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Development of Patchouli Extraction with Quality Control and Isolation of Active Compounds with Antibacterial Activity

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ABSTRACT

Patchouli (*Pogostemon cablin*), is a shrub with fragrant-smelling leaves from which patchouli oil is extracted. The oil has properties suitable for aromatherapy, relief of stress and anti-inflammatory activity. Water-steam distillation is the best method for extraction of patchouli oil. Fresh patchouli leaves gave much lower yield than dried leaves. Dried leaves fermented for 77 days at room temperature produced the highest yield (2.48% dry wt), which was much higher than from drying in an oven at 50°C for 40 h (0.30% dry wt). Soxhlet extraction of dried patchouli leaves with hexane for 15 h provided crude hexane extract (4.97% dry wt). The hexane extract was further separated and purified to obtain patchouli alcohol (0.05% dry wt), a mixture of β-sitosterol and stigmasterol (0.09% dry wt) and 7,3′,4-tri-*O*-methyleriodictyol (0.04% dry wt). Antibacterial activity assay showed that patchouli oil could inhibit *Staphylococcus aureus* and *Bacillus subtilis* better than the hexane extract. Patchouli alcohol, a major component in patchouli oil, and the extract showed higher antibacterial activity than the mixture of β-sitosterol and stigmasterol and 7,3′,4-tri-*O*-methyleriodictyol. Therefore, patchouli alcohol could be used as a marker for quality control of patchouli oils and the extracts. Quantitative determination of patchouli alcohol in the patchoulil oils and the hexane extract was performed by GC-MS using the new optimum conditions. The results showed that patchouli oil contained more patchouli alcohol than the hexane extract. Time of leaf harvesting (3 months, 6 months and 9 months) was also important. Three-month harvesting of patchouli leaves gave the highest amount of patchouli oil and patchouli alcohol.

Key words: patchouli (*Pogostemon cablin*), water–steam distillation (hydrodistillation), patchouli alcohol (patchoulol), quantitative determination, gas chromatography–mass spectroscopy (GC-MS)

INTRODUCTION

Patchouli (*Pogostemon cablin*) is a bush herb in the Libiatae family. This plant originated in Southeast Asia. Patchouli leaves contain patchouli oil (essential oil) as the major constituent. It has been reported that the essential oil from patchouli consists of patchouli alcohol (patchoulol) as a major component and several other minor components such as caryophyllene,

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α-, β-, γ- and δ-patchoulene, pogostol, seychellene, cycloseychellene, α- and β-bulnessene, α- and δ-guaiene and norpatchoulenol (Akhila and Nigam, 1984; Akhila *et al*., 1988). In the genus *Pogostemon*, the other species (*P. horthensis, P. hyneanus and P. plectranthoide*) also yielded patchouli oil but in lesser amounts than *P.cablin*.

Patchouli oil is used for the reduction of stress without any allergic reaction, stimulation of the nervous system to a normal condition and relief of stress. Moreover, it also possesses anti-inflammatory activity. The fresh leaves are very effective in healing burns, calming nerves, controlling appetite, relieving depression and improving sexual interest. In Thailand, patchouli oil is used in perfume formulation as a fixative and in drugs formulation and aromatherapy.

The extensive cultivation of *Pogostemon cablin* is practiced in Indonesia, Malaysia, China and Brazil. The aerial part of this plant can be used as an antifungal agent in traditional medicine and has been used against the common cold (Ichikawa *et al*., 1989). Moreover, the patchouli oil is an important ingredient for the perfume industry. Therefore, research and development of the extraction of patchouli oil, isolation of pure active constituents and quality control are very important.

