ภาคผนวก
ผลงานจากโครงการวิจัย

Article 1: Published

Sanubol, S., Chaveerach, A.*, Sudmoon, R., Tanee, T., Noikotr, K. and Chuachan, C. 2014. Betel-like-scented *Piper* plants as diverse sources of industrial and medicinal aromatic chemicals. Chiang Mai Journal of Science 41(5.1): 1171–1181. (ISI impact factor 0.516).

Chiang Mai J. Sci. 2014; 41(5.1) : 1171-1181
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Betel-like-scented *Piper* Plants as Diverse Sources of Industrial and Medicinal Aromatic Chemicals

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Received: 21 May 2013
Accepted: 10 August 2013

ABSTRACT

Piper betle (Piperaceae) or betel leaf, known locally as "*Phlu*" has been used by people in Thailand for chewing for a long time. Additionally, the leaves are used for traditional remedies and folk customs, such as for weddings and housewarming ceremonies. More recently, the aromatic oil industry has used the leaves for oil distillation. Moreover, the oils are used in several household products. Over the past 12 years of our research on *Piper* species, we found that among the more than 43 species recorded, there are some plants other than *P. betle* that possess a betel-like scent, viz. *P. betloides*, *P. crocatum*, *P. maculaphyllum*, *P. rubroglandulosum*, *P. semiimmersum*, *P. submultinerve*, *P. tricolor*, and *P. yinkiangense*. As it was expected that these plants would contain similar useful chemicals, their extracts were screened for the chemical contents by GC-MS. The extracts contain some important chemical substances that are similar to the betel extract, namely, eugenol, isoeugenol, chavicol, caryophyllene, sabinene, phellandrene, germacrene A and germacrene D, and sesquiterpenes. The results indicate that the eight plant species would have as high a potential as *P. betle* for industrial purposes. Moreover, as the plants are wild species they have a greater vigor, thus growing well and with more branching than betel. The diverse *Piper* species studied and documented are important for sustainable uses and can enable conservation management for posterity.

Keywords: betel plant, betel-like-scented plants, GC-MS, *Piper* species, Thailand

1. INTRODUCTION

The plants in the genus *Piper* are of great interest because they are useful in many functional aspects, for instance, as spices, medicines, and insecticides [1-3]. The betel plant, *P. betle*, is a well-known and important species that contains important chemical

substances, including essential oils and substances, such as chavicol, cineol, and eugenol which can be used for medicinal and insecticidal purposes [4,5]. Indeed, it has been reported that eugenol has anti-oxidant and anti-inflammatory properties [4]. It is also known to be stimulating, anesthetic, and psychoactive [6,7]. Even though the betel plant is of great economic importance, there are inherent problems with its cultivation, with one common cause being that the roots and leaves often rot because of infection by Phytophthora parasitica Dast [8,9]. Banka and Teo [9] have reported that leaf spots caused by bacteria are another problem.

Two *Piper* species from Cameroon, *P. nigrum* and *P. guineenense*, were studied [10] to identify the aromatic target components responsible for the characteristic odor of these valuable spices and food-flavoring products. The main compounds detected were as follows: *P. nigrum* contains germacrene D (11.01%), limonene (10.26%), β-pinene (10.02%), α-phellandrene (8.56%), β-caryophyllene (7.29%), α-pinene (6.40%) and cis-beta-ocimene (3.19%); *P. guineense* (black) contains β-caryophyllene (57.59%), β-elemene (5.10%), bicyclogermacrene (5.05%) and α-humulene (4.86%); and *P. guineense* (white) contains β-caryophyllene (51.75%), cis-β-ocimene (6.61%), limonene (5.88%), β-pinene (4.56%), linalool (3.97%) and α-humulene (3.29%).

Investigations of the genus *Piper* in Thailand [11,12] reveals that of the 43 known *Piper* species, there are numerous species that possess a betel-like scent. All of these species are blossoming, wild species that produce abundant branches and leaves. Furthermore, these species are hardy and resistant to diseases, and some of them have a stronger scent than the betel plants. Therefore, these species are predicted to have an economic potential greater than that of the betel plants.

Betel oil contains several substances, such as chavicol, terpene, and sesquiterpene [13], hydroxychavicol, hydroxychavicol acetate, allylpyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A, and piperol B [14], chavibetol, chavibetol acetate and caryophyllene [15], allopyrocatecholdiacetate, safrole and B-phellandrene [16], and eugenol and hydroxychavicol [17]. Both the crude extracts of *P. betle* leaves and the purified compounds have been found to possess important antiseptic (e.g., oral hygiene), anti-diabetic, cardiovascular, anti-inflammatory/ immunemodulatory, antiulcer, hepato protective, and anti-infective properties [14] and are also active against many species of bacteria and fungi [16,18]. The chemical composition of natural products was successfully accessed by gas chromatography-mass spectrometry (GC-MS) analysis as reported by Phutdhawong et al. [19], Quang et al. [20], and Norkaew et al. [21]. The properties of the major compounds are as follows. Isoeugenol, an isomer of eugenol, is a phenylpropene that is synthesized from eugenol and is a constituent of the essential oils of plants. Chavibetol, another isomer of eugenol, is an aromatic compound with a spicy odor [22]. Caryophyllene is a spicy, clove-like aroma [23]. Phellandrenes are used in fragrances because of their pleasant aromas; the odor of β-phellandrene has been described as peppery-minty and slightly citrusy [24]. Sabinene is a natural bicyclic monoterpene, which is one of the chemical compounds that contributes to the spiciness of black pepper (*P. nigrum*) [25]. Sesquiterpenes, a class of volatile compounds, are typically produced for their antimicrobial and insecticidal properties [26,27].

In Thailand, betel oil has been used in modern medicine as an antiseptic component in gels, balms, and cosmetics for hands, feet, and body, as an anti-inflammatory, and as a

treatment for many diseases. Examples of applications of betel oil are as follows: "Plumix", a product by Information Service Center, Institute of Science and Technology Research, Thailand for inhibition of gastrointestinal pathogens in chickens [28]; "betel oil", by Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Thailand, claimed to have an anti-microorganism effect [29]; "Plugenol gel", a product by Thai Herbal Products Co., Ltd, for treatment of microbial infection and inflammatory [30]. The research in this study aims to investigate various betel-like-scented species for a preliminary chemical information analysis using hexane extracts.

2. MATERIALS AND METHODS

2.1 Plant Materials

The 43 *Piper* species in Thailand were explored in the areas as described by Chaveerach et al. [11,12] and screened for their betel scent. Species identifications were done following the literature and compared with the specimens kept at Khon Kaen University. Leaf samples from several individuals of the betel-like-scented species were collected for chemical extraction. Voucher specimens were kept at the Department of Biology, Faculty of Science, Khon Kaen University. No specific permits were required for the described field studies because the locations are not privately owned nor are they protected in any way. Moreover the field studies did not involve endangered or protected species.

2.2 Preparation of Chemical Extracts

The extracts were prepared, and the chemical contents were analyzed by gas chromatography-mass spectrometry (GC-MS). The leaf samples were rinsed with water and air-dried to get rid of water. The 25 g of leaf samples were ground into a powder, mixed with a 120 mL hexane solvent (analytical grade), and filtered at room temperature. A 90 mL filtrate was obtained and stored at -20°C until used for the GC-MS analysis.

2.3 GC-MS Analysis and Identification of Components

The GC-MS analysis of the crude extracts was performed using an Agilent Technologies GC 6890 N/5973 inert MS fused with a capillary column (30.0 m × 250 mm × 0.25 mm). Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injection and mass-transferred line temperature were set at 280°C. The oven temperature was programmed for 70°C to 120°C at 3°C/min, then held isothermally for 2 min, and finally raised to 270°C at 5°C/min. A 1 μL aliquot of the crude extract was injected in the split mode. The relative percentage of the crude constituents was expressed as the percentage using peak area normalization. Identification of the components of the crude extracts was assigned by comparison of the mass spectra obtained with those of the reference compounds stored in the Wiley 7N.1 library.

