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Chemical Activities of Nine Betel-like scented *Piper* Species Against Pathogenic Microorganisms

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ABSTRACT

Nine betel-like scented *Piper* species were tested for their activity against pathogenic bacteria and fungi. The crude extracts of *P. betle*, *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. rubroglandulosum*, *P. semiimmersum*, *P. submultinerve*, *P. tricolor* and *P. yinkiangense* were tested on *Pseudomonas aeruginosa*, *Bacillus thuringiensis*, *Escherichia coli*, *Staphylococcus aureus*, *Trichoderma harzianum* and *Aspergillus flavus*. The crude extracts of the nine *Piper* species were extracted with hexane and filtrated. The filtrates were evaporated to remove the solvent and then dissolved with dimethyl sulfoxide (DMSO). The extracts were tested on bacteria and fungi at 0 (undiluted), 2×, 4× and 8× dilutions, with DMSO alone as a control. Effectiveness against *P. aeruginosa* was found with undiluted *P. rubroglandulosum* and *P. yinkiangense* extracts, and effectiveness against *S. aureus* was observed with undiluted *P. betloides* extract and all concentrations of *P. maculaphyllum* extract. Therefore, the most interesting species is *P. maculaphyllum*, as it was effective against *S. aureus*, which causes many human diseases. Therefore, the human population can benefit from both local and industrial medicines produced from these studied plants, which to date have only been produced from betel leaves.

Keywords: crude extract; fungi; hexane; *Piper* species; *Pseudomonas aeruginosa*; *Staphylococcus aureus*.

INTRODUCTION

Medicinal and aromatic plants have been used by humans for purposes such as food, treating disease, beauty and cultural activities since pre-history. Plant-derived chemicals are the basis of many compounds used in healthcare in both industrialized and developing countries, in both processed and traditional forms. Plant-derived chemicals are produced by secondary metabolic activities involved in environmental interactions. They have been used in medicines such as analgesics (morphine), antitussives (codeine), antihypertensives (reserpine), cardiotonics (digoxin), antineoplastics (vinblastine and paclitaxel), and antimalarial compounds (artemisinin). These chemicals represent some of the many irreplaceable medicinal products derived from plants. Over 25% of new drugs approved over the last 30 years are based on molecules of plant origin, and approximately 50% of the top-selling chemicals are derived from knowledge on plant secondary metabolism (Terryn *et al*., 2006; Gomez-Galera *et al*., 2007). Additionally, seven plant-derived compounds with anticancer activity have received FDA approval for clinical use: taxol/paclitaxel from *Taxus brevifolia*, vinblastine and vincristine from *Catharanthus roseus*, topotecan and irinotecan from *Camptotheca acuminata*, and etoposide and teniposide from *Podophyllum peltatum* (Gomez-Galera *et al*., 2007). Additionally, the US National Cancer Institute has screened over 40,000 plant samples since 1986 and found five chemicals with significant anti-AIDS activity (http://www.cancer.gov; Gomez-Galera *et al*., 2007).

Plants in the genus *Piper* have been used since prehistoric times for a variety of human activities. They are used as spices, in traditional and processed forms of medicines, in cosmetic compounds, in cultural activities and as insecticides (Chaveerach *et al*., 2006; Scott *et al*., 2008; Fan *et al*., 2011). *Piper betle*, the betel plant, is one of the most important and well-known species of the genus. It contains important chemical substances, such as chavicol, cineol and eugenol, used in essential oils, medicines and insecticides (Yusoff *et al*., 2005; Misra *et al*., 2009). Eugenol has been reported as having anti-oxidant and anti-inflammatory properties (Misra *et al*., 2009). Although the betel plant is of great economic importance, it is challenging to cultivate. The main problem is foot and leaf rot, which caused by the fungus *Phytophthora parasitica* Dast

(Silvayoi *et al*., 1985; Banka and Teo, 2000). In addition, the plant is subject to leaf spot, caused by bacteria (Banka and Teo, 2000). Investigations of the genus *Piper* in Thailand (Chaveerach *et al*., 2008, 2009) have found that among the 43 *Pip*er species, some produce a betel-like scent. Of these, all are wild species and hardy, producing numerous branches and leaves. They are tolerant and resistant to disease. Some produce a stronger scent than betel. Therefore, these species might be equally or more economically beneficial than the betel plant.

Crude hexane *Piper* extracts were tested on several types of bacteria and fungi that are common pathogens of humans: *Pseudomonas aeruginosa*, *Bacillus thuringiensis*, *Escherichia coli*, *Staphylococcus aureus*, *Trichoderma harzianum* and *Aspergillus flavus*. *Pseudomonas aeruginosa* is an opportunistic pathogen of humans. It causes urinary tract, soft tissue, bone, joint, gastrointestinal and respiratory system infections; systemic infections; bacteremia; and dermatitis. Patients with severe burns, and cancer and AIDS patients who are immunosuppressed, are particularly affected, and serious infections arise in patients hospitalized with cancer, cystic fibrosis and burns. The case fatality rate in these patients is close to 50% (Todar, 2009). *Bacillus thuringiensis* which produces insecticidal toxins is used as an alternative insecticide. Most *E. coli* strains are harmless in healthy humans, but some serotypes can cause serious food poisoning and are occasionally responsible for product recalls due to food contamination. Some strains have been reported to be colonic pathological strains borne from surgical excisions and autopsies of acutely infected patients (Kelly, 1990). Others are opportunistic pathogens for infection in patients with hemorrhagic colitis (Attar, 1998). *Staphylococcus aureus* is a major cause of nosocomial infections, pyoarthritis, endocarditis and other disorders (Siriwong *et al*., 2009). It is considered a transient pathogenic organism of the skin. It can express a variety of virulence factors and can secrete exotoxins and enzymes that cause cutaneous and systemic infections, such as impetigo, subcutaneous abscesses, furuncles, staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS) (Brewer *et al*., 2008). *Trichoderma harzianum*, a filamentous fungus, is an effective biological control agent. It is used to control a variety of soil borne plant pathogens, such as *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Sclerotium rolfsii* (Harman *et al*., 2004). *Trichoderma harzianum* has been shown to induce defense responses and systemic resistance as well as control

plant pathogens (Alfano *et al*., 2007). *Aspergillus flavus* is a major problem in the storage of food and feedstuffs. There are reports of severe cases of mycotoxicoses in humans and livestock due to the consumption of commodities contaminated with *A. flavus*. Aflatoxins produced by toxigenic strains of *A. flavus* have received much attention throughout the world because of their hepatocarcinogenic, teratogenic, mutagenic and immunosuppressive properties (Aoudou *et al*., 2010). Approximately five billion people have been exposed to aflatoxins in developing countries, and aflatoxicosis is ranked 6th among the ten most important human health risks identified by WHO (Prakash *et al*., 2010).

