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LIPOXYGENASE INHIBITING FLAVONOIDS FROM *INDIGOFERA HETRANTHA*

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Abstract-Hetranthins A (**1**) and B (**2**), new flavonoids, together with known compounds 7,3′,4′-trihydroxyflavanone (**3**) and 3,5,7,3′,4′-pentahydroxyflavanone **(4)** have been isolated from the ethyl acetate fraction of *Indigofera hetrantha*. Their structures have been assigned on the basis of spectral analysis. Compounds (**1-4**) showed inhibitory activity against lipoxygenase enzyme.

INTRODUCTION

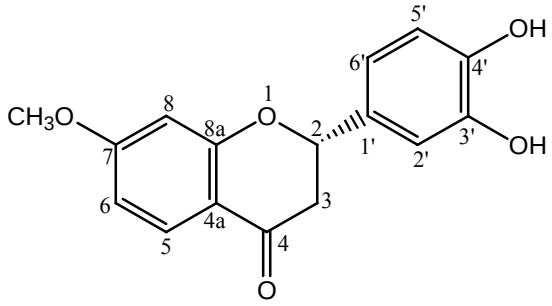
The genus *Indigofera*, belonging to the family Leguminosae, comprises of 300 species. All of these are herbs or shrubs, distributed throughout the tropical regions of the globe. In Pakistan it is represented by 24 species.[1] The family Leguminosae is known to be a rich source of flavonoids and most of the phenyl derivatives have been found in this family.[2,3] The flavonoids exhibit diverse biological activities and recent interest has been focussed on their medicinal and nutritional values.[4] Recently, it was found that the prenyl groups on the flavonoid skeleton play an important role in anti-HIV activity.[5] *Indigofera hetrantha* Wall is widely distributed in northern parts of Pakistan and finds various medicinal uses in the indigenous system of medicine. The sister species of *Indigofera hetrantha* were found to contain toxic nitro compounds of insecticidal activity. Previously triterpenes, steroids and flavonoids have been reported from *Indigofera hetrantha.*[6] In the present investigation, a methanolic extract of the stem of *Indigofera hetrantha* showed strong cytotoxcity in the brine shrimp lethality test.[7] Further biological screening of the methanolic and ethyl acetate fractions revealed significant inhibitory activity against the enzyme lipoxygenase. Herein we report the isolation and structural elucidation of flavonoids hetranthins A (**1**) and B (**2**), along with the known compounds 7,3′,4′-trihydroxyflavanone (**3**) and 3,3′,4′,5,7-pentahydroxyflavanone (**4**) reported for the first time from this species.[8,9] The compounds (**1-4**) showed inhibitory activity against lipoxygenase enzyme.

Lipoxygenases (EC 1.13.11.12) constitute a family of non-heme iron containing dioxygenases that are widely distributed in animals and plants. In mammalian cells these are key enzymes in the biosynthesis of variety of bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines.[10] It has been found that these lipoxygenase products play a role in a variety of disorders such as bronchial asthma, inflammation[11] and tumor angiogenesis.[12] Lipoxygenases are therefore potential target for the rational drug design and discovery of mechanism-based inhibitors for the treatment of bronchial asthma, inflammation, cancer and autoimmune diseases.

RESULTS AND DISCUSSION

Ethylacetate soluble fraction of the methanolic extract of the stem of *Indigofera hetrantha* was subjected to column chromatography over flash silica eluting with different mobile phases. Compounds (**1-4**) were finally obtained and their structures established by UV, IR, MS and NMR spectroscopy.

