Lignans from Cell Suspension Cultures of *Phyllanthus niruri*, an Indonesian Medicinal Plant

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Cell suspension cultures of *Phyllanthus niruri* were used to study the lignan profiles and biosynthesis. Suspension cultures yielded two lignans: the new cubebin dimethyl ether (**1**) and urinatetralin (**2**), a new lignan from *P. niruri*, but reported earlier from *P. urinaria*. This is the first report of cell suspension cultures of *P. niruri* that successfully produce lignans. Feeding 0.5 mM ferulic acid or 0.5 mM caffeic acid, being early precursors of lignan biosynthesis, resulted in an increase up to 0.7 mg g^{-1} DW of **1** (control value 0.1 mg g^{-1} DW) and up to 0.3 mg g^{-1} DW of **2** (control value 0.2 mg g^{-1} DW). Comparison of the lignan profiles of cell suspensions, callus cultures, aerial plant parts, roots, and seeds showed significant differences.

Phyllanthus niruri L. (Euphorbiaceae) is a small plant widely distributed in tropical and subtropical regions in Central and South America and Asia (including India and Indonesia). Whole plants have been used in traditional medicine for treatment of jaundice, asthma, hepatitis, and malaria and because of diuretic, antiviral, and hypoglycemic properties.[1−3] To scientifically support the traditional use, several pharmacological studies have been carried out with plant extracts and with isolated compounds. *P. niruri* extract shows potential therapeutic actions in the management of hepatitis B.[2,3] Its antiviral activity extends to HIV-1 RT inhibition.[4,5] Its role in urolithiasis is related to the inhibition of calcium oxalate endocytosis by renal tubular cells.[6,7] In vitro antiplasmodial activity of this plant extract has been described.[8] *P. niruri* extract also showed inhibitory activities against angiotensin converting enzyme (ACE) and rat lens aldose reductase (AR), which play a significant role in the reduction of aldose to alditol under abnormal conditions such as diabetes.[9] Immunomodulating effects in the treatment of cancer by influencing the function and activity of the immune system[10] and lipid lowering activity[11] have been reported. An extract of the callus culture of *P. niruri* showed analgesic activity.[12]

The aerial parts of *P. niruri* have been reported to contain alkaloids, flavonoids, phenols, coumarins, tannins, terpenoids, and lignans. Several of these isolated compounds have been tested for their pharmacological activities.[3,5,13,14] Lignans from this plant have been studied most intensively; 17 different lignans have been found so far. Several of these lignans were tested for cytotoxicity and other biological activities in vitro. Phyllanthin and hypophyllanthin were protective against carbon tetrachloride- and galactosamine-induced cytotoxicity in primary cultured rat hepatocytes.[15] 3,4-Methylenedioxybenzyl-3′,4′-dimethoxybenzylbutyrolactone has been reported to possess antitumor activity.[16] Nirtetralin and niranthin were tested against human hepatitis B virus in vitro.[13]

The aims of this study were to establish the cell suspension cultures of *P. niruri*, to study the production and biosynthesis of lignans, and to compare the lignan profile of cell suspensions with callus cultures, aerial plant parts, roots, and seeds.

Results and Discussion

From the various medium compositions tested, the B5 medium resulted in the best growth of callus and cell suspension cultures of *P. niruri*. This medium was used in all experiments. Cell suspension cultures of *P. niruri* had a growth cycle of 21 days. During this time, conductivity decreased from 3.2 ± 0.01 mS on day 1 to 1.5 ± 0.02 mS on day 15 and slightly increased to 2.0 mS on day 21. This indicates that cell lysis occurred between day 15 and 21. During the growth cycle, a slow increase in pH of the growth medium was observed. Fresh weight (FW) increased from 105.2 ± 3.2 g L^{-1} to 202.8 ± 19.2 g L^{-1}, and dry weight (DW) increased from 6.0 ± 1.1 g L^{-1} to 12.9 ± 0.1 g L^{-1}.

