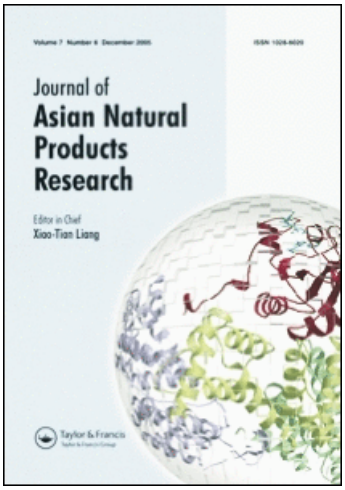

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Two new lignans from *Phyllanthus amarus*

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Two new lignans, 3-(3,4-dimethoxy-benzyl)-4-(7-methoxy-benzo[1,3]dioxol-5-yl-methyl)-dihydrofuran-2-one (**1**) and 4-(3,4-dimethoxy-phenyl)-1-(7-methoxy-benzo[1,3]dioxol-5-yl)-2,3-bis-methoxymethyl-butan-1-ol (**2**), were isolated from the leaves of *Phyllanthus amarus* and their structures were established by spectral analysis. Additionally, eight known lignans were also isolated and characterized.

Keywords: *Phyllanthus amarus*; Euphorbiaceae; γ-butyrolide lactones; lignans

1. Introduction

The genus *Phyllanthus*, belonging to the family Euphorbiaceae, comprises over 600 species in the world. These species are widely distributed in most tropical and subtropical countries and have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes, and liver diseases [1]. The most widespread species of genus *Phyllanthus* is *P. amarus*, which is commonly known as 'Bhuiamlaki.' It is a small annual herb that grows 15–50 cm in height. Phytochemical investigation of *P. amarus* has resulted in the isolation of a variety of bioactive compounds including lignans, such as phyllanthin and hypophyllanthin, flavonoids, triterpenes, alkaloids, and hydrolyzable tannins.

Lignans are a large class of bioactive natural products formed by the dimerization of two phenylpropanoid units. Many lignans are considered as an interesting source for lead molecules toward the discovery of new anticancer drugs [2,3]. Cytotoxic effect of *Phyllanthus* lignans on two human leukemia cell lines, K-562 and leucena-1, suggests their potential action as multidrug resistance reversing agents [4]. Recently, *P. amarus* extract administration has been shown to inhibit the liver tumor development induced by *N*-nitrosodiethylamine in rats and increase the life span of hepatocellular carcinoma harboring animals [5]. Pharmacological effects of *P. amarus*, such as antibacterial [6,7], anti-inflammatory [8,9], antihepatotoxic [3,10–12] in addition to their anticancer [13,14], antiviral [15,16], and antihyperglycemic [17] activities, have largely been documented.

On the basis of the therapeutic potential of *Phyllanthus* sp. as herbal drug, we initiated to investigate *P. amarus* for new lignans, to serve as marker chemicals for qualitative and quantitative evaluation of plant. This effort led us to the isolation and identification of 10 lignans in *P. amarus*; out of these, a lignan lactone (**1**) and a hydroxy lignan (**2**) are being

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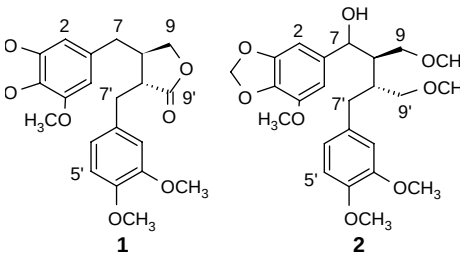

Figure 1. Chemical structures of compounds **1** and **2**.

reported for the first time (Figure 1). Existence of these lignans is of chemotaxonomic importance.

2. Results and discussion

Ethanolic extract of the aerial part of *P. amarus* was divided into *n*-hexane, chloroform, *n*-butanol, and water-soluble fractions by solvent partitioning. Hexane fraction was subjected to column chromatography on silica gel eluting with increasing percentage of ethyl acetate in hexane (from 90:10 to 75:25).

Compound **1** was isolated from the fractions eluted with hexane/ethyl acetate (75:25). It gave a positive ESI molecular ion peak at *m/z* 423 $[M+Na]^+$ and a negative molecular ion peak at *m/z* 399 $[M-H]^+$, suggesting the molecular weight to be 400. The DEPT experiment indicated the presence of three methyl, four methylene, seven methine, and eight quaternary carbons. Thus, on the basis of the MS data and DEPT experiment, the molecular formula of **1** was deduced as $C_{22}H_{24}O_7$.

