Phytochemical and Biosynthetic Studies of Lignans, with a Focus on Indonesian Medicinal Plants

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Chapter 4

Lignan profile of *Piper cubeba*, an Indonesian medicinal plant

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Abstract

The lignan profiles of aerial part of *Piper cubeba* L. (Piperaceae) was determined using GC, GC-MS and HPLC. The number of lignans found in the leaves was 15, followed by berries and the stalks with respectively 13 and 5 lignans. This is the first investigation of lignans from the leaves and the stalks of *P. cubeba*. Cubebin, hinokinin, yatein, isoyatein are common lignans in the genus *Piper* and appeared as major components in all part of *P. cubeba* investigated.

Introduction

Piper cubeba L. (Piperaceae) inhabits Java, Sumatra, Southern Borneo, and other isles in the Indian Ocean. The berries of *P. cubeba* are commonly known as cubeb (in Indonesia known as *kemukus*) and used in Indonesian traditional medicine to treat gonorrhea, dysentery, syphilis, abdominal pain, diarrhea, enteritis and asthma (Eisai, 1995, Satroamidjojo, 2001). Phytochemical and biological investigations have been carried out in order to prove its traditional use. In comparison to other species of genus *Piper*, *P. cubeba* has received less attention so far. Only three groups of secondary metabolites have been reported from the berries of *P. cubeba*, i.e. alkaloids, lignans and terpenoids (essential oil). The lignans and the essential oil have been more intensively investigated, since *P. cubeba* accumulates both groups of compounds in relatively high amounts. Economically, *P. cubeba* is important as a source of pepper (the dried berries) for the worldwide spice market (Usia et al., 2005). Piperine is an abundant alkaloid in the berries of this species (Parmar et al., 1997).

Twenty four lignans have so far been reported from *P. cubeba* (Fig. 1) (Prabhu and Mulchandani, 1985, Badheka et al., 1986, 1987, Koul et al., 1996, Parmar et al., 1997, Usia et al,. 2005). Lignans are an important group of secondary metabolites that are also assumed to contribute to the biological activity. Some of these lignans showed inhibitory activity against cytochrome P450 enzymes that are involved in the metabolism of all currently used drugs (Usia et al., 2005a, 2005b). Yatein, hinokinin, cubebin, dihydrocubebin have been reported to have antifeedant activity against a number of stored product insects. This activity is comparable to podophyllotoxin (Harmatha and Nawrot, 2002). Hinokinin has been reported to have anti-inflammatory and analgesic effect. Because of the structural relationship, hinokinin can be synthesized using cubebin as precursor (Da Silva et al. 2005). Cubebin has been shown to possess anti-inflamatory, analgesic and trypanocidal activities (Borsato et al., 2000, Bastos et al., 2001, De Souza et al., 2005). Yatein is also an interesting lignan due to its biological activity and its function as a biosynthetic precursor of deoxypodophyllotoxin and podophyllotoxin that are well known for their anticancer properties. Methanol and water extract of *P. cubeba* berries have been shown to display an inhibitory effect against the hepatitis C virus (Hussein et al., 2000). Anti-inflammatory, antioxidant, anti-allergic and analgesic activities of *P. cubeba* have been studied using chemically-induced edema and arthritis *in vivo* (Choi and Hwang, 2003 and 2005).

In this study we made a lignan profile of aerial parts of *P. cubeba* using GC, GC-MS and HPLC and compared the data from berries, stalks and leaves.

Materials and methods

Plant material, solvents and chemicals

Piper cubeba L. (Piperaceae) was collected in April 2002 from Jatiroto, Temanggung, Central Java, Indonesia, and authenticated at the Department of Biology, Institut Teknologi Bandung (Indonesia), based on the Flora of Java (Backer and Van de Brink, 1968). A voucher specimen (HBG10PC01) is deposited at the Herbarium Bandungense, Indonesia. The collected plant material was air-dried. All solvents and chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Extraction and isolation

