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Flavonoids, triterpenoids and a lignan from *Vitex altissima* ☆

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Abstract

A new tetrahydrofuranoid lignan, altissinone (**1**) and a new acylated flavone *C*-glucoside, 2″-*O*-*p*-hydroxybenzoylorientin (**2**), were isolated in addition to several known triterpene acids and flavonoids from the ethyl acetate extractives of the leaves of *Vitex altissima*. The structures of the compounds were established based on interpretation of high resolution NMR (HMQC, HMBC and NOESY) spectral data. The ethyl acetate extract exhibited significant anti-inflammatory activity in rat paw edema model. The flavonoids and triterpene acids showed moderate antioxidant and 5-lipoxygenase enzyme inhibitory activities, respectively.

Keywords: *Vitex altissima*; Leaves; Lignan; Altissinone; Flavonoids; Triterpenoids; Anti-inflammatory

1. Introduction

Vitex altissima Linn. belongs to the family Verbenaceae. It is a moderate to large sized tree found in Eastern Ghats and Deccan plateau in India (Pullaiah and Sandhyarani, 1999). The leaves are reported to be useful in the treatment of rheumatism (Narayana Rao and Thammanna, 1990). We reported recently (Sridhar et al., 2004) the isolation and characterization of several new iridoids from the ethyl acetate extractives of the leaves of *V. altissima*. Further investigation on the ethyl acetate extractives of the same plant has led to the isolation of a new lignan, named altissinone (**1**) and a new flavonoid, 2″-*O*-*p*-hydroxybenzoylorientin (**2**), along with nine known triterpene acids and two flavonoids.

2. Results and discussion

Dried and powdered leaves of *V. altissima* were extracted with *n*-hexane, ethyl acetate, methanol and 70% methanol, successively. The ethyl acetate extractives which exhibited potent anti-inflammatory activity were subjected to silica gel column chromatography and reversed phase preparative HPLC to give a lignan (**1**), and a flavonoid (**2**) in addition to nine triterpene acids and two flavonoids. The triterpenoids have been identified as ursolic acid (Alves et al., 2000), corosolic acid (Murkami et al., 1993), epicorosolic acid (Bhandari et al., 1990), maslinic acid (Murkami et al., 1993), epimaslinic acid (Cheung and Yan, 1972), euscaphic acid (Kuang et al., 1989), euscaphic acid glucoside ester (Guang-Yl et al., 1989), 2α,3α,24-trihydroxyurs-12-en-28-oic acid (Jung et al., 2004), 2α,3α,24-trihydroxyurs-12,20(30)-dien-28-oic acid (Kojima et al., 1987) by comparison of spectral data with those reported in the literature. The flavonoids were identified as vitexin (Tomczyk et al., 2002), and luteolin 7-*O*-glucoside (Markham et al., 1978), by comparison with the literature data.

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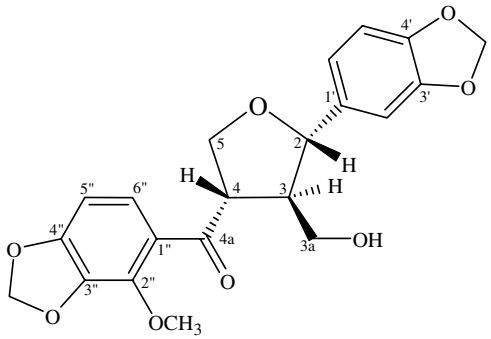

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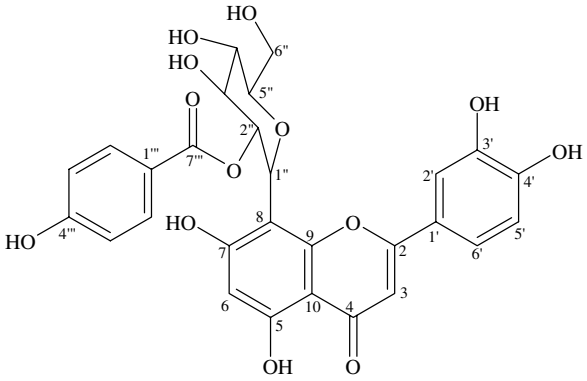

