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Chemical Constituents from Leaves and Cell Cultures of *Pogostemon cablin* and Use of Precursor Feeding to Improve Patchouli Alcohol Level

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ABSTRACT: The study on the chemical constituents of an essential oil of *Pogostemon cablin* was carried out by hydrodistillation of leaf explants and the oil analysed by Gas Chromatography Mass Spectrometry (GC/MS). The oil yield was found to be 0.30 % (v/w) of fresh weight. Twenty two compounds were identified by GC/MS as eighteen sesquiterpenes and three oxygenated sesquiterpenes. Among these, patchouli alcohol (60.30 %) was the major component, followed by germacrene A (11.73 %). In order to study the chemical constituents of the essential oil of plant cell cultures, leaves were surface sterilised and callus cultures initiated on MS media containing naphthaleneacetic acid (0.5 mg/l), and benzyladenine (1 mg/l), followed by incubation in suitable culture conditions. Cell suspension cultures were initiated by subculturing callus cultures into new liquid media and maintained in the same conditions. Chemical constituents of the essential oils produced by both callus and cell suspension cultures were extracted with dichloromethane and analysed by GC and GC/MS. The results showed that essential oil obtained from these cultures contained the same major constituents, namely patchouli alcohol, as in the intact plant, but the level was low, and also contained a small amount of minor constituents. Feeding cis-farnesol, the precursor of patchouli alcohol, to cell suspension cultures resulted in the patchouli alcohol being increased from 19.5 mg/l to 25.5 mg/l.

KEYWORDS: *Pogostemon cablin*, chemical constituents, patchouli alcohol, cis-farnesol, precursor feeding.

INTRODUCTION

Pogostemon cablin (Blanco) Benth. (Syn. *Pogostemon patchouli* Hook.) belongs to the family Lamiaceae and is commonly known as patchouli. It is native to subtropical Himalayas, Southeast Asia and the Far East, and has been cultivated extensively in Indonesia, Malaysia, China, and Brazil for the essential oil namely "patchouli oil".[1] This oil has been used in the food industry and as fixative in perfumery raw material.[2] It also has therapeutic properties, namely antidepressant, anti-inflammatory, antiseptic, aphrodisiac, astringent, carminative, diuretic, febrifuge, fungicide, insecticide, sedative and tonic. The chemical constituents of patchouli oil are almost entirely composed of sesquiterpenes with patchouli alcohol (Fig. 1) being the most abundant.[2,3] This compound is biosynthesised by cyclisation of cis-farnesyl pyrophosphate,[4] and synthesised by several steps.[5] Some researches on cell cultures of *P. cablin* [6-10] have been reported, but the chemical constituents from these cultures have not been investigated. The purpose of this study was to identify chemical constituents from *P. cablin* intact plant and cell cultures, and improve the yield of patchouli alcohol by precursor feeding.

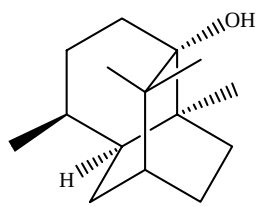

Fig 1. The chemical structure of patchouli alcohol.

MATERIALS AND METHODS

Plant Materials

Leaves of *Pogostemon cablin* (Blanco) Benth. were collected from the plant garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University,

Bangkok, Thailand. These explants were identified and authenticated by comparison with the herbarium specimen (SN 030264) at the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Hydrodistillation of Leaves of *Pogostemon cablin*

Fresh leaves of *P. cablin* were hydrodistilled by a Clevenger-type apparatus. The essential oil was collected and stored at 4 °C until being analysed for its chemical constituents by Gas Chromatography/Mass Spectrometry (GC/MS).

Plant Cell Cultures

Leaf explants of *P. cablin* were surface sterilised by shaking in 5% Clorox® for 5 min and then washed three times with redistilled water. Sterilised explants were then callus initiated by placing on MS[11] basal medium which was made with 3% (w/v) sucrose, 0.5% (w/v) ascorbic acid, 0.5 (mg/l) naphthaleneacetic acid, 1 (mg/l) benzyladenine, solidified with agar (1%), and adjusted to pH 5.75 before autoclaving at 121 °C for 15 min. Callus cultures were subcultured every 21-28 days. The suspension cultures initiated from the fourth generation of callus were maintained in the same media as callus (except without the agar) under continuous fluorescent light at 25±2 °C on an orbital shaker (100 rpm). Suspension cultures were subcultured every 21 days by transferring 10 ml of the old suspension cultures to 90 ml of new media.