3. RESULTS AND DISCUSSION

3.1 The Betel-Like-Scented Piper Species

There were nine species found to be aromatic betel-like plants, viz. *P. betle* L., *P. betloides* Chaveer. & Tanomtong, *P. crocatum* Ruiz et Pavon, *P. maculaphyllum* Chaveer. & Sudmoon, *P. rubroglandulosum* Chaveer. & Mokkamul, *P. semiimmersum* C.DC., *P. submultinerve* C.DC., *P. tricolor* Y.C.Tseng, and *P. yinkiangense* Y.C.Tseng. The voucher specimen numbers and sites of specimen collection are shown in Table 1. *Piper betle* is found as a cultivated plant in all regions of Thailand. Except for *P. betle*, most of the species are wild, however *P. maculaphyllum*

and *P. crocatum* are often collected from the wild and grown as decorative plants. The images of all of the betel-like-scented *Piper* species are shown in Figure 1.

Table 1. Details of specimen collection.

Species	Voucher specimen number	Site of specimen collection
P. betle L.	A. Chaveerach 16	Cultivated, Muang District, Khon Kaen Province, Northeastern Thailand
P. betloides Chaveer. & Tanomtong	A. Chaveerach 171	Doi Suthep-Pui National Park, Chiang Mai Province, Northern Thailand
P. crocatum Ruiz et Pavon	A. Chaveerach 12	Garden and Development Department, Queen Sirikit Botanic Garden, Chiang Mai Province, Northern Thailand
P. maculaphyllum Chaveer. & Sudmoon	A. Chaveerach 126	Punyaban Waterfall, Lum Nam Kraburi National Park, Ranong Province, Southern Thailand
P. rubroglandulosum Chaveer. & Mokkamul	A. Chaveerach 319	Khao Phra Thaeo Wildlife Conservation Development and Extension Center, Phuket Province, Southern Thailand
P. semiimmersum C.DC.	A. Chaveerach 115	Sri Pungnga National Park, Pungnga Province, Southern Thailand
P. submultinerve C.DC.	A. Chaveerach 223	Doi Suthep-Pui National Park, Chiang Mai Province, Northern Thailand
P. tricolor Y.C.Tseng	A. Chaveerach 64	Sri Pungnga National Park, Pungnga Province, Southern Thailand
P. yinkiangense Y.C.Tseng	A. Chaveerach 133	Khao Sok National Park, Surat Thani Province, Southern Thailand

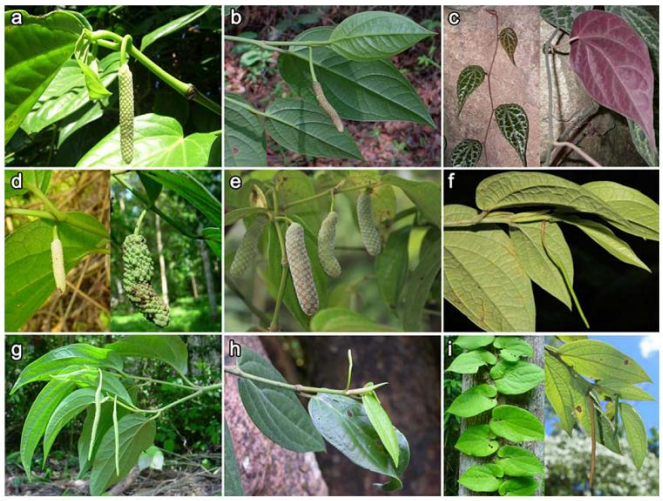

Figure 1. *Piper betle* (a) and the eight betel-like-scented species: *P. betloides* (b), *P. crocatum* (c), *P. maculaphyllum* (d), *P. rubroglandulosum* (e), *P. semiimmersum* (f), *P. submultinerve* (g), *P. tricolor* (h), and *P. yinkiangense* (i).

3.2 Chemical Identification of the Betel-Like-Scented Species Using GC-MS

The preliminary phytochemical screening of the hexane crude extract of nine *Piper* species shows the presence of different aromatic compounds. The total ionic chromatographs (TIC), showing the peak identities of the compounds from the nine individual species are given in Figure 2. The identified chemical compounds and their relative contents in each species are provided in Table 2.

Piper betle contains major chemicals, such as 80.52% eugenol (or isoeugenol, 4-cyclopropyl-2-methoxyphenol or chavibetol), which are identical to the constituents in earlier studies [15-17]. It also contains caryophyllene and some other minor chemicals, as shown in Table 2.

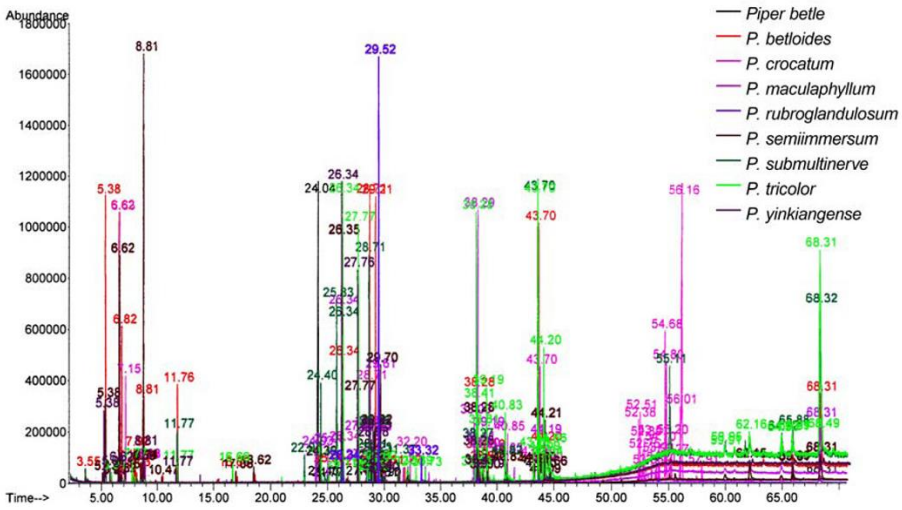

Figure 2. GC-MS chromatograms of hexane crude extracts from the leaves of the nine examined *Piper* species.

Table 2. Preliminary phytochemicals identified in the hexane crude extracts from the leaves of the nine examined *Piper* species.

