New Anti-HIV-1, Antimalarial, and Antifungal Compounds from *Terminalia bellerica*

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A bioactivity-guided fractionation of an extract of *Terminalia bellerica* fruit rind led to the isolation of two new lignans named termilignan (**1**) and thannilignan (**2**), together with 7-hydroxy-3′,4′-(methylenedioxy)flavan (**3**) and anolignan B (**4**). All four compounds possessed demonstrable anti-HIV-1, antimalarial, and antifungal activity in vitro.

Terminalia bellerica Roxb. (Combretaceae) is one of the most commonly used plants in Indian traditional systems of medicine. The fruit rind is used in different preparations, for example, as one of the ingredients of "Triphala" (three fruits), used in Ayurveda for the treatment of fever, cough, diarrhea, dysentery, and skin diseases.[1,2] Antiviral activity[3−7] including anti-HIV-1 activity, antibacterial, and antifungal activity[8,9] of crude extracts of *T. bellerica* fruits have been reported. However, no active constituents have been isolated in a pure state from this plant to date.

An 80% EtOH extract of the fruit rind of *T. bellerica* was included in our antimicrobial screening program of Indian medicinal plants and was found to be active when screened against *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (IMI 349010), and *Aspergillus niger* (IMI 76837). As one of the most active extracts out of the 80 tested, it was selected for further studies. This article reports the isolation and structure elucidation of three lignans (**1**, **2**, and **4**) and one flavan (**3**) from *T. bellerica* all showing a significant antimalarial and antifungal activity. A weak anti-HIV-1 activity could be demonstrated. However, none of compounds **1**−**4** showed significant activity against the above-mentioned bacteria. The structures of the new compounds **1** and **2** were determined by spectroscopic methods.

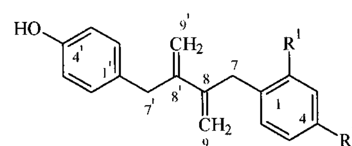
The bioactivity-guided fractionation of the CH_2Cl_2 extract of the fruit rind of *T. bellerica* led to the isolation of four compounds (**1**−**4**), all showing antifungal activity.

The ^{1}H-NMR and ^{13}C-NMR spectra of compound **1** (Tables 1 and 2) were very similar to the spectra of anolignan B,[10] except for the appearance of signals corresponding to a methoxy group and the pattern of the signals originating in one of the two phenyl rings, which disclosed the presence of two *meta*-disposed oxygens. The assumption that **1** is anolignan B hy-

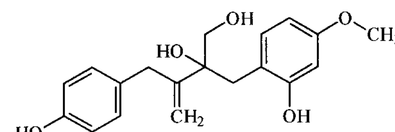
Table 1. ^{1}H-NMR Data for Lignans **1** and **2** Recorded in CD_3CN at 200 MHz[a]

proton	1	2
3	6.38 (d, 1.5)	6.37 (d, 2.0)
5	6.33 (dd, 1.5, 7.0)	6.35 (dd, 2.0, 7.0)
6	6.84 (d, 7.0)	6.97 (d, 7.0)
7a	3.44 (br s)	3.04 (d, 14.5)
7b	3.44 (br s)	3.18 (d, 14.5)
9a	5.26 (br s)	3.68 (d, 12.5)
9b	4.90 (br s)	3.69 (d, 12.5)
2′+ 6′	7.00 (m)	6.97 (m)
3′ + 5′	6.72 (m)	6.70 (m)
7′	3.50 (br s)	3.31 (br s)
9a′	5.22 (br s)	5.19 (br s)
9b′	4.78 (br s)	4.60 (br s)
CH_3O	3.68 (s)	3.75 (s)

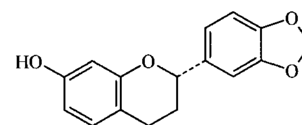
[a] In parentheses are given the multiplicities of the signals and the coupling constants in Hz, (br s: broad singlet; d, doublet; dd, double doublet; m, multiplet; s, singlet).