Given that these pathogens affect human health, that plants act as sources of chemicals for medicinal drugs, and that Thailand has many *Piper* betel-like scented species, the present study aims to test the activities of crude hexane extracts of nine *Piper*, betel-like-scented species against microorganisms that cause disease. Our results will help guide their potential use, in combination with, or instead of, the betel plant.

MATERIALS AND METHODS

Plant materials. Leaves of nine *Piper* species were collected, including those from both sexes of *P. betle* and *P. rubroglandulosum* (Figure 1). These included *P. betle* from Khon Kaen province, northeastern Thailand; *P. betloides*, *P. crocatum* and *P. submultinerve* from Chiang Mai province, northern Thailand; *P. maculaphyllum* from Ranong province, southern Thailand; *P. rubroglandulosum* from Phuket province, southern Thailand; *P. semiimmersum* and *P. tricolor* from Phang Nga province, southern Thailand; and *P. yinkiangense* from Surat Thani province, southern Thailand. These were used for the anti-microorganism test using the agar-well diffusion method, as explained below.

Crude extract preparation. Leaves from 11 individuals of the nine sampled species were air-dried. Twenty-five grams of each were then ground, added to 120 ml hexane (analytical grade) solvent, and filtered at room temperature. Ninety milliliters of filtrate was collected and maintained at -20 °C. Fifteen milliliters of the solution was then divided among new tubes, and the hexane was evaporated following centrifuging with a vacuum concentrator (ScanVac LaboGene, Denmark) at 20 °C and 200 rpm for 2 h. A dark green, thick, viscous crude extract was obtained. Fifteen milliliters of

dimethyl sulfoxide (DMSO) was added to each crude extract tube, and the tubes were maintained at -20 °C until antimicrobial activity testing. Upon testing, the crude extracts were variously diluted to yield undiluted, 2×, 4× and 8× dilutions.

Microorganism and inoculum preparation for testing. The microorganisms used for crude extract testing were *Pseudomonas aeruginosa* TISTR 78, *Bacillus thuringiensis* TISTR 126, *Escherichia coli* TISTR 780, *Staphylococcus aureus* TISTR 118, *Trichoderma harzianum* TISTR 3553 and *Aspergillus flavus* TISTR 3041. They were obtained from the culture collection at the Thailand Institute of Scientific and Technological Research (TISTR). The bacterial and fungal cultures were cultivated at 37 °C for 24 h on LB (Luria-Bertani) agar (Sambrook and Russell, 2001) and at 28 °C for 5 days in Potato dextrose agar (PDA), respectively. Bacteria were then isolated from a single colony and cultivated at 37 °C for 24 h on LB agar. Cell densities of approximately 1×10^1 CFU mL^{-1} were prepared from the cultures by dilution with distilled water. The fungi were cultivated at 28 °C for 5 days.

Testing activities. The antimicrobial activities of the crude extracts of the nine *Piper* species were investigated using well plate diffusion. Petri dishes, each containing 20 mL of LB agar, were used to cultivate the bacteria and fungi. Seven 5-mm-diameter wells were bored into each agar plate. Two-fold serial dilutions of the crude extracts were made with DMSO: 0, 2×, 4× and 8×. The negative controls were DMSO and distilled water. The positive controls consisted of 50 µg/mL of antibiotics, including itraconazole for the fungi *Aspergillus flavus* and *Trichoderma harzianum*, ampicillin for the bacteria *Pseudomonas aeruginosa*, *Bacillus thuringiensis* and *Escherichia coli*, and clindamycin for *Staphylococcus aureus*.

1.1 Anti-bacterial test

Twenty microliters each of crude extract, negative controls and positive controls were added to each well in the petri dishes of LB agar, containing bacteria growing at a density $1x10^1$ CFU mL^{-1}. Each experiment was performed in triplicate, and the plates were incubated at 37 °C for 24 hours. The clear zone within the sample wells, caused by the activity of phytochemicals against the microorganisms, was measured as the diameter of the clear zone minus the diameter of the well, a method modified from Suppakul *et al*. (2006). The results were categorized into three levels: no inhibition zone (or no clear zone), no inhibition and inhibition. Each level was defined or sub classified

as follows: no inhibition zone (0 mm; i.e., no phytochemical activities against the microorganisms); no inhibition or clear zones without inhibition (<5 mm); and inhibition, classified as mild inhibition (5-10 mm), moderate inhibition (>10-15 mm) and strong inhibition (>15 mm).

1.2 Anti-fungal test

The fungi in each 5-mm-diameter well of the active plate was transferred and placed in the middle of experimental plate. The plates were incubated at 28 °C to cultivate fungi for 5 days. Next, 20 µl of crude extract, negative controls and positive controls were added to each well. Each experiment was performed in triplicate, and the plates were then further incubated at 28 °C for 7 days. The classifications are as in section 4.1.

Determination of minimum inhibitory concentration. The agar dilution method of the European Society of Clinical Microbiology and Infectious Diseases (2000) was adopted to determine the minimum inhibitory concentration (MIC). Here, the minimum inhibitory concentration at which no visible growth observed was defined as the MIC of the *Piper* species crude extracts that resulted in a zone of inhibition.

Statistical analysis. Data points were indicated by the means of the measured values and subjected to analysis of variance (ANOVA) using the IBM program SPSS Statistics 19.

RESULTS

Undiluted extracts

Phytochemical activities on *Bacillus thuringiensis*

Eight *Piper* species; namely *P. betle* (male and female), *P. betloides*, *P. crocatum*, *P. submultinerve*, *P. maculaphyllum*, *P. rubroglandulosum* (male and female), *P. semiimmersum* and *P. yinkiangense* produced clear zones with *B. thuringiensis* but were not inhibitory.

The effects of the different dilutions of crude hexane extracts from the leaves of *Piper* species on the selected microorganisms are shown in Figure 2, and the width of the inhibition zones produced by each *Piper* species on their respective bacteria and fungi are shown in Table 1.

Phytochemical activities on *Pseudomonas aeruginosa*

Seven *Piper* species, including *P. betle* (both ♂ and ♀), *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. rubroglandulosum* (both male and female), *P. semiimmersum* and *P. yinkiangense* showed clear zones with *P. aeruginosa*. Additionally, two species; namely, *P. rubroglandulosum* (♀) and *P. yinkiangense*, had mild inhibitory effects, producing inhibition zones of 5.17 ± 0.29 and 5.17 ± 0.29 mm, respectively; there was no significance difference between the two species ($p > 0.05$).

Phytochemical activities on *Staphylococcus aureus*

The crude hexane extracts of *P. betloides* and *P. maculaphyllum* had mild inhibitory effects on *S. aureus*, with no significance difference between the two species ($p > 0.05$). The zones of inhibition were 5.83 ± 0.76 and 6.00 ± 1.00 mm, respectively.

All nine *Piper* species had no activity, with no clear zone, on *E. coli*, *T. harzianum* and *A. flavus*.