One gram of dried plant material (either berries, leaves or stalks) was grinded together with quartz (Merck, Darmstadt, Germany) and extracted under sonification (1 h) using 2 mL of 80% MeOH. The resulting extract was fractionated using 4.0 mL of CH_2Cl_2 and 4 mL of water, shaken for 5 s and centrifuged at 1,500 g for 5 min. For the determination of lignans, 2.0 mL of CH_2Cl_2 phase were taken and evaporated to dryness. The residue was redissolved in 1.0 mL methanol and centrifuged. These

samples were ready to be analyzed by GC, GC-MS, HPLC. The isolation of lignans was achieved by preparative thin layer chromatography (TLC) using silica gel F254 plate (Merck, Germany) and toluene : acetone (85 : 15 v/v) as eluent. Bands corresponding to lignans were scraped off and extracted with 2 mL MeOH. The methanolic solutions were submitted to further analysis. The most abundant lignans, cubebin, hinokinin and yatein, were isolated as >95% pure compounds (as checked by GC and HPLC) and used for quantitative purposes.

Analysis of lignans using GC, GC-MS, HPLC, TLC

Gas chromatography (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 x 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.

GC-MS analysis was performed on a Shimadzu QP5000 GC-MS system equipped with a 17A GC, an AOC-20i autoinjector, and the GCMS solution software 1.10. GC conditions: WCOT fused silica CPsil 5 CB lowbleed/MS, CP7810, film thickness 0.10 μm; 15 m x 0.25 mm ID (Varian Middelburg, The Netherlands). Temperature program: 150°C – 320 °C at 15 °C min^{-1}. Injector temperature: 275°C; inlet pressure: 75 kPa; column flow: 2.1 mL min^{-1}: linear velocity: 75.5 cm sec^{-1}; split ratio: 20:1; total flow: 46.2 mL min^{-1}; carrier flow (He): 46.2 mL min^{-1}; injection volume: 2 μL; temperature program 35 min at 90 °C, 90-170 °C at 4 °C min^{-1}. MS conditions: ionization energy, 70 eV; ion source temperature, 250°C; interface temperature, 250°C; scan speed, 2 scans s^{-1}; mass range, 34-600 u

HPLC analysis was performed using a Shimadzu-VP system (Shimadzu, 's-Hertogenbosch, The Netherlands) consisting of a LC-10AT *vp* pump, a Kontron 360 auto sampler, a SPD-M10A *vp* DAD detector (200 – 340 nm, band with: 4 nm) , a FCV-10AL *vp* low pressure gradient mixer, a SCL-10A *vp* system controller, a FIAtron systems CH-30 column heater (USA), and CLASS-VP software, version 6.12SP4. ProFill 25 mm syringe HPLC filters, nature, PTFE, pore size 0.45 μm, part nr. AF223471-13208 (Alltech/Applied Science Group, Breda) were used, together with 2 mL autosampler vials, cat. nr. 151123; Brown Chromatography Supplies). As crimp seals, 11 mm with rubber/PTFE septa (cat. nr. 151216; Brown Chromatography Supplies) were used.

The column was a Luna C18(2), 250 x 4.6 mm, 5μm, 00G-4252-E0, nr.: 279423-28, together with a Phenomenex guard cartridge C18 (ODS, 4x3 mm), AJ0-4287. (Phenomenex, Bester, the Netherlands). The injection volume was 20 μL with a flow rate of 1 mL min^{-1} using a time program of 30 minutes consisting of 5 min 95% solvent A (5 mM ammonium formate (0.05% formic acid) : acetonitrile = 800 : 156 (w/w)) and 5% solvent B (acetonitrile : MeOH (0.05% formic acid) : 5 mM ammonium formate (0.05% formic acid) = 585 : 40 : 200 (w/w)), followed by a gradient to 100% solvent B in 19 min, 2 min 100% solvent B, a gradient back to 5 % solvent B in 2 min and remaining on this final concentration for another 2 min, based on a method described by Walsky and Obach (2004)

Analytical thin layer chromatography (TLC) was performed using silica gel 60-F254 (5 x 10 cm, 0.25 mm thickness, Merck, Darmstadt, Germany) and toluene: acetone (85:15) as the eluent. The elution length was 8 cm in a saturated chamber. The bands were detected by UV light at 254 nm.