(**1**) R¹=OH, R²=OCH₃
(**4**) R¹=H, R²=OH



(**2**)



(**3**)

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Table 2. ^{13}C NMR Data for Lignans **1** and **2** Recorded in CD_3CN at 50 MHz

carbon	**1**	**2**
1	117.6	118.3
2	158.5	159.7
3	100.5	95.2
4	154.5	159.9
5	104.4	104.9
6	130.1	124.4
7	32.2	35.4
8	146.0	93.7
9	113.6	54.6
1′	130.6	129.2
2′ + 6′	129.1	129.9
3′ + 5′	114.6	114.7
4′	154.4	155.4
7′	38.7	36.6
8′	145.1	150.4
9′	113.6	111.3
CH_3O	54.2	54.6

droxylated at position C-2 and methylated at one of the three hydroxy groups yields the empirical formula $C_{19}H_{20}O_3$, which was confirmed by HRMS. The location of the methoxy group was established by a difference NOE spectrum. We suggest the name termilignan for the new compound **1** [2-(2-hydroxy-4-methoxybenzyl)-3-(4-hydroxybenzyl)butadiene].

The ^1H-NMR and ^{13}C-NMR spectra of compound **2** were similar to the spectra of compound **1** except for missing signals corresponding to one of the two terminal double bonds. Instead, two AB systems were recognized, one centered at 3.10 ppm and the other at 3.69 ppm. The latter was assigned to the two protons of a primary alcohol and the former to a benzylic methylene group next to a tertiary alcohol group, as suggested in structure **2**. The introduction of a stereogenic center at C-8 explains that the two pairs of protons in the two methylene groups become diastereotopic. The mass spectrum did not reveal a molecular ion but an ion at *m/z* 312, corresponding to loss of a water molecule. A coupling between the protons of the methylene group at 3.10 ppm and the protons of the trisubstituted aromatic nucleus as revealed by a COSY spectrum located the glycol as depicted in formula **2**. The methoxy group was located by a NOESY spectrum disclosing a coupling between the methyl group and the two aromatic protons at 6.37 and 6.35 ppm. The absolute configuration of **2** has not been established. We suggest the name thannilignan for the new compound **2** [2-(2-hydroxy-4-methoxybenzyl)-3-(4-hydroxybenzyl)-but-3-ene-1,2-diol].

The ^1H-NMR and ^{13}C-NMR spectra of **3** revealed that this compound was 7-hydroxy-3′,4′-(methylenedioxy)-flavan, previously isolated from *Zephyranthes flava* (Amaryllidaceae).[10] Flavans are generally assumed to possess the *S*-configuration, but this has only been established in a few cases. The presence of two phenyl groups with a well-defined angle between the directions of the electric transition dipole moments should make it possible to determine the absolute configuration by the exciton-split Cotton effect.[11] The previous CD spectra of flavans[12] were recorded in MeOH, which has a cut off at approximately 210 nm. The use of MeCN expanded the measurable range to below 200 nm. The additional 10 nm revealed a positive Cotton effect at 210 nm ($\Delta\epsilon$ 4.52) and a negative cotton effect at 202 nm ($\Delta\epsilon$ −4.52). The presence of two bands of opposite sign and equal intensity is characteristic for exciton-split Cotton effects. Consequently the nonempirical-coupled oscillator theory[11] established the absolute configuration at C-2 as *S*. By comparison with CD spectra of other flavans, a moderate-intensity band of negative sign at 285 nm is in agreement with an *S*-configuration of C-2 for **3**.[12] From a chemotaxonomic point of view the presence of a flavan in Combretaceae is interesting because flavans are an uncommon group of natural products previously not found in this family.

Table 3. Antimalarial Activity of Compounds **1**−**4** When Tested against the Chloroquine-Susceptible Strain 3D7 of *Plasmodium falciparum*

compd	IC_{50} (μM)[a]
1	9.6 ± 1.2
2	> 50
3	> 50
4	20.5 ± 10.0
chloroquine	0.15

[a] The values are expressed as means ± standard deviation of four experiments each performed in triplicate.

