Enzymes Inhibiting Lignans from *Vitex negundo*

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Two new lignans trivially named negundins A (1) and B (2), were isolated along with (+)-diasyringaresinol (3), (+)-lyoniresinol (4), vitrofolal E (5) and vitrofolal F (6), reported for the first time from this species. The structures of the new compounds were established through spectral studies. Compound 2 showed potent inhibitory activity against lipoxygenase enzyme, while 5 showed moderate activity against butyryl-cholinesterase.

Key words *Vitex negundo*; Verbinaceae; lignan; lipoxygenase; cholinesterase inhibition

Vitex negundo Linn. (syn: *V. inesia* Lam.) a deciduous shrub belonging to family Verbenaceae which comprises 75 genera and nearly 2500 species, chiefly occurring in Pakistan, India and Ceylon.[1,2] Though almost all plant parts are used, the extract from leaves and the roots is the most important in the field of medicine and is sold as drugs.[3] The leaf extract is used in Ayurvedic and Unani system of medicine.[4] The decoction of leaves is considered as tonic, vermifuge and is given along with long pepper in caterrhal fever.[3] Water extract of mature fresh leaves exhibited anti-inflammatory, analgesic and antihistamine properties.[5] The methanolic root extract possessed potent snake venom neutralizing capacity. The plant extract significantly antagonized the *Vipera russellii* and *Naja kaouthia* venom-induced lethal activity both in *in vitro* and *in vivo* studies. *V. russellii* venom-induced haemorrhage, coagulant, defibrinogenating and inflammatory activity was neutralized by the plant extract.[6] The acetone extract of *Vitex negundo* was found to possess insecticidal, ovicidal, feeding deterrence, growth inhibition and morphogenetic effects against various life stages of a noxious lepidoteron insect-pest, *Spilarctia obliqua* Walker.[7]

Literature survey of *V. negundo* revealed the presence of volatile oil,[8] triterpenes,[9] diterpenes,[10] sesquiterpenes,[11] lignan,[12] flavonoids,[13] flavone glycosides,[14] iridoid glycosides,[15—17] and stilbene derivative.[18] In the present investigation, a crude methanolic extract of the roots of *Vitex negundo* showed strong cytotoxicity in brine shrimp lethality test. Further biological screening of various fractions revealed significant inhibitory activity in the $CHCl_3$ extract against lipoxygenase and acetylcholinestrase enzymes. This prompted us to carry out isolation studies on the $CHCl_3$ soluble fraction of this plant. Herein we report the isolation and structure elucidation of two new lignans, **1** and **2** along with four known lignans which could be identified as (+)-diasyringaresinol (**3**), (+)-lyoniresinol (**4**), vitrofolal E (**5**) and vitrofolal F (**6**) through comparison of their physical and spectral data with those reported in literatures.[19—21]

Results and Discussion

Compounds **1**—**6** were isolated from the $CHCl_3$ soluble fraction of the methanolic extract of ground and shade dried roots. Negundin A was found to have the molecular ion peak in HR-MS at *m/z* 352.0911 corresponding to the molecular formula $C_{20}H_{16}O_6$ (Calcd for $C_{20}H_{16}O_6$, 352.0942). The IR spectrum contained absorption bands at 3266—3530 cm^{-1}, 1680 cm^{-1}, and 1241 cm^{-1} characteristic of phenolic groups, the carbonyl of γ-lactone and the methoxyl groups, respectively. The ^{1}H-NMR spectrum showed two methoxyl groups at δ 3.92 and 3.72 along with three aromatic signals at δ 8.66, 7.72 and 7.62, respectively. The three other aromatic protons showed ABX type substitution pattern forming a doublet at δ 7.36 (J=7.9 Hz), a doublet at δ 7.10 (J=1.5 Hz) and a doublet of doublets at δ 7.06 (J=7.9, 1.5 Hz). In addition, a methylene singlet was observed at δ 4.44.