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Table 1
NMR spectral data of compound **1** ($CDCl_3$, 500 MHz)[a]

Position	^1H	^{13}C	HMBC
2	4.57 *d* (9.0)	83.9	C-3, C-3a, C-1′, C-2′, C-6′
3	2.85 *m*	52.7	
3a	3.63 *dd* (11.0, 6.5) 3.73 *dd* (11.0, 5.0)	62.2	C-4, C-2
4	4.03 *m*	55.1	C-3, C-3a, C-5, C-4a
4a		200.3	
5	4.17 *dd* (9.0,6.0) 4.22 *t* (9.0)	70.8	C-3, C-2, C-4a
1′		134.7	
2′	6.97 *d* (1.5)	107.2	C-4′, C-6′
3′		147.3	
4′		147.8	
5′	6.77 *d* (8.0)	108.0	C-1′, C-3′,C-4′
6′	6.85 *dd* (8.0,1.5)	120.3	C-2, C-4′
1″		125.3	
2″		143.0	
3″		136.6	
4″		152.9	
5″	6.59 *d* (8.5)	103.3	C-3″, C-1″
6″	7.27 *d* (8.0)	125.6	C-2″, C-4″, C-4a
–OCH_3	4.11 *s* 3H	60.1	C-2″
–OCH_2O–	5.95 *s* 2H	101.0	C-3′, C-4′
–OCH_2O–	6.03 *s* 2H	101.8	C-3″, C-4″

[a] Chemical shifts (δ) are in ppm, and coupling constants (J in Hz) are given in parentheses.

Structures of the new compounds (**1** and **2**) have been deduced by the interpretation of high-resolution NMR data and the details are presented below.

(−)-Altissinone (**1**) was obtained as pale green flakes, m.p. 151–152 °C. The molecular formula $C_{21}H_{20}O_8$ was deduced from microanalytical and LC–MS [*m*/*z* 423, $(M + Na)^+$] data. IR spectrum of **1** showed bands at 3483 (hydroxyl), 1654 (carbonyl), 1592, 1485 (aromatic), 1041 and 942 cm^{-1} (methylenedioxy). The ^1H and ^{13}C NMR spectral data (Table 1) of **1** showed signals characteristic of a 2,3,4-trisubstituted furanoid lignan (Klemm, 1978; Banerji et al., 1984). The ^1H NMR data revealed the presence of an oxymethine proton at δ 4.57 (1H, *d*, J = 9.0 Hz) characteristic of H-2 of tetrahydrofuranoid lignans (Lin-Gen et al., 1983; Yu et al., 1998), two methine protons (1H, δ 2.85, *m*, H-3 and 1H, δ 4.03, *m*, H-4), and two oxymethylene groups [(1H, δ 3.63, *dd*, J = 11.0, 6.5 Hz, H_a-3a and 1H, δ 3.73, *dd*, J = 11.0, 5.0 Hz, H_b-3a) and (1H, δ 4.17 *dd*, J = 9.0, 6.0 Hz, H_a-5 and 1H, δ 4.22, *t*, J = 9.0 Hz, H_b-5)]. The ^1H NMR data also showed the presence of a piperonyl unit constituted by a 1,2,4-trisubstituted phenyl moiety [δ 6.77 (1H, *d*, J = 8.0 Hz), 6.85 (1H, *dd*, J = 8.0, 1.5 Hz) and 6.97 (1H, *d*, J = 1.5 Hz)] and a methylenedioxy group at δ 5.95 (2H, *s*). In addition, the ^1H NMR spectrum contained a methoxyl group (3H, δ 4.11, *s*), a methylenedioxy group (2H, δ 6.03, *s*) and two aromatic AB protons at δ 6.59 (1H, *d*, J = 8.5 Hz, H-5″) and 7.27 (1H, *d*, J = 8.0 Hz, H-6″), suggesting the presence of a 1,2,3,4-tetrasubstituted phenyl unit. From the above data, the structure of **1** was derived as a 2,3,4-trisubstituted tetrahydrofuranoid lignan (Jung et al., 1998) having a piperonyl and 2-methoxy-3,4-methylenedioxyphenyl moieties. The IR (1654 cm^{-1}) and ^{13}C NMR data (δ 200.3) suggested the presence of a ketone carbonyl. The HMBC data (Table 1) of **1** has shown correlations between H-6″, H-4, and H-5 protons and the ketone carbonyl (δ 200.3), suggesting the location of ketone on C-4a. Further, the H-3a, H-5 and H-6′ protons showed correlations with C-2 (δ 83.9). Based on the above, the structure of the lignan has been deduced as 2-(3′,4′-methylenedioxyphenyl)-3-hydroxymethyl-4-(2″-methoxy-3″,4″-methylenedioxybenzoyl)tetrahydrofuran, a new lignan named altissinone (**1**). The configuration of **1** at C-2, C-3 and C-4 was proposed to be identical with those of (−)-sesaminone (Maioli et al., 1997), based on the observed similarity in chemical shifts and optical rotation ($[\alpha]_D^{25} - 40.3°$) data. Compound **1** could have formed biosynthetically through an oxidative cleavage of a previously known 2-methoxysesamin (Jaensch et al., 1989).