Table 4. Antifungal Activity of Compounds **1**−**4**

compd	*Penicillium expansum*[a]	*Candia albicans*[b]
1	1	10
2	2	80
3	3	6
4	5	>200
amphotericin B		1
nystatin	0.5	

[a] Minimum amount (μg) of compound to inhibit growth of fungal cultures on a TLC plate by a bioautographic method.[18] [b] Minimum amount (μg) of compound needed to inhibit growth of yeast culture on a TLC plate by an agar overlay technique.[19]

The ^1H-NMR and ^{13}C-NMR spectra of compound **4** revealed that this compound was anolignan B previously isolated from *Anogeissus acuminata* (Combretaceae).[13]

Although previously found in anolignan B, the presence of a 2,4-dioxygenated phenyl group in lignans is remarkable, because lignans are believed to be derived from shikimic acid, most frequently leading to 3,4,5-, 3,4- or 4-oxygenated phenyl groups. In general 2,4-oxygenated phenyl groups are of polyacetate origin. A number of reviews give no additional examples of lignans with 2,4-dioxygenated phenyl groups.[14−16]

Previously anolignan B has been shown to possess some HIV-1−reverse transcriptase inhibitory activity.[13] This finding and the previous reports on anti-HIV activity of *T. bellerica* extracts[6,7] and anti-HIV activity of flavans[17] encouraged us to test compounds **1**−**4** for anti-HIV-1 activity. All four compounds only showed a poor anti-HIV effect, with effective concentrations for obtaining 50% reduction of HIV antigen production in the range 7 to 16 μM and selectivity indices between 3 and 7. All compounds were tested for antimalarial activity. Two lignans (**1** and **4)** showed significant antimalarial activity (Table 3). All four compounds showed significant antifungal activity against *Penicillium expansum* (IMI 960902), and compounds **1** and **3** showed significant activity against *Candida albicans* (IMI 349010), as shown in Table 4.

Experimental Section

General Experimental Procedures. The NMR spectra were recorded on a Bruker AC-200F spectrometer at 200 and 50 MHz for the ^1H NMR and ^{13}C-NMR

spectra, respectively. Mass spectra were recorded on a JEOL AX505W mass spectrometer.

Plant Material. *T. bellerica* (local name "Thanni" in Malayalam) fruits were collected at Shenkotta Pass, Tirunelveli District, India, in July 1996, and identified by Dr. V. Chelladivari, Research Officer at CCRAS. Additional fruits were bought at the market in Nagercoil, Tamil Nadu, India. The presence of compounds **1**−**4** in the collected as well as the bought material was verified by TLC. A voucher specimen (22980) is kept at the Royal Danish School of Pharmacy.

Extraction and Isolation. Powdered fruit rind of *T. bellerica* (10 kg) was extracted with CH_2Cl_2 (3 × 14 L). The concentrated extract was fractionated by flash chromatography over Si gel (ICN silica TSC, 60 A) using eluents of increasing polarity starting with CH_2Cl_2, adding EtOAc and later MeOH. All of the 12 fractions obtained were tested for antifungal activity against *P. expansum* using a direct bioautographic method.[18] Inhibition zones were located mainly in fractions 4, 6, and 7. Fraction 4 was subjected to chromatography over Si gel (Merck, 0.063−0.200 mm) using heptane to which increasing amounts of EtOAc (10−100%) were added to isolate compound **3**. Compounds **1** and **4** were isolated from fraction 6 after repeated chromatography over Si gel using CH_2Cl_2−EtOAc (5−100%) followed by chromatography over silanized Si gel (Merck, RP-2) using CH_3CN−H_2O (1:1) as eluent. Fraction 7 was repeatedly chromatographed on Si gel columns using gradient mixtures of CH_2Cl_2−EtOAc (5−100%) to isolate compound **2**.

Termilignan (1): colorless crystalline powder; mp 106 −107 °C; MS *m/z* 296 $[M]^+$ (20), 189 $[M - HOC_6H_4CH_2]^+$ (100), 137 $[HO(CH_3O)C_6H_3CH_2]^+$(49), 107 $[HOC_6H_4CH_2]^+$ (18); ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; HREIMS *m/z* 296.1385 (calcd for $C_{19}H_{20}O_3$, 296.1412).

Thannilignan (2): colorless crystalline powder; mp 127−129 °C; $[\alpha]^{24}_D$ −63.9° (*c* 0.3, $CHCl_3$); UV (MeCN) λ_{max} (ϵ) 285 (5.2 × 10^3), 222 (1.17 × 10^4), 195 (6.70 × 10^4) nm; CD (MeCN) λ_{max} ($\Delta\epsilon$) 287 (−0.49), 235 (0.96), 222 (0) nm; MS *m/z* 312 $[M - H_2O]^+$ (50), 281$[M - H_2O - CH_3O]^+$ (100), 205 $[M - H_2O - HOC_6H_4CH_2]^+$ (10), 187 $[C_{11}H_{12}O_2]^+$ (30), 137 $[HO(CH_3O)C_6H_3CH_2]^+$ (20), 107 $[HOC_6H_4CH_2]^+$ (18); ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; HREIMS *m/z* 312.1339 (calcd for $C_{19}H_{22}O_5 - H_2O$, 312.1362).