Isolate **1** showed a positive optical rotation. The IR spectrum exhibited characteristic absorption band at 1773 cm^{-1}, which is indicative of the γ-butyrolide group [18]. The bands at 1608 and 1513 cm^{-1} were attributed to the phenyl group [19]. The UV spectrum showed absorption maxima at 232 and 279 nm for the butyrolactone-type lignan [18]. The ^1H and ^{13}C NMR spectra of **1** also bear a close resemblance to that of butyrolactone-type lignans [19]. The ^1H NMR spectrum gave a characteristic signal for the methylene dioxy function at δ 5.93 [20]. The position of the methylene dioxy function was confirmed by the HMBC correlation of C-3 and C-4 with the protons at δ 5.93. Appearance of three singlets at δ 3.84, 3.85, and 3.89 in the ^1H NMR spectrum confirmed the presence of three methoxyl groups in the benzene rings of compound **1** [19]. The DEPT spectra revealed the presence of three methylene carbons; out of these, the value at δ 101.7 was attributed to the methylene dioxy carbon on the basis of a single-quantum coherence result [19]. The other two methylene carbons were displayed at δ 39.1 and 35.4. The signal at δ 39.1, corresponding to proton signals at δ 2.58 and 2.44, showed a long-range correlation with the carbon at δ 132.7 (C-1). Thus, this signal was attributed to C-7. The *trans*-junction of the γ-lactone group was deduced from the coupling constant ($J_{8-8'} = 9.3$ Hz) resulted after irradiation of H-7′ in the ^1H homonuclear decoupling experiment [21]. The other two benzylic protons attached to C-7′ were observed at δ 2.90 and 2.96. The downfield shift of these two protons can clearly be explained by the proximity of the carbonyl group with C-7′ than with C-7. The methylene group of γ-lactone gave characteristic signals at δ 3.87 and 4.14 [19]. The signal at δ 3.87 overlapped with methoxyl signals of the benzene ring, while the signal at δ 4.14 appeared as a double doublet of 6.9 and 9.0 Hz due to geminal coupling with H-9′ and vicinal coupling with H-8. Compound **1** showed an aromatic proton signal less than bursehernin in the ^1H NMR spectrum. Instead, **1** gave two sets of aromatic proton signals in the aromatic region. Two broad singlets at δ 6.16 and 6.14 were due to H-2 and H-6, respectively. The other set appeared as an ABX system, which is characteristic of dimethoxy-substituted aromatic group of lignan lactones [19]. On the basis of the HMBC and HSQC

correlations, the signals at δ 6.16 and 6.14 were attributed to H-2 and H-6, respectively.

In the ^{13}C NMR spectrum, the signal at δ 178.8 confirmed the presence of the γ-lactone ring carbonyl group [18]. The carbonyl carbon of lactone ring correlated with the proton signal at δ 2.57 (H-8′), which further correlated with the carbon at δ 35.4 (C-7′). Thus, the location of the carbonyl group was confirmed as 9′. Three aromatic methoxyl groups were confirmed in compound **1** but only two methoxyl groups bearing aromatic carbons appeared in the ^{13}C NMR spectra. The value at δ 148.5 was fixed as C-4′ due to its long-range correlation with H-2′ and H-5′. Carbon signal at δ 149.6 was correlated with signals at δ 5.93 and 6.14 (H-6) as well as with δ 6.68 (H-2′) and δ 6.69 (H-5′). This is only possible if three methoxy-substituted aromatic carbons are present in isolate **1**, among them two resonated at an identical value at δ 149.6 and their positions were confirmed as C-5 and C-3′. The results obtained from the ^{1}H–^{1}H correlation in the COSY spectrum were also in good agreement with the assigned values. Thus, on the basis of the above spectroscopic analysis, compound **1** (5-methoxy bursehernin) was assigned as 3-(3,4-dimethoxy-benzyl)-4-(7-methoxy-benzo[1,3]dioxol-5-yl-methyl)-dihydrofuran-2-one.

Compound **2** was isolated from later fractions eluted with the same ratio of hexane/ethyl acetate and purified by means of preparative thin-layer chromatography. It was obtained as a viscous mass and showed a positive molecular ion peak at *m/z* 471 $[M+Na]^+$ in the ESI-MS spectrum, while in the EI-MS spectrum, it gave a peak at 448 $[M]^+$, showing the molecular weight to be 448. The DEPT spectra combined with other spectroscopic analysis revealed the presence of five methyl, four methylene, eight methine, and seven quaternary carbon atoms. Thus, on the basis of the MS and NMR spectral data, the molecular formula of isolate **2** was assigned as $C_{24}H_{32}O_8$.