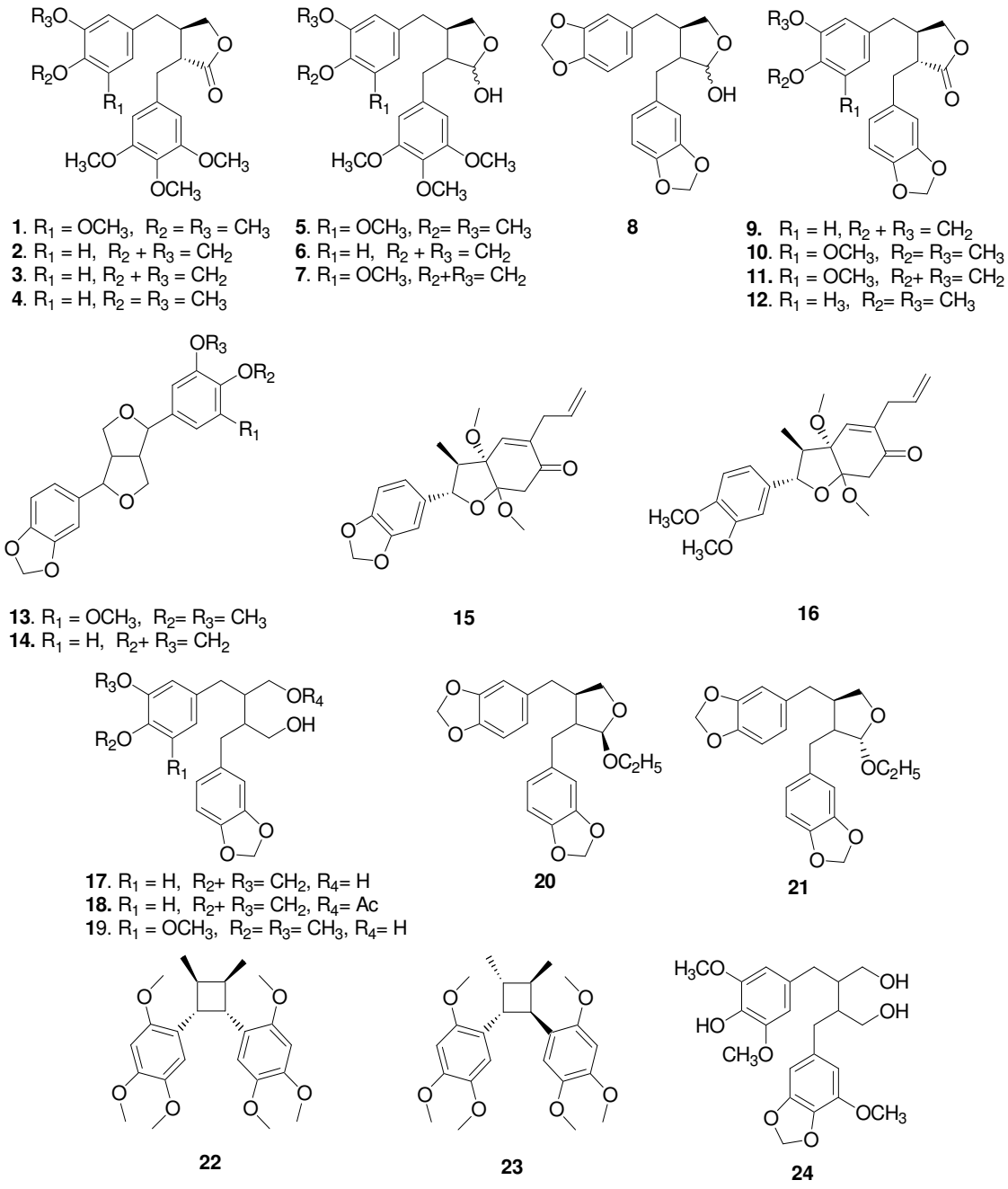

Fig. 1. Survey of lignans from *Piper cubeba* reported in the literature; cubebininolide (**1**)**,** cubebinone (**2**), yatein (**3**)**,** thujaplicatin trimethylether (**4**)**,** cubebinin (**5**), clusin (**6**), 5-methoxyclusin (**7**), cubebin (**8**), hinokinin (**9**), isoyatein (**10**), 5'-methoxyhinokinin (**11**), 2-(3",4"-methylenedioxy-benzyl)-3-(3',4'-dimethoxybenzyl) butyrolactone (**12**), aschantin (**13**), sesamin (**14**), kadsurin A (**15**), piperenone (**16**) dihydrocubebin (**17**), hemiarensin (**18**), dihydroclusin (**19**), β-O-ethylcubebin (**20**), α-O-ethylcubebin (**21**), heterotropan (**22**), magnosalin (**23**), 4-hydroxycubebinone (**24**).