Compound	Formula	Relative content**								
		P. betle	*P. betloides*	*P. crocatum*	*P. maculaphyllum*	*P. rubroglandulosum*	*P. semiimmersum*	*P. submultinerve*	*P. tricolor*	*P. yinkiangense*
Eugenol or Isoeugenol or Chavibetol or 4-cyclopropyl-2-methoxyphenol	$C_{10}H_{12}O_2$	80.52	-	-	3.24	-	-	-	-	-
Germacrene-D or β-cubebene or α-cubebene or α-ylangene or Naphthalene, 1,2,3,4,4a,5,6, 8a-octahydro or Bicyclo [4.4.0]dec-1-ene, 2-isopropyl-5-m	$C_{15}H_{24}$	5.21	13.29	4.59	3.78	-	-	12.13	-	-
Germacrene-A	$C_{15}H_{24}$	-	-	-	24.26	-	-	-	-	-
Trans-caryophyllene	$C_{15}H_{24}$	3.83	5.89	2.06	13.44	5.33	15.30	8.56	14.59	39.44
α-amorphene	$C_{15}H_{24}$	3.32	-	-	-	-	-	-	-	-
α-humulene or α-caryophyllene or cis,cis,cis-1,1,4,8-tetramethyl-4, 7, 10-cycloundecatriene	$C_{15}H_{24}$	-	-	1.12	3.61	-	5.21	-	9.43	35.03
Phytol or Phytol isomer	$C_{20}H_{40}O$	1.67	8.93	4.37	-	-	-	24.75	-	-
Bicyclogermacrene or 1H-cycloprop[e]azulene or Naphthalene,1,2,3,4,4a,5,6, 8a-octahydro	$C_{15}H_{24}$	1.24	12.55	-	10.98	-	-	-	-	-
α-pinene or Tricyclene or γ-terpinene or 4-carene or Tricycle[2.2.1.02,6] heptane,1,7,7-trimethyl-3-carene	$C_{10}H_{16}$	-	9.42	-	-	-	3.40	-	-	6.03
Sabinene or β-phellandrene or β-terpinene or β-thujene or β-pinene or Cyclohexane or 4-methylene-1-(1-methylethyl)	$C_{10}H_{16}$	-	18.43	10.17	-	-	32.91	-	-	3.65
1,3-dimethyl-4-azaphenanthrene or 4'methy-2 phenylindole	$C_{15}H_{13}N$	-	9.16	-	-	-	-	-	-	-
L-linalool	$C_{10}H_{18}O$	-	4.38	-	-	-	-	2.38	-	-
1,8-cineole	$C_{10}H_{18}O$	-	-	-	1.39	-	-	-	-	-
4-allyloxy-6-methoxy-N,	$C_9H_{14}NO_2$	-	-	6.54	-	-	-	-	-	-
N-dimethyl-1,3,5-										
β-myrcene	$C_{10}H_{16}$	-	-	4.01	-	-	-	-	-	-
β-elemene	$C_{15}H_{24}$	-	-	-	3.01	-	-	-	-	-
β-bisabo	$C_{15}H_{24}$	-	-	-	-	-	5.98	-	-	-
Methyl (25R)-5-oxo-A-nor-3, 5-secospirost	$C_{27}H_{42}O_5$	-	-	3.65	-	-	-	-	-	-
Palmitic acid	$C_{16}H_{32}O_2$	-	-	2.26	-	-	-	-	2.71	-
24(Z)-methyl-25-homocholesterol or 1,5-dimethyl-6-(1,5-dimethylhexyl) -15,16	$C_{29}H_{50}O$	-	-	-	9.66	-	-	19.15	-	-
1-(1,5-dimethyl-4-hexenyl) -4-methylbenzene	$C_{15}H_{22}$	-	-	-	3.39	-	-	-	-	-
2,6-bis(1,1-dimethylethyl) -4-methylphenol (butylated hydroxytoluene)	$C_{15}H_{24}O$	-	-	-	-	89.83	-	-	-	-
9-octadecenoic acid or Heptadecene-(8)-carbonic acid-(1)	$C_{18}H_{34}O_2$	-	-	-	-	-	5.00	-	-	-
or 9,12,15-octadecatrien-1-ol,(z,z,z)- Neophytadiene	$C_{20}H_{38}$	-	4.13	-	-	-	4.06	-	13.85	-

Table 2. Continued.

Compound	Formula	Relative content**								
		P. betle	*P. betloides*	*P. crocatum*	*P. maculaphyllum*	*P. rubroglandulosum*	*P. semiimmersum*	*P. submultinerve*	*P. tricolor*	*P. yinkiangense*
Vitamin e	$C_{29}H_{50}O_2$	-	-	-	-	-	2.66	-	-	-
α-gurjunene	$C_{15}H_{24}$	-	-	-	-	-	-	9.20	-	-
α-copaene	$C_{15}H_{24}$	-	-	-	-	-	-	5.52	-	3.19
(23s)ethylcholest-5-en-3. β-ol	$C_{29}H_{50}O$	-	-	-	-	-	-	-	18.66	-
9,12,15-octadecatrien-1-ol,(z,z,z)- or 7,10,13-hexadecatrienoic acid	$C_{18}H_{32}O$	-	-	-	-	-	-	-	7.03	-
3,7,11,15-tetramethyl-2-hexadecene	$C_{20}H_{40}$	-	-	-	-	-	-	-	-	1.52
4-allyl-1, 2-diacetoxy benzene	$C_{13}H_{14}O_4$	-	-	-	-	4.84	-	-	-	-
unknown*		4.20	13.83	61.24	23.24	-	25.48	18.32	33.73	11.15

*Unknown from each species is not the only one compound and may not be the same compound through all species.

** - means not detected

Isoeugenol and chavibetol, isomers of eugenol, compounds of the phenylpropanoid group are some of the primary constituents in the extract of *P. betle*. *Piper maculaphyllum* also contains isougenol or chavibetol (3.24%), though in smaller amounts.

Caryophyllene is an important constituent that was found in all nine of the studied species, though in different quantities: *P. yinkiangense* (39.44%); *P. semiimmersum* contains trans-caryophyllene (15.30%) and α-caryophyllene (5.21%); *P. tricolor* (14.59%); *P. maculaphyllum* (13.44%); *P. submultinerve* (8.56%); *P. betloides* (5.89%); *P. rubroglandulosum* (5.33%); *P. betle* (3.83%) and *P. crocatum* (2.06%).

Sabinene and phellandrene are monoterpene constituents of essential oils and were found in relatively high percentage in all four of the studied species, viz. *P. semiimmersum*, (32.91%); *P. crocatum* (10.17%); *P. betloides* (18.43%); and *P. yinkiangense* (3.65%). Phellandrene is a constituent of the essential oil of Eucalyptus dives [24]. Phellandrenes are used in fragrances because of their pleasant aromas; the odor of β-phellandrene has been described as peppery-minty and slightly citrusy. Sabinene is a natural bicyclic monoterpene, isolated from the essential oils of a variety of plants, including holm oak (*Quercus ilex*) and Norway spruce (*Picea abies*), which are not grown in Thailand. In addition, sabinene is one of the chemical compounds that contributes to the spiciness of black pepper (*Piper nigrum*) and is a major constituent of carrot seed oil. It also occurs in tea tree oil at a low concentration and is present in the essential oil obtained from nutmeg [25].

Germacrene A and germacrene D, sesquiterpenes, are significant components of *P. maculaphyllum* containing 24.26% and 3.78% respectively. The sesquiterpenes are also present in *P. betloides* (13.29%), *P. submultinerve* (12.13%), *P. betle* (5.21%), and *P. crocatum* (4.59%).

The minor common volatile aromatic compounds are found in *P. betloides* (9.42% α-pinene and 4.38% L-linalool), *P. semiimmersum* (3.40% α-pinene), *P. submultinerve* (2.38% L-linalool), and *P. yinkiangense* (6.03% α-pinene). These compounds are components in both branded and imitation perfumes [31]. *Piper rubroglandulosum* contains significantly

high amounts of butylated hydroxytoluene (89.83%) also known as butylhydroxytoluene (BHT) or 2,6-bis (1,1-dimethylethyl)-4-methylphenol. BHT is a lipophilic (fat-soluble) organic compound, a derivative of phenol that is useful against viruses in the herpes family, for its antioxidant properties, as a food additive, and as a prevention for apoptosis in etiolated seedlings. BHT induces large structural changes in the organization of all cellular organelles and the formation of new unusual membrane structures in the cytoplasm [32,33]. This is very interesting, because people are very close to the species. The people in Southern Thailand have been using *P. rubroglandulosum* for chewing (instead of *P. betle*) and for medicinal purposes for long time. This high content compound may be a key active ingredient which should be further examined.