The IR spectrum band at 3400 cm^{-1} is indicative of the hydroxyl group in the molecule. The bands at 1510, 1627, and 1496 cm^{-1} were attributed to aromatic rings. Compound **2** gave a characteristic signal at δ 101.5 for methylene carbon of the methylene dioxy function in its ^{13}C NMR spectrum. The ^{1}H NMR spectrum also confirmed the presence of a methylene dioxy function (δ 5.93). Two methoxy-bearing methylene carbons appeared at δ 71.9 and 72.6. The other methylene carbon signal appeared at δ 37.1 and was connected with the proton signal at δ 2.60 in the HSQC spectrum. Instead of two carbon signals at δ 35.0 and 35.5 for two methylene carbons of niranthin corresponding to C-7 and C-7′, only a single methylene carbon appeared in this region, which suggested that out of these two carbons (C-7 and C-7′) one is substituted [22]. Two quaternary aromatic carbons appeared at δ 134.1 and 134.5 which were attributed to C-1 and C-1′, respectively, and their positions were confirmed by the comparison of chemical shifts with niranthin and by analyzing the HMBC correlations. Two non-aromatic methine carbons appeared at δ 41.1 and 46.2; the signal at δ 41.1 showed a long-range correlation with the proton signal at δ 2.60. The signal of δ 2.60 further correlated with the carbon signal at δ 134.5 (C-1′), thus the proton signal at δ 2.60 was given to H-7′. Three quaternary carbon signals at δ 145.5, 148.0, and 148.7 were assigned to C-5, C-4′, and C-3′, respectively, due to long-range connectivity with methoxyl protons.

Analysis of IR and mass spectra confirmed the presence of a hydroxyl group in compound **2**. The notable differences between ^{13}C NMR spectra of isolate **2** and niranthin were appearance of the signal at δ 76.6 and disappearance of the signal in the range δ 35–39 for C-7 [22]. The position of the hydroxyl group

at C-7 was confirmed by the analysis of 2D NMR spectra of compound **2**. The signal at δ 76.6 was connected with the proton at δ 4.85 in HSQC and showed a long-range correlation signal with C-9. The downfield proton signal (δ 4.85) also confirmed it as proton attached to the hydroxyl group bearing benzylic carbon; moreover, the proton at δ 4.85 gave an intense signal in correlation with H-8 in the homonuclear $^1H-^1H$ COSY spectrum.

Three methoxyl groups appeared in a relatively upfield region at δ 56.1, 56.3, and 56.9 and two appeared in the downfield region at δ 59.3 and 59.1. The chemical shifts were compared with niranthin and downfield values were given to the methoxy groups attached to C-9 and C-9′ [22]. Two aromatic protons appeared in a comparatively upfield region at δ 6.29 and 6.25 as broad singlets and were coupled in the COSY spectrum. Both of these protons correlated with the ^{13}C NMR signal at δ 134.1 (C-1) in the HMBC spectrum and thus assigned as H-2 and H-6, respectively. The signals at δ 6.78 and 6.80 were given to H-6′ and H-2′ due to the HMBC correlations with the carbon at δ 134.5 (C-1′). Proton and carbon NMR data of compound **2** suggested a close resemblance of structure and stereochemistry with niranthin [20]. Comparison of these data revealed a *trans* correlation between H-8 and H-8′. A low value of coupling constant (5.4 Hz) of H-7 suggested a *cis* relation with H-8. The *cis* relationship of H-7 and H-8 was also confirmed by the NOE enhancement of H-8 upon irradiating at the frequency of H-7 (δ 4.85). Position of the hydroxyl group was also confirmed by EI-MS spectrum. A fragment at *m/z* 430 $[C_{24}H_{30}O_7]^+$ indicated the loss of a water molecule, 398 indicated loss of a water and a methanol molecule $[C_{23}H_{26}O_6]^+$, 367 indicated loss of a water and methanol molecule and a methoxy group $[C_{22}H_{23}O_5]^+$, 353 indicated loss of a water and methanol molecule and a methoxy methylene group $[C_{21}H_{21}O_5]^+$. A fragment at *m/z* 181 arose due to bond rupture between C-7 and C-8 $[C_9H_9O_4]^+$ and 151 due to bond rupture between C-1 and C-7 $[C_8H_7O_3]^+$. Hence, on the basis of the above discussion, the structure of compound **2** (7-hydroxy niranthin) was confirmed as 4-(3,4-dimethoxy-phenyl)-1-(7-methoxy-benzo[1,3]dioxol-5-yl)-2,3-bis-methoxymethyl-butan-1-ol, a new natural product.

