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Visconata: A rare flavonol having long chain fatty acid from *Dodonaea viscosa* which inhibits Human neutrophil elastase (HNE)

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ABSTRACT

A series of flavonoids were isolated from *Dodonaea Viscosa* and tested for inhibition of human neutrophil elastase (HNE), enzyme involved in inflammatory disorders. Isolated compounds were identified as a novel flavonol (**1**) along with eight known flavonoids (**2**–**9**). Novel flavonol, visconata (**1**) has a very rare skeleton having odd numbered long chain (C19) fatty acid, which was completely identified by mass fragmentation and 2D NMR analysis. All compounds (**1**–**9**) inhibited HNE in dose dependent manner with IC_{50}s ranging between 2.4 and 150 μM. Visconata (**1**) emerged to be the most potent compound with 2.4 μM of IC_{50}. In kinetic studies, compound (**1**) was observed to be reversible, noncompetitive inhibitor having K_i = 1.8 μM, whereas other flavonoids (**2**–**9**) displayed mixed type inhibition.

Introduction

Dodonaea Viscosa (L.) Jacq is an ever green medicinal plant, belonging to Sapindaceae family and is widely distributed in tropical and subtropical countries including India and Pakistan.[1] The aerial parts of this plant has been used in folk medicine to suppress diabetes, bacterial and fungal infections, skin diseases and a variety of inflammatory disorders.[2,3] Previous phytochemical studies of *D. viscosa* have resulted in isolation of flavonoids, triterpenoids, labdane diterpenoids, and saponines.[4] Several researchers have explored the biological effectiveness of the whole extract and individual phytochemicals of *D. viscosa* such as gastroprotective, hepatoprotective, biofilm suppression, antiviral and antibacterial activities.[4–6] But still much work is needed to disclose its further biological effects especially based on enzyme inhibition. During our ongoing research on exploration of human neutrophil elastase (HNE) inhibitors from natural sources, we found that the methanol extract of aerial parts of *D. viscosa* showed potent inhibition against HNE.

Human neutrophil elastase (HNE, EC 3.4.21.37) is a serine protease, belonging to the chymotrypsin family, and is stored in azurophilic granules of neutrophil.[7] This enzyme has a broad substrate specificity, causing enzymatic cleavage of elastin, but also hydrolyzes other components of extracellular matrix including proteoglycan, collagen and fibronectin, hence leading to connective tissues degradation and inflammation.[8] The uncontrolled elastolytic activity has been implicated in several diseases such

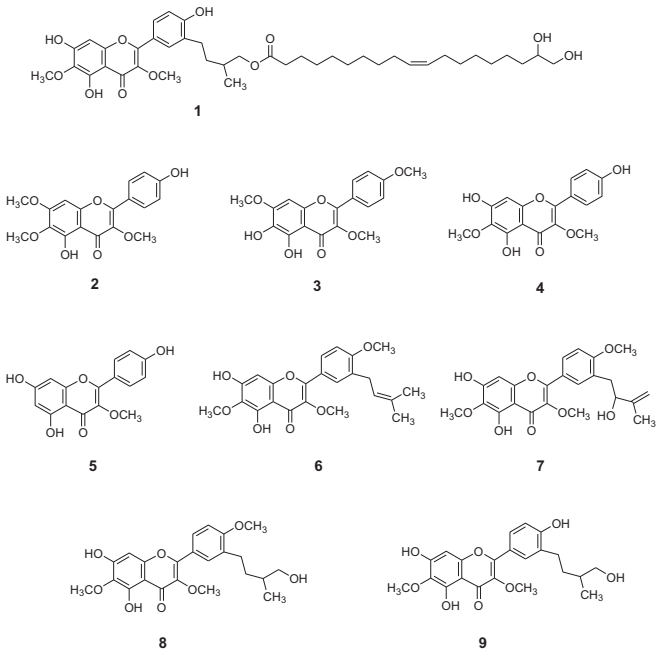

Fig. 1. Chemical structures of isolated compounds (**1**–**9**) from *Dodonaea viscosa*.

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as cystic fibrosis, acute respiratory distress syndrome, chronic obstructive pulmonary disease, acute lung injury, atherosclerosis and other inflammatory diseases such as rheumatoid arthritis.[9,10] Recently several studies has also reported its role as key mediator of secondary pathogenesis in spinal cord injury, brain ischemia, obesity and insulin resistance [8,11]. Being a multiple player, discovery of novel HNE inhibitors which targets its substrates could be a promising strategy to contribute in the ongoing research against inflammatory diseases.