The preliminary study on the chemical constituents of betel-like-scented *Piper* species (screened down to nine species) uses the non-polar solvent hexane for the extraction process, thus isolating the major monoterpenes (sabinene and phellandrene), sesquiterpenes (germacrene A, germacrene D, and caryophyllene), and phenylpropenes (eugenol, isoeugenol, and chavibetol). Even though hexane was used to extract chemical substances for the GC-MS analysis rather than the essential oils, the chemical constituents found tended to be similar, for instance among *P. betle*, *P. nigrum*, and *P. guineense.* A study of *P. nigrum* and *P. guineense* essential oils also provided evidence of the similarity among the chemical constituents [10].

3.3. Uses of Piper Species

Many research studies have been conducted on various *Piper* species, such as *P. nigrum*, *P. guineense* and *P. chaba* to investigate their potential uses. *Piper betle* has been studied for many years, particularly for its compounds and usefulness in treating diseases [10,34,35]. The plants are still of interest among researchers for their pharmacological activities, antimicrobial, and insecticidal properties, however, more information about the other Piper species with a similar aroma could be obtained. The study, therefore, examines whether the eight species identified as aromatic betel-like plants have any important identical chemical substances. The research findings would contribute to the data on aromatic plants and provide the basis for further research investigations.

In addition to eugenol (or isoeugenol, 4-cyclopropyl-2-methoxyphenol or chavibetol) reported in P. betle [15,16,17], caryophyllene is another compound found from this study.

The main compounds, which include β-caryophyllene, germacrene D, limonene, β-pinene, α-phellandrene and α-humulene, are identical to the compounds found in the Piper species from Cameroon [10]. The minor constituents, including δ-carene, β-phellandrene, isoborneol, α-guaiene, sarisan, elemicin, calamenene, caryophyllene alcohol, isoelemicin, T-muurolol, cubenol, and bulnesol, are of great importance for the characteristic pepper odor of these *Piper* species. Additionally, minor common volatile aroma compounds, including α-pinene and L-linalool found in *P. betloides*, *P. semiimmersum*, *P. submultinerve*, and *P. yinkiangense* are components found in both branded and imitation perfumes [31].

4. CONCLUSION

The GC-MS analysis of the hexane crude extracts of different *Piper* species shows the presence of a composition of volatile chemicals from the phenylpropene, monoterpene, and sesquiterpene groups. The identification of economically beneficial chemicals existing in many other plant species is important for industry and is also

beneficial in providing a growing population with supplements from natural sources.

Many plant species in the wild are not exploited or used because they are not known or well characterised. The hardiness, good growth, and abundance of branches of the wild species should be studied for uses in native forms, modified forms, and purified chemicals. The diversity of betel-like-scented species and the chemical components could be useful for industries to produce flavors, fragrances, cosmetics, drugs, perfumes, and food preservatives.

ACKNOWLEDGEMENTS

The authors are thankful for the financial support provided by the Thailand Research Fund and Khon Kaen University through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0027/2553) to Arisa Sanubol and Arunrat Chaveerach and the Research Career Development Grant (Grant No. RSA5580054). We wish to acknowledge the support of the Khon Kaen University Publication Clinic, Research and Technology Transfer Affairs, Khon Kaen University for their assistance.

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Article 2: In Press

Sanubol, A., Chaveerach, A.*, Sudmoon, R., Tanee, T., and Liehr, T. Verification of selected *Piper* species (Piperaceae) using morphological characters, molecular data, and chemical constituents. Malayan Nature Journal (SNIP 0.146).

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Title: Verification of selected *Piper* species (Piperaceae) using morphological characters, molecular data, and chemical constituents

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Running head: Verification of *Piper* species based on molecular and chemical data

Verification of selected *Piper* species (Piperaceae) using morphological characters, molecular data, and chemical constituents

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Abstract

Piper argyritis Ridl. ex C.DC., *P. betle* L., *P. maculaphyllum* Chaveer. & Sudmoon, *P. pendulispicum* C.DC., and *P. rubroglandulosum* Chaveer. & Mokkamul are very important species for biodiversity, food, and medicine. Accordingly, proper identification should be verified using available data, especially since some controversy exists concerning the distinctiveness of certain species pairs (e.g., *P. argyritis/ P. maculaphyllum*, *P. betle/ P. rubroglandulosum*, and *P. pendulispicum/ Piper* sp.) based solely on morphology. In this study, a dendrogram constructed from 1,015 random amplified polymorphic DNA (RAPD) banding patterns revealed this method to be a powerful and efficient tool to distinguish among *Piper* species. Individuals of *P. betle* and *P. rubroglandulosum* are grouped in each species clade, with genetic similarity (S) levels of 0.84-0.91 and 0.87, respectively, and all studied species are recognized as separate species, with S values of 0.57 to 0.82. The S levels of males and females of *P.*

betle and *P. rubroglandulosum* are 0.84-0.91, within the level considered as identical species. Moreover, an analysis of chemical constituents provided additional species distinctions, as *P. rubroglandulosum* contains 2,6-bis(1,1-dimethylethyl)-4-methylphenol, trans-caryophyllene, and 4-allyl-1,2-diacetoxy benzene, while *P. betle* contains eugenol, isoeugenol, and chavibetol.

Key words: chemical constituents, genetic similarity, *Piper*, RAPD

1. Introduction

The genus *Piper* in Thailand has been studied with regard to species diversity, usage, new species, and molecular markers (Chaveerach et al., 2002, 2006a, 2006b, 2007a, 2007b, 2008a, 2010; Suwanphakdee & Chantaranothai, 2008, 2011; Sudmoon et al., 2011, 2012; Suwanphakdee & Simpson, 2012). Because pipers are of great interest for use as spices, medicines, and insecticides (Chaveerach et al., 2006; Scott et al., 2008; Fan et al., 2011), correct species identification is important. This is commonly based on morphological characteristics alone, but for some pipers, such as dioecious species, molecular data should be considered because morphology among and within species can be rather similar (Chaveerach et al., 2008a). Taxonomists who specifically concentrate on the specimen type might encounter identification biases in species overlap and might not consider such data as molecular evidence. For example, based on morphological characteristics, *P. maculaphyllum* Chaveer. & Sudmoon has been reduced to synonymy under *P. argyritis* Ridl. ex C.DC., and *P. rubroglandulosum* Chaveer. & Mokkamul has been synonymized under *P. betle* L. (Suwanphakdee & Chantaranothai, 2011). Therefore, to overcome potential identification problems associated with a uniform, worldwide species type and to enable the use of these species in domestic and industrial applications, other characteristics, such as chemical composition and molecular data, must be used. DNA fingerprinting, which is based on the polymerase chain reaction (PCR), is an efficient and reliable technology used to classify plant groups, including apples (Koller et al., 1993), chestnuts (Galderisi et al., 1998), *Curcuma* species (Chaveerach et al., 2008b), and *P. protrusum* Chaveer. & Tanee (Sudmoon et al., 2011).

Several substances have been found in *P. betle*, depended on the studies, namely eugenol and hydroxychavicol (Amonkar et al., 1986), allopyrocatecholdiacetate, safrole,

and B-phellandrene (Arambewela et al., 2005), chavibetol, chavibetol acetate, and caryophyllene (Suppakul et al., 2006), chavicol, terpene, and sesquiterpene (Manigauha et al., 2009), and hydroxychavicol, hydroxychavicol acetate, allylpyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A, and piperol B (Kumar et al., 2010). These compounds possess important anti-septic, anti-diabetic, cardiovascular protective, anti-inflammatory, anti-immunomodulatory, anti-ulcer, hepato-protective, and anti-infective properties (Kumar et al., 2010). The chemical composition of natural products has been successfully elucidated by gas chromatography-mass spectrometry (GC-MS) analysis, as reported by Andersen & Taglialatela-Scafati (2005), Quang et al. (2008), Schierling et al. (2011), and Demiray et al. (2013).