Hetranthin A (**1**) was isolated as a colorless crystalline solid, m.p. 141-142°C. The molecular formula was assigned $C_{16}H_{14}O_5$ by HREIMS showing a $[M]^+$ ion at *m/z* 286.0841 (calcd for $C_{16}H_{14}O_5$, 286.0844). The IR spectrum revealed the presence of hydroxyl groups (3452 cm^{-1}), a methoxyl group (1588, 1112 cm^{-1}), and a conjugated carbonyl group (1670 cm^{-1}) in the molecule. The UV spectrum exhibited characteristic absorption peaks of the flavanone moiety at 228, 273, and 296 nm.[13] The presence of flavanone structure was also indicated by an AMX system with resonance at δ 5.20 (1H, dd, J = 12.6, 3.1 Hz, H-2), 2.81 (1H, dd, J = 17.0, 12.6 Hz, H-3_{ax}) and 2.44 (1H, dd, J = 17.0, 3.1 Hz, H-3_{eq}).[13,14,15] In the aromatic region of the ^{1}H-NMR spectrum of **1** further signals which appeared at δ 6.67 (1H, d, J = 1.8 Hz, H-2′), 6.62 (1H, d, J = 8.0 Hz, H-5′) and 6.44 (1H, dd, J = 8.0, 1.8 Hz, H-6′) were assigned to the protons of 1, 3, 5-trisubstituted ring B and the signals appearing at δ 7.71 (1H, d, J = 8.5 Hz, H-5), 6.59 (1H, d, J = 1.8 Hz, H-8) and 6.46 (1H, dd, J = 8.5, 1.8 Hz, H-6) were assigned to the protons of mono-substituted ring A. The methoxyl protons appeared at δ 3.90 (3H, s, OMe-7). The ^{13}C-NMR (BB, DEPT) spectra of **1** corroborated the presence of one methyl, one methylene, seven methine and seven quaternary carbons. The signals at δ 78.4, 41.3 and 201.7 were typical of C-2, C-3 and C-4 carbons of the flavanone skeleton.[14,15] The EIMS gave a distinct peak at *m/z* 286 and further two fragment ions were observed at *m/z* 150 and 136 due to retro-Diels-Alder fragmentation, confirming the presence of one methoxyl groups in ring A and two hydroxyl groups in ring B of the flavanone skeleton. The position of the hydroxyl and methoxyl groups were further confirmed by HMBC (Figure 1). The absolute configuration at C-2 was assigned '*S*' on the basis of CD spectrum which showed similar Cotton effects as reported literature for related flavonoids.[16,17] On the basis of these evidence, the structure of **1** was assigned as (2*S*)-3′,4′-dihydroxy-7-methoxyflavanone.



1

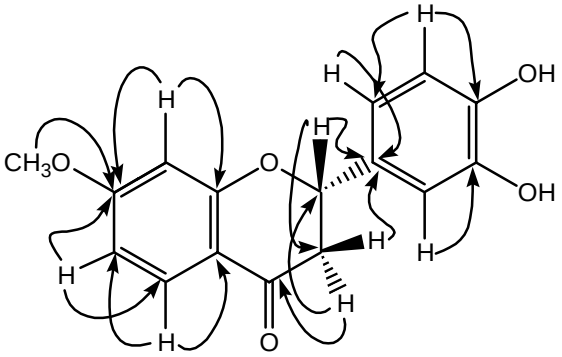
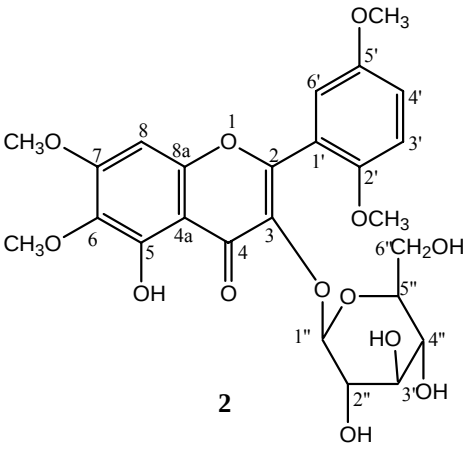