Aim of the present work was to investigate HNE inhibitory activity of phytochemicals of *D. viscosa*, in this regard the methanol extract of aerial parts gave nine phenolic compounds (**1**–**9**), especially a novel compound (**1**) having a unique odd number long chain fatty acid (C19). We elucidated compound **1** to be a flavonol having odd number long chain fatty acid by mass fragmentation using HREIMS and detailed 1D and 2D NMR analysis. Furthermore inhibitory mechanisms of isolated inhibitors were ascertained by kinetic plots.

Results and discussion

In preliminary experiment, the methanol extract of the areal parts of *D. viscosa* exhibited a good potential activity against HNE (100 μg/ml, 75% inhibition). Successive purification of methanol extract over dianion HP-20, silica gel, Octadecyl-functionalized silica gel, and Sephadex LH-20 gave nine flavonoids (**1**–**9**) (Fig. 1), which were responsible for HNE inhibition. These compounds were identified as penduletin (**2**), 5,6-dihydroxy-3,4′,7-trimethoxyflavone (**3**), viscosine (**4**), isokaemferide (**5**), viscosol (**6**), 5,7-dihydroxy-3′-(2-hydroxy-3-methylbutenyl)-3,6,4′-trimethoxy-flavone (**7**), 5,7-dihydroxy-3′-(3-hydroxy-methylbutyl)-3,6,4′-trimethoxy-flavone (**8**), and 5,7,4′ -trihydroxy-3′-(3-hydroxymethylbutyl)-3,6-dimethoxyflavone (**9**), through analysis of spectroscopic data and comparison with previous studies, (Supplementary material)[12,13,14a].

Compound **1** was isolated as a pale yellow sticky oil with molecular formula $C_{41}H_{58}O_{11}$ by the [M$^+$] ion at 726.3980 (Calcd