MATERIALS AND METHODS

General

Melting points were measured on a Fisher Johns Apparatus. ^{1}H NMR and ^{13}C NMR spectra, operating at 400 and 100 MHz, respectively, were determined on a VARIAN^{UNITY} INOVA 400 MHz spectrometer. MS data were obtained from GC-MS –QP-5050, MSD trap AGILENT 122-5532. Silica gel column chromatography exployed Kieselgel 60 (70-230 mesh) from Merk and Fluka. Kieselgel 60, F_{254} (230-400 mesh) from Merck was used for the TLC analysis. Visualization of compounds on TLC plates was achieved by viewing under 254 nm UV light (model UVGL-58) followed by spraying with vanillin reagent and heating for 5-10 min at 115°C.

Plant materials

Leaves of *Pogostemon cablin* were collected in June 2004 from Chacheongsao province, Thailand. A voucher specimen (Ngampong Kongkathip-1 (BK 64105)) was deposited at the Bangkok Herbarium, Department of Agriculture, Bangkok, Thailand.

Pogostemon cablin extraction

Two methods of extraction are available.

1. Water-steam distillation (hydro-distillation) of fresh leaves and dried leaves of *P. cablin*.

Fresh leaves (200 g) of *P. cablin* were finely ground and then extracted by water-steam distillation.

Dried leaves were prepared by two methods. In the first method, *P. cablin* leaves (995 g) were dried in the oven at 50°C for 40 h, finely ground and then extracted by water-steam distillation. In the second method, leaves were fermented for various periods (11 days, 34 days, 44 days and 77 days) at room temperature, finely ground and then extracted by water-steam distillation.

The effect of leaf harvesting time of *P. cablin* planted at 30×30 cm spacing was studied. *P. cablin* leaves were harvested after 3 months, 6 months and 9 months. Each sample (200 g leaves) was fermented at room temperature for 77 days, finely ground and then extracted by water-steam distillation.

2. Soxhlet extraction

Dried leaves (1.5 kg) of *P. cablin* were finely ground and then extracted with hexane (6 L) by Soxhlet extraction for 15 h. The hexane solution was evaporated to dryness under vacuum at 45°C using a rotary evaporator to produce the

crude hexane extract.

Isolation and purification of crude hexane extract

The crude hexane extract (45.0 g) was separated by vacuum liquid chromatography (VLC) on a silica gel column (270 g) using gradient elution with hexane, dichloromethane, ethyl acetate and lastly ethanol to give six fractions (fractions A1-A6) based on their TLC patterns. Fraction A2 (2.5 g) was further subjected to chromatography on a silica gel column (88 g) using gradient elution with hexane and ethyl acetate to afford five fractions (fractions B1 to B5). Fraction B4 showed one spot on the TLC chromatogram which was patchouli alcohol (24 mg, R_f = 0.42, 20% ethyl acetate in hexane). Fraction A5 (3.0 g) was subjected to chromatography on a silica gel column (150 g) using gradient elution with hexane and ethyl acetate to give five fractions (fractions C1 to C5). Fraction C2 after storing at room temperature overnight gave a white precipitate which was recrystallized from ethanol and identified as a mixture of β-sitosterol and stigmasterol (40 mg, R_f = 0.67, 30% ethyl acetate in hexane). After standing at room temperature for a while, fraction C4 provided a white crystal which was recrystallized from ethanol and identified as 7, 3′, 4-tri-*O*-methyleriodictyol (18 mg, R_f = 0.37, 40% ethyl acetate in hexane).

GC-MS analysis

A gas chromatography-mass spectroscopy (GC-MS) technique was used to analyze the chemical composition of patchouli oil and the crude hexane extract. Patchouli oil and the crude hexane extract were separated on a 30m × 0.25 mm (lx i.d.) capillary column coated with a 0.25 mm film of 5% phenyl methyl siloxane at a column temperature of 80°C for injection. Temperature programming started at 10°C min^{-1} to 150°C and then from 5°C min^{-1} to 250°C and finally from 10°C min^{-1} to 280°C and held for 5 min. Using splitless injection, (2 μL), helium was employed as the carrier gas with a flow rate of 1 mL/min. The spectrometer was operated in electron-impact (EI) mode, with electron energy of 70 eV and a scan range of 50- 550 amu. The inlet and ionization source temperatures were 240°C and 280°C, respectively.