We found qualitatively and quantitatively different lignan profiles comparing seeds, roots, and aerial plant parts and callus and suspension cultures as determined by GC-MS. All lignans found in aerial plant parts (phyllantin, 3,4-methylenedioxybenzyl-3′,4′-dimethoxybenzylbutyrolactone, niranthin, phyltetralin, seco-isolariresinol trimethyl ether, nirtetralin, lintetralin, isolintetralin, hypophyllantin, seco-4-hydroxylintetralin), seed (phyllantin, niranthin and nirtetralin), root (demethylenedioxyniranthin, lintetralin and isolintetralin), and callus culture (3,4-methylenedioxybenzyl-3′,4′-dimethoxybenzylbutyrolactone) have been described previously in *P. niruri*.[3,16−18] None of these lignans was present in cell suspension cultures. However, four other lignans were found in cell suspension cultures. Compound **1** was a lignan that was not found in the plants before, and compound **2** has been reported earlier as a new compound from *P. urinaria*.[19] Two more compounds with MS fragmentation patterns resembling lignans were found. These last two compounds could not be separated. Compounds **1** and **2** were purified by preparative HPLC and their structures determined and confirmed (Figure 1). This is another example showing that plant cell cultures do not always accumulate, either qualitatively or quantitatively, the same compounds found in the plant from which they are established.

Compound **2** was identified as urinatetralin, which was confirmed by comparison of NMR and MS spectra with published data.[19] The relative configuration derived from the ^{1}H NMR coupling constants was in agreement with the (7′*R**,8*S**,8′*S**) configuration of **2** from *P. urinaria*.[19]

Compound **1** was a white amorphous solid with a molecular formula of $C_{22}H_{26}O_6$, as shown by HRMS ([M^+]: *m*/*z* 386.1723). The molecular mass of **1** is two mass units higher than that of compound **2**. Fragments at *m*/*z* 354 and 322 correspond to the loss of two molecules of MeOH [M − $HOCH_3$], as shown by HRMS. The base peak at *m*/*z* 135 corresponded to a methylenedioxy benzyl fragment ($C_8H_7O_2$), typical for lignan structures. These data indicated an urinatetralin-like structure for **1**, i.e., lacking the carbocylic ring at C-2 and C-7′. The ^{1}H NMR data exhibited two

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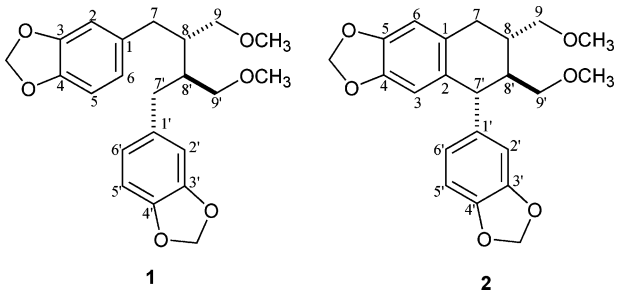

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Figure 1. Lignans from cell suspension cultures of *P. niruri*: cubebin dimethyl ether (**1**) and urinatetralin (**2**).

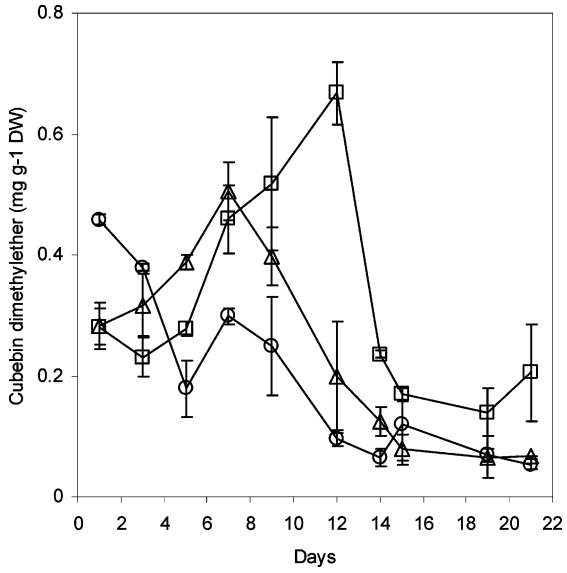

Figure 2. Cubebin dimethyl ether (**1**) accumulation of *P. niruri* cell suspension cultures after feeding of 0.5 mM caffeic acid (□), 0.5 mM ferulic acid (△), and in control cultures (○). Individual values expressed as means ± standard deviation in mg g^{-1} DW are averages of three independent experiments.

methylenedioxy groups at δ_H 5.92 (4H, s), two methoxy groups at δ_H 3.28 (6H, s), and six aromatic protons (δ_H 6.5−6.7) as two ABM spin systems. Due to the symmetry of the molecule, only one set of signals is observed. The structure for **1** was further supported by H,H-COSY, HMQC, and HMBC spectroscopic analysis. This compound, called cubebin dimethyl ether, is a new natural product but has been synthesized from dihydrocubebin.[20] Although the relative configuration could not be determined unambiguously, a (8*S**,8′*S**) configuration is assumed from a common biogenesis of **1** and **2**. This is the first report of cell suspension cultures of *P. niruri* that successfully produce lignans.