Compound (**2**) was obtained as a pale yellow amorphous powder from methanol. The molecular formula $C_{28}H_{24}O_{13}$, was deduced from microanalytical and LC–MS [*m*/*z* 567 $(M - H)^-$] data. The absorption maxima at 267 and 342 nm in the UV spectrum are attributable to a flavonoid skeleton. IR spectrum of 2 showed

bands at 3345 (hydroxyl), 1645 (α,β-unsaturated ketone), and 1565 and 1468 cm^{-1} (aromatic). The ^1H NMR spectral data (Table 2) showed the presence of a sharp one-proton singlet at δ 6.69 attributable to H-3 of flavone (Koteswara Rao et al., 2002). The ^1H NMR spectrum showed the presence of an ABX system [δ 7.65 (1H, br *d*, J = 8.5 Hz), δ 7.57 (1H, br *s*) and δ 6.92 (1H, *d*, J = 8.5 Hz)] characteristic of a 1,2,4-trisubstituted phenyl unit. In addition, the ^1H NMR spectrum showed a series of signals between δ 4.99 and 3.40 indicative of a sugar moiety. The coupling constant (J = 10.0 Hz) of the anomeric proton (δ 4.99) is consistent with the presence of a β-C-glucoside in **2**. A fact supported further by the ^{13}C NMR signals (δ_C 71.6, 72.9, 76.5, 71.2, 82.7 and δ_C 61.8) of the sugar unit

Table 2
NMR spectral data of compound **2** (d_6-DMSO, 500 MHz)[a]

Position	^1H	^{13}C	HMBC
2		164.6	
3	6.69 *s*	102.9	C-1′, C-10
4		182.4	
5		161.0	
6	6.08 *s*	98.1	C-8, C-10
7		162.5	
8		104.3	
9		156.8	
10		102.8	
1′		122.4	
2′	7.57 br *s*	114.5	C-2, C-4′, C-6′
3′		146.3	
4′		150.1	
5′	6.92 *d* (8.5)	116.2	C-1′, C-3′
6′	7.65 *d* (8.5)	119.9	C-2, C-2′, C-4′
1″	4.99 *d* (10.0)	71.6	C-7, C-9
2″	5.50 *t* (9.5)	72.9	C-7‴, C-4″
3″	3.65 *m*	76.5	
4″	3.50 *m*	71.2	
5″	3.40 *m*	82.7	
6″	3.87 *m*, 3.65 *m*	61.8	
1‴		120.9	
2‴ and 6‴	7.59 *d* (8.5)	131.8	C-2‴, C-4‴, C-6‴, C-7‴
3‴ and 5‴	6.75 *d* (8.5)	115.5	C-1‴, C-3‴, C-5‴
4‴		162.1	
7‴		165.1	

[a] Chemical shifts (δ) are in ppm, and coupling constants (J in Hz) are given in parentheses.