Eight known lignans, viz., phyllanthin (**3**), hypophyllanthin (**4**), niranthin (**5**), nirtetralin (**6**), 5-demethoxy niranthin (**7**), virgatusin (**8**), heliobuphthalmin lactone (**9**), and bursehernin (**10**), were also isolated and characterized by a direct comparison of their physical data ($[\alpha]_D$, PMR, CMR, HSQC, HMBC, COSY, MS, and UV data) [20,23].

3. Experimental

3.1 General experimental procedures

The optical rotations were measured on a Horiba polarimeter (SEPA-300). UV spectra were recorded on a Spectronic Genesys-2 spectrometer. IR spectra were obtained on a Perkin-Elmer-spectrum-BX spectrophotometer. The ^1H NMR (300 MHz) spectra were recorded in $CDCl_3$ with tetramethyl silane as an internal standard using a Bruker Avance instrument. ^{13}C NMR and DEPT spectra were recorded at 75 MHz. The DEPT experiments were used to determine multiplicities of carbon atoms. Chemical shifts are given in parts per million. COSY, HSQC, and HMBC were performed using standard Bruker pulse programs. ESI-MS were measured on a Shimadzu LCMS-2010EV spectrometer (70 eV) and EI-MS were obtained on a Perkin-Elmer Turbo mass spectrometer. Elemental analysis was performed on a Euro-EA-3000 CHNS analyzer, Eurovector (Milan, Italy). Preparative HPLC was performed on Shimadzu LC-8A using reverse phase column: 21.2 mm × 25 cm (PLC-18, Supelcosil™, 12 μm; Supelco,

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** and **2**.

	Compound **1**				Compound **2**			
Position	δ_H (m, *J*, Hz)	δ_C	HMBC	COSY	δ_H (m, *J*, Hz)	δ_C	HMBC	COSY
1	–	132.7	–	–	–	134.1	–	–
2	6.16 (brs)	102.8	C-3, C-6, C-7	H-6	6.29 (brs)	103.6	C-1, C-3, C-6	H-6
3	–	143.9	–	–	–	147.8	–	–
4	–	134.4	–	–	–	135.9	–	–
5	–	149.6	–	–	–	145.5	–	–
6	6.14 (brs)	109.4	C-2, C-4, C-5, C-7	H-2	6.25 (brs)	108.8	C-1, C-2, C-5	H-2
7	2.58 (m), 2.44 (m)	39.1	C-1, C-2, C-6, C-8, C-9	H-9, H-8	4.85 (d, 5.4)	76.6	C-8	H-8
8	2.48 (m)	41.6	C-1, C-7, C-8′, C-9	H-7, H-9, H-8′	2.05 (m)	46.2	C-8′	H-7
9	3.87 (m), 4.14 (dd, 9.0, 6.9)	71.5	C-7, C-8	H-8	3.50 (m)	71.9	C-7, C-8, 9 OMe	9 OMe
1′	–	130.6	–	–	–	134.5	–	–
2′	6.68 (brs)	111.8	C-1′, C-3′, C-4′, C-7′	H-6′	6.80 (brs)	111.2	C-3′	–
3′	–	149.6	–	–	–	148.7	–	–
4′	–	148.5	–	–	–	148.0	–	–
5′	6.69 (d, 9)	112.9	C-3′, C-4′, C-6′	H-6′	6.78 (brs)	109.6	C-4′, C-6′	H-6′
6′	6.76 (d, 9)	121.7	C-1′, C-2′, C-5′, C-7′	H-5′	6.78 (brs)	118.9	C-1′	H-5′
7′	2.90 (m), 2.96 (m)	35.4	C-1′, C-2′, C-6′, C-8′, C-9′	H-8′	2.60 (m)	37.1	C-1′, C-8′, C-9′	H-6′
8′	2.57 (m)	46.9	C-1′, C-7, C-7′, C-8, C-9′	H-7′, H-8	2.00 (m)	41.1	C-7′	H-8
9′	–	178.8	–	–	3.30 (brs)	72.6	C-7′, C-8′, 9′ OMe	H-8′
$-OCH_2O-$	5.93 (s)	101.7	C-3, C-4	H-2	5.93 (s)	101.5	C-3, C-4	H-2
3′ OMe	3.85 (s)	56.2	C-3′	H-2′	3.83 (s)	56.3	C-3′	–
4′ OMe	3.84 (s)	56.3	C-4′	H-5′	3.87 (s)	56.1	C-4′	–
5 OMe	3.89 (s)	57.2	C-5	H-6	3.85 (s)	56.9	C-5	–
9 OMe	–	–	–	–	3.31 (s)	59.3	C-9	H-9
9′ OMe	–	–	–	–	3.32 (s)	59.1	C-9′	H-9′