Feeding of early precursors of lignans to cell suspension cultures, 0.5 mM caffeic acid or 0.5 mM ferulic acid, enhanced the production of **1** and **2**. While feeding 0.1 mM of either compound had no effect, 1 mM appeared to be toxic for cell suspension cultures (causing growth inhibition). In the control cells, **1** reached a maximum at 0.46 mg g^{-1} DW on day 1 after inoculation, decreased to 0.18 mg g^{-1} DW on day 5, then increased on day 7 and further decreased to 0.05 mg g^{-1} DW on day 21. By feeding 0.5 mM caffeic acid, **1** increased to 0.67 mg g^{-1} DW on day 12 (7-fold compared to control cells). Feeding 0.5 mM ferulic acid caused an increase of the amount of **1** to 5.0 mg g^{-1} DW on day 7, and maximum enhancement was reached on day 7 (2-fold compared to control cells) (Figure 2).

In control cells, the amount of **2** increased from 0.09 mg g^{-1} DW on day 1 after inoculation to 0.20 mg g^{-1} DW on day 7, then decreased until day 14, and from day 14 to 21, the content remained constant. By feeding 0.5 mM caffeic acid, the amount of **2** increased

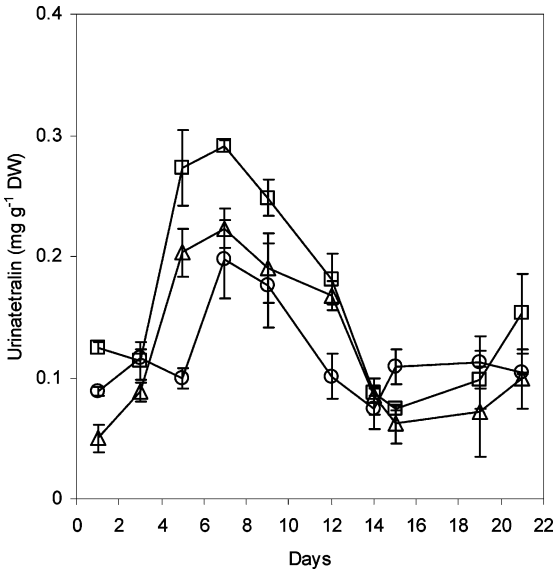

Figure 3. Urinatetralin (**2**) accumulation of *P. niruri* cell suspension cultures after feeding of 0.5 mM caffeic acid (□), 0.5 mM ferulic acid (△), and in control cultures (○). Individual values expressed as means ± standard deviation in mg g^{-1} DW are averages of three independent experiments.

to 0.3 mg g^{-1} DW on day 7, and a maximal enhancement was found on day 5 (2.5-fold compared to control cells). Feeding of 0.5 mM ferulic acid caused an increase of the amount of **2** to 0.22 mg g^{-1} DW on day 7, and maximal enhancement was shown on day 5 (2-fold compared to control cells) (Figure 3). Addition of 0.5 mM caffeic acid or 0.5 mM ferulic acid did not inhibit growth of cell suspension cultures of *P. niruri*. By adding 0.5 mM caffeic acid, the conductivity decreased from 3.5 ± 0.05 mS to 1.9 ± 0.01 mS, FW increased from 117.0 ± 7.0 g L^{-1} to 208.1 ± 7.4 g L^{-1}, and DW increased from 7.5 ± 0.3 g L^{-1} to 15.6 ± 0.9 g L^{-1}. Adding 0.5 mM ferulic acid, conductivity decreased from 3.4 ± 0.01 mS to 1.8 ± 0.01 mS, FW increased from 118.7 ± 6.1 g L^{-1} to 203.2 ± 3.8 g L^{-1}, and DW increased from 8.0 ± 0.05 g L^{-1} to 15.9 ± 2.2 g L^{-1}. These data show that after feeding caffeic and ferulic acid in concentrations of 0.5 mM, cell suspension cultures grew with a rate comparable to control cultures.