Diluted extracts (2×, 4×, and 8×):

The crude hexane extracts of nine *Piper* species prepared as 2×, 4×, and 8× dilutions were tested on the selected microorganisms. The results are shown below (Table 1).

Phytochemical activities on *Bacillus thuringiensis*

The 2×, 4×, and 8× dilution crude hexane extracts of *P. betle* (male), *P. betloides*, *P. crocatum, P. maculaphyllum*, ♀ and ♂ *P. rubroglandulosum*, *P. semiimersum*, *P. submultinerve* and *P. yinkiangense* showed clear zones in *B. thuringiensis* treatment, as did the 2× and 4× extracts of ♀ *P. betle*; however, these were not inhibitory.

Phytochemical activities on *Pseudomonas aeruginosa*

The 2×, 4×, and 8× dilution crude hexane extracts of ♂ *P. rubroglandulosum*; the 2× and 4× diluted extracts of *P. betloides*, *P. crocatum*, ♀ *P. rubroglandulosum*, *P. semiimmersum* and *P. yinkiangense*; and the 2× diluted extracts of ♀ and ♂ *P. betle* and *P. maculaphyllum* all showed clear zones with *P. aeruginosa*, but without inhibition. In contrast, the 2×, 4×, and 8× diluted crude hexane extracts of *P. submultinerve* and *P. tricolor* did not produce clear zones with *P. aeruginosa*.

Phytochemical activities on *Staphylococcus aureus*

The 2×, 4×, and 8× dilution crude hexane extracts of *P. betloides* and *P. maculaphyllum* produced clear zones; however, those of *P. betloides* were not inhibitory. For *P. maculaphyllum*, inhibition zones of 5.67 ± 0.58, 6.33 ± 1.15 and 5.67 ± 0.58, for the 2×, 4×, and 8× dilutions, respectively. These values were not significantly different ($p > 0.05$).

No clear zones were produced from the 2×, 4×, and 8× diluted crude hexane extracts of ♀ and ♂ *Piper betle*, *P. crocatum*, ♀ and ♂ *P. rubroglandulosum*, *P. semiimersum*, *P. submultinerve*, *P. tricolor* or *P. yinkiangense.*

In addition, no phytochemical activity and no clear zones were observed in any of the *Piper* extracts against *E. coli*, *T. harzianum* or *A. flavus.*

DISCUSSION

This study identified *Piper* species from a high biodiversity country that, in addition to *P. betle*, may provide medical benefits. The most promising species are *P. rubroglandulosum* (♂), *P. yinkiangense*, *P. betloides* and *P. maculaphyllum.* Undiluted extracts of *P. rubroglandulosum* and *P. yinkiangense* were effective against *P. aeruginosa*. Undiluted extracts of *P. betloides* and all concentrations of *P. maculaphyllum* were effective against *S. aureus*. The most interesting species is *P. maculaphyllum*, as it was effective against *S. aureus*, which causes many human diseases. Therefore, the human population can benefit from local and commercial medicines made from these plants, which formerly were made only from betel leaves.

The two bacteria species *P. aeruginosa* and *S. aureus* are common pathogens in houses and hospitals in tropical countries. They are also opportunistic pathogens of humans and cause many topical and internal diseases, particularly when the human immune system is compromised, as mentioned in the introduction.

Traditional and pharmaceutical use of *P. betle* is widespread in many countries due to several important substances, including xydroxychavicol, chavicol, eugenol, terpene, sesquiterpene, safrole, and allypyrocatechol diacetate. Several different extracts and solvents have been studied, and several compounds with high biological activity have been identified The phenolic compounds eugenol and hydroxychavicol are not mutagenic in various strains of *Salmonella typhimurium* (Amonkar, 1986). Betel leaves are reported to possess antioxidant activity against tobacco carcinogens (Chang *et al.*,

2002). Ethanol extracts have antibacterial activity against both *Staphylococcus aureus*, which causes skin disease (Sukatta *et al*., 2004). Aqueous extracts have been shown to have antioxidant activity (Dasgupta, 2004). Antidiabetic activities of aqueous and ethanolic extracts have been observed in rats (Arambewela *et al*., 2005). Betel leaves have antiseptic activities on skin when ground with 40% ethyl alcohol solvent (Chaveerach *et al*., 2006). Methanolic extracts of *P. betle* have broad spectrum activities, including antifungal, antibacterial, antitumour, hypotensive, respiratory depressant, anthelminthic, carditonic and antifertility; their antioxidant activity scavenges free radicals and reduces free-radical induced cell injury (Manigauha *et al*., 2009). The leaf extracts, fractions and purified compounds play a role in oral hygiene and have positive cardiovascular effects. They also have anti-diabetic, anti-inflammatory/immunomodulatory, anti-ulcer, hepato-protective, anti-infective, and high antioxidant activities. Important compounds include hydroxychavicol, hydroxychavicol acetate, allypyro-catechol, chavibetol, piperbetol, methylpiperbetol, and piperols A and B (Kumar *et al*., 2010). Other works discuss the optimum environmental conditions for growth in *P. betle* and review its medicinal and nutritional benefits (2011).

Sanubol *et al*. (2014) described some of the major chemicals within the nine *Piper* betel-like scented species; specifically, sabinene, β-phellandrene, 4-allyloxy-6-methoxy-N, N-dimethyl-1,3,5-germacrene-D, β-cubebene and others. A high amount, 34.835% of an unknown compound was also identified. These compounds seem to be different from those of *P. betle* (Sanubol *et al*., 2014) that are active against many species of bacteria and fungi (Arambewela *et al*., 2005; Charoenwattana, 2007).

In Thailand, traditional and pharmaceutical use of *P. betle* has occurred from ancient times into the present. Traditional Thai forms include fresh leaves and capsules. It has antiseptic and anti-inflammatory activities on skin diseases, presumably from *P. aeruginosa* and *S. aureus*. The prepared form is made by grinding as many leaves as needed with 40% ethyl alcohol to produce a concentrated extract. The extract is then applied to the skin.

However, our results show clear zones of *P. betle* on *P. aeruginosa*, but without inhibitory effects; we also observed no inhibitory activity on *S. aureus*. Possible reasons include our use of different solvent extracts or concentrations. In addition, *P. rubroglandulosum*, *P. yinkiangense*, *P. betloides* and *P. maculaphyllum* had greater

inhibitory activities on *P. aeruginosa* and *S. aureus* than did *P. betle*, a finding consistent with its hotter and spicier attributes when chewed. Therefore, the human population can benefit from both local and industrial medicines from these plants, originally derived only from betel leaves.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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Table 1. Antimicrobial activity of different dilution levels of crude hexane extracts from the leaves of *Piper* species using an agar well diffusion assay.