Figure 1: Improtant HMBC correlations of **1**

Hetranthin B (**2**) was isolated as colorless gummy solid. The molecular formula $C_{25}H_{28}O_{13}$ was established by HR-FAB-MS showing $[M+H]^+$ peak at *m/z* 537.1523 (calcd for $C_{25}H_{27}O_{13}$, 537.1529). Positive Shinoda test,[18] and negative Quastel test indicated the absence of an ortho dihydroxyl moiety[19] and the UV absorption maxima in MeOH at 273 and 342 nm suggested that compound (**2**) was a flavonoid.[20] The IR spectrum showed the α, β–unsaturated carbonyl group at 1728 and 1645 cm^{-1}, methoxyl group at 2925 and 1190 cm^{-1}, hydroxyl group at 3396 cm^{-1} and aromatic ring at 1600 cm^{-1}. In the presence of $AlCl_3$-HCl band **2** showed a bathochromic shift of about 18 nm relative to the spectrum in pure methanol. The magnitude of this shift indicated the presence of hydroxyl group at position 5 and a further methoxyl group at position 6.[21] The ^{1}H-NMR spectrum provided signals of functional groups including a chelated hydroxyl group at δ 12.71 (1H, s) and four aromatic methoxyl groups appeared at δ 3.90, 3.92, 3.95 and 3.96, each integrated for three protons. The signal at δ 6.51 (1H, s, H-8) could be assigned to the aromatic proton of ring A and the aromatic protons of ring B resonated at δ 7.32 (1H, d, *J* = 8.5 Hz, H-3′), 6.98 (1H, dd, *J* = 8.5, 2.2 Hz, H-4′) and 7.50 (1H, d, *J* = 2.2 Hz, H-6′). The presence of sugar moiety in β configuration was revealed by a doublet of anomeric proton at 5.01 (1H, d, *J* = 7.5 Hz, H-1″). The signals of four protons geminal to hydroxyl groups appeared at [δ 3.54 (1H, t, *J* = 9.5 Hz H-4″), 3.67 (1H, m, H-5″), 3.85 (1H, t, *J* = 9.5 Hz H-3″), 3.97 (1H, dd, *J* = 9.5, 3.7 Hz H-2″)], the methylenic protons at δ 3.65 (1H, dd, *J* = 11.2, 4.5 Hz H-6″a) and 3.75 (1H, dd, *J* = 11.2, 5.1 Hz H-6″b). The sugar moiety could be identified through acid hydrolysis, which provided the glycone being identified as D-glucose through sign of its optical rotation and comparison of retention time of its TMS ether with that of standard in GLC. The ^{13}C-NMR spectrum (BB, DEPT) of **2** corroborated the presence of four methyls, one methylene, nine methines and eleven quaternary carbons. The signals at δ 149.5, 134.9 and 180.4 being the typical signals of C-2, C-3 and C-4, indicated that **2** is a 3-flavonol glycoside (Table 1). The position of glucose moiety was confirmed at C-3 by HMBC experiments; the important correlations illustrated in Figure 2. The EI-MS spectrum showed a fragment at *m/z* 374 due to the loss of sugar moiety from the molecule. The other two characteristic fragments were observed at *m/z* 196 and 178 due to the retro-Diels-Alder fragmentation, confirming the one hydroxyl and methoxyl groups in ring A, and two methoxyl groups in ring B of the

flavonoid skeleton. The position of the hydroxyl and methoxyl groups was confirmed by long-range HMBC experiments in (Figure 2). On the basis of these evidence, the structure of **2** could be assigned as 3-*O*-*β*-D-gluco-5-hydroxy-6,7,2′,5′-tetramethoxyflavone.



2

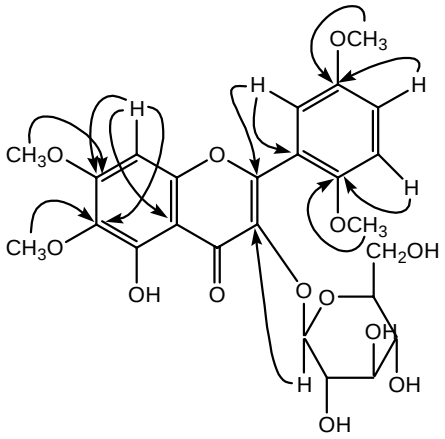


Figure 2: Important HMBC correlations of **2**

Table 1 ^{1}H (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) spectral data for **1** and **2**