Feeding caffeic and ferulic acid increased the concentration of compounds **1** and **2** in *P. niruri* cell suspensions. This may be due to the incorporation of these precursors into the secondary metabolite biosynthetic pathway. Studies with labeled precursors may confirm this. Caffeic and ferulic acid have the necessary phenolic groups required for the oxidative coupling mechanism. Other compounds lacking a phenolic group, such as 3,4-dimethoxy and 3,4,5-trimethoxycinammic acid, are not converted into lignans.[21] Caffeic acid enhanced the formation of urinatetralin and cubebin dimethyl ether better than ferulic acid. This may have been caused by the different oxygenation pattern of the compounds. Caffeic acid, with its 3′,4′-dihydroxy substitution pattern, is apparently more easily incorporated than ferulic acid, which has a 4′-hydroxy-3′-methoxy substitution pattern. The coupling of phenylpropanoid monomers leading to urinatetralin and cubebin dimethyl ether in *P. niruri* cell suspension cultures appears to involve two precursors with a 4′-hydroxy-3′-methoxy substitution pattern or 3′,4′-dihydroxy substitution pattern. These compounds can be replaced by a 3′,4′-methylenedioxy substitution pattern. This agrees with the incorporation of labeled ferulic acid into podophyllotoxin and deoxypodophyllotoxin in *L. album*.[22]

Accumulation of **2** on days 2−7 was followed by accumulation of **1** on days 5−12 after feeding 0.5 mM caffeic acid. This suggests that **1** is a precursor for **2** in cell suspension cultures of *P. niruri* following cyclization involving C-2 and C-7′. This reaction has

Table 1. ^{1}H and ^{13}C NMR Data of Cubebin Dimethyl Ether (**1**) and Urinatetralin (**2**) (500 MHz, TMS, $CDCl_3$)

	1		**2**	
position	^{1}H (J/Hz)	^{13}C	^{1}H (J/Hz)	^{13}C
1		134.9 s		130.1 s
2	6.7 d (7.8)	121.8 d		133.4 s
3		108.1 d	6.21 s	109.5 d
4		145.6 s		145.8[a] s
5	6.59 d (1.3)	147.5 d		145.8[a] s
6	6.56 dd (7.9, 1.6)	109.5 d	6.56 s	108.0 d
7	3.26 m, 3.30 m	35.1 t	2.78 m	33.8 t
8	2.02 m	40.8 d	2.13 m	36.4 d
9	2.55 dd (13.9, 8.0)	72.7 t	3.40 dd (6.5, 9.3)	75.5 t
	2.65 dd (13.5, 5.9)		3.45 dd (4.0, 9.3)	
1′		134.9 s		139.4 s
2′	6.59 d (1.3)	109.5 d	6.55 d (1.7)	108.6 d
3′		147.5 s		146.4 s
4′		145.6 s		147.9 s
5′	6.7 d (7.8)	108.1 d	6.74 d (7.9)	107.6 d
6′	6.56 dd (7.85, 1.55)	121.8 d	6.64 dd (7.9, 1.7)	122.6 s
7′	3.26 m, 3.30 m	35.1 t	3.93 d (10.8)	47.7 d
8′	2.02 m	40.8 d	1.78 ddt (10.8, 10.8, 3.2)	45.1 d
9′	2.55 dd (13.9, 8.0)	72.7 t	3.10 dd (9.6, 3.4)	71.2 t
	2.65 dd (13.5, 5.9)		3.36 m	
9-OMe	3.28 s	58.8 q	3.35 s	59.0 q
9′-OMe	3.28 s	58.8 q	3.26 s	59.1 q
4,5-OCH_2O-	5.92 s	100.7 t	5.83 s	99.8 t
3′,4′-OCH_2O−	5.92 s	100.7 t	5.93 s	100.2 t

[a] Signals coincide.

also been shown by cyclization of the quinone methide as an intermediate to desoxypodophyllotoxin.[23]