(Abou-Zaid et al., 2001). The aromatic proton signal located at δ 6.08 (1H, *s*), suggested the presence of a trisubstituted A ring in **2** (Abou-Zaid et al., 2001). The ^1H NMR spectrum also showed the presence of signals attributable to a *p*-hydroxybenzoyl unit [δ 7.59 (2H, *d*, J = 8.5 Hz) and δ 6.75 (2H, *d*, J = 8.5 Hz)] (Hirobe et al., 1997).

The observed ^{13}C NMR chemical shifts (Table 2) of methine (δ_C 98.1, C-6) and the C-glycosylated carbon (δ_C 104.3, C-8) of ring A revealed the presence of orientin, a C-8 glycosylated flavonoid skeleton in **2** (Agrawal, 1989). The presence of C-8 glycosyl moiety was supported further by the HMBC (Table 2) correlations observed between anomeric proton [δ 4.99 (1H, *d*, J = 10.0 Hz)] and the quaternary carbons C-7 (δ_C 162.5) and C-9 (δ_C 156.8) of ring A. The upfield shift to the extent of 2.0 ppm observed for C-1″(δ_C 71.6), in comparison with orientin (δ_C 73.6), indicated the location of *p*-hydroxybenzoyl unit on C-2″. A fact supported further by the HMBC correlations between H-2″(δ 5.50, *t*, J = 9.5 Hz) and the ester carbonyl (δ_C 165.1, C-7‴). Based on the foregoing, the structure of compound 2 was characterized as 2″-*O*-*p*-hydroxybenzoylorientin, a new flavonoid glycoside. Its isomer, 2″-*O*-*p*-hydroxybenzoylisoorientin, was isolated from *Gentiana asclepiadea* earlier (Goetz and Jacot-Guillarmod, 1978).

The ethyl acetate extract of the leaves of *V. altissima* exhibited significant anti-inflammatory activity in carrageenan induced rat paw edema model (Table 3). Among the isolates, the triterpene acids exhibited moderate 5-lipoxygenase enzyme inhibitory activity, corosolic acid (80%), epicorosolic acid (79%), ursolic acid (70%), maslinic acid (72%), and euscaphic acid (55%) at a dose of 500 μM, in comparison to nordihydroguaiaretic acid (70%, 100 μM). The flavonoids, vitexin and luteolin 7-*O*-glucoside showed moderate antioxidant activity, both in a superoxide free radical scavenging test (NBT method), (IC_{50} 62 and 8 μg/mL) and in a DPPH-radical scavenging test (IC_{50} 43 and 7.4 μg/mL), in comparison to the known antioxidants, vitamin-C (NBT, IC_{50} 150 μg/mL and DPPH, IC_{50} 6.1 μg/mL) and butylated hydroxyanisole (BHA) (NBT, IC_{50}174 μg/mL and DPPH, IC_{50}3.2 μg/mL). The new lignan, altissinone (**1**) did not

Table 3
Anti-inflammatory activity of *V. altissima* extracts on carrageenan-induced paw edema in rats

S. no	Test material	Dose (mg/kg)	Mean edema[a]±SEM (mL)	Inhibition (%)
1	Control group		0.70 ± 0.02	
2	Diclofenac sodium	25	0.26 ± 0.03	62[b]
3	*V. altissima* hexane extract	250	0.59 ± 0.02	15
4	*V. altissima* ethyl acetate extract	250	0.43 ± 0.03	39[b]
5	*V. altissima* methanol extract	250	0.60 ± 0.01	13
6	*V. altissima* 70% methanol extract	250	0.56 ± 0.02	20

[a] n = 6.
[b] $p < 0.001$ vs control. Student's test.

exhibit significant 5-lipoxygenase inhibitory or antioxidant activities. Due to paucity, the new flavonoid (**2**) could not be tested for its antioxidant potential.