The aim of this study was to verify the identification of pairs of presumably different species, *P. argyritis* (De Candolle, 1912) and *P. maculaphyllum* (Chaveerach et al., 2008a), and *P. betle* and *P. rubroglandulosum* (Chaveerach et al., 2008a), using a combination of morphological characteristics, genome analyses with random amplified polymorphic DNA (RAPD) marker, and their chemical constituents. The RAPD marker has been proved to be a powerful tool for taxon identification (Chalageri & Babu, 2012; Muthiah et al., 2013). The species pair female *P. pendulispicum* and *Piper* sp., firstly identified as male *P. pendulispicum* by Chaveerach et al. (2008a), was also compared.

2. Materials and methods

2.1. Plant collection

Plants were sampled at the different sites and provinces shown in Table 1. Figure 1 shows a map of Thailand and highlights the different provinces where specimens were collected. *Piper betle* and *P. rubroglandulosum* were transferred from southern

Thailand and planted in Khon Kaen province under same environmental conditions for a period of two years before collecting of leaves for chemical extraction to eliminate the climate factors that are related to the production of aromatic compounds.

2.2. Morphological comparisons

The morphological characteristics of each pair of species, *P. betle* and *P. rubroglandulosum* published by Chaveerach et al. (2008a), and *P. argyritis* published by De Candolle (1912) and *P. maculaphyllum* published by Chaveerach et al. (2008a), were compared.

2.3. Molecular analyses

The molecular characteristics were assessed following DNA extraction, DNA fingerprinting with RAPD, and dendrogram construction.

2.3.1. DNA extraction

Genomic DNA was extracted from all collected samples using the Plant Genomic DNA Extraction Kit (RBC Bioscience). The extracted DNA was analyzed by 0.8% agarose gel electrophoresis and ethidium bromide staining. The quality and quantity of DNA were determined using a gel documentation instrument. The DNA samples were diluted to a final concentration of 20 ng/μL in Tris-EDTA buffer (TE; consists of 10 mM Tris-Cl, 1 mM EDTA, pH 8.0), and these dilutions were used as the DNA template in PCR reactions.

2.3.2. DNA fingerprinting using RAPD marker and dendrogram construction

Amplifications were performed for all species in 25-μL reactions consisting of GoTaq Green Master Mix (Promega), 0.5 μM primer, and 5 ng DNA template. Thirty-six RAPD primers were screened, and the 22 primers successfully amplified were as follows (5′ to 3′): GGTGGTCAAG, TGCCGAGCTG, AATCGGGCTG,

GGGTAACGCC, CAATCGCCGT, GTTGCGATCC, CAAACGTCGG, CACAGGCGGA, TGAGCGGACA, GTACGCCCGA, CTCCTGCCAA, TGGGCGTCAA, CAGGCCCTTC, GTTTCGCTCC, GGACTGGAGT, GGTGACGCAG, GTCCACACGG, CTGCTGGGAC, GTAGACCCGT, TCCGCTCTGG, GGAGGGTGTT, and AGGGAACGAG. Each reaction mixture was incubated at 94 °C for 3 min, and the amplification was performed with the following thermal cycles using a Swift Maxi Thermal Cycler (Esco Micro Pte., Ltd): 35 cycles of denaturation for 1 min at 94 °C, 2 min annealing at 40 °C, 2 min extension at 72 °C, and a 7-min final extension at 72 °C. The amplification products were detected by 1.2% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) and visualized using ethidium bromide staining. The RAPD bands resulting from successfully amplified primers and discerned by agarose gel electrophoresis were documented as diallelic characters (present = 1, absent = 0). These bands were used to construct a dendrogram with the NTSYS-pc 2.1 program (Rohlf, 1998).

2.4. Comparison of the chemical constituents by gas chromatography-mass spectrometry (GC-MS)

Samples of *P. betle* ♂, *P. betle* ♀, *P. rubroglandulosum* ♂, and *P. rubroglandulosum* ♀ were prepared for chemical extraction, and the chemical constituents were analyzed by GC-MS.

2.4.1. Preparation of chemical extracts

The air-dried leaves (25 g) were ground, added to 120 mL hexane solvent (analytical grade), and filtered at room temperature. Ninety mL of filtrate was obtained and stored at -20 °C until the GC-MS analysis.

2.4.2. GC-MS analysis

The GC-MS analysis of the crude extracts was performed using an Agilent Technologies GC 6890 N/ 5973 inert Mass Selective Detector fused with a capillary column (30.0 m × 250 µm × 0.25 µm). Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The injection and mass-transfer line temperatures were set to 280 °C. The oven temperature was programmed from 70 to 120 °C at 3 °C/min, held isothermally for 2 min, and then raised to 270 °C at 5 °C/min. A 1-µL aliquot of crude extract was injected in the split mode. The relative percentage of crude constituents was expressed as a percentage by peak area normalization. The identity of the components of the crude extract was assigned by a comparison of their GC-MS spectra to the Wiley 7N.1 library.

3. Results

The morphological characteristics of *P. argyritis* and *P. maculaphyllum*, including plant sexuality (monoecious vs. dioecious), leaf shape and type, flowering spike, and fruiting spike, are shown in Table 2, confirming that these plants are, indeed, different species. The significant morphological characteristics of *P. maculaphyllum* that differed from those of *P. argyritis* are shown in Figure 2 (A-C). The main differences involved leaf shape, leaf dappling, and spikes of both male and female flowers in *P. maculaphyllum*, a monoecious plant, and the male plant of *P. argyritis* (a dioecious plant). In addition to different distributions, morphological data revealed different characteristics for *P. rubroglandulosum* and *P. betle*, including the node and internode, leaf apex, arrangement of flowers along the rachis, and bearing fruiting spike, as shown in Table 3

and Figure 2 (D-I). Morphological characters of *P. pendulispicum* and *Piper* sp. are shown in Figure 2 (J-L).

A total of 1,015 RAPD bands, comprising 383 characters (Figure 3), was produced from the 22 successful primers. The resulting dendrogram (Figure 4) separated the outgroup *Peperomia pellucida* and appropriately grouped each studied species in distinct lineages.

The genetic similarity (S) levels of the six different species are shown in Table 4, ranging from 0.57 between *Piper* sp. and *P. betle* ♀ 3 to 0.82 between *P. argyritis* and *P. pendulispicum* ♀. Individuals of the same species showed S levels ranging from 0.84 to 0.97 for *P. betle* and 0.87 for *P. rubroglandulosum*. The S levels of the males and females were 0.84 for *P. betle* and 0.87 for *P. rubroglandulosum*, indicating an identical species, whereas those of *P. betle* and *P. rubroglandulosum*, 0.64-0.75, indicated different species. An S value for *P. argyritis* and *P. maculaphyllum* of 0.67 confirmed their different species designation. An S value for *Piper* sp. and *P. pendulispicum* of 0.81 is in the range of different species.

The preliminary phytochemical screening of the crude hexane extract of male and female plants of *P. betle* and *P. rubroglandulosum* demonstrated the presence of different chemical compounds. The chemical compounds, their retention times, and relative contents for each species are provided in Table 5. The total ion chromatographs (TICs), showing the peak identities of the compounds identified, are provided in Figure 5. The major chemicals of both male and female *P. betle* were eugenol, isoeugenol, and chavibetol, whereas 2,6-bis(1,1-dimethylethyl)-4-methylphenol, trans-caryophyllene, and 4-allyl-1,2-diacetoxy benzene were abundant in both male and female *P. rubroglandulosum*. The other compounds from *P. betle* are unknown.

