Combination of capillary GC, GC/MS and ¹³C-NMR for the characterization of the rhizome oil of *Piper betle* L. (Piperaceae) from Vietnam

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Abstract. The essential oil from the rhizomes of *Piper betle* L. (betel), collected around Hue, was obtained in 0.20% yield. The oil was examined by a combination of capillary GC and GC/MS. ¹³C-NMR studies confirmed the structure assignments proposed by retention data and mass spectra of the components with a content higher than 1%. In some instances the structure elucidation based on GC and GC/MS data had to be corrected. More than forty constituents were found of which the major ones were α -cadinol (26.2%), δ -cadinene (11.7%), and about equal amounts of T-cadinol and T-muurolol (unseparated, together 20.7%). This study clearly illustrates the advantage of complementary identification techniques.

1. Introduction

Piper betle L. (betel, local name 'trau let'), Piperaceae, is widespread in damp forests and is cultivated in Vietnam and other countries in South-East Asia, such as India and China, and also in Central and South America and Africa. The flowering time in Vietnam is between May and August.

A concoction of indigenous Indian drugs containing *P. betle* dry extract was found to be an effective long-lasting oral contraceptive [1]. The flowers of this plant are used as ingredient for the chewing

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food known as betel quid in South-East Asia [2]. Mouthwashes and tablets containing pulverized betel nuts were used for the treatment of dental and periodontal diseases [3]. Betel leaves were reported to have high antioxidant effects [4,5]. The leaves possess antibacterial properties and are beneficial in the treatment of purulent parodontosis in the form of a collutory made of the juice or extract. A poultice of the leaves and a wash with the decoction are used in treating wounds, burns, impetigo, furunculosis, eczema and lymphangitis. The leaves if topically applied to the chest cure cough and asthma and if applied to the breast arrest lactation. Friction of the spinal column with the leaves is recommended for treating colds. The roots (8 to 12 g) are used in treating rheumatism [6]. The essential oil of *P. betle* showed hypotensive, cardiac and respiratory depressant effects [7]. Eugenol was identified as the antifungal principle in the oil [8].

The betel leaves, in vernacular pan, are used as leaf morsel by people in the Indian subcontinent. It is believed that more than 100 types are cultivated on a commercial scale in India. On the basis of flavor characteristics of the essential oil the existing types are grouped into five distinguishable cultivars, viz. 'Bangla', 'Desawari', 'Kapoori', 'Meetha' and 'Sanchi'. The distinction in flavor is marked by the varying concentration of mono- and sesquiterpenoids [9].

The chemical composition of the essential oils from various *Piper betle* species has been reported [10–19]. M.L. Sharma et al. have identified terpinyl acetate (22%), eugenol (16%) and 1,8-cineole (6%) as major components of the Kapoori leaf oil [11]. The same authors found eugenol (82–90%) and methyleugenol (4–7%) as major components of Bangla leaf oils, in addition to p-cymene, α -terpineol, terpinyl acetate and caryophyllene [12]. α -Pinene, sabinene, 1,8-cineole, p-cymene, γ -terpinene, γ -cadinene, methylchavicol and terpinyl acetate were detected as major constituents of Desawari leaf oils [13].

A.K.S. Rawat et al. found anethole (32%), eugenol (19%) and terpinyl acetate (16%) to constitute the major part of the Meetha betel leaf oil [14]. In a subsequent paper these authors reported that eugenol is the major and common compound among the leaf oils of all cultivars, being dominant in Bangla (64%) [15,16]. Desawari and Meetha leaf oils contained respectively mainly 5-(2-propenyl)-1,3-benzodioxole (45%) and anethole (19%). The leaf oil from the Kapoori cultivar was marked by a larger number of constituents such as α -thujene, (E)- β -ocimene, terpinolene, allo-ocimene, δ cadinene, terpinen-1-ol, α -costol, δ -cadinol, methyl-2-hexadecan-1-ol, geraniol, hexadecanoic acid, methyl benzoate, etc. The Sanchi leaf oil was marked by the presence of stearaldehyde which was absent in other cultivars [15,16].