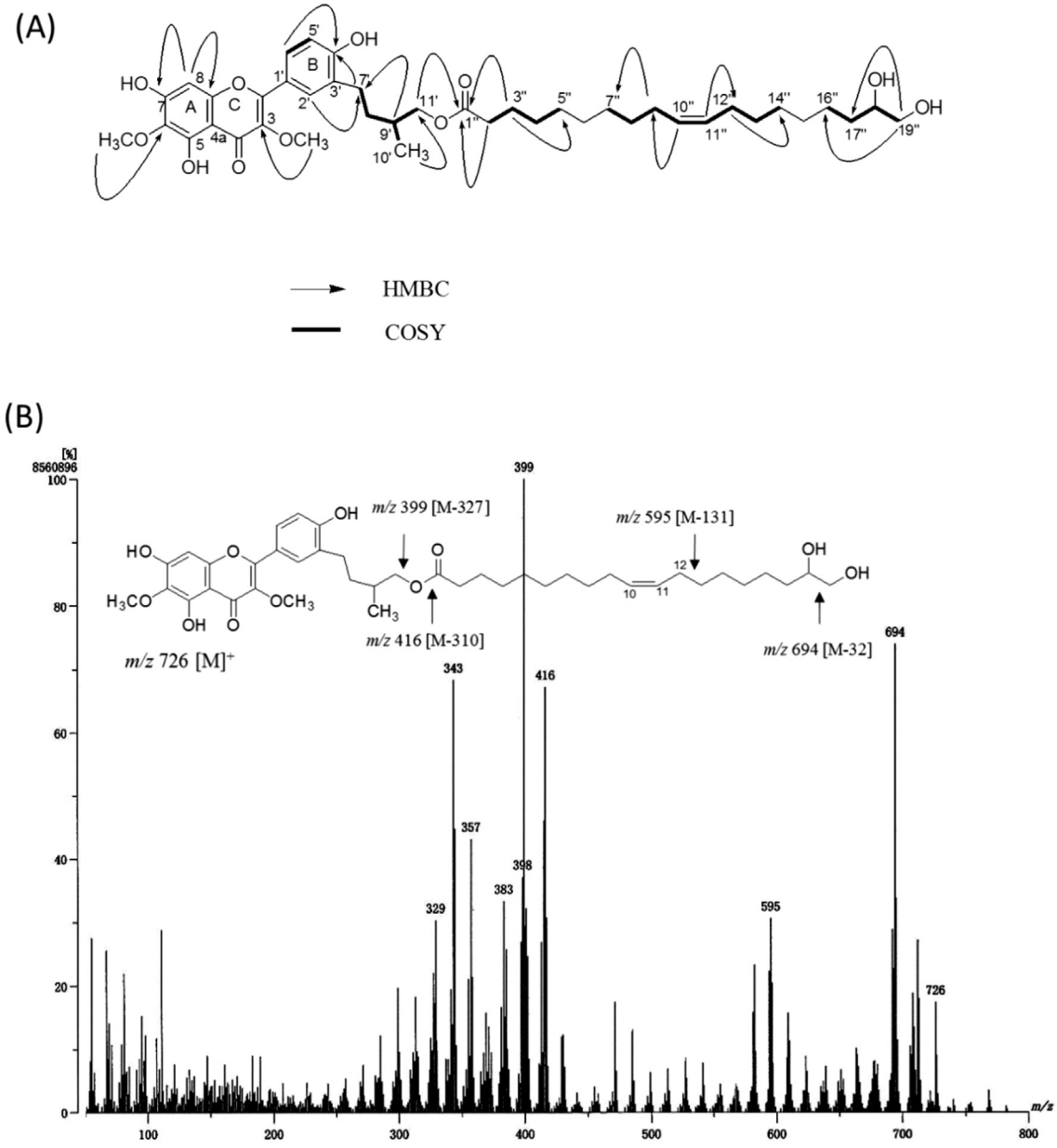

Fig. 2. Key ^{1}H-^{1}H COSY and HMBC correlations of compound **1** (A). Proposed fragmentation pattern of compound **1**, deduced by EI-MS (B).

726.3979) in the HREIMS[14b]. The IR spectrum showed absorption for OH (3450 cm^{-1}) and two carbonyls (1750 and 1660 cm^{-1}). The UV spectrum resembled that of flavonol derivatives with λ_{max} 270 nm (log ε = 4.0) and 350 nm (log ε = 4.2). DEPT experiments in conjunction with ^{1}H and ^{13}C NMR data indicated the presence of 41 carbon atoms consisting of the following carbon characteristics: 18 methylenes (sp^3), 2 methines (sp^3), 6 methines (sp^2), 3 methyles and 12 quaternary carbons. The extra three degrees of unsaturation after counting C-C and C-O double bonds were ascribed to tricyclic skeleton of flavonol structure. The overall NMR characteristics suggested that **1** bears a flavonol backbone. A characteristic hydrogen bonded proton signal of C-5 (δ_C 152.2) hydroxyl group was observed at δ_H 12.94. The 5, 6, 7-trisubstitution of A-ring was deduced by a singlet of H-8 (δ_H 6.40) that has a strong HMBC correlation with oxygenated carbons C-7 (δ_C 152.4) and C-8a (δ_C 157.7). The presence of C6-OCH_3 was proved by HMBC correlation of OCH_3 (δ_H 3.78) and C-6 (δ_C 131.3). The location of C3-OCH_3 in C-ring was pointed by strong correlation of OCH_3 (δ_H 3.66) with C-3 (δ_C 137.7). The features of B-ring were confirmed by ABX coupling between H-2′ (δ_H 7.76, d, J = 2.0 Hz), H-5′ (δ_H 6.78, d, J = 8.5 Hz), and H-6′ (δ_H 7.71, dd, J = 2.5, 8.5 Hz). The position of C4′-OH was deduced by HMBC correlation between oxygenated carbon C-4′ (δ_C 158.2) and H-5′/H-6′ (δ_H 6.78 / 7.71). The presence of 3-hydroxymethylbutyl motif on C-3′ was deduced from successive protons network across H-7′ (δ_H 2.60), H-8′a/H-8′b (δ_H 1.36, 1.65), H-9′ (δ_H 1.75), H-10′ (δ_H 0.93), and H-11′ (δ_H 3.89) in the COSY spectrum (Fig. 2A, supplemental). The location of this functionality was confirmed by HMBC correlation of H-7′ (δ_H 2.60) with C-2′ (δ_C 130.0), C-3′ (δ_C 129.0), and C-4′ (δ_C 158.2). Thus the above obtained spectral data showed that **1** has 5,7-dihydroxy-2-(4-hydroxy-3-(4-hydroxy-3-methylbutyl) phenyl)-3,6-dimethoxy-4H-chromen-4-one moiety. As shown in Fig. 2B, odd numbered long chain (C19) fatty acid motif was elucidated by the fragment ions at m/z 399 [M-327] and 416 [M-310], which were selected as main diagnostic product ions in EI-Mass analysis (Fig. 2B). The position of double bond in C19 chain was determined by the fragment ion at m/z 595 [M-131], which was formed by the α-cleavage of alkene at a position between C12″ and C13″ (Fig. 2B). The J value of 10.5 Hz pointed that there is a *cis*-configuration between H-10″ and H-11″. Terminal diol functionality was confirmed by successive protons connectivity between oxygenated atom H-19″ (δ_H 3.44), H-18″ (δ_H 3.40), H-17″ (δ_H 1.24–1.45) and H-16″ (δ_H 1.24–1.45), as well as HMBC correlation of H-19″ (δ_H 3.44) with C-16″ (δ_C 21.7), and C-17″ (δ_C 32.3). The attachment of fatty acid chain with main skeleton(flavonol) was proved by a strong HMBC correlation between H-11′ (δ_H 3.89) and carbonyl C-1″ (δ_C 174.2). Base hydrolysis of **1** by LiOH yielded mother skeleton **9** that was ascertained by UPLC-Q-TOF/MS analysis with reference to an authentic sample. Specific rotation value was measured as $[\alpha]_D$ = 6.4 (*c* 0.56, MeOH). Thus the compound was identified as (*Z*)-4-(5-(5,7-dihydroxy-3,6-dimethoxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-2-methylbutyl-18,19-dihydroxy-nonadec-10-enoate, named visconata (**1**). Spectral data of all the detected carbons, protons, COSY and HMBC of **1** are given in Table 1 and Fig. 2A. To the best of our knowledge, flavonoid skeleton having long chain fatty acid has never been reported yet. As well as occurrence of odd numbered long chain fatty acid is rare in terrestrial plants[15], therefore these results are striking, which could be a starting for further similar discoveries.

