroxyl proton at δ 12.94 (5-OH) showed HMBC interaction with δ 153.64 (C-5), δ 132.2 (C-6) and δ 105.7 (C-10), indicating that the hydroxyl group at C-5 position is chelated. The position of other methoxyl groups and aromatic protons were also confirmed through HMBC cross peaks (Fig. 2). Thus, compound **1** was assigned as 5, 7-dihydroxy-3'-(4"-acetoxy-3'-methylbutyl)-3, 6, 4'-trimethoxyflavone.

Urease inhibitors have attracted considerable attention as potent anti-ulcer drugs (Atta-ur-Rahman and Choudhary, 2001). A large number of synthetic compounds such as imidazoles, hydroxamic acids and phosphazenes have proven to be potential urease inhibitors, but few studies have been done on natural products associated with this activity. Here, we isolated five C-alkylated flavonoids, and all were screened for urease inhibitory activities. All these flavonoids showed very good activities (Table I). The activities of all these

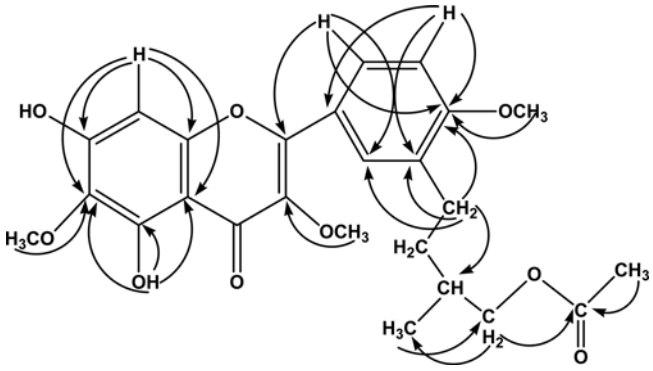

Fig. 2. Important HMBC interactions of compound **1**

flavonoids may be due to the presence of hydroxyl groups in the compounds, which can easily chelate with the Ni in the urease active site. The most active compounds **3** and **4** possess four and three hydroxyl groups, respectively. As the number of hydroxyl groups decreases the inhibition activities also decreases as exhibited in case of compounds **2** and **1**. Although the compound **5** also contains three hydroxyl groups, but the decrease in the activity in this compound may be due to the two bulky methyl groups at the end of the side chain, which can interfere during chelation process. All compounds were inactive in case of α-chymotrypsin (Table II). The α-chymotrypsin is a protease enzyme and digests protein debris of the ulcer; also it had been used for treatment of peptic ulcer (Coblentz, 1968; Atta-ur-Rahman and Choudhary, 2001).

Hence, compounds (**1**-**5**) are selective inhibitor of urease (Table I), but not α-chymotrypsin (Table II), which may enhance the healing rate of peptic ulcer and could be a potential antiulcer therapeutic.

Table I. Urease inhibitory effect of compounds **1**-**5**

Compound no.	Concentration used (mM)	% Inhibition	IC_{50} ± S.E.M. (μM)
1	0.5	57.1	316.13 ± 2.48
2	0.5	72.2	300.8 ± 2.45
3	0.5	73.6	118.4 ± 2.75
4	0.5	61.1	144.7 ± 1.39
5	0.5	75.2	348.33 ± 2.95
Standard (Thiourea)	0.5	98.2	21 ± 0.011

Thiourea, standard inhibitor of urease enzyme; SEM, standard error of the mean

Table II. Chymotrypsin inhibitory effect of compounds **1**-**5**

Compound no.	Concentration used (mM)	% Inhibition	IC_{50} ± S.E.M. (μM)
1	0.5	-	-
2	0.5	-	-
3	0.5	33.0	-
4	0.5	34.3	-
5	0.5	26.8	-
Chymostatin (Standard)	0.125	98.6	5.7 ± 0.013

Chymostatin, standard inhibitor of urease enzyme; SEM, standard error of the mean

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