Quantification of patchouli alcohol in patchouli oils and the crude hexane extract by GC-MS

Quantitative analysis was performed by GC-MS using external calibration. The relationship between the amount of patchouli alcohol and the peak area was evaluated by analyzing a series of standard solutions of patchouli alcohol in hexane (at various concentration 25, 50, 100, 200 and 400 ppm). The patchouli alcohol showed a single peak at the retention time of 30.59 ± 0.03 min. The calibration curve was prepared to determine the patchouli alcohol content in the crude samples. This was derived from three injections of five concentrations (25, 50, 100, 200 and 400 ppm) of patchouli alcohol versus their peak area responses. Linearity of the amount of patchouli alcohol in the dose-response curve was observed in this concentration range with the linear regression equation, y = 17190x-143879 and a very high coefficient of determination (R^2) value at 99.99% (Figure 1). The calibration range was chosen to calculate patchouli alcohol concentration in *P. cablin* samples.

Determination of patchouli alcohol in patchouli oils and the crude hexane extract

The sample volume was 2 μL. Patchouli alcohol concentration was calculated on the basis of the linear calibration function. The content of patchouli alcohol was expressed as grams per 100 g of dry weight.

Antibacterial activity assay

Patchouli oil, the crude hexane extract and pure compounds were tested for antibacterial

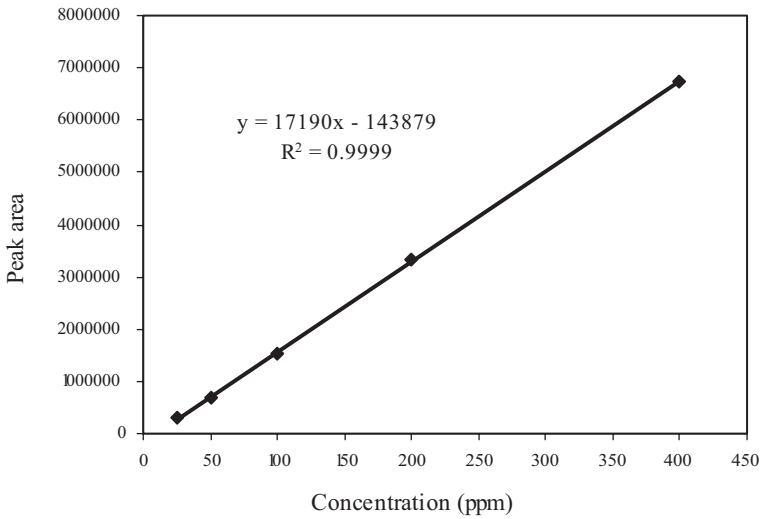

Figure 1 Calibration curve of patchouli alcohol.

activity by comparing minimal inhibitory concentrations (MIC). The MIC was evaluated by the broth microdilution method using 96-well microtitre plates (Deguerry *et al.*, 2006). The extracts were dissolved in DMSO at 1000 *m*g/mL. Twofold serial dilution of each extract was carried out. Each well was inoculated with 10^4 cfu of test bacteria (*Staphylococcus aureus,* ATCC 25923 and *Bacillus subtilis,* ATCC 6633) and incubated at 37°C for 18 h. MIC was defined as the lowest concentration of extract at which no turbidity or no bacterial growth occurred. Streptomycin and enrofloxacin were used as the control antimicrobial agents.

RESULTS AND DISCUSSION

Chemistry

Water-steam distillation of fresh patchouli leaves and dried leaves was studied. Dried patchouli leaves were obtained by two processes, firstly, oven drying at 50°C for 40 h and secondly, fermentation at room temperature for various periods. Fermentation of the leaves for 77 days produced the highest yield of patchouli oil (2.48% dry wt.) when compared with the non-fermented fresh leaves and those from the other fermentation periods. The yields of patchouli oils from water-steam distillation of all samples are shown in Table 1. Dried patchouli leaves were also extracted with hexane by Soxhlet extraction to

Table 1 Extraction of patchouli oils from fresh patchouli leaves and dried leaves.