3. Experimental

3.1. General

Melting points were recorded on a Mel-Temp apparatus and are uncorrected.Optical rotations were measured on a JASCO DIP-370 polarimeter. UV and IR spectra were recorded on Varian (Cary-50) and Perkin–Elmer BX FT-IR spectrophotometers, respectively. The ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Varian Unity INOVA 500 MHz spectrometer with standard pulse sequences. Mass spectra were recorded on Agilent 1100 series LC/MSD and elemental analysis was carried out on a Vario El Elementar instrument. Preparative HPLC was carried out on Shimadzu HPLC system (LC-8A pump, SPD-10A UV–visible detector) using Luna C_{18} (10 M, 21.2×250 mm, Phenomenex) column. Silica gel (100–200 mesh, ACME) was used for open column chromatography.

3.2. Plant material

The leaves of *Vitex altissima* L. were collected from Seshachalam Hill ranges (The Tirumala forest) of the Eastern Ghats in Andhra Pradesh, India, during January 2001. The leaves were authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, India. The voucher specimens (VA-010222) are on deposit at the Herbarium of Sri Venkateswara University, Tirupati, India.

3.3. Extraction and isolation

The shade-dried and milled leaves of *V. altissima* (2.8 kg) were extracted successively with *n*-hexane (5×5 L), ethyl acetate (5×5 L), methanol (5×5 L), and 70% methanol (5×5 L) in a reflux apparatus. The combined extracts of each solvent were concentrated in vacuo, to give dark gummy residues of *n*-hexane (60 g), ethyl acetate (150 g), methanol (400 g), and 70% methanol (275 g). The ethyl acetate extractives, which showed potent anti-inflammatory activity (39% inhibition against carrageenan-induced paw edema in rats, $p < 0.001$), were subjected to column chromatography over silica gel with different solvents of increasing polarity (*n*-hexane–EtOAc; $CHCl_3$–MeOH). The selected fractions were combined into eight subfractions (1–8) based on TLC, using mixtures of $CHCl_3$–MeOH (9.6:0.4), $CHCl_3$–acetone (8:2), and EtOAc–MeOH (9:1) as solvent systems. Fraction 1 was rechromatographed over silica gel column with increasing polarities of *n*-hexane–EtOAc to give ursolic acid (45 mg). Fraction 2 was subjected to column chromatography over silica gel with increasing polarities of $CHCl_3$–MeOH mixtures as an eluent to give epicorosolic acid (25 mg) and epimaslinic acid (16 mg). Fraction 3 was rechromatographed over silica gel column with increasing polarities of acetone–$CHCl_3$ mixtures as an eluent to yield compound **1** (180 mg), euscaphic acid (250 mg) and maslinic acid (30 mg). Fraction 4 was subjected to column chromatography over silica gel with increasing polarities of $CHCl_3$–MeOH mixtures as an eluent to give corosolic acid (100 mg). Fraction 5 was subjected to reversed-phase preparative HPLC (Luna C_{18}, 10 μm, 250×21.2 mm, 20 mL/min), using CH_3CN–H_2O (1:1) as solvent system, to give 2α,3α,24-trihydroxyurs-12,20(30)-dien-28-oic acid (15 mg, t_R8.0 min) and 2α,3α,24-trihydroxyurs-12-en-28-oic acid (25 mg, t_R10.7 min). Fraction 6 was rechromatographed over silica gel column with increasing polarities of $CHCl_3$–MeOH mixtures as an eluent to yield euscaphic acid glucoside ester (45 mg). Fraction 7 was rechromatographed over silica gel column using ethyl acetate as an eluent to yield vitexin (11 mg). Fraction 8 was rechromatographed over silica gel column using ethyl acetate as an eluent to yield compound **2** (9 mg) and luteolin 7-*O*-glucoside (140 mg).

3.4. Compound *1*

(−)-Altissinone (**1**). Pale green flakes, m.p. 151–152 °C (Found: C, 62.99; H, 5.36. $C_{21}H_{20}O_8$ requires: C, 63.00; H, 5.00%); $[\alpha]_D^{25}$: −40.3° ($CHCl_3$; *c*, 0.5); UV $\lambda_{max}^{CHCl_3}$ nm(log ε) : 226(7.0); IR ν_{max}^{KBr} cm^{-1}: 3483 (OH), 1654 (C=O), 1592, 1485, 1246, 1041; ^{1}H, ^{13}C NMR and HMBC: Table 1; LC–MS *m*/*z*: 423 $[M + Na]^+$.