Precursor Feeding

cis-Farnesol (purity over 98%) was selected to be a precursor in the feeding experiment in order to improve the yield of patchouli alcohol. It was mixed with 70% ethanol and added to the suspension cultures by passing through a preautoclaved Mobile Phase Filter (0.45 μm pore size, Whatman). The control reading experiments were made without substrate added to cultures, and media added substrates without cell cultures. The cultures were incubated under the same conditions as above.

Extraction

Cell suspension cultures were taken aseptically and extracted with redistilled dichloromethane and centrifuged 76.9 mg/l n-Pentadecane was added prior to extraction as an internal standard. The dichloromethane layer was evaporated under N_2 to 100 μl prior to GC analysis.

GC Analysis

A Hewlett Packard 5890A with FID detector was used for GC analysis. The column was a ZB-5 capillary fused silica column (25 m X 0.32 mm i.d., 0.25 mm film thickness); oven temperature programming was 50-250 °C, at 7 °C/min; injector and detector temperatures were 250 °C and 280 °C, respectively; injection volume was 1 *m*l; split ratio was 1:9; and carrier gas was N_2 at 20 psi.

GC/MS Analysis

A Matt 95 spectrometer equipped with Sun Mash-3 computer data output was used for GC/MS analysis. The operating conditions were similar to those used in GC analysis, except with a HP-5MS column and helium as carrier gas. MS conditions were as follows: ionisation current, 1A; ionisation potential, 70 eV; source temperature 150 °C; resolution, 1000; and scan speed, 1 sec/decade.

Compound Identification

Compounds were identified by comparing the Kovats GC retention indices of the peaks on the HP-5MS column with literature values, computer matching using the Masslynx database, and comparison of the fragmentation patterns of the mass spectra with those reported in the literature.[12,13] The Kovats index of patchouli alcohol was 1659 and an MS fragmentation pattern in the order of decreasing m/z value was (222 [M^+], 205, 189, 161, 138, 125, 109, 95, 81, 67, 55, 41). Identification of compounds was also checked by using the standard patchouli alcohol. The concentration of patchouli alcohol was calculated with respect to the internal standard.

Results

Chemical Constituents Obtained from Hydrodistillation of *Pogostemon cablin* Leaves

The oil yield was found to be 0.30 % (v/w) of fresh weight. Twenty two compounds were identified by GC/MS as eighteen sesquiterpenes, and three oxygenated sesquiterpenes. Among of these, patchouli alcohol (60.30 %) was the major component, followed by germacrene A (11.73 %). The list of essential oil compositions is shown in Table 1.

Identification the Chemical Constituents in *P. cablin* Cell Cultures by GC/MS

Chemical constituents produced by callus and cell suspension cultures, were extracted and analysed by the methods described above. The results revealed that patchouli alcohol, the major constituents of this essential oil, could be produced in both callus and cell suspension cultures. Yields of patchouli alcohol in callus and cell suspension cultures were 19.9 mg/l and 19.5 mg/l, respectively. However, some minor constituents such as seychellene, α-patchoulene, β-patchoulene, γ-patchoulene, and δ-patchoulene could

Table 1. Chemical constituents obtained from *P. cablin* leaves by hydrodistillation.

Compound	Kovat's Index	% Area
Sesquiterpenes		
δ-elemene	1339	t
β-patchoulene	1380	t
β-elemene	1391	0.33
cis-thujopsene	1429	0.25
trans-caryophyllene	1418	2.24
α-guaiene	1439	7.22
γ-patchoulene	1441	3.89
α-humulene	1454	0.48
α-patchoulene	1456	2.27
seychellene	1460	0.98
valencene	1491	0.85
germacrene D	1480	0.15
β-selinene	1485	t
α-selinene	1494	0.23
viridiflorene	1493	1.91
germacrene A	1503	11.73
α-bulnesene	1505	0.86
7-epi-α-selinene	1517	0.17
Oxygenated sesquiterpenes		
longipinanol	1566	t
globulol	1583	4.62
patchouli alcohol	1659	60.30
Others		
1-octen-3-ol	0978	0.20

t = trace (less than 0.01).

also be found in trace amounts.

Time-Course Studies of Patchouli Alcohol Content During the Growth Cycle of *P. cablin* Cell Cultures

The time-course of patchouli alcohol content during the growth cycle was studied in order to determine the best time to harvest cells. Levels of patchouli alcohol and the growth rate of callus and suspension cultures were determined as shown in Tables 2 and 3 and Figs. 2 and 3.