Plants and level diluted crude hexane extracts	Zone of inhibition[a] / mm		
	Bt	*Pa*	*Sa*
♀ *Piper betle*			
Un-dilution	2.00 ± 0.00 [A]	2.67 ± 0.58 [A]	0.00
2×-dilution	2.17 ± 0.29	2.00 ± 0.00	0.00
4×-dilution	2.00 ± 0.00	0.00	0.00
8×-dilution	0.00	0.00	0.00
♂ *P. betle*			
Un-dilution	2.50 ± 0.50 [A]	3.67 ± 0.58 [ABD]	0.00
2×-dilution	2.33 ± 0.29	3.00 ± 0.00	0.00
4×-dilution	2.00 ±0.00	0.00	0.00
8×-dilution	2.00 ±0.00	0.00	0.00
P. betloides			
Un-dilution	2.67 ± 0.58 [AB]	4.33 ± 0.58 [BCDE]	5.83 ± 0.76 [A]
2×-dilution	2.17 ± 0.29	3.33 ± 0.29	3.83 ± 0.76
4×-dilution	2.17 ± 0.29	2.50 ± 0.87	3.67 ± 1.53
8×-dilution	2.00 ± 0.00	0.00	2.67 ± 1.15
P. crocatum			
Un-dilution	3.75 ± 0.35 [BC]	4.50 ± 0.87 [BCE]	0.00
2×-dilution	3.33 ± 0.58	3.17 ± 1.04	0.00
4×-dilution	3.33 ± 0.29	2.67 ± 0.58	0.00
8×-dilution	2.83 ± 0.76	0.00	0.00
P. maculaphyllum			
Un-dilution	2.33 ± 0.29 [A]	3.33 ± 1.15 [AB]	6.00 ± 1.00 [A]
2×-dilution	2.50 ± 0.00	3.00 ± 0.00	5.67 ± 0.58
4×-dilution	2.50 ± 0.50	0.00	6.33 ± 1.15
8×-dilution	2.00 ± 0.00	0.00	5.67 ± 0.58
♀ *P. rubroglandulosum*			

Un-dilution	3.50 ± 0.87 ^{BCD}	5.17 ± 0.29 ^{C}	0.00
2×-dilution	3.17 ± 0.58	4.00 ± 0.00	0.00
4×-dilution	3.17 ± 0.76	3.00 ± 0.00	0.00
8×-dilution	2.67 ± 1.15	0.00	0.00
♂ *P. rubroglandulosum*			
Un-dilution	2.67 ± 0.58 ^{ACD}	5.00 ± 0.00 ^{CD}	0.00
2×-dilution	2.33 ± 0.58	4.00 ± 0.00	0.00
4×-dilution	2.83 ± 1.04	3.17 ± 0.29	0.00
8×-dilution	3.00 ± 0.00	2.00 ± 0.00	0.00
P. semiimersum			
Un-dilution	2.17 ± 0.29 ^{A}	3.67 ± 1.53 ^{ABD}	0.00
2×-dilution	2.33 ± 0.58	4.33 ± 2.31	0.00
4×-dilution	2.00 ± 0.00	3.00 ± 0.00	0.00
8×-dilution	2.00 ± 0.00	0.00	0.00
P. submultinerve			
Un-dilution	2.00 ± 0.00 ^{A}	0.00	0.00
2×-dilution	2.00 ± 0.00	0.00	0.00
4×-dilution	2.33 ± 0.58	0.00	0.00
8×-dilution	2.33 ±0.58	0.00	0.00
P. yinkiangense			
Un-dilution	3.67 ± 0.76 ^{C}	5.17 ± 0.29 ^{CE}	0.00
2×-dilution	3.00 ± 0.50	4.00 ± 0.00	0.00
4×-dilution	2.33 ± 0.58	2.67 ± 0.58	0.00
8×-dilution	2.00 ± 0.00	0.00	0.00

Figure legend

Figure 1. The nine *Piper* species investigated: ♀ *Piper betle* (A), ♂ *P. betle* (B), *P. betloides* (C), *P. crocatum* (D), *P. maculaphyllum* (E), ♀ *P. rubroglandulosum* (F), ♂ *P. rubroglandulosum* (G), *P. semiimmersum* (H), *P. submultinerve* (I), *P. tricolor* (J-K) and *P. yinkiangense* (L).

Figure 2. Examples of different dilution levels and clear zones of crude hexane extracts from the leaves of *Piper* species against *Pseudomonas aeruginosa* (♀ *Piper rubroglandulosum* (A) and *P. yinkiangense* (B)) and *Staphylococcus aureus* (*Piper betloides* (C) and *P. maculaphyllum* (D)). Treatments: antibiotic (1), sterile distilled water (2), undiluted extractions (3), 2×-diluted extractions (4), 4×-diluted extractions (5), 8×-diluted extractions (6) and DMSO (7).

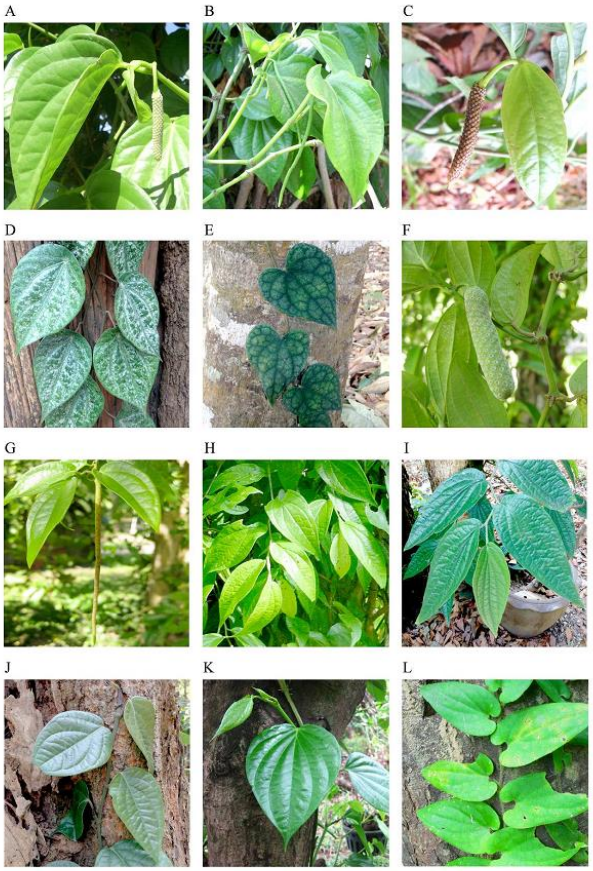

Figure 1. The nine Piper species investigated: ♀ Piper betle (A), ♂ P. betle (B), P. betloides (C), P. crocatum (D), P. maculaphyllum (E), ♀ P. rubroglandulosum (F), ♂ P. rubroglandulosum (G), P. semiimmersum (H), P. submultinerve (I), P. tricolor (J-K) and P. yinkiangense (L).
284x414mm (300 x 300 DPI)