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4. Discussion

Piper argyritis and *P. maculaphyllum* are unquestionably distinct species, with different important morphological characteristics, as shown in Table 2. These differences were, in particular, with regard to the male flowers of dioecious *P. argyritis*; *P. maculaphyllum* is monoecious. In summary, the morphological data of *P. argyritis* published by De Candolle in 1912 support the differences between *P. maculaphyllum* and *P. argyritis*, which were identified as distinct species in the present study.

It is clear that only the important morphological characteristics (Table 3) of the pair of species compared, *P. betle* and *P rubroglandulosum*, can be used to distinguish these two species. We assume that *P. betle* and *P. rubroglandulosum* were once the same species, as indicated by the similar female flowers and other characters, but that geographical barriers led to morphological changes for long period, particularly with regard to fruiting spikes. *Piper rubroglandulosum* is specifically distributed in southern Thailand, whereas *P. betle* is distributed throughout all Thailand regions and shows no fruiting spikes, even in the southern Thailand in the same growing area as *P. rubroglandulosum*.

Therefore, we propose that morphological characterization should be the first tool used for plant identification, because of it is the easiest aspect for distinguishing species. In some cases, incomplete investigation or incomplete plant characteristics might influence the interpretation of the various morphological traits in plants, as demonstrated by Suwanphakdee & Chantaranothai (2011), who mistakenly concluded that *P. maculaphyllum* and *P. argyritis*, and *P. betle* and *P. rubroglandulosum*, were identical species.

Thus, identification of morphologically similar pipers should be corroborated using additional characterization tools because certain species pairs have several variable features and are widely distributed with overlapping geographical locations, often resulting in species confusion. To overcome these problems and accurately identify plants for domestic and industrial applications, we suggest that characterization of the genus *Piper* should be investigated based on genetic information. The present research used molecular data obtained by RAPD fingerprinting, which indicated different species with S values of 0.57-0.82 and identical species with S values of 0.84-0.97.

The S values of 0.67 between *P. argyritis* and *P. maculaphyllum* and 0.64-0.75 between *P. betle* and *P. rubroglandulosum* indicate different species and support the morphological data used to identify distinct species. Further support for this designation arises from the dendrogram (Figure 4), which clearly indicates that each taxon formed a monophyletic group. In addition, based on the dendrogram, *P. argyritis* appears to be more similar genetically to *P. pendulispicum* than *P. maculaphyllum* this emphasizes that they are distinct taxa.

The S value of 0.81 between *Piper* sp. and *P. pendulispicum* indicated that they are different species, as also suggested by many morphological characters. Formerly *Piper* sp. was identified by Chaveerach et al. (2008a) as *P. pendulispicum* ♂ based on several features, particularly leaf shape; after revisiting the distribution areas years later, some differences, such as leaf color, usage, distribution area, and sex, confirmed the conclusion of the present study. *Piper* sp., locally called ‘sakhan daeng’, has leaves that are darker green, a male spike, and red wood. The root and stem of *Piper* sp. are soaked in bottles of Thai white whisky until the color of the whisky becomes red, and the local men often drink a small glass of this whisky a day to improve blood circulation and

virility. The distribution areas of *Piper* sp. include high elevations on mountains and in evergreen forests in northeastern and northern Thailand. *Piper pendulispicum* ♀ presents female spikes becoming fruiting spikes, even when there are no male plants nearby, and cream-colored wood. Its soft stem is often used in a soup called 'kaeng khae' both in northern and northeastern Thailand. It is popularly cultivated by local people in home gardens in northern Thailand, whereas, in Loei province of northeastern Thailand, it always grows in forests and is not cultivated. Although chemicals were not extracted from these two species, based on morphological and genetic differences it is likely that the chemical composition is also different. We propose that *Piper* sp. is not *P. pendulispicum* ♂, and upon further analysis it may be determined that this is a new *Piper* species.

Although they were grown in the same environment, different chemicals were found between *P. betle* and *P. rubroglandulosum* whereas similar compounds were found between the males and females of each species. The major chemicals of both male and female *P. betle* were eugenol/isoeugenol/chavibetol, whereas 2,6-bis(1,1-dimethylethyl)-4-methylphenol, trans-caryophyllene, and 4-allyl-1,2-diacetoxy benzene were found in male and female *P. rubroglandulosum*. The major chemicals contained in *P. betle* are identical to the constituents reported in several previous studies (Amonkar et al., 1986; Arambewela et al., 2005; Suppakul et al., 2006). *Piper betle* also contains caryophyllene and some other minor chemicals.

Currently, all the data indicate that *P. rubroglandulosum* and *P. betle*, and *P. argyritis* and *P. maculaphyllum*, represent distinct species. This finding is in contrast to the conclusions of Suwanphakdee & Chantaranothai (2011) that *P. maculaphyllum* is a synonym of *P. argyritis* and *P. rubroglandulosum* is a synonym of *P. betle*.

Additionally, the data for *P. pendulispicum* ♀ and *Piper* sp., including morphological characters, usages, and RAPD banding, suggest that these are distinct species, in contrast to Chaveerach et al. (2008a) who first identified *Piper* sp. as *P. pendulispicum* ♂. The different sex *Piper* species plants should be carefully observed before determining the same or different species.

The present study used a small number of individuals for the genetic similarity analysis. However, we employed several methods, including morphological characteristics, chemicals, usages, and molecular data, and the results are all congruent. The molecular study, a subset of a genomic study, used 1-2 individual species; indeed, such studies are usually much smaller than morphological studies, often as small as a single individual (Hillis, 1987). Analyses using a large sample size are often limited by the availability of specimens, the expense of the analysis, and the time-consuming nature.

Many species of the genus *Piper* are dioecious (e.g., *P. argyritis*, *P. caninum*, *P. colubrinum*, *P. montium*, *P. pendulispicum*, *P. semiimmersum*, and *P. tricolor* (Chaveerach et al., 2008a)), which complicates identification based on morphological characteristics. Using molecular and chemical techniques similar to those of the present study, we hope to verify the identification of all dioecious *Piper* species in Thailand.

Acknowledgements

The authors are thankful for the financial support provided by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0027/2553) and Khon Kaen University to Arisa Sanubol and Arunrat Chaveerach. Part of the

research is also supported by the Research Career Development Grant (Grant No. RSA5580054).

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Table 1. *Piper* species collected from various provinces and regions of Thailand. All voucher specimens were kept at Department of Biology, Faculty of Science, Khon Kaen University.

Plant sample	Voucher number	Province of specimen collection	Region of Thailand
P. argyritis	AC11	Chiang Mai	Northern Thailand
*P. argyritis**	-	-	-
P. betle ♂	AC341	Si Sa Ket	Northeastern Thailand
P. betle 1 ♀	AC16.1	Khon Kaen	Northeastern Thailand
P. betle 2 ♀	AC16.2	Khon Kaen	Northeastern Thailand
P. betle 3 ♀	AC16.3	Sa Kaeo	Central Thailand
P. betle 4 ♀	AC16.4	Loei	Northeastern Thailand
P. maculaphyllum	AC127	Ranong	Southern Thailand
P. pendulispicum ♀	AC82	Chiang Mai	Northern Thailand
P. rubroglandulosum ♂	AC316	Phuket	Southern Thailand
P. rubroglandulosum ♀	AC340	Phuket	Southern Thailand
Piper sp.	AC868	Chiang Mai	Northern Thailand

*Latinized description published by De Candolle (1912)

Table 2. Comparison of principal morphological characteristics of *Piper argyritis*, firstly described by De Candolle (1912), and *P. maculaphyllum*, firstly described by Chaveerach et al. (2008a).