Sample	Sample weight (g)	Amount of patchouli oil (g)	% Patchouli oil /dry wt. of sample
Fresh leaves	200	0.30	0.60
Leaves dried in the oven at 50°C	995	2.99	0.30
Fermented dried leaves for 11 days	400	7.682	1.92
Fermented dried leaves for 34 days	200	2.07	1.04
Fermented dried leaves for 44 days	200	4.19	2.10
Fermented dried leaves for 77 days	200	4.96	2.48

provide the crude hexane extract (4.97% dry wt). Patchouli alcohol (0.05% dry wt), the mixture of β-sitosterol and stigmasterol (0.09% dry wt) and 7,3′, 4-tri-*O*-methyleriodictyol (0.04% dry wt), were isolated from the crude hexane extract (Figure 2). The structures of all isolated components were identified by comparing mp, IR, ^{1}H-NMR, ^{13}C-NMR and MS spectral data with those reported in the literature (Nishiya *et al*., 1995; Alam *et al*., 1996; Miyazawa *et al*., 2000). In addition, GC-MS data revealed that patchouli oils and the crude hexane extract contained patchouli alcohol as the major component while the minor components were 4,7-methanoazulene, trans-caryophyllene, a-guaine, seychellene, azulene, α-patchoulene and pogostol (Hu *et al*., 2006).

Antibacterial activity

It was found that patchouli oil, which contained the highest amount of patchouli alcohol showed much better activity against *S. aureus* and *B. subtilis* than the crude hexane extract. Furthermore, patchouli alcohol showed better antibacterial activity against *Bacillus subtilis* than against *Staphylococcus aureus*, and was much better than the mixture of β-sitosterol and stigmasterol, and 7, 32 , 4-tri-*O*-methyleriodictyol (Table 2).

The above results showed that patchouli alcohol is the major component and most active compound in patchouli oil and the extract. Therefore, patchouli alcohol could be used as a marker for quality control. The combination of GC-MS and the new optimum conditions for extraction were used to determine the amount of patchouli alcohol in the samples as shown in Table 3.

Study on harvesting time

The leaves from the 30×30 cm planting were used to study the impact of harvesting periods of 3 months, 6 months and 9 months. The results showed that the sample harvested after 3 months (3.45% dry wt) gave a higher yield of patchouli oil than after 6 months (3.36% dry wt) and 9 months (2.38% dry wt).

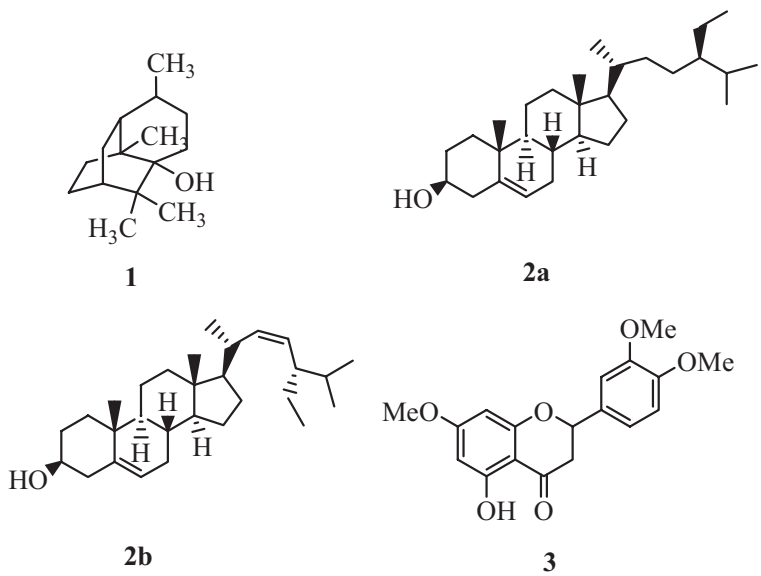

Figure 2 Structures of compounds isolated from *P. cablin* leaves: (1) patchouli alcohol; the mixture of (2a) β-sitosterol and (2b) stigmasterol; and (3) 7,3′,4-tri-*O*-methyleriodictyol.