Phillipine *P. betle* leaf oil contained mainly chavibetol (53%) and its acetate (16%), and caryophyllene (4%). Minor components included chavibetol methyl ether, eugenol, α - and β -pinene, limonene, safrole, 1,8-cineole, p-cymene and allylpyrocatechol monoacetate [17]. The leaf oil of Malaysian *P. betle* also contained mainly chavibetol (69%) [18]. In the *P. betle* leaf oil from Kampuchea over fifty components were found, of which α -pinene (7%), sabinene (8%), 1,8-cineole (10%), methylchavicol (9%) and terpinyl acetate (19%) were the main components [19].

The essential oil of Taiwanese *P. betle* flowers contained mainly safrole (28%) and myrcene (26%), as measured by GC/MS. HPLC and TLC analysis of that oil showed safrole, hydroxychavicol, eugenol, isoeugenol and eugenol methyl ether as major phenolic compounds [2].

Apart from these studies on the leaf and flower oils, no other plant parts of betel cultivars have been investigated thus far. In the present paper, the results of a study on the characterization of the rhizome oil of *Piper betle* L. from Vietnam by a combination of capillary GC, GC/MS and ¹³C-NMR is discussed.

2. Experimental

2.1. Plant material

The rhizomes studied were collected from plants grown in Huê, Vietnam. A voucher specimen has been deposited at the Herbarium of the Huê University. The essential oil was obtained from the fresh rhizomes by steam distillation for 4 h. as previously described [20]. The species produced a colorless rhizome oil ($n_D = 1.4935$) with a pleasant odor in 0.20% yield (based on fresh weight).

2.2. Compositional analysis

A Hewlett-Packard 5890 Series II Chromatograph equipped with a FID detector and HP-1 or HP-2 fused silica columns (25 m \times 0.32 mm, 0.25 μ m film thickness) was used. The samples, dissolved in hexane, were injected in the splitless mode into helium carrier gas. Injector and detector temperatures were maintained at 250°C. The column temperature was programmed from 60°C (after 2 min) to 220°C at 4°C/min, and the final temperature was held for 20 min. Peak areas and retention times were measured by electronic integration or by computer. The relative amounts of individual components are based on the peak areas obtained, without FID response factor correction.

GC/MS analyses were carried out on a Hewlett-Packard 5970A mass selective detector (MSD), directly coupled to a HP 5790A gas chromatograph. A 26 m \times 0.22 mm column, coated with 0.13 μ m of CP-Sil 5CB was employed, using helium carrier gas. The oven temperature program was 60°C (3 min), then 5°C/min to 250°C (30 min). Other conditions were the same as described under GC. Electron Ionization (EI) mass spectra were acquired over a mass range of 10–400 Da at a rate of 2/s.

The carbon-13 NMR spectrum of the whole oil was recorded on a Bruker AC 200 Fourier Transform spectrometer operating at 50.323 MHz, in deuterated chloroform, with all shifts referred to internal tetramethylsilane and with the following parameters: pulse width 3.0 μ s (flip angle 45°), acquisition time 1.3 s for a 32 K data table with a spectral width of 12,500 Hz (250 ppm), CPD mode decoupling, digital resolution 0.763 Hz/pt. The number of accumulated scans was 10,000 (22 mg of the oil in 0.5 ml CDCl₃).

The constituents of the oil were identified by matching their 70 eV EI mass spectra and GC retention indices with reference libraries [21–29]. Computer aided analysis of the carbon-13 NMR spectrum of the oil by a custom-made experimental procedure [30] enabled the unambiguous identification of the major individual constituents. A data bank containing around 350 carbon-13 NMR spectra of pure mono-, sesqui- and diterpenes was used.

3. Results and discussion

Table 1 reports the composition of the rhizome oil of *Piper betle* L. from Huê. More than forty compounds have been identified in this oil, which was characterized by the presence of a high content of δ -cadinene (11.7%), α -cadinol (26.2%), and unseparated T-cadinol plus T-muurolol (20.7%). All other constituents amounted to less than 5% each. Predominant minor constituents were camphene, α -copaene, β -caryophyllene, γ -muurolene, germacrene D, α -muurolene, γ -cadinene, (E)-nerolidol, epicubenol and δ -cadinol.