Character	***P. argyritis***	***P. maculaphyllum***
Plant nature	dioecious	monoecious
Stem	branchlet glabrous	with white short hairs
Leaves	**One type**: oblong-ovate, base with slight equilateral rounded, apex sharply acuminate, 18 cm. long and 6 cm. wide.	**Two types**: Leaves on epiphytic branches - leaf blade thick, leathery, adaxially light green to dark pink dapple, abaxially green, ovate to broadly lanceolate, 7–15 cm wide, 20–22 cm long; apex acuminate; base cordate. Leaves on free branches - leaf blade thick, leathery, with scattered pellucid and brownish-red glands, shape and size same as on epiphytic branches; apex acuminate; base sub-rounded or subcordate with rounded and unequal lobes, basal lobes sometimes overlapping, both sides glabrous.
Spike	**Male**: 8 cm long, 3 cm thick, rachis hirsute; bracts glabrous, rhachis adnate and free margin, up to 1-5 mm. long; stamens 2, anthers reniform, 4-valvate, filament equilateral.	**Male:** pendulous, approx. 2 cm long, 0.2 cm in diameter; peduncle approx. 1 cm long; bract elliptic, peltate, stalk short and hairy, margin ciliate; stamens 2, with flat and unequal filament long. **Female:** similar to male spike, 2–8 cm long; stigmas 4. **Fruiting**: 2–10 cm long, 0.4–1 cm in diameter; peduncle 1.2–2.2 cm long; drupe hairy.

Table 3. Comparison of principal morphological characteristics of *P. betle* and *P. rubroglandulosum*, firstly described by Chaveerach et al. (2008a).

Character	***P. betle*** ♂	***P. betle*** ♀	***P. rubroglandulosum*** ♂	***P. rubroglandulosum*** ♀
Distribution area in Thailand	All Thailand regions	All Thailand regions	Southern Thailand	Southern Thailand
Stem	Stout with pinkish-stripe, straight node-internode branching on free branch, length of node on free branch 3.4-9.2 cm long.	Stout with pinkish-stripe, straight node-internode branching on free branch, length of node on free branch 2.5-10.5 cm long.	Slender to stout, stiff, often pinkish-green stripes, zigzag node-internode branching on free branch, length of node on free branch 2-7.7 cm long.	Slender to stout, stiff, often pinkish-green stripes, zigzag node-internode branching on free branch, length of node on free branch 1.5-2.8 cm long.
Leaves	petiole 1.7-3.4 cm long shape broadly ovate, 7.3-10.3 cm wide, 13-16 cm long, lacking reddish glands; base oblique cordate, apex acuminate	petiole 1.5-3.5 cm long shape broadly ovate, 7.7-11.3 cm wide, 12.7-14.3 cm long, lacking reddish glands; base cordate to oblique cordate; apex acuminate	petiole 0.7-1 cm long; shape ovate or elliptic, 4-5 cm wide, 7.5-11 cm long, densely reddish glandular base shallowly cordate, often oblique apex acute or cuspidate	petiole 0.5-1 cm long; shape ovate or elliptic, 4-5 cm wide, 7.5-11 cm long, densely reddish glandular base shallowly cordate, often oblique apex acute or cuspidate

	veins 7-8, two pairs basal, apical pair arising 2.4-3.3 cm above base.	veins 9, three pairs basal, apical pair arising 1.2-2.2 cm above base.	veins 7, two pairs basal, apical pair arising 0.3-0.9 cm above base.	veins 7, two pairs basal, apical pair arising 0.2-0.5 cm above base.
Spike	Cylindric, slender, pendulous, 0.2 cm wide, 6-6.5 cm long	Cylindric, slender, pendulous, 0.4-0.5 cm wide 3.1-3.7 cm long	Cylindric, slender, pendulous, 0.2 cm wide, 5.5-10 cm long	Cylindric, stout, pendulous, 0.3-0.5 cm wide 1.7-3.2 cm long
	Peduncle 2-3 cm long, glabrous	Peduncle 2-3 cm long, glabrous	Peduncle 1-2 cm long, rachis hairy	Peduncle 1-1.2 cm long, glabrous
	stamen 2	-	stamen 2	-
	stigma -	stigma 4-6, pubescent, female flowers spirally arranged on rachis	-	stigma 3-5, pubescent, female flowers not spirally arranged on rachis
	bract orbicular, peltate	bract orbicular, peltate	bract subrounded, margin free, stalk short and pubescent	bract subrounded, margin free, stalk short and pubescent
Fruiting spike	-	not fruiting, flower wilted and dried	-	pendulous, 3-7 cm long, drupe embedded in rachis, pubescent

Table 4. Similarity coefficients of six *Piper* species based on RAPD fingerprint data using 22 primers and the NTSYS-pc 2.1 program.

	P. betle ♂	*P. betle*1♀	*P. betle* 2 ♀	*P. betle* 3 ♀	*P. betle* 4 ♀	*P. rubroglandulosum* ♂	*P. rubroglandulosum* ♀	*Piper* sp.	*P. pendulispicum* ♀	*P. argyritis*	*P. maculaphyllum*	*Peperomia pellucida*
P. betle ♂	1.00											
*P. betle*1♀	0.91	1.00										
P. betle 2 ♀	0.88	0.92	1.00									
P. betle 3 ♀	0.88	0.92	0.97	1.00								
P. betle 4 ♀	0.84	0.87	0.89	0.91	1.00							
P. rubroglandulosum ♂	0.70	0.69	0.69	0.69	0.75	1.00						
P. rubroglandulosum ♀	0.68	0.67	0.65	0.64	0.72	0.87	1.00					
Piper sp.	0.60	0.58	0.58	0.57	0.60	0.62	0.63	1.00				
P. pendulispicum ♀	0.61	0.61	0.60	0.58	0.62	0.64	0.66	0.81	1.00			
P. argyritis	0.65	0.64	0.64	0.62	0.64	0.66	0.67	0.78	0.82	1.00		
P. maculaphyllum	0.66	0.64	0.65	0.64	0.65	0.65	0.67	0.63	0.64	0.67	1.00	
Peperomia pellucida	0.61	0.61	0.59	0.58	0.61	0.58	0.62	0.56	0.58	0.64	0.61	1.00

Table 5. Preliminary phytochemicals identified in crude hexane extracts of the leaves of *Piper betle* and *P. rubroglandulosum*.

Plant	**RT (min)**	**Compound**	**Formula**	**Mw**	**Relative content (%)**
P. betle ♂	24.030	Eugenol or chavibetol	$C_{10}H_{12}O_2$	164	39.273
	68.308	(23s)-ethylcholest-5-en-3.β.-ol	$C_{29}H_{50}O$	414	8.042
	31.780	1-hydroxy-1,7-dimethyl-4-Isopropyl-2,7-cyclodecadiene	$C_{15}H_{26}O$	222	7.443
	43.702	Phytol isomer	$C_{20}H_{40}O$	296	7.229
	6.627	Sabinene or β-phellandrene	$C_{10}H_{16}$	136	5.125
	28.513	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro or α-amorphene or bicyclo[4.4.0]dec-1-ene,2-isopropyl-5-m or α-copaene	$C_{15}H_{24}$	204	4.027
	38.278	Neophytadiene	$C_{20}H_{38}$	278	4.020
		Unknown	-	-	24.841