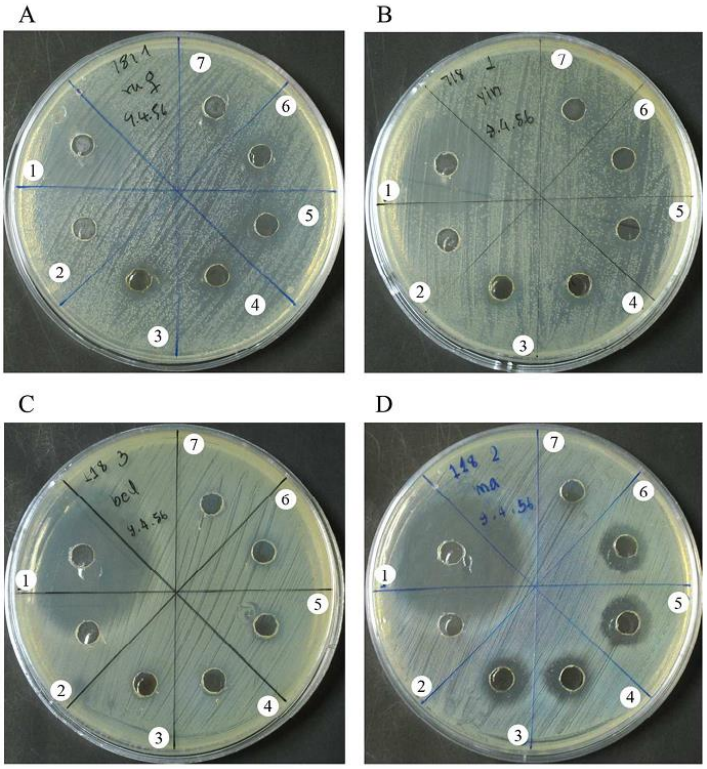

Figure 2. Examples of different dilution levels and clear zones of crude hexane extracts from the leaves of Piper species against Pseudomonas aeruginosa (♀ Piper rubroglandulosum (A) and P. yinkiangense (B)) and Staphylococcus aureus (Piper betloides (C) and P. maculaphyllum (D)). Treatments: antibiotic (1), sterile distilled water (2), undiluted extractions (3), 2×-diluted extractions (4), 4×-diluted extractions (5), 8×-diluted extractions (6) and DMSO (7).
164x180mm (300 x 300 DPI)

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Quantity and components of the volatile oils of nine betel-like scented *Piper* plants and the mixture of these oils with commercial products

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ABSTRACT

Volatile oil quantity and chemical components were detected in the leaves of a cultivated betel species including *Piper betle* and eight wild betel-like-scented species by GC-MS. The oil quantity is higher in the commercially cultivated species at 2.15-2.73 mL/kg, while wild species had a lower amount, 0.03-0.76 mL/kg. However, when some wild species are grown in sunlight-exposed areas around households and office buildings, they produce higher oil amounts e.g., the species *P. rubroglandulosum*, *P. tricolor* and *P. yinkiangense*. The reason for the increased production is attacks by pathogens and insects, which is a route for secondary metabolite synthesis in plants. Interestingly, species with high oil amounts synthesize several chemicals with a low percentage for each chemical, while species with lower oil amounts synthesize less diverse chemicals but at a higher percentage. Substances found in a satisfactory amount in each species include the following: eugenol/isoeugenol and 4-allyl-1,2-diacetoxybenzene in *P. betle*, L-linalool, α-terpineol, β-selinene and butylated hydroxytoluene in *P. betloides*, L-linalool, β-terpineol, α-terpineol and butylated hydroxytoluene in *P. crocatum*, trans-caryophyllene, germacrene D and butylated hydroxytoluene in *P. maculaphyllum*, 4-allyl-1,2-diacetoxybenzene in *P. rubroglandulosum*, butylated hydroxytoluene in *P. semiimersum*, β-elemene, trans-caryophyllene, β-selinene, 4-

allyl-1,2-diacetoxybenzene and germacrene D in *P. submultinerve*, 4-terpineol, eugenol/isoeugenol, 4-allyl-1,2-diacetoxybenzene and bicyclogermacrene in *P. tricolor*, and α-caryophyllene, 4-allyl-1,2-diacetoxybenzene and α-selinene in *P. yinkiangense*. The oils were added to anti-inflammatory and anti-itching soap and foot deodorant spray products.

Keywords: betel-like-scented *Piper* species, GC-MS, phytochemicals, stream distillation, volatile oils

1. Introduction

Essential oils or volatile oils are aroma oils physically extracted by various methods from aromatic plants popularly obtained by stream distillation. Medicinal and aromatic plants have always been related to current human health systems including traditional usage in households and industry. Plant-derived medicines and aromatic oils constitute substantial components that are products of plant secondary metabolism and involved in many other aspects of a plant's interaction with its immediate environment [1,2]. The oils are used for fragrances in-house products, such as green pesticides and soaps, flavorings in perfume, cosmetics and seasoning in food at the household and industry levels, and more recently, in aromatherapy and as herbal medicines. Plant essential oils are commercially produced from several botanical sources, and many are members of the mint family (Lamiaceae). The oils are generally composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes. Examples include 1,8-cineole, a major constituent of oils from rosemary and eucalyptus, eugenol, which is from clove oil, thymol, which is from garden thyme, menthol, which is from various mint species, asarones, which is from calamus, and carvacrol and linalool, which come from many plant species [3]. The oils comprise various substances depending on the plant species and are used for diverse options. Two classes of compounds, terpenoids and phenyl propenes, make up the bulk of plant volatile oils and define the particular properties of many species and herbs, e.g., terpenoid menthol, a major component of peppermint, provides this herb with its cool, peppery aroma and flavor. In contrast, the major constituent of cloves is phenylpropene eugenol, which provides this spice with its pungent, distinctive aroma. Eugenol makes up 70 to 90% of essential oils and 15% of the dry weight of clove buds (Myrtaceae). Eugenol is also found in significant amounts in cinnamon and cinnamon leaves and at a lesser amount in nutmeg and pepper [4]. β-

Caryophyllene, which is found in *Helichrysum italicum* and *Achille millefolium*, has anti-inflamatory activity [1].