Position	**1**		**2**	
	δC (mult.)	δH (mult., *J* in Hz)	δC (mult.)	δH (mult., *J* in Hz)
2	78.4 (d)	5.20 (dd, *J* = 12.6, 3.1)	149.5 (s)	
3	41.3 (t)	2.81 (dd, *J* = 17.0, 12.6) 2.44 (dd, *J* = 17.0, 3.1)	134.9 (s)	
4	201.7 (s)		180.4 (s)	
4a	115.9 (s)		106.2 (s)	
5	134.4 (d)	7.71 (d, *J* = 8.5)	153.2 (s)	OH 12.71 (s)
6	109.5 (d)	6.46 (dd, *J* = 8.5, 1.8)	126.5 (s)	
7	162.9 (s)		152.5 (s)	
8	99.8 (d)	6.59 (d, *J* = 1.8)	92.7 (d)	6.51 (s)
8a	165.7 (s)		158.8 (s)	
1′	131.0 (s)		124.0 (s)	
2′	116.0 (d)	6.67 (d, *J* = 1.8)	153.2 (s)	
3′	145.9 (s)		120.1 (d)	7.32 (d, *J* = 8.5)
4′	144.7 (s)		111.4 (d)	6.98 (dd, *J* = 8.5, 2.2)
5′	117.5 (d)	6.62 (d, *J* = 8.0)	153.7 (s)	
6′	121.8 (d)	6.44 (dd, *J* = 8.0, 1.8)	110.2 (d)	7.50 (d, *J* = 2.2)
OMe-6			56.6 (q)	3.90 (s)
OMe-7	56.7 (q)	3.90 (s)	56.5 (q)	3.92 (s)
OMe-2′			56.3 (q)	3.95 (s)
OMe-5′			56.1 (q)	3.96 (s)
1″			101.8 (d)	5.01 (d, *J* = 7.5)
2″			74.8 (d)	3.97 (dd, *J* = 9.5, 3.7)
3″			78.3 (d)	3.85 (t, *J* = 9.5)
4″			71.1 (d)	3.54 (t, *J* = 9.5)
5″			78.3 (d)	3.67 (m)
6″			62.4 (t)	3.65 (dd, *J* = 11.2, 4.5) 3.75 (dd, *J* = 11.2, 5.1)

Baicalein was used as positive control in lipoxygenase inhibiting assay. From the results (Table 2) it is apparent that compounds (**1**, **3** and **4**) have significant lipoxygenase inhibitory potential. This may be due

to the presence of stereo-center as well as higher number of hydroxyl groups on the aromatic ring as compared to the compound (**2**), which has only one hydroxyl moiety.

Table 2: *In vitro* inhibition of lipoxygenase by compounds (**1-4**)

Compounds	IC_{50} (µg/ mL)± SEM[1]
1	2.1 ± 0.06
2	59.8 ± 1.6
3	3.7 ± 0.01
4	6.9 ± 0.05
Baicalein[2]	22.5 ± 0.5

[1] Standard mean error of five assays

[2] Standard inhibitor of lipoxygenase

EXPERIMENTAL

General: Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectra were recorded on a 460 Shimadzu spectrometer. EIMS and HRFABMS were recorded on JMS-HX-110 and JMS-DA 5000 mass spectrometers. The ^{1}H-^{13}C NMR, HMQC, and HMBC spectra were recorded on Bruker spectrometers operating at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C-NMR, respectively. The chemical shift values are reported in ppm (δ) units and the coupling constants (*J*) are in Hz. Aluminum sheets precoated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E-Merck) were used for TLC and flash silica (230–400 mesh) was used for column chromatography. Visualization of the TLC plates was carried out under UV at 254 and 366 nm and by spraying with ceric sulfate reagent solution (with heating). For enzyme inhibition assay, all chemicals used and lipoxygenase (1.13.11.12) type I-B were purchased from Sigma (St. Louis, MO, USA). The GC was performed on a Shimadzu gas chromatograph (GC-9A) (3% OV-1 silanized *chromosorb W,* column temperature 180 ^{o}C, injection port and detector temperature 275–300 ^{o}C, flow rate 35 ml/min, flame-ionization detector).