Table 2 Antibacterial activity of patchouli oil, the crude hexane extract and three compounds against *Staphylococcus aureus (*ATCC, 25923) and *Bacillus subtilis* (ATCC 6633).

Sample	MIC value[a] (μg/mL)	
	S. aureus (ATCC 25923)	*B. subtilis* (ATCC 6633)
Patchouli oil	390	100
Crude hexane extract	3906	1950
Patchouli alcohol (**1**)	125	50
Mixture of *β*-sitosterol (**2a**) and stigmasterol (**2b**)	3130	780
7,3′,4-tri-*O*-methyleriodictyol (**3**)	3130	200
Streptomycin[b]	60	20
Enrofloxacin[c]	<0.12	<0.12

[a] Minimal inhibition concentration for the growth of bacteria.

[b, c] Reference samples

Table 3 GC-MS quantitative analysis of patchouli alcohol in patchouli oils and the crude hexane extract of patchouli leaves.

Sample	Concentration of sample (ppm)	Peak area of patchouli alcohol	Amount of patchouli alcohol from calibration curve (ppm)	% Patchouli alcohol / dry wt. of sample
Patchouli oil from fresh l leaves	200	2,852,841	174.32	0.13
Patchouli oil from dried leaves (fermented 77 days)	400	3,650,150	220.71	2.19
Crude hexane extract	100	249,157	22.86	1.17
Patchouli oil at 3 months harvesting	200	2,863,945	174.97	3.01
Patchouli oil at 6 months harvesting	200	2,656,885	162.93	2.74
Patchouli oil at 9 months harvesting	200	2,437,067	150.14	1.78

CONCLUSIONS

The extraction method that produced the highest yield of patchouli oil was water-steam distillation of dried patchouli leaves after fermentation for 77 days at room temperature. Fresh leaves gave a lower yield than dried leaves. Leaves dried in the oven at 50°C for 40 h showed the lowest yield of patchouli oil. The optimum conditions for GC-MS analysis were studied and verified that patchouli oils and the crude hexane extract contained patchouli alcohol as the major component, whereas the minor components were 4,7-methanoazulene, trans-caryophyllene, a-guaine, seychellene, azulene, a-patchoulene and pogostol. Patchouli oil contained a higher amount of patchouli alcohol (2.19% dry wt) than the crude hexane extract (1.17% dry wt). Two pure compounds, patchouli alcohol (0.05% dry wt) and 7,3′,4-tri-*O*-methyleriodictyol (0.04% dry wt), as well as the mixture of *β*-sitosterol and stigmasterol (0.09% dry wt) were isolated from the crude hexane extract.

Antibacterial activity assay showed that patchouli oil inhibited *Staphylococcus aureus* and *Bacillus subtilis* with an MIC value of 390 and

100 μg/mL, respectively. This result was better than for the hexane extract with an MIC value of 3906 and 1950 μg/mL, respectively. In addition, patchouli alcohol (with an MIC value of 125 and 50 μg/mL for *S. aureus* and *B. subtilis*, respectively) showed better activity than the mixture of β-sitosterol and stigmasterol (with an MIC value of 3130 and 780 μg/mL, respectively) and 7,3′,4-tri-*O*-methyleriodictyol (with an MIC value of 3130 and 200 μg/mL, respectively). Therefore, it could be concluded that patchouli alcohol, which was the major component and the most active compound from the *P. cablin* extract, can be used as a marker for quality control of *P. cablin* extracts. The time of leaf harvesting was also important, with leaf harvesting after 3 months (3.45% dry wt) giving a higher yield of patchouli alcohol than after 6 months (3.36 % dry wt) and after 9 months (2.38 % dry wt).

Therefore, the study's result should be very useful for the production of patchouli oil in the aromatherapy industry and for other purposes.

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