3.5. Compound *2*

2″-O-p-hydroxybenzoylorientin (**2**). Yellow amorphous powder, (Found: C, 60.00; H, 4.50. $C_{28}H_{24}O_{13}$ requires: C, 59.14; H, 4.22%); UV λ_{max}^{MeOH} nm : 267 and 342; IR $\nu_{max}^{KBr}cm^{-1}$: 3483 (OH), 1654 (C=O), 1592, 1485, 1246, 1041; ^{1}H, ^{13}C NMR and HMBC: Table 2; LC–MS *m*/*z*: 567 $[M - H]^-$.

3.6. Biological activity

3.6.1. Anti-inflammatory activity

The extracts were screened for anti-inflammatory activity by carrageenan induced rat paw edema method (Winter et al., 1962). Wistar rats of either sex weighing between 180 and 220 g were divided into six groups, with each group consisting of six animals. One group served as negative control (received 1% Tween-80, 10 mL/kg), and a second group, which served as positive

control received 25 mg/kg, diclofenac sodium suspended in 1% Tween-80. The third, fourth, fifth, and sixth groups were treated with 250 mg/kg of the hexane, ethyl acetate, methanol, and 70% methanol extracts suspended in 1% Tween-80, respectively, by the oral route. Edema was produced after 1 h of drug treatment, by injecting 0.1 mL (1% w/v in saline) carrageenan solution to the sub planter region of the left hind paw of rats of all groups. The paw volume was measured by a plethysmometer at zero and 3 h after carrageenan injection. The difference between the initial and final paw volume was considered as the edema volume. The percent inhibition of paw edema was calculated by comparing the mean edema volume of treated group and control group.

3.6.2. Lipoxygenase enzyme inhibitory activity

Test compounds were screened for 5-lipoxygenase enzyme inhibitory activity by the modified ferric-xylenol orange peroxide assay (Gay and Gebicki, 2002). The assay mixture contained 50 mM phosphate buffer (pH 6.3), 5-lipoxygenase, various concentrations of test substances, and linoleic acid (80 mM), in a total volume of 0.5 mL. After 5 min of incubation, to the above reaction mixture, 0.5 mL ferric-xylenol orange reagent (in perchloric acid) was added and absorbance was measured after 2 min at 585 nm on a spectrophotometer. Nordihydroguaiaretic acid (NDGA) (100 μM) was used as a positive control (70% inhibition). Percent inhibition was calculated by comparing absorbance of test substances with that of the control. All the tests were run in triplicate and averaged.

3.6.3. Superoxide free-radical scavenging activity

Superoxide radical-scavenging activity of the test compounds isolated from *V. altissima* was determined by the method of McCord and Fridovich (McCord and Fridovich, 1969). The assay mixture contained EDTA (6.0 μM), NaCN (3 μg), riboflavin (2 μM), NBT (50 μM), and various concentrations of the test substances in methanol and phosphate buffer (58 mM, pH 7.8), in a final volume of 3 mL. The tubes were shaken well and the absorbance was measured before and after illumination at 560 nm. The percent inhibition of superoxide radical generation was measured by comparing the mean absorbance values of control and those of the test substances. IC_{50} values were obtained from the plot drawn concentration in μM verses percentage inhibition. The known antioxidants vitamin C and butylated hydroxyanisole (BHA) were used as positive controls.

3.6.4. DPPH radical-scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity was measured by the method of Lamaison et al. (1991) The reaction mixture contained 1.0×10^{-4} mM methanolic solution of DPPH and various concentrations of the test substances and kept in a dark area for 50 min. The absorbance of the samples was measured on a spectrophotometer at 517 nm against a blank. All tests were run in triplicate and averaged. The known antioxidants vitamin-C and butylated hydroxyanisole (BHA) were used as positive controls.

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