Plant Material: The whole plant material was collected from Swat and identified as *Indigofera hetrantha* by Mr. Habib Ahmad. A voucher specimen is deposited in the herbarium of the Department of Botany, Government, Postgraduate Jahanzeb Degree College, Saidu Sharif, Swat, Pakistan.

Extraction and Isolation: The shade dried whole plant material (30 kg) was extracted thrice with MeOH (80 L) at rt for 192 h . The combined methanolic extract (800 g) was partitioned between n-hexane and water. The water fraction was further extracted out with $CHCl_3$ and EtOAc. The EtOAc fraction (250 g) was subjected to VLC (vacuum liquid chromatography) over plate silica and eluted with $CHCl_3$, $CHCl_3$-MeOH and MeOH in increasing order of polarity to obtain six fractions (A-F). The fraction B (35 mg) obtained from $CHCl_3$-MeOH (9.5:0.5) was further subjected to medium pressure liquid chromatography over flesh silica eluting with $CHCl_3$-MeOH in increasing order of polarity. The fraction (21 mg) obtained from $CHCl_3$-MeOH (9:1) was further purified by column chromatography using silica gel (70-230 mesh) eluting with $CHCl_3$-MeOH (9.8:0.2) to furnish hetranthin A (**1**), (10 mg). The fraction C (45 mg) obtained from $CHCl_3$-MeOH (8.5:1.5) was a mixture of two components, which were separated by column chromatography over flash silica using solvent system $CHCl_3$-MeOH (9:1) to afford compounds (**3**) (12 mg) and (**4**) (15mg). The fraction D (26 mg) obtained from $CHCl_3$-MeOH (8:2) was rechromatographed over flash silica and eluted with $CHCl_3$-MeOH (8.5:1.5) to afford the hetranthin B (**2**) (18 mg).

Acid Hydrolysis of hetranthin B (2)

A solution of compound (**2**) (8 mg) in MeOH (5 mL) containing 1 N HCl (4 mL) was refluxed for 4 h, concentrated under reduced pressure, and diluted with H_2O (8 mL). It was extracted with EtOAc. The aqueous phase was concentrated and D-glucose was identified by the sign of its optical rotation ($[\alpha]_D^{20}$+ 52^o). It was also confirmed based on the retention time of its TMS ether (α-anomer 4.1 min, β-anomer 7.8 min) with a standard.

Hetranthin A (1): Colorless crystalline solid; mp 141-142°C ($CHCl_3$); $[\alpha]_D^{25}$ -8.2^o (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 246 (4.36), 273 (3.92) and 298 (4.15) nm; IR (KBr) ν_{max}: 3452, 1670, 1588, 1112 cm^{-1}; CD: $\Delta\varepsilon_{282}$ –5.6, $\Delta\varepsilon_{310}$ +2.8; ^{1}H and ^{13}C-NMR (CD_3OD), see Table 1; EIMS *m/z* (rel. int.) 286 (46), 151 (69), 136 (36), 107 (53); HREIMS *m/z* 286.0841 (calcd for $C_{16}H_{14}O_5$, 286.0844).

Hetranthin B (2): Colorless gummy solid; $[\alpha]_D^{25}$ +28.2^o (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 273 (261) and 342 (2.48) nm with $AlCl_3$-HCl 286 nm, 341 (sh); IR (KBr) ν_{max}: 3396, 2925, 1728, 1645, 1190 cm^{-1}; ^{1}H and ^{13}C-NMR (CD_3OD), see Table 1; EIMS *m/z* (rel. int.) 374 (15), 359 (81), 342 (76), 196 (10), 178 (7); HR-FAB-MS *m/z* 537.1523 $[M+H]^+$ (calcd for $C_{25}H_{27}O_{13}$, 537.1529).