Experimental Section

General Experimental Procedures. NMR spectra were recorded on Bruker WM 400 (400 MHz) and Bruker DRX 500 (500 MHz) spectrometers in $CDCl_3$. TMS was used as an internal standard. GC analysis was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 injector and a Hewlett-Packard 3365 Series II Chemstation, under the following conditions: column, WCOT fused-silica CP-Sil 5 CB (15 m × 0.31 mm i.d.; film thickness 0.25 μm; Chrompack, Middelburg, The Netherlands); oven temperature program, 150−320 °C at 15 °C min^{-1} and maintained at 320 °C for 5 min; injector temperature, 260 °C; detector (FID) temperature, 300 °C; helium was used as carrier gas; inlet pressure, 5 psi; linear gas velocity, 32 cm s^{-1}; split ratio, 20:1; injected volume, 2.0 μL. Gas chromatography−mass spectrometry (GC-MS) analysis was performed on a Shimadzu QP5000 GC-MS system equipped with a 17A GC, an AOC-20i autoinjector, and the GC-MS solution software 1.10. The GC conditions were the same as for GC analysis. MS conditions: ionization energy, 70 eV; ion source temperature, 250 °C; interface temperature, 280 °C; scan speed, 2 scans s^{-1}; mass range, 34−600 u. The HPLC system consisted of a Spectra-Physics (model SP 8810) liquid pressure pump, a Rheodyne high-pressure valve equipped with a 100 μL sample loop, and a Lichrospher RP-18 column (250 × 4 mm i.d., Merck, Damstradt, Germany). The mobile phase consisted of C_2H_3N/H_2O (45:55; v/v) containing 0.1% H_3PO_4 at a flow of 1.0 mL min^{-1} and a Shimadzu photodiode array UV−vis SPD-M6A detector (Shimadzu, 's-Hertogenbosch, The Netherlands) at 290 nm. Analytical thin-layer chromatography (TLC) was performed using silica gel 60-F254 (5 × 10 cm, 0.25 mm thickness, Merck, Darmstadt, Germany) and toluene/acetone (50:1) as the eluent. The elution length was 8 cm in a saturated chamber. The bands were detected by 254 nm UV light.

Plant Material, Solvents, and Chemicals. *Phyllanthus niruri* L. (Euphorbiaceae) was collected from a wild habitat in Bandung, West Java, Indonesia, and authenticated at the Department of Biology, Institut Teknologi Bandung (Indonesia), based on the Flora of Java.[24] A voucher specimen (HBG10PN12) is deposited in the Herbarium Bandungense. The age of the plant material was 0.5−1 year. All solvents and chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). All media and supplements used to grow the callus and the suspension culture were obtained from Duchefa (Haarlem, The Netherlands).

Cell Suspension Culture of *P. niruri*. Seeds of *P. niruri* were grown in a plant container under an L/D regime (16/8 h: 3000 lux) at 25 °C. Small plants developed after 3−4 weeks. The leaves were sterilized by dipping them into a 3% w/v NaOCl solution for 3 min, followed by bathing in sterile H_2O for 10 min. They were rinsed three times with sterile twice-deionized H_2O. The sterile leaves were cut into slices, and callus induction was obtained using media with different compositions. These media were modifications of the Murashige and Skoog medium[25] or the Gamborg's B5 medium.[26] Murashige and Skoog (MS) medium was supplemented with 1.0 mg L^{-1} indole-3-acetic acid and 1.0 mg L^{-1} 6-benzylaminopurin. Gamborg's B5 (B5) was supplemented with 4 mg L^{-1} α-naphthalene acetic acid. The so-called MS−B5, a combination of the macronutrients of MS and the micronutrients of B5 medium, was also used. It was supplemented with 3 mg L^{-1} α-naphthalene acetic acid. All media were supplemented with 4% (w/w) sucrose and solidified with 0.9% agar. The callus cultures were grown under an L/D regime (16/8 h: 3000 lux) at 26 °C. Callus developed after 6 weeks. Friable callus appeared as clumps, varying in color from dark brown to yellow.

Cell suspension cultures were initiated by transferring callus clumps into 100 mL sterile conical flasks with 50 mL of liquid medium of the same composition as described above but without agar. Cultures were incubated on a rotary shaker (175 rpm) at 26 °C under an L/D regime (16/8 h: 3000 lux, daylight L 36W/10, OSRAM, Germany). After 1 month, 50 mL of cell suspension cultures was transferred into a 500 mL conical flask, with fresh medium yielding a total volume of 300 mL. Subcultures were prepared every 3 weeks by adding 100 mL of a full-grown cell suspension culture to 200 mL of fresh medium.

Feeding Experiment. Caffeic and ferulic acid were added to reach three different final concentrations: 0.1, 0.5, and 1 mM. These compounds were dissolved using 0.2 mL of 96% EtOH and transferred to a sterile 500 mL Erlenmeyer flask containing the cell suspension cultures of *P. niruri* after inoculation into fresh medium (at the start of the growth cycle). Suspension-grown cells were harvested each 2 days during the growth cycle of 21 days. Samples of about 10 mL were taken aseptically and transferred into a calibrated conical tube and centrifuged for 5 min at 1500*g*. To monitor the viability and growth of the cell cultures, medium pH and conductance were routinely measured in the supernatant. Cells were filtered using a Büchner funnel. FW was determined and the cells were put overnight into the freezer and then freeze-dried. DW was also determined. Lignans were analyzed by GC and GC-MS.