The isolated compounds (**1**–**9**) were analyzed for their inhibitory potential toward HNE. The enzyme inhibition was assayed according to a standard literature procedure by following hydrolysis of methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide spectrophotometrically[16]. As shown in Table 2 and Fig. 3A, all isolated

Table 2
Inhibitory effects of compounds **1**–**9** on HNE activities.

Compounds	Human neutrophil elastase	
	IC_{50} (μM)[a]	Type of inhibition (K_i [b], μM)
1	2.4 ± 0.2	Noncompetitive (1.8 ± 0.1)
2	65.4 ± 0.1	Mixed (44.9 ± 0.3)
3	25.4 ± 0.4	Mixed (23.2 ± 0.2)
4	150.2 ± 1.2	NT[c]
5	93.9 ± 0.6	Mixed (74.6 ± 0.4)
6	10.9 ± 0.3	Mixed (8.0 ± 0.5)
7	114.7 ± 0.2	NT[c]
8	33.4 ± 0.5	Mixed (22.0 ± 0.1)
9	74.7 ± 0.3	Mixed (54.8 ± 0.6)
Caffeic acid[d]	67.7 ± 0.9	NT[c]

[a] All compounds were examined as set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss.
[b] Values of inhibition constant.
[c] NT: not tested.
[d] Positive control

Table 1
^{1}H and ^{13}C NMR data of new Compound **1**.