Results and discussion

The extraction methods applied and the further isolation conditions assured that most lignans were exhaustively extracted from the plant material. The simple isolation of lignans using preparative TLC provided satisfactory results. On the chromatogram eleven bands from the leaves extract and seven bands from the fruit extract, all clearly separated, corresponded to lignans. The major lignans could be isolated up to 95% pure, although some lignans co-existed with others in the same bands. The isolated

lignans were used to identify lignan peaks in the GC, GC-MS and HPLC chromatograms. Mass spectra of the analyzed lignans were compared to earlier published data (see Table 2). Different parts of *P. cubeba* (leaves, berries, stalks) showed different lignan profiles (see Table 2) and identification took place based on comparison of the mass fragmentation patterns. Thirteen lignans were detected in the berries, fifteen in the leaves and only five lignans in the stalks. The stereochemsitry of these compounds surveyed in Fig. 1 was taken from the literature. Mass spectral data can not be used to determine the stereochemistry of the isolated compounds.

The structures of the lignans found in *P. cubeba* show a broad variation. All types of lignan structures that are commonly distinguished could be identified. Furanofuran lignans such as cubebin, hinokinin, yatein, isoyatein that are common lignans found in the genus *Piper* also appeared as major lignans in all parts of *P. cubeba*. Neolignans with an unusual structure such as kadsurin A and piperenone that have also been reported from *P. cubeba* (Koul et al., 1996) could not be identified in our material. Yatein was the most abundant lignan found in the berries at concentrations 2.0 times higher than hinokinin and 1.3 times higher than cubebin. Hinokinin was the most abundant lignan in the leaves and the seeds (see Table 1).

For the quantitative determination of cubebin, hinokinin and yatein, calibration curves were prepared using MeOH solutions of the isolated lignans at concentrations ranging from 2.5 to 25 µg mL^{-1}. Each concentration was prepared in triplicate. Regression equations were y = 123516 x – 218 (R^2 = 0.9885), y = 117773x - 6397.4 (R^2 = 0.997) and y = 111833x - 558.67 (R^2 = 0.995) for cubebin, hinokinin and yatein, respectively. The limit of detection (LOD) was established as the amount of analyte that provided a signal-to-noise ratio of 3. LOD were 0.1 µg for cubebin, and 0.2 for hinokinin and 0.25 for yatein. 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 0.3 µg for cubebin, 0.9 µg for hinokinin and 0.1 µg for yatein

So far, phytochemical and pharmacological studies have only been done with the berries of *P. cubeba* that are used in traditional medicine, including *jamu*. Based on the present inventory of lignans in the aerial parts of the plant we conclude that in addition to the berries also the leaves may be used for medical purposes. This knowledge can be used for the further development of (rationally designed) phytomedicines from *P. cubeba*. It might be attractive from an economical point of view to harvest the leaves earlier, in a stage in which the plant has not yet developed flowers and later berries. This may be of economical benefit. It should however be taken into account that the concentrations of the major lignans in the leaves are considerably lower than in the berries.

Table 1. Concentration of major lignans in *Piper cubeba* berries, leaves, and stalks (means from three independent determinations).

Compound	Concentration (mg DW^{-1})		
	Berries (± SD)	Leaves (± SD)	Stalks (± SD)
Yatein	23.9 ± 4.9	2.4 ± 0.1	0.2 ± 0.0
Hinokinin	18.9 ± 5.6	3.7 ± 0.4	0.6 ± 0.0
Cubebin	12.3 ± 1.9	2.8 ± 0.3	0.4 ± 0.0

Table 2. Occurrence of lignan in *Piper cubeba* berries, leaves, and stalks.