The ^{13}C-NMR and DEPT experiments displayed the signals for six methines, one methylene, two methyls and eleven quaternary carbons. The downfield signal at δ 171.9 was due to the lactone functionality while the signal at δ 45.3 could be attributed to the methylene group adjacent to the carbonyl of the lactone functionality. The spectral data showed close agreement to those of detetrahydroconidendrin[27,28] particularly the signals of phenyl naphthalene moiety were very identical. This allowed us to place the two methoxyl groups to C-6 and C-3′ and the phenolic groups at C-7 and C-4′ respectively. This could be confirmed through HMBC correlations and NOE interactions. The methoxyl protons at δ 3.72 showed ^3J correlation with C-3′ while methoxyl protons at δ 3.92 showed ^3J correlation with C-6. Moreover, NOE interaction was observed between methoxy protons at C-3′ with aromatic proton at C-2′. A similar interaction was observed between methoxyl proton at C-6 and the aromatic proton at C-5. The H-5′ at δ 7.36 showed ^2J correlations with C-4′ (δ 147.9) and C-6′ (δ 123). The H-8 at δ 7.72 showed ^2J correlations with C-7 (δ 150.3) and C-8a (δ 131.8), thereby confirming the loci of phenolic groups at C-4′ and C-7. Although spectral data are very similar to deterahydrconidendrin but ^{1}H- and ^{13}C-NMR signals of **1** showed notable differences from latter particularly the significant upfield shifts of C-9, C-2, downfield shift of C-3 and the upfield shift of H_2-9 by *ca.* 0.9 ppm. This allowed us to place the γ-lactone moiety with oxygen directly attached to C-3. Negundin A is therefore, a positional isomer of deterahydrconidendrin. This is also revealed by wide variation in melting points of negundin A (125 °C) and detetrahydroconidendrin (254 °C). The HMBC correlations were in accordance to the assigned structure. The H_2-9 at δ 4.44 showed ^2J correlations with lactone carbonyl (δ 171.9), C-2 (δ 128.9) and ^3J correlations with C-3 (δ 136.9) and C-1 (δ 133.3). The H-4 at δ 8.66 showed ^2J correlations with C-3

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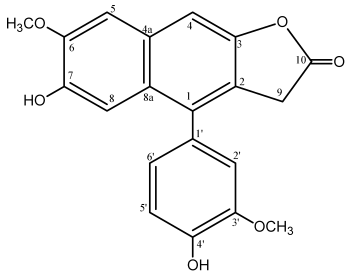

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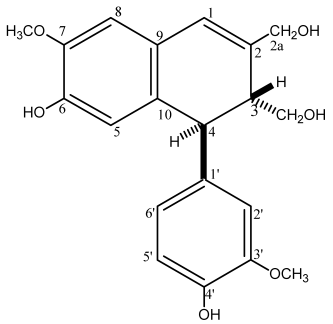

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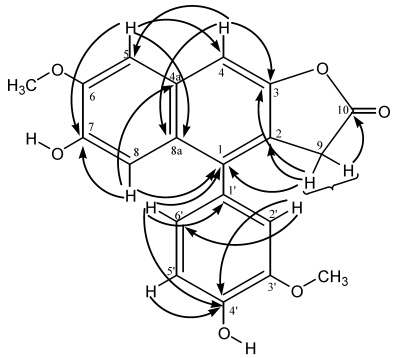
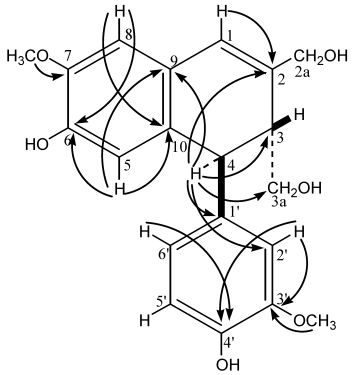
Fig. 1



important HMBC Interactions of **1**



important HMBC Interactions of **2**

Fig. 2

(δ 136.9), C-4a (δ 129.6) and ^3J correlations with C-2 (δ 128.9) and C-8a (δ 131.8). All these evidences led to the structure **1** for negundin A.

Negundin B (**2**) was obtained as white amorphous solid. The molecular formula $C_{20}H_{22}O_6$ was deduced from HR-EI-MS. Its UV spectrum (MeOH) exhibited absorption maxima at 283 and 222 nm. Its IR exhibited bands at 3340 cm^{-1} (hydroxyl group), 1225 (methoxyl group), 1599 and 1512 (aromatic nucleus). Consonant with the presence of phenolic functions the spectrum underwent a bathochromic shift upon addition of alkali.

The ^{1}H-NMR spectrum showed two methoxyl groups at δ 3.85 and 3.71. Of the five aromatic protons two were singlets (δ 6.75, 6.44), while the remaining three span the 2′, 5′ and 6′ positions of the lower pendant phenyl ring. The singlet at δ 6.56 could be attributed to the olefinic proton.