Assay of Lipoxygenase Inhibition

Lipoxygenase-inhibiting activity was conveniently measured by slightly modifying the spectrometric method developed by Tappel.[22] Lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma Chemicals. A mixture of 160 µL of 0.1 mM sodium phosphate buffer (pH 7.0), 10 µL of test compound solution, and 20 µLof lipoxygenase solution was incubated for 5 min at 25 ^{o}C. The reaction

was then initiated by the addition of linoleic acid 10 µL (substrate) solution, with the formation of (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoate. The change in absorbance was followed for 10 min. Test compounds and the control were dissolved in 50% EtOH. All the reactions were performed in triplicate. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, MA, USA).

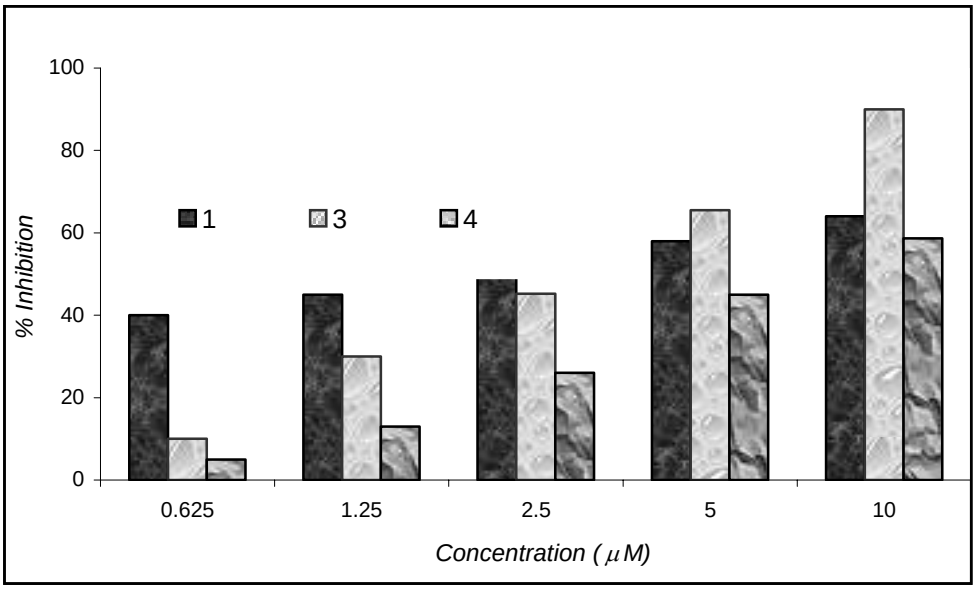


Figure 1. Inhibition [%] of lipoxygenase at various concentrations of compounds (**1**, **3** and **4**)

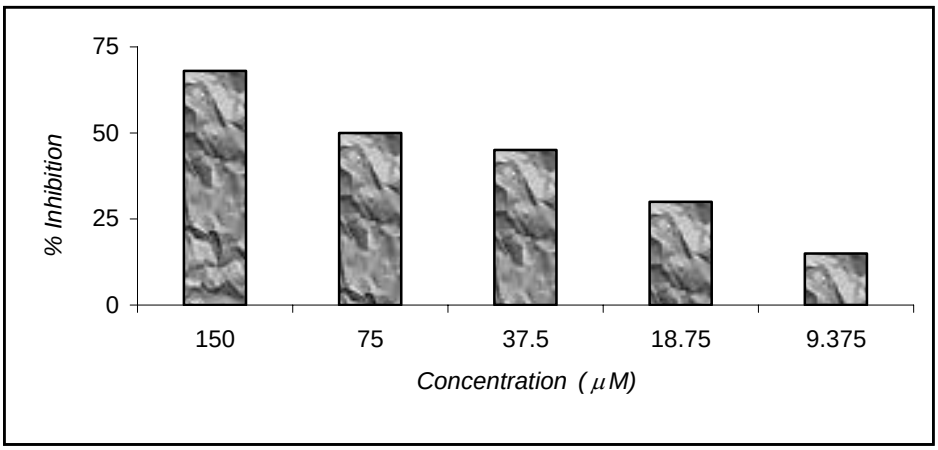


Figure 2. Inhibition [%] of lipoxygenase at various concentrations of compound (**2**)

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