Compound (nr. compare Fig. 1)	Mass spectrum *m/z* (rel. int.)*	Plant part			Reference
		Berry	Leaf	Stalk	
Ashantin (**13**)	400 (27), 370 (4), 219 (8), 182 (51), 181 (100), 167 (10), 151 (15), 135 (67)	+	+	nd**	Parmar et al., 1997
Clusin (**6**)	402 (3), 358 (5), 248 (2), 182 (14), 181 (9), 136 (30), 135 (15)	+	+	nd	Prabhu et al., 1985
Cubebin (**8**)	356 (13), 203 (10), 161 (11), 136 (51), 135 (100), 131 (15)	+	+	+	Prabhu et al., 1985
Cubebinin (**5**)	448 (41.5), 430 (24.5), 249 (32), 182 (13.6), 181 (100)	+	nd	nd	Prabhu et al., 1985
Cubebininolide (**1**)	446 (64), 265 (4.5), 238 (1.5), 223 (3.6), 219 (3), 182 (60), 181 (100)	+	+	+	Badheka et al., 1986
Cubebinone (**2**)	430 (24), 249 (2), 248 (3), 235 (2), 222 (2), 207 (12), 203 (3), 194 (3), 182 (49), 181 (100), 166 (51), 165 (70)	+	+	nd	Badheka et al., 1986
Dihydroclusin (**19**)	404 (41), 386 (10), 250 (2.7), 225 (3.6), 182 (100), 181 (98), 136 (16), 135 (56)	nd	nd	nd	Prabhu et al., 1985
Dihydrocubebin (**17**)	358 (9), 204 (7), 187 (7), 136 (29), 135 (100)	nd	+	nd	Prabhu et al., 1985
α-*O*-Ethylcubebin (**21**)	384 (14), 339 (7), 338 (21), 203 (21), 173 (10), 161 (41), 148 (12), 136 (28), 135 (6)	+	+	nd	Badheka et al., 1987
β-*O*-Ethylcubebin (**20**)	384 (16), 339 (7), 338 (31), 203 (54), 173 (22), 162 (8), 161 (12), 145 (12), 136 (23), 135 (100)	+	+	nd	Badheka et al., 1987
Hemiarensin (**18**)	400 (50), 382 (1), 340 (7), 322 (3), 204 (15), 192 (9), 187 (25), 179 (6), 161 (12), 136 (33), 135 (100)	nd	+	nd	Badheka et al., 1987
Heterotropan (**22**)	na***	nd	nd	nd	Badheka et al., 1987
4-Hydroxycubebinone (**24**)	na	nd	nd	nd	Usia et al., 2005
Hinokinin (**9**)	354 (13), 284 (7), 162 (7), 136 (36), 135 (100),	+	+	+	Badheka et al., 1987
Isoyatein (**10**)	400 (71), 219 (2), 207 (4), 206 (4), 194 (1), 192 (1), 181 (100), 136 (17), 135 (94)	+	+	+	Badheka et al., 1986
Kadusrin A (**15**)	372 (3), 210 (82), 179 (28), 165 (15), 162 (50), 151 (100), 138 (11), 135 (13)	nd	nd	nd	Koul et al., 1996
Magnosalin (**23**)	na	nd	nd	nd	Badheka et al., 1987
5'-Methoxyhinokinin (**11**)	384 (56), 249 (2), 225 (6), 222 (4), 166 (59), 165 (100), 136 (21), 135 (53)	+	+	nd	Badheka et al., 1987
5-Methoxyclusin (**7**)	na	nd	nd	nd	Usia et al., 2005
2-(3',4'-Methylenedioxybenzyl) -3-(3',4'-dimethoxybenzyl)-butyrolactone (**12**)	370 (8), 235 (3), 182 (10), 15 (19), 151 (35), 135 (100)	+	+	nd	Badheka et al. 1986
Piperenone (**16**)	388 (100), 347 (40), 210 (6), 179 (12), 178 (72), 166 (8), 152 (8), 151 (67), 138 (9)	nd	nd	nd	Koul et al., 1996
Sesamin (**14**)	na	nd	nd	nd	Parmar et al., 1985
Di-*O*-methyl thujaplicatin methylether (**4**)	400 (88), 265 (3), 264 (2), 251 (5), 238 (3), 219 (2), 182 (38), 181 (100), 177 (11), 167 (8), 165 (3), 152 (16), 152 (41)	+	+	nd	Badheka et al., 1986
Yatein (**3**)	400 (26), 182 (100), 181 (77), 151 (31), 135 (88)	+	+	+	Badheka et al., 1986

* Mass spectra data are taken from the literature and compared to the fragmentation patterns of thelignans found in our study

**nd = not detected

***na = not available

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