The ^{13}C-NMR (Table 2) and DEPT spectra showed the signals for two methylenes, eight methines, two methoxy and eight quaternary carbons. The spectral data of the compound **2** was very similar to (+)-isolariciresinol.[29] The notable difference being the presence of olefinic bond between C-1 and C-2 in compound **2**. This close agreement between compound **2** and isolariciresinol helped us to assign the two methoxyl groups to C-7 and C-3′ and the two hydroxyl groups to C-6 and C-4′, respectively. The HMBC correlations and NOE interactions further supported these assignments. The methoxyl protons at δ 3.85 showed ^3J correlation with C-3′, while methoxyl protons at δ 3.71 showed ^3J correlations with C-7. An NOE interaction was observed between methoxyl protons at C-3′ with aromatic proton at C-2′. A similar interaction was found between methoxyl protons at C-7 and the aromatic proton at C-8. Moreover, H-5′ at δ 6.61 showed ^2J correlations with C-4′ (δ 145.6) and C-6′ (δ 124.2). Similarly H-5 at δ 6.44 showed ^2J correlations with C-6 (δ 147.0) and C-10 (δ 127.0) and ^3J correlation with C-9 (δ 137.1). The H-4 (δ 4.1) showed ^2J correlations with C-3 (δ 48.5), C-10 (δ 127.0), C-1′ (δ 130.0) and ^3J correlations with C-2 (δ 138.1), C-9 (δ 137.1), C-3a (δ 63.5) and C-2′ (δ 112.7).

The relative stereochemistry at C-4 and C-3 could be assigned in analogy to 7,8,9,9-tetradehydroisolariciresinol[12] and confirmed by the signal of H-4 at δ 4.32 which did not couple with H-3, revealing a dihedral angle of *ca.* 90° between H-4 and H-3 (*i.e.* equatorial–equatorial interaction) and ruled out other possibilities (*i.e.* transdiaxial, axial–equatorial and equatorial–axial interactions).[12]

Lipoxygenase (LOX) is involved in arachidonic acid metabolism, generating various biologically active leukotrienes that play important role in inflammation.[22] Angiogenesis, the formation of new capillary vessels from pre-existing ones, underpins a number of physiological processes and participates in the development of several pathological conditions such as arthritis, cancer.[23] Lipoxygenase are therefore potential target for the rational drug design and discovery of mechanism-based inhibitors for the treatment of variety of disorders such as bronchial asthma, inflammation, cancer and autoimmune diseases. Only compound **2** showed significant inhibitory activity against lipoxygenase (Table 1).

Acetylcholinestrase (AChE) is a key component of cholin-

Table 1. *In Vitro* Quantitative Inhibition of Lipoxygenase, Acetylcholinesterase and Butyrylcholinesterase by Compounds **1**—**5**

Compound	IC_{50}±S.E.M.[a] (μM)		
	LOX	AChE	BChE
1	99.5±2.0	>300	85.0±0.8
2	6.25±0.5	254±0.5	194.0±4.4
3	68.1±0.7	—	300±1.5
4	—	263.3±3.5	—
5	—	106.5±4.0	35.0±1.5
Galanthamine[b]		0.5±0.0001	8.7±0.01
Baicalein[c]	22.5±0.25	—	—

a) Standard error of the mean of five assays. *b*) Positive control used in acetyl cholinesterase and butyrylcholinesterase inhibiting assay. *c*) Positive control used in lipoxygenase inhibiting assay.

Table 2. ^{13}C-NMR Data of Compounds **1**, **2** and Detetrahydroconidendrin

Carbon	**1**	**2**	Detetrahydro-conidendrin
1	133.3 (C)	121.3 (CH)	132.4
2	128.9 (C)	138.1 (C)	138.3
2a		65.9 (CH_2)	
3	136.9 (C)	48.5 (CH)	121.3
3a		63.5 (CH_2)	
4	121.7 (CH)	45.1 (CH)	124.4
4a	129.6 (C)		130.1
5	108.5 (CH)	115.8 (CH)	108.7
6	150.2 (C)	147.0 (C)	150.9
7	150.3 (C)	148.6 (C)	151.9
8	109.3 (CH)	111.5 (CH)	109.2
8a	131.8 (C)		133.2
9	45.3 (CH_2)	137.1 (C)	70.0
10	171.9 (C)	127.0 (C)	172.2
1′	129.1 (C)	130.1 (C)	127.9
2′	113.9 (CH)	112.7 (CH)	123.7
3′	148.9 (C)	147.8 (C)	149.2
4′	147.9 (C)	145.6 (C)	148.4
5′	116.9 (CH)	118.0 (CH)	117.1
6′	123.0 (CH)	124.2 (CH)	123.0
(6-OMe)	55.8 (CH_3)		56.0
(7-OMe)		56.6 (CH_3)	
(3′-OMe)	55.9 (CH_3)	56.3 (CH_3)	56.2

ergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine.[24] According to the cholinergic hypothesis, the memory impairment in the patients with snile dementia of Alzheimer's type results from a deficiency in cholinergic function in the brain.[25] Hence the most promising therapeutic strategy for activating central cholinergic functions has been the use of cholinomimetic agents. The aim of acetylcholinesterase inhibitors is to boost the endogenous levels of acetylcholine in the brains of Alzheimer disease (AD) patients and thereby to boost cholinergic neurotransmission. It has also been found that butyrylcholinesterase (BChE) inhibition may also be an effective tool for the treatment of AD and related dementias.[26] From the inhibition studies (Table1) it is clear that compound **2** posseses potent inhibitory potential against LOX enzyme as compared to baicalein which was used as positive control. The compo°unds **1**—**3** showed weak activity while **4**, **5** were found inactive. Against AChE **3** was inactive while remaining compounds displayed weak inhibitory potential as compared to galanthamine which was used as a positive control. In case of BChE inhibition, compound **5** displayed moderate activity, compounds **1**—**3** showed weak activity compared to galanthamine, while **4** was inactive. These compounds were also screened against chymotrypsine, urease and α-glucosidase but no activity could be observed.

Experimental

General 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. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) are in Hz. MS and HR-MS were obtained on a JMS-HX-110 with a data system and on JMS-DA 500 mass spectrometers. Flash silica (230—400 mesh) was used in flash column chromatography. Visualization of the TLC plates was carried out 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.

Plant Material The roots of *Vitex negundo* LINN. were collected 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.

Extraction and Isolation The shade dried root of *Vitex negundo* was chopped and soaked in methanol for a period of 10 d and extraction was repeated three times. The combined methanolic extract was evaporated in vacuo. The resulting residue (1.5 kg) was suspended in H_2O and extracted successively with *n*-hexane and $CHCl_3$ respectively. The chloroform soluble fraction (50 g) was subjected to column chromatography using silica gel (70—270 mesh) and *n*-hexane–$CHCl_3$ and $CHCl_3$–MeOH gradient systems as mobile phase. As a result fractions A—D were obtained. Repeated column chromatography of these fractions using hexane–ethyl acetate (3 : 1) solvent system resulted in isolation of compounds **1**—**6**.

Compound (**1**): Amorphous white solid, 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. HR-EI-MS: *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), 7.72 (1H, s, H-8), 7.62 (1H, s, H-5), 7.36 (1H, d, J=7.9 Hz, H-5′), 7.10 (1H, d, J=1.5 Hz, H-2′), 7.06 (1H, dd, J=7.9, 1.5 Hz, H-6′), 4.44 (2H, br s, H_2-9), 3.92 (3H, s, MeO-6), 3.72 (3H, s, MeO-3′); ^{13}C-NMR (100 MHz, pyridine-d_5): Table 2.

Compound (**2**): Amorphous white solid. $[\alpha]_D^{26}$ −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. HR-EI-MS: *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, J=1.8 Hz H-2′), 6.61 (1H, d, J=8.5 Hz, H-5′), 6.56 (1H, s, H-1), 6.44 (1H, s, H-5), 6.41 (1H, dd, J=8.5, 1.8 Hz, H-6′), 4.11 (br s, H-4), 4.01 (2H, dd, 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): Table 2.

***In Vitro* Lipoxygenase Inhibition Assay** Lipoxygenase inhibiting activity was conveniently measured by slightly modifying the spectrometric method developed by A. L. Tappel.[30] Soybean lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. In assay protocol, 160 μl of 100 mM sodium phosphate buffer (pH 8.0), 10 μl of test-compound solution and 20 μl of lipoxygenase solution were mixed and incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 μl linoleic acid (substrate) solution, with the formation of (9*Z*,11*E*)-(13*S*)-13-hydroperoxyoctadeca-9,11-dienoate, the change in absorbance at 234 nm was followed for 6 min. Test compounds and the positive control were dissolved in ethanol. All the reactions were performed in triplicate in 96-well micro-plate in SpectraMax 384 Plus (Molecular Devices, U.S.A.). The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

***In Vitro* Cholinesterase Inhibition Assay** Electric-eel acetylcholinesterase (EC 3.1.1.7), horse-serum butyrylcholinesterse (E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured by the spectrophotometric method developed by Ellman *et al.*[31] Acetylthio-

choline iodide and butyrylthiocholine chloride was used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. The reaction mixture contained 150 μl of (100 mM) sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 10 μl of test-compound solution and 20 μl of acetylcholinesterase or butyrylcholinesterase solution, which were mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μl acetylthiocholine or butyrylthiocholine, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively at a wavelength of 412 nm (15 min). Test compounds and the positive control were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro-plate in SpectraMax 384 Plus (Molecular Devices, U.S.A.). The percentage inhibition was calculated as follows $(E-S)/E\times100$, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

Estimation of IC_{50} Values The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

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