Analysis. Lignan profiling was performed according to Koulman et al.[27] Dried and powdered material (100 mg) was weighed in a Sovirel tube. MeOH (2 mL, 80%) was added, and the mixture was sonicated

for 1 h. CH_2Cl_2 (4 mL) and distilled H_2O water (4 mL) were added. The tube was closed, vortexed, and centrifuged at 1500*g* for 5 min. The aqueous layer was discarded, and 2 mL of the organic layer was transferred into a 2 mL microtube. The CH_2Cl_2 was evaporated gently using a flow of nitrogen, and the residue was redissolved in 200 μL of MeOH. The tube was centrifuged at 15000*g* for 5 min in an Eppendorf 5414 centrifuge, and part of the liquid was transferred into a 0.8 mL crimp neck vial (Art. No. 98819, Alltech/Applied Science B.V. Breda, The Netherlands) and sealed immediately. For quantitative determination of **1** and **2**, calibration curves were prepared using MeOH solutions with concentrations ranging from 2.5 to 25 μg mL^{-1}. Each concentration was done in triplicate. Regression equations were $y = 143229x - 264$ ($r^2 = 0.9902$) and $y = 123516x - 218$ ($r^2 = 0.9885$) for **1** and **2**. The limit of detection (LOD) was established as the amount of analyte that provided a signal-to-noise ratio of 3. LODs were 0.12 μg for **1** and 0.25 for **2**. The limit of quantification (LLOQ) was defined as the lowest calibration standard that could be quantified with an accuracy of 90−110% and a precision of 15%. LLOQ was 2.5 μg for both **1** and **2**.

Extraction and Isolation. For the isolation of lignans, three-week-old suspension cultures were harvested and filtered through Miracloth (Lot B43936, Calbiochem, Behring Diagnostics, La Jolla, CA). A total of 500 g of fresh cells was extracted (3×) using 1 L of 80% MeOH each time. To disrupt cells, mixtures were sonicated for 1 h. The MeOH extracts were combined, and the volume was reduced to 100 mL under reduced pressure. The remaining extract was partitioned (3×) between 250 mL of H_2O and 250 mL of CH_2Cl_2. All organic phases were combined and concentrated. The resulting extract was fractionated on a column (75 × 2.5 cm) filled with silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany). Fractions were eluted by subsequent use of the following eluents: *n*-hexane/CH_2Cl_2 (9:1, 500 mL), CH_2Cl_2 (500 mL), and CH_2Cl_2/MeOH (25:1, 500 mL). Fractions of 20 mL each were collected and were profiled by TLC and GC-MS. Fractions with corresponding profiles were combined and concentrated. This resulted in a total of 16 fractions. The lignan-containing fraction was further separated by preparative TLC on preparative silica gel plates (20 × 20 cm, 1 mm thickness, Merck, Darmstadt, Germany) using toluene/acetone (50:1) as eluent. The elution length was 18 cm in a saturated chamber. Bands were detected by 254 nm UV light. Three bands were scraped off and analyzed by GC and GC-MS. R_f values were 0.28, 0.31, and 0.43, respectively.

For further purification of the lignans obtained by preparative TLC, semipreparative HPLC was used. Eluting substances were collected in glass tubes according to their retention times. Organic solvents were evaporated, and the aqueous residue was partitioned with CH_2Cl_2. The CH_2Cl_2 fraction yielded pure compounds **1** (3.0 mg) and **2** (2.5 mg).

Cubebin dimethyl ether (1): amorphous solid; ^{1}H and ^{13}C NMR data, see Table 1; MS (EI, 70 eV) *m*/*z* (rel int): 386 $[M]^+$ (2), 354(3), 322 (2), 187 (19), 135 (100), 77 (29), 45 (56); HRMS: *m*/*z* 386.1723 $[M^+]$ (calc for $C_{22}H_{26}O_6$, 386.1729).

Urinatetralin (2): amorphous solid; ^{1}H and ^{13}C NMR data, see Table 1; MS (EI, 70 eV) *m*/*z* (rel int) 384 $[M]^+$ (5), 352(6), 320 (7), 185 (12), 135 (26), 77 (8), 45 (100).

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