Members of the *Piper* species also include medicinal and aromatic plants. Many species, particularly cultivated species such as *P. betle*, *P. nigrum*, *P. chaba*, and *P. sarmentosum*, have long been used both in households and industry worldwide, including in Asia, due to their high species diversity. Additionally, these plants contain some chemicals with benefit to humans for the numerous usages mentioned above for instances citronella, eugenol, cineole and many others found in many plants species, such as *Piper betle*, *P. betloides*, *P. crocatum*, are used for insect and mosquito repellant [3,5]. Moreover, isoeugenol, chavibetol, caryophyllene, sabinene, phyllandrene and germacrene A and D are major components found in hexane crude extracts by gas chromatography-mass spectrometry (GC-MS) of the nine betel-like-scented *Piper* species. A number of these compounds have long been used for several treatments including isoeugenol, an isomer of eugenol that is a phenylpropene synthesized from eugenol and a constituent of essential oils in plants; chavibetol, another eugenol isomer that is an aromatic compound with a spicy odor [6]; caryophyllene, which has a spicy and clove-like aroma [7]; sabinene, a natural bicyclic monoterpene that is one of the chemical compounds that contributes to the spiciness of *P. nigrum* black peppers [8]; phyllandrene, which is used in fragrances for its pleasant aroma; β-phyllandrene, which has been described to be a peppery-mint and slightly citrus [9]; and germacrenes, which are sesquiterpenes, a class of volatile organic hydrocarbons that are typically produced for their antimicrobial and insecticidal properties [10,11].

GC-MS is a method that has been accepted for phytochemical identification and has been used in conjunction with the analysis of the essential oils of *Piper* species. For example, this method identified chemicals contained in the essential oils of *P. nigrum* and *P. guineense* [12], *P. nigrum* and *P. longum* [13], *P. capense*, *P. guineense*, *P. nigrum*, and *P. umbellatum* [14], and *P. betle* [15].

Because of the many uses for volatile oils, including the various chemicals contained in *Piper* crude extract species, we aimed to study volatile oil quantity concentrated from wild species. Additionally, the chemical components in the oils were elucidated by GC-MS. Soap, liquid soap and foot deodorant spray were produced with a mixture of the oils obtained via stream distillation in the laboratory.

2. Experimental

98 *2.1 Plant samples and collection*

99 The leaves of the nine species studied including eight wild aromatic betel-like plants, viz.
100 *P. betloides* Chaveer. & Tanomtong, *P. crocatum* Ruiz et Pavon, *P. maculaphyllum* Chaveer.
101 & Sudmoon, *P. rubroglandulosum* Chaveer. & Mokkamul, *P. semiimmersum* C.DC., *P.*
102 *submultinerve* C.DC., *P. tricolor* Y.C.Tseng, *P. yinkiangense* Y.C.Tseng, and one from the
103 more cultivated *P. betle* L. species were collected from the various native growing areas
104 described in the Table 1. The morphological characteristics of these species are shown in Fig.
105 1. Species identification was performed according to Chaveerach et al. [16,17] and compared
106 with the specimens maintained kept at Khon Kaen University. The research study collection
107 took place between 2012 and 2014. Some species, such as *P. betle*, *P. rubroglandulosum*,
108 *P. submultinerve* and *P. tricolor*, were easier/possible to collect, and more than one was for
109 amount clarification and to sort chemicals in various times and areas collected.

110 *2.2 Stream distillation and oil quantity comparison for each of the studied species*

111 Freshly matured leaves (1 kg) of each of the nine studied samples were subjected to
112 stream distillation with 3.8 L water using WHM12017 Heating Mantles (Daihan Scientific
113 Co. Ltd., Korea) for 3 hours at 25 °C. After distillation, the oils were collected in two parts:
114 the first was floated on top of water in a flask, and the second was mixed with water in a
115 flask. The oils on top of water were transferred to a new bottle by an auto pipette that was
116 maintained at -20 °C. The oil and water mixture was divided in a separation funnel by adding
117 dichloromethane (CH_2Cl_2) to the mixture in a 25:500 mL proportion. Then, the funnel was
118 checked to determine whether it was a homogeneous mixture, and it was left for 1 h at room
119 temperature. Dichloromethane and the volatile oil remained at the bottom of the funnel. The
120 dichloromethane was evaporated using a Rotary Evaporator (Buchi Rotavapor R-210,
121 Switzerland); thus, the remainder was the oil only, which was transferred it to the supplement
122 in the bottom of the first part and maintained at -20 °C until further use of the products and
123 chemicals for identification by GC-MS.

124 *2.3 Chemical identification of the oils of the betel-like scented species using GC-MS*

125 GC-MS analysis of the oils was performed using an Agilent Technologies GC 6890
126 N/5973 inert MS fused with a capillary column (30.0 m × 250 mm × 0.25 mm). Helium gas
127 was used as the carrier gas at a constant flow rate of 1 mL/min. The injection and mass-
128 transferred line temperature were set at 280 °C. The oven temperature was programmed at 70

°C to 120 °C at 3 °C/min. The temperature was then held for 2 min and finally raised to 270 °C at 5 °C/min. A 1-µL aliquot of oil was injected in the split mode. The relative percentage of the oil constituents was expressed as the percentage using peak area normalization. The identification of the oil components was determined by comparison of the mass spectra obtained with those of the reference compounds stored in the Wiley 7N.1 library.

2.4 Products with a mixture of volatile oils

The volatile oils from the nine species sampled were obtained via stream distillation in the laboratory and the oil of a species was used in mixtures for soap, liquid soap and foot deodorant spray. Medicinal properties were tested by 60 volunteers.

3. Results

The oil quantity of the nine *Piper* species, including eight wild and one cultivated, was determined by stream distillation (Table 2).

The cultivated plant *P. betle* produced oil in the highest amount (2.73 mL/kg) compared with the wild species. However, there was a difference between the commercial individual/cultivars *P. betle* 1* and *P. betle* 2**. These cultivars were purchased from a market, and *P. betle* 1* which is commercially cultivated individuals gave high oil amounts ranging from 0.79 to 2.73 mL/kg. For these plants, the two individuals collected on 9/7/2013 had low oil amounts in the range of 0.79 -0.84. For *P. betle* 2**, growth under natural conditions for one plant produced a low amount of oil 0.95 mL/kg.

For the eight wild species, low oil amounts were obtained, starting with 0.03 mL/kg for *P. crocatum*, which was from the forest of the Garden and Development Department, Queen Sirikit Botanic Garden, to 0.76 mL/kg for *P. tricolor* 1***. Additionally, the eight wild species were divided into two groups.

The first group included three individual species viz. species that were transferred from the forest for growth in houses under natural conditions: *P. tricolor* 1***, which provided oil that was 0.76 mL/kg higher than the native wild individual; *P. tricolor* 2, which grows in forests; the individual species *P. rubroglandulosum* 2#, which grows around office areas under natural conditions, providing an oil content higher to 0.72 mL/kg than the native wild individual; *P. rubroglandulosum* 1, which grows in forests; and *P. yinkiangense*#, which grows around office areas under natural conditions and provides a high oil content (0.62 mL/kg).

The second group comprised individual *P. tricolor* 2 and *P. rubroglandulosum* 1 species, and the other five species with several individuals included *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. semiimersum*, *P. submultinerve* 1, *P. submultinerve* 2, *P. submultinerve* 3, and *P. submultinerve* 4. These plants had oil contents, starting at 0.03 mL/kg for *P. crocatum*, *submultinerve* 3, and *P. submultinerve* 4, to 0.47 mL/kg for *P. rubroglandulosum* 1.