7-Hydroxy-3′,4′-(methylenedioxy)flavan (3): colorless crystalline powder; mp 130−133 °C (lit. 122−124 °C)[10], $[\alpha]^{24}_D$ −20.9° (*c* 0.2, $CHCl_3$), (lit.[10] $[\alpha]^{24}_D$−14.2°); ^{1}H-NMR and ^{13}C-NMR data as previously reported;[10] UV (MeCN) λ_{max} (ϵ) 284 (8.14 × 10^3) nm; CD (MeCH) λ_{max} ($\Delta\epsilon$) 292 (−1.51); 210 (4.52); 202 (−4.51) nm.

Anolignan B (4): colorless crystalline powder; mp 160−160.5 °C (lit.[13] mp 147 °C); ^{1}H-NMR and ^{13}C-NMR data as previously reported.[13]

Antifungal Assays. A direct bioautographic method[18] was used to determine the activity against *P. expansum*. The components of the fraction to be tested were separated on TLC plates. A suspension of fungal spores were sprayed onto the TLC plate, and after incubation in a humid atmosphere for 72 h at room temperature, inhibition was observed as reduced or lack of mycelial growth. A thin-layer chromatographic agar overlay technique[19] was used to determine the activity against *C. albicans*. A seeded agar medium was applied over the TLC plates, and the plates were incubated overnight at room temperature in a square dish. After incubation, the TLC plates were sprayed with an aqueous solution (1 mg/mL) of dimethylthiazolyldiphenyltetrazoliumbromide (MTT). Inhibition zones were observed as yellow spots against a blue background. Minimum amount of compounds **1**−**4** to inhibit growth were determined by a TLC agar overlay technique against *C. albicans* using amphotericin B as a reference and by a direct bioautographic method against *P. expansum* using nystatin as a reference. The results are shown in Table 4.

Anti-HIV-1 Assay. Compounds **1**−**4** were examined for anti-HIV-1 activity using MT-4 cells[20] as infected cells. The HIV-1 strain HTLV-IIIB[21] was propagated in H9 cells at 37 °C, in 5% CO_2 using RPMI 1640 with 10% heat-inactivated fetal calf serum and antibiotics. MT-4 cells were incubated with virus for 2 h, washed, and afterwards added in a proportion of 1:10 to uninfected cells, which had been preincubated in growth medium containing these test compounds for 2 h. Cultures were maintained with the test compounds for 7 days in parallel with virus-infected control cultures without test compounds added. Expression of HIV in the culture medium was quantitated by HIV-1 antigen detection ELISA.[22] Compounds mediating 30% or more reduction of antigen expression were considered as active. These were examined for cytotoxic effect using concentration-dependent inhibition of MT-4 cell proliferation as a measure of cytotoxicity using the MTT assay.[23]

Antimalarial Assay. A chloroquine-susceptible strain (3D7) of *Plasmodium falciparum* was used for the in vitro assay. The effects of all four compounds were assessed by a modified method originally described by Jensen *et al.*[24] Into each well of 96-well microtiter plates 50 μL of parasitized erythrocytes (1%) at a concentration of 5 × 10^8 cells/mL and 50 μL of medium containing the test compounds at different concentrations were added . The cultures were incubated at 37 °C for 48 h in an atmosphere of 2% oxygen, 5% carbon dioxide, and 93% nitrogen. After half of the incubation period before termination of incubation, 20 μL of [^{3}H]-hypoxanthine (40 μCi/mL) were added into each well. The effect of compounds in cultures were monitored by the measurement of [^{3}H]hypoxanthine incorporation into the parasite nucleic acids. The cultures were harvested on filter paper by a cell harvester and counted in a liquid scintillation counter. All cultures were performed in triplicates. The percent of growth inhibition was calculated as follows: growth inhibition = 1 − (cpm in drug treated parasite culture − cpm background)/(cpm in control culture − cpm background) × 100. From these the IC_{50} values were calculated.

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