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Compound	Retention index ^{a)}	Percentage ^{b)}	Identification methods
α -pinene	928	0.7	GC. MS
camphene	940	1.8	GC. MS. NMR
sabinene	962	0.1	GC MS
β -ninene	967	0.5	GC MS
myrcene	982	0.2	GC MS
o-terpinene	1008	tr	GC MS
n cymana	1010	tr	GC MS
p-cymene 1.8 sincele	1010	u 0.0	CC MS
limonono	1010	0.9	CC MS
	1019	0.1	GC, MS
γ -terpinene	1047	0.2	GC, MS
terpinolene	10//	tr	GC, MS
linalool	1084	tr	GC, MS
camphene hydrate	1127	0.2	GC, MS
borneol	1144	0.1	GC, MS
terpinen-4-ol	1158	0.6	GC, MS
α -terpineol	1169	0.3	GC, MS
safrole	1259	tr	GC, MS
bornyl acetate	1265	tr	GC, MS
p-eugenol	1327	tr	GC, MS
δ-elemene	1330	tr	GC, MS
o-eugenol	1339	0.9	GC, MS
α -cubebene	1344	0.4	GC, MS
α -copaene	1370	1.7	GC. MS. NMR
β -bourbonene	1377	tr	GC. MS
β -elemene	1384	0.3	GC. MS
β -carvophyllene	1411	16	GC MS
α -humulene	1444	0.6	GC MS
allo-aromadendrene	1451	tr	GC MS
	1466	23	GC MS NMP
germacrene D	1470	2.5	GC MS NMR
	1470	4.0	CC MS
a-induforene	1409	2.7	CC MS NMP
y-cadifiene	1505	5.5	CC MS
cis-calamenene	1505	0.8	GC, MS
o-cadinene	1510	11./	GC, MS, NMK
cubenene	1520	0.6	GC, MS
α -cadinene	1525	0.7	GC, MS
(E)-nerolidol	1547	1.7	GC, MS, NMR
ledol	1585	0.8	GC, MS
epicubenol	1609	4.7	NMR
T-cadinol + T-muurolol	1621	20.7	NMR
δ -cadinol	1623	3.5	GC, MS
α -cadinol	1632	26.2	GC, MS, NMR
cadalene	1650	0.4	GC, MS
other compouds		3.5	

 Table 1

 Chemical composition of the rhizome oil of *Piper betle* L. from Vietnam

^{a)} Linear retention index relative to n-alkanes on HP-1.

^{b)} Area percent; tr = trace = < 0.1%.

In order to confirm the structure of the main compounds, this oil was studied by ¹³C-NMR. The chemical shift of each carbon in the experimental spectrum was compared with those of the spectra of pure compounds in the laboratory spectral data bank containing around 350 spectra, and with literature data, with the help of laboratory-made software. The spectral parameters (concentration, low power decoupling, temperature) were optimized to provide good accuracy of the chemical shift values. Each

compound was unambiguously identified, taking into account i) the number of identified carbons, ii) the number of overlapping signals, and iii) the difference in chemical shift of each resonance in the mixture and in the reference spectrum. This method allows for precise identification of major terpenes present in the oil. Structurally, closely related molecules such as stereo-isomers, which exhibit insufficiently resolved mass spectral patterns [30–32], and compounds inseparable by GC can be identified in this way. The method is very well suited for chemical polymorphism studies [33,34].

Using the procedure outlined above, the presence of the following compounds was confirmed by carbon-13 NMR: camphene, α -copaene, γ -muurolene, germacrene D, γ -cadinene, δ -cadinene, (E)-nerolidol, epicubenol, T-cadinol, T-muurolol and α -cadinol. It appeared that epicubenol was wrongly identified (as cubenol) by means of GC retention indices and mass spectra, while γ -muurolene was overlooked by GC/MS. Worse, however, was the false identification of T-muurolol and T-cadinol, which could not be separated on HP-1 and HP-2 columns, as δ -cadinol. Carbon-13 NMR clearly revealed the presence of T-muurolol and T-cadinol in amounts of about 10% each, but could not verify the presence of δ -cadinol, the spectrum of which was missing in the data bank. At lower temperature programming rates (3°C/min) the respective GC peak splitted into two peaks, the first representing still unseparated T-cadinol plus T-muurolol and the second could unequivocally be assigned to δ -cadinol by GC/MS.

4. Conclusion

This study shows that analysis of this oil is quite dificult, particularly by the presence of many sesquiterpenes, among which several substituted octahydro-naphthalenol isomers. This is a good example to demonstrate the complementarity of capillary GC, GC/MS and NMR.

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