The GC-MS results of each species volatile oil are listed in Table 3, which shows the different aromatic compounds. Total ionic chromatographs (TICs) showing the peak identities of compounds from nine individual *Piper* species are shown in Fig. 2. The summary of the types and content of compounds compared with the rate of essential oil (mL/kg) are shown in Table 4. The compound contents are listed beginning with a 10% increase (13.629%) e.g., L-linalool, which was found in *P. betloides* at the highest amount and 4-allyl-1,2-diacetoxybenzene, which was found in *P. rubroglandulosum* 2 at 60.089%. By comparing the chemical content and volatile amounts of the group of individual species that produce low volatile amounts, we observed a release of high amounts of important chemicals.

Three products with mixtures of volatile oils of a species studied were shown in Fig. 3. When tested on 60 volunteers, the soaps and liquid soaps showed significant anti-inflammatory activity on the skin e.g., anti-inflammatory and anti-itching activity, and the foot deodorant spray decreased/removed bad smells with $p<0.05$.

4. Discussion and conclusions

Individual plant samples were collected in unequal numbers because of two reasons. The first is oil quantity, as it was required that the plants be of the same species, and the analysis would be better when many individuals of commercially cultivated species were studied e.g., *P. betle* 1. The second reason is that wild species collections are difficult to obtain from distant forests. Thus, the authors attempted to collect as many as possible. Some species are easier e.g., *P. submultinerve*, and some species are harder to obtain, such as *P. maculaphyllum* and *P. yinkiangense*. However, the species with the most numbers showed the best results reaching for the record summary and study in the species with small numbers.

The yields of volatile oils varied according to many factors, such as type of species, cultivated and wild individual species, and growth conditions, including cultivars and optimum growth conditions for leaf production in relation to the season and temperature in

addition to suitable factors such as water, light, fertilizer and pathogenic and insect protection.

The volatile oil quantity of one of the commercially cultivated *P. betle* species and eight wild species showed that *P. betle* 1 grown under the cultivated condition produces a high volatile oil amount ranging from 2.15-2.73 mL/kg, and with the exception of a *P. betle* 1 individual purchased on 9/7/2013, it produces low volatile oils amount in the range of 0.79-0.84 mL/kg, which may be due to its growth in the raining season with increased water and other suitable factors; thus, the individual species synthesizes a less volatile oil amount but increases primary substances for growth. In contrast, *P. betle* 2 was grown in the absence of commercially cultivated conditions, under natural conditions without factors such as fertilizer and water; thus, the individual produced a less volatile oil amount.

The volatile oil amount may depend on species cultivars, optimum growing conditions, season, and temperature with fertilizer and water supplementation. Although *P. betle* can be grown in a wide temperature range, there is actually a different degree of rain and dry seasons, but the effects on volatile oil production in the case of plants depend on secondary metabolite synthesis, which is induced by insect and pathogen attacks. For example, *P. betle* 1 was collected during the dry season, which has an average temperature of 37-40 °C, on 5/8/2013, and the volatile amount collected increased to 2.15-2.73 mL/kg. This result means that there is a small amount of water in leaves including those with optimum growing conditions under commercially cultivated conditions. During the same dry season, with the exception of the *P. betle* 1 collected on 29/11/2012, the lower temperature averaged 25-30 °C under optimum commercially cultivated conditions, producing volatile oil at 1.84 mL/kg. The *P. betle* individual collected on 9/7/2013 provided a low volatile oil amount (0.79-0.84 mL/kg), and it was taken from a different cultivated source farm, which had suboptimal growth conditions.

P. betle 2 grows under natural conditions, and it produces a lower volatile oil amount than *P. betle* 1 compared with the eight wild species because their growth depends on natural conditions. Presumably, volatile oil synthesis in wild species should also depend on human management. Additionally, the eight wild species have more advantages than cultivated the *P. betle* species such as growing well, strength, abundant branching, and disease and insect resistance. Accordingly, the three wild individuals species, including *P. rubroglandulosum* 2#, grow around office areas under natural conditions and release a higher volatile amount (0.72 mL/kg) than the collected wild individual *P. rubroglandulosum* 1, which releases a volatile amount of only 0.47 mL/kg. Similarly, *P. tricolor* 1***, which was collected from a

forest and grown in a house area under natural conditions, produces a volatile oil amount of 0.76 mL/kg, which is greater than that of the collected wild individual *P. tricolor* 2, which produced only 0.03 mL/kg volatile oil. Another species, *P. yinkiangense* #, which was grown in an office area under natural conditions, produced a volatile oil amount of 0.62 mL/kg. Chaveerach et al. [16] reported that wild *Piper* can grow in a wide range of conditions, including tropical rainforests and dry evergreen forests, where there is less light due to trees and moisture. Thus, the author suggested that when the wild *Piper* species is moved for growth in sunlight, it has the ability to synthesize oil similar to the three species *P. rubroglandulosum* 2#, *P. tricolor* 1*** and *P. yinkiangense* #. The reason for this phenomenon is pathogens and insects.

Although wild individual species produce a smaller volatile oil amount than individual cultivated species, the results from GC-MS show several highly important chemical components (Table 3). Starting with 10% quantitation, *P. betle* 2 produces a volatile oil amount of 0.95 mL/kg, but eugenol synthesis is increased to 53.559%, while *P. betle* 1 produces a volatile oil amount of 2.15-2.73 mL/kg but synthesizes eugenol/isoeugenol at only 29.739% (7.847). This result is similar to *P. rubroglandulosum* 1, 2, which produces a volatile oil amount of 0.47-0.72 mL/kg but it produces a yields an allyl-1, 2-diacetoxybenzene amount of 60.089% (Table 4). In summary, cultivated and wild species can be used for processes depending on chemical requirement and volatile oil quantity for sufficient wild individual species support. Thus, our research revealed that the eight wild species can serve the same purpose as *P. betle* for various human purposes including those for household products and industry e.g., drug development, the food industry, and the aroma industry. The results of this study are invaluable as it demonstrated more of an advantage in the choice of using more *P. betle* species; additionally, volatile oils from *P. betle* species are very expensive.

As shown in Table 4, substances found in a satisfactory amount for each species include the following: eugenol/Isoeugenol and 4-Allyl-1,2-diacetoxybenzene in the commercially cultivated *P. betle*, L-linalool, β-terpineol, α-terpineol and butylated hydroxytoluene in *P. crocatum*, L-linalool, α-terpineol, β-selinene and butylated hydroxytoluene in *P. betloides*, 4-terpineol, eugenol/isoeugenol, 4-allyl-1,2-diacetoxybenzene and bicyclogermacrene in *P. tricolor*, β-elemene, trans-caryophyllene, β-selinene, 4-allyl-1,2-diacetoxybenzene and germacrene D in *P. submultinerve*, trans-caryophyllene, germacrene D and butylated hydroxytoluene in *P. maculaphyllum*, α-caryophyllene, 4-Allyl-1,2-diacetoxybenzene and α-

selinene in *P. yinkiangense*, and 4-allyl-1,2-diacetoxybenzene in *P. rubrograndulosum* and butylated hydroxytoluene in *P. semiimersum*.