In callus cultures of *P. cablin*, patchouli alcohol reached the maximum concentration (19.9 mg/l) at day 35 of the growth cycle, while in cell suspension cultures, it reached the maximum concentration (19.5 mg/l) at day 21 of the growth cycle.

Table 2. Fresh weight, dry weight and patchouli alcohol content in *P. cablin* callus cultures (SD<5%, n=3).

Day	Fresh weight (FW) (g)	Dry weight (DW) (g)	Patchouli alcohol content (mg/l)
0	2.8	0.31	0.65
7	2.9	0.34	2.15
14	3.2	0.37	5.5
21	4.1	0.54	12.55
28	7.5	1.05	14.8
35	8.5	1.1	19.85
42	8.9	1.15	16.75

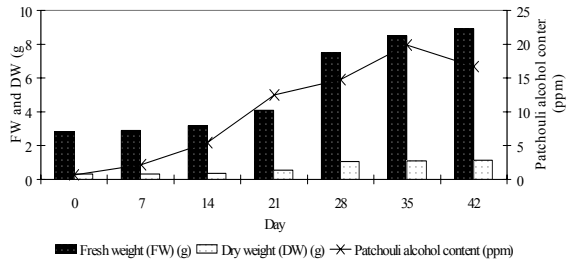

Fig 2. Time-course of growth and the formation of patchouli alcohol in *P. cablin* callus cultures (SD<5%, n=3).

Table 3. Fresh weight, dry weight and patchouli alcohol content in *P. cablin* cell suspension cultures (SD<5%, n=3).

Day	Fresh weight (FW) (g)	Dry weight (DW) (g)	Patchouli alcohol content (mg/l)
0	1.12	0.14	0.85
7	2.47	0.37	1.35
14	3.89	0.58	4.5
21	6.62	1.19	19.5
28	7.74	1.39	15.55

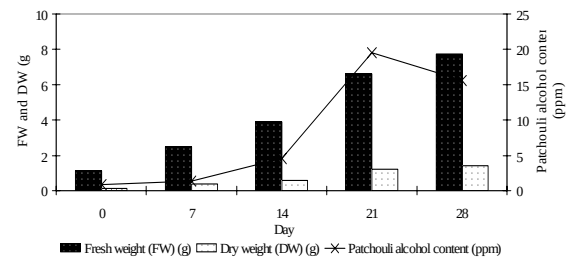

Fig 3. Time-course of growth and the formation of patchouli alcohol in *P. cablin* cell suspension cultures (SD<5%, n=3).

Feeding Precursors of Patchouli Alcohol to Cell Suspension Cultures of *P. cablin* to Increase Yield of Patchouli alcohol

cis-Farnesol (100 mg/l), was fed into cell suspension cultures at day 20 of the stationary phase. Cells were harvested at day 21 for the maximum yield of patchouli alcohol. The level of patchouli alcohol was increased from 19.5 mg/l to 25.5 mg/l.

Discussion

The chemical constituents of the essential oil, obtained from hydrodistillation of *P. cablin* leaves and

identified by GC/MS, were found to be twenty-two sesquiterpenoids, of which patchouli alcohol was the major constituent. This compound had shown interesting pharmacological activities, such as antifungal[14], antibacterial[14], anti-emetic[15] activities, and IL-4 production inhibition.[16] Cell cultures of *P. cablin* were established and their chemical constituents were analysed. Patchouli alcohol could be detected as the major compound in both callus and cell suspension cultures. Its yield in callus and cell suspension cultures was 19.9 mg/l and 19.5 mg/l, respectively. Some minor constituents such as seychellene, α-patchoulene, β-patchoulene, γ-patchoulene, and δ-patchoulene could be also found in trace amounts in the cell extracts. Attempts were made to increase the yield of patchouli alcohol in cell cultures of *P. cablin*, by using precursor feeding in the early part of the stationary phase of growth, and cells were harvested when yield was at the maximum concentration. Biotransformation using an exogenous supply of biosynthetic precursors may improve the accumulation of secondary metabolites.[17-19] Terpenoids are cytotoxic to plant cells, causing a decrease in cell membrane permeability and inhibiting respiration and photosynthesis. Thus the excessive concentration of cis-farnesol may cause cell death. The appropriate concentration of cis-farnesol fed into these cells was 100 mg/l. It did not effect cell growth and remained in the cell cultures until the end of experiment. This is the first report on the yield of patchouli alcohol produced by callus and cell suspension cultures of *P. cablin*, which was successfully improved after feeding cis-farnesol into cell cultures.

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