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) recorded in $CDCl_3$; chemical shifts are expressed in ppm and *J* values in Hz.

Bellefonte, PA, USA), PDA detector. Column chromatography was performed using silica gel (60–120 mesh; E-Merck, Mumbai, India) and preparative TLC was carried out on silica gel 60 F_{254} (20 × 20 cm, 0.5 mm; E-Merck, Darmstadt, Germany) plates.

3.2 Plant material

Leaves of *P. amarus* were harvested from our experimental farm at Lucknow, India. A herbarium specimen (voucher specimen no. CIMAP-1421) and seeds of *P. amarus* are available in the National Gene Bank of the Institute.

3.3 Extraction and isolation

Air dried and finely powdered leaves of the plant (4 kg) were exhaustively extracted at room temperature (25 ± 5°C) with ethanol (71 × 4) and the ethanolic extract was concentrated *in vacuo* to give a residue (804 g). Water (250 ml) was added and it was then partitioned with *n*-hexane, chloroform, and *n*-butanol. A part (190 g) of the hexane extract (290 g) was subjected to column chromatography on silica gel (1.5 kg, 60–120 mesh); elution was carried out with varying percentages of ethyl acetate in *n*-hexane. Fractions eluted with hexane/ethyl acetate (75:25) afforded compounds **1** (10 mg) and **2** (8 mg).

Compound **1** was purified by preparative HPLC: mobile phase, MeOH/H_2O (65:35); flow rate, 15 ml/min, RT, 21.00 min. Detection was carried out at 210 nm. Compound **2** was obtained as a viscous mass by PTLC using hexane/ethyl acetate/acetone (70:24:6) as the mobile phase and spots were viewed in UV light (254 nm).

Column chromatography with an increasing gradient of *n*-hexane/ethyl acetate mixture afforded compounds **3** (6.1 g), **4** (1.65 g), **5** (100 mg), **6** (110 mg), **7** (7 mg), **8** (10 mg), **9** (8 mg), and **10** (11 mg).

3.3.1 Compound (*1*)

A viscous mass, 10 mg; $[\alpha]_D^{28}$ +13.30 ($c = 0.104$, $CHCl_3$); UV (MeOH) λ_{max} (log *e*) 232 (4.08), 279 (3.79) nm; IR (KBr) ν_{max} 3020, 2928, 2856, 1773, 1608, 1544, 1513, 1452, 1262, 1241, 1031, 933 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z*: 423 $[M+Na]^+$, 399 $[M-H]^+$. Elemental analysis: found: C, 66.20; H, 6.14; calcd for $C_{22}H_{24}O_7$: C, 66.00; H, 6.00%.

3.3.2 Compound (*2*)

A viscous mass, 8 mg; $[\alpha]_D^{28}$ −19.58 ($c = 0.120$, $CHCl_3$); UV (MeOH) λ_{max} (log *e*) 226 (3.75), 276 (3.25); IR (KBr) ν_{max} 3400, 1510, 1543, 1627, 1496, 1262, 1237, 1047, 968 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z*: 471 $[M+Na]^+$; EI-MS *m/z*: 448 $[C_{24}H_{32}O_8]^+$, 430 $[C_{24}H_{30}O_7]^+$, 398 $[C_{23}H_{26}O_6]^+$, 367 $[C_{22}H_{23}O_5]^+$, 353 $[C_{21}H_{21}O_5]^+$, 181 $[C_9H_9O_4]^+$, 151 $[C_8H_7O_3]^+$. Elemental analysis: found: C, 64.58; H, 7.20; calcd for $C_{24}H_{32}O_8$: C, 64.28; H, 7.14%.

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