Industries require certain chemicals in volatile oils and use them in different quantities. Moreover, plants can serve as sources for homemade products produced in the laboratory. The products have several activities including those that inhibit inflammation, acne, itching, and bacteria. For example, some oils have activity against *Staphylococcus aureus*, which causes skin disease [18]. Others have antiseptic activities for skin for conditions caused by *S. aureus* and *Pseudomonas aeruginosa* [19], whereas others have antifungal, antitumor, hypotensive, and respiratory depressant effects [20]. The important compounds related to these treatments include hydroxychavicol, hydroxychavicol acetate, allpyro-catechol, chavibetol, piperbetol, methylpiperbetol, and piperols A and B [21]. Additionally, there are more chemicals within the hexane crude extracts of the nine betel-like scented species, including sabinene, β-phellandrene, 4-allyloxy-6-methoxy-N, N-dimethy-1,3,5-germacrene-D, and β-cubebene. These are not found in *P. betle* but in other species [5] and have activity against many bacteria and fungi species [22,23].

Thus, the substances contained in the volatile oils from these nine sample species, as well as their bioactivities, are important to determine their benefits when used in soap, liquid soap and foot deodorant. However, we have concentrated only on the quantity mixture for efficiency.

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343 **Table legends**

344 **Table 1** The nine investigated samples of *Piper* species with leaves collections and growing
345 areas which are collected sites.

346 **Foot note**

347 * Purchased from a market, indicating that it grows in cultivated conditions

348 ** Grows under natural conditions

349 *** Transferred from forest to grow in house area under natural conditions

350 # Grows around office areas under natural conditions

351 **Table 2** The amount of essential oils from the leaves of the examined *Piper* species

352 **Table 3** Preliminary phytochemicals identified in essential oil from the leaves of the
353 examined *Piper* species

354 **Table 4** The summary of substances, both in type and quantity, compared to the amounts of
355 volatile oils

356

357 **Figure legends**

358 **Fig. 1.** The nine *Piper* species investigated: A. *Piper betle* 1; B. *P. betle* 2; C. *P. betloides*; D.
359 *P. crocatum*; E. *P. maculaphyllum*; F. *P. rubroglandulosum* 1; G. *P. rubroglandulosum* 2; H.
360 *P. semiimmersum*; I. *P. submultinerve* 1; J. *P. submultinerve* 2; K. *P. submultinerve* 3; L. *P.*
361 *submultinerve* 4; M. *P. tricolor* 1; N. *P. tricolor* 2; O-P. *P. yinkiangense*

362 **Fig. 2.** GC-MS chromatograms of volatile oils of the nine *Piper* species here are *P. betle* 1-2
363 (A-B); *P. betloides* (C); *P. crocatum* (D); *P. maculaphyllum* (E); *P. rubroglandulosum* 1 (F).

364 **Fig. 2 cont.** GC-MS chromatograms of volatile oils of the nine *Piper* species: shown here are
365 the chromatograms for *P. rubroglandulosum* 2 (G); *P. semiimmersum* (H); *P. submultinerve*
366 1-4 (I-L).

367 **Fig. 2 cont.** GC-MS chromatograms of volatile oils of the nine *Piper* species: shown here are
368 the chromatograms for *P. tricolor* (M-N); *P. yinkiangense* (O)

369 **Fig. 3.** The three products with the mixture of volatile oils, namely, soap (A), liquid soap (B)
370 and foot deodorant spray (C)


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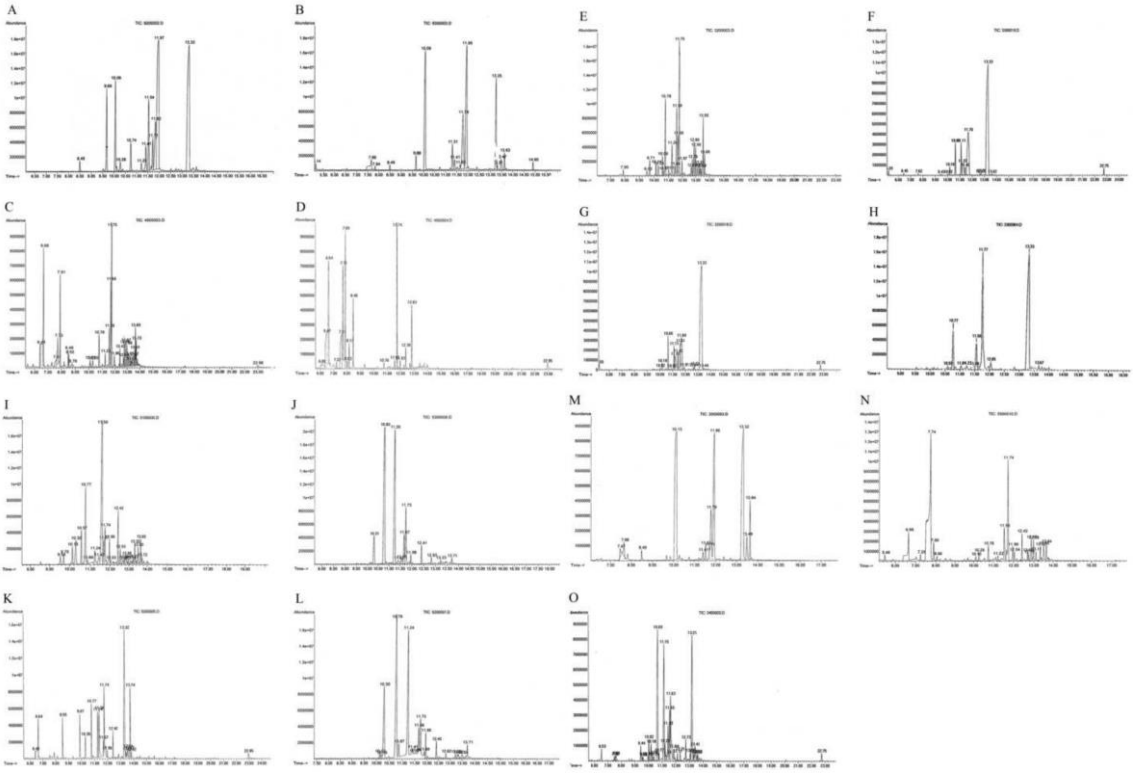
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A


สบู่สมุนไพร
น้ำมันหอมพลู
ลดสิวและผดผื่น 150.-
เหมาะสำหรับผิวธรรมดา-ผิวมัน

B


เจลอาบน้ำสมุนไพร
น้ำมันหอมพลู

C


สเปรย์สมุนไพร