Position	δ_H J (Hz)	δ_C m	Position	δ_H J (Hz)	δ_C m
2		156.9 s	1″		174.2 s
3		137.7 s	2″	2.20 t, (7.5)	33.8 t
4		178.8 s	3″	1.48 t, (7.5)	24.7 t
5		152.2 s	4″[b]	1.14–1.17 m	28.7 t
6		131.3 s	5″[b]	1.14–1.17 m	28.8 t
7		152.4 s	6″	1.24–1.29 m	36.8 t
8	6.40 s	93.7 d	7″	1.32–1.36 m	36.6 t
8a		157.7 s	8″[b]	1.14–1.17 m	28.7 t
4a		104.8 s	9″	1.84–1.92 m	26.7 t
1′		121.1 s	10″	5.17 m (10.5)	129.2 d
2′	7.76 d (2.0)	130.0 d	11″	5.22 m (10.5)	129.5 d
3′		129.0 s	12″	1.84–1.92 m	26.7 t
4′		158.2 s	13″[b]	1.14–1.17 m	29.3 t
5′	6.78 d (8.5)	114.6 d	14″	1.24–1.45 m	25.5 t
6′	7.71 dd (2.5, 8.5)	127.6 d	15″[b]	1.14–1.17 m	28.8 t
7 '	2.60 m	27.0 t	16″	1.24–1.45 m	21.7 t
8′a, 8′b	1.36,1.65 m	33.0 t	17″	1.24–1.45 m	32.3 t
9′	1.75 m	32.2 d	18″	3.40 m	70.8 d
10′	0.93 d (7.0)	15.8 q	19″	3.44 t (6.5)	61.5 t
11′	3.89 dd (1.0, 6.0)	68.6 t	C3-OCH_3	3.66 s	59.1 q
5-OH	12.94 s ($CDCl_3$)[a]		C6-OCH_3	3.78 s	59.5 q

[a] $CDCl_3$ solvent was used only to detect 5-OH signal at 12.94 ppm.
[b] Overlapped signals.

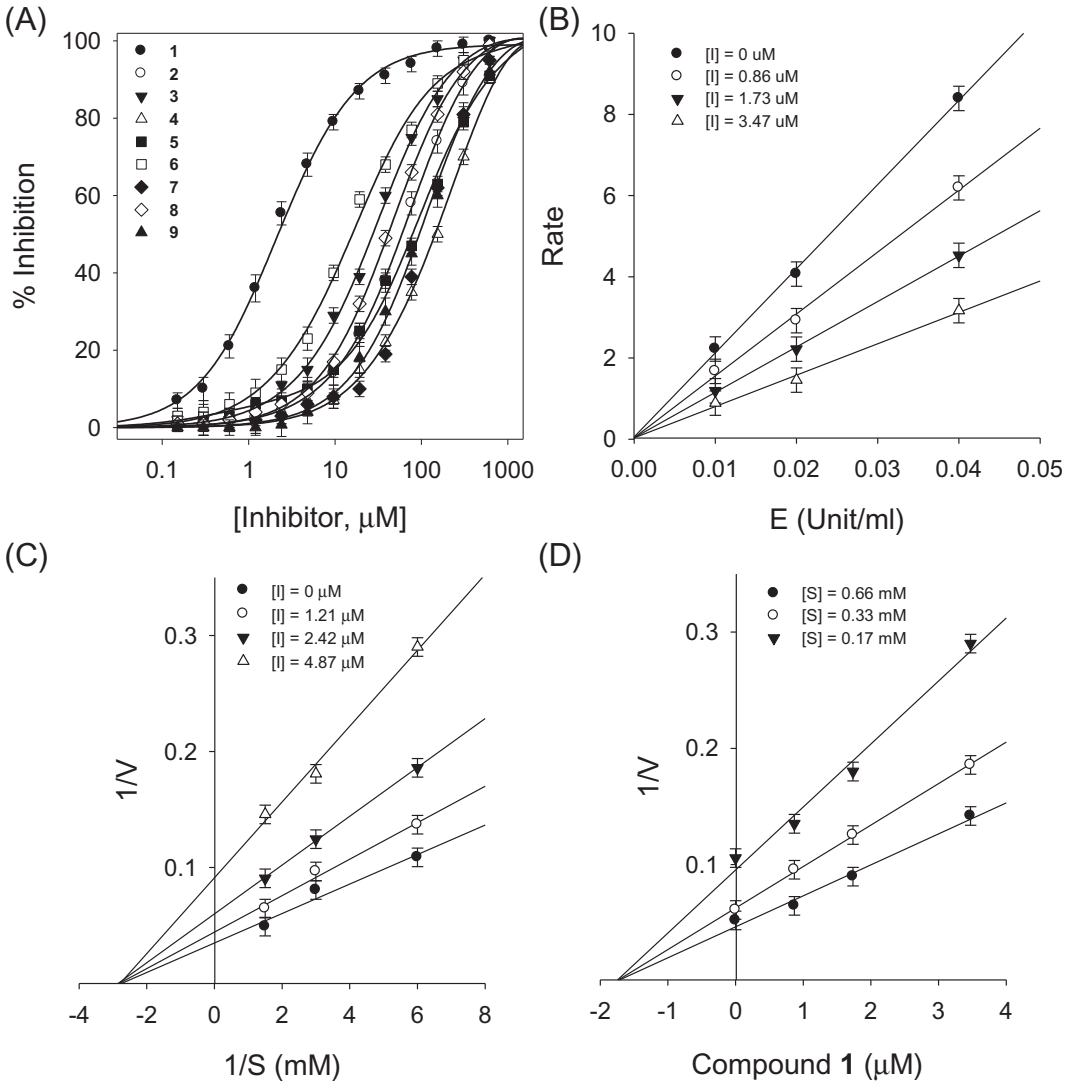

Fig. 3. (A) Dose-dependent inhibitory effects of isolated compounds (**1**–**9**) on HNE. (B) Determination of the reversible inhibitory mechanism of visconata (**1**). (C) Lineweaver-Burk plot for HNE inhibition by compound **1**. (D) Dixon plot for HNE inhibition by compound **1**.

flavonols inhibited HNE activity dose-dependently with IC_{50} values of 2.4–150 μM. The potencies of flavonols were affected by subtle changes in compounds structures. It appears that better inhibition is observed when the flavonol B-ring bears methoxy group and prenyl group. This is apparent from the comparison of compounds **6** (IC_{50} = 10.9 μM), **3** (IC_{50} = 25.4 μM) and **4** (IC_{50} = 150.2 μM). The most striking aspect of this result is the long chain fatty acid which is pivotal in HNE inhibition, due to which compound **1** (IC_{50} = 2.4 μM) was 30-fold more potent than mother molecule **9** (IC_{50} = 74.7 μM) shown in Table 2. Further kinetic analysis showed that compound **1** exhibited reversible noncompetitive inhibitory behavior, because increasing inhibitor concentrations resulted in a family of straight lines with different slopes but a common x-axis intercept (Fig. 3B,C). Dixon plot unveiled the inhibitory constant to be K_i = 1.8 μM, given in Fig. 3D. Whereas other flavonols exhibited mixed inhibition kinetics. For example, flavonol **6** showed that with increasing concentration of inhibitors resulted in a family of lines which shared a common intercept on left side of the vertical axis and above the horizontal axis (Supplementary material). The inhibitory constants (K_i) values of tested compounds were also determined by Dixon plots which are given in Table 1.

In conclusion, we isolated a new compound along with eight known flavonoids (**1**–**9**) which inhibited HNE in dose dependent manner. The novel compound, named visconata, has an odd number long chain fatty acid (C19). This is the first report on flavonoid attached long chain fatty acid, which has never been reported so far. In evaluation of HNE inhibition, the most active inhibitor was found to be compound **1** (IC_{50} = 2.4 μM). In kinetic study compound **1** emerged to be a reversible, noncompetitive inhibitor with (K_i = 1.8 μM), whereas rest of flavonoids (**2**–**9**) displayed mixed type inhibition.

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Supplementary data

Supplementary data (UV, IR, MS, NMR and kinetic data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2017.05.059.

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14. (a) Sachdev K, Kulshreshtha DK. *Phytochemistry*. 1986;25:1967–1969(b) The aerial parts of *Dodonaea Viscosa*, were collected at hilly mountains in Malakand, Pakistan, in May 2014. The voucher specimens (SWAT00261) were identified by Professor Zahid Ullah, and deposited for future references at Herbarium of University of Swat, KPK, Pakistan. Plant material (1.2 kg) was extracted with methanol at room temperature for ten days to give an extract (180 g). After removing chlorophyll with Diaion HP-20, the extract (25 g) was subjected to silica gel and eluted with a gradient of hexane in ethylacetate (100–0%), to give ten different fractions (A-J). Fraction F (1.2 g) was fractionated through MPLC using C18 column (130 g) with gradient elution of MeOH in H2O (0–100%) having flow rate of 15 ml/min, to give the fraction enriched with compound **1**, which was subjected to MPLC repeatedly eluting with MeOH in H2O (0-100%). This isolated fraction was further chromatographed over a sephadex LH-20 column eluting with MeOH-H2O (90:10) to yield compound **1** (11 mg).
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