P. betle ♀	24.042	Eugenol or isoeugenol or chavibetol	$C_{10}H_{12}O_2$	164	80.524
	28.707	Germacrene-D	$C_{15}H_{24}$	204	5.210
	26.339	Trans-caryophyllene	$C_{15}H_{24}$	204	3.833
	28.513	α-amorphene	$C_{15}H_{24}$	204	3.319
	43.702	Phytol	$C_{20}H_{40}O$	296	1.669
	29.213	Bicyclogermacrene	$C_{15}H_{24}$	204	1.241
		Unknown	-	-	4.024
P. rubroglandulosum ♂	29.518	2,6-bis(1,1-dimethylethyl)-4-methylphenol	$C_{15}H_{24}O$	220	89.828
	26.339	Trans-caryophyllene	$C_{15}H_{24}$	204	5.330
	33.319	4-allyl-1,2-diacetoxy benzene	$C_{13}H_{14}O_4$	234	4.842
P. rubroglandulosum ♀	29.518	2,6-bis(1,1-dimethylethyl)-4-methylphenol	$C_{15}H_{24}O$	220	87.694
	26.340	Trans-caryophyllene	$C_{15}H_{24}$	204	6.167
	33.326	4-allyl-1,2-diacetoxy benzene	$C_{13}H_{14}O_4$	234	6.139

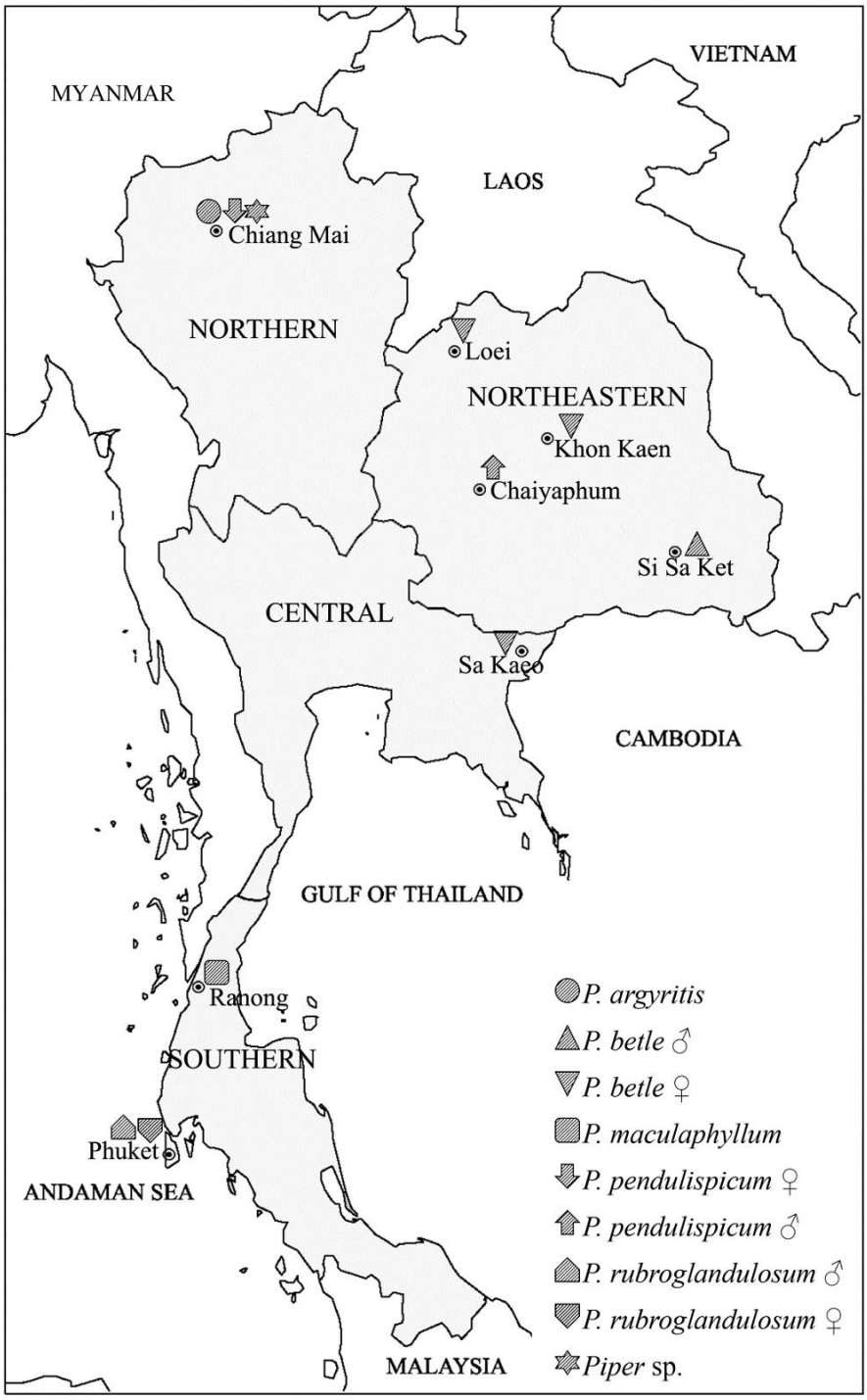

Figure 1. Map of Thailand showing the different provinces where the *Piper* species were collected.

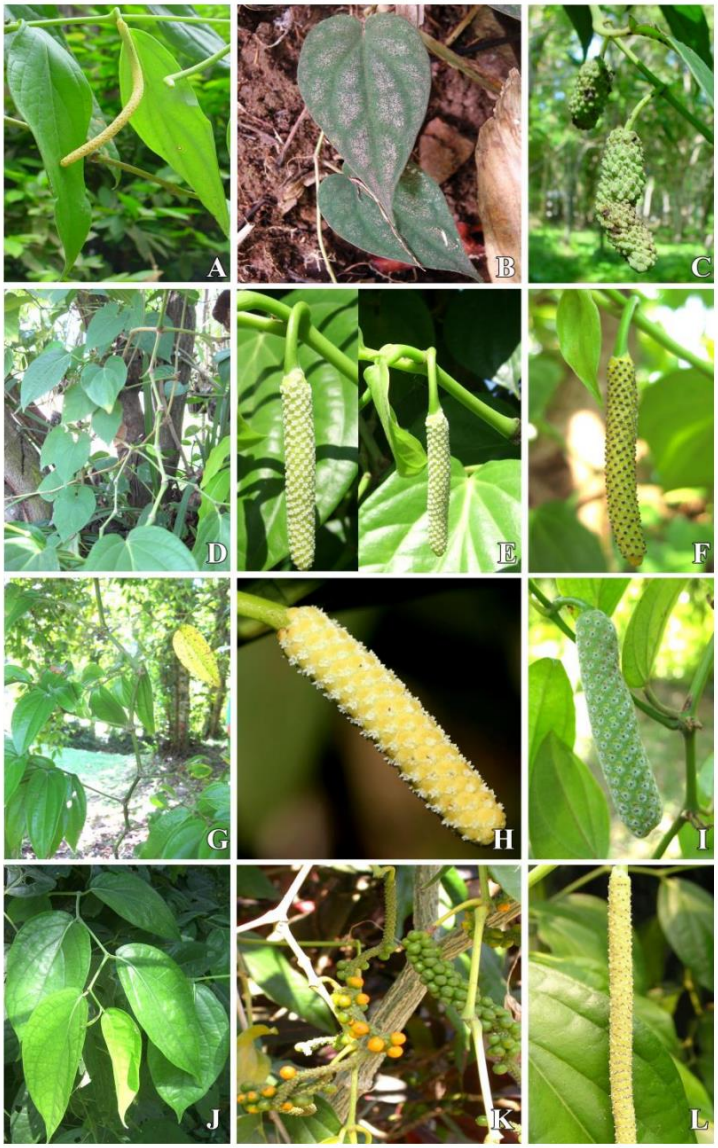

Figure 2. Photographs to illustrate important morphological characteristics of *Piper argyritis* (A. habit showing leaves on free branch and male spike), *P. maculaphyllum* (B. dappled leaves, C. fruiting spikes), *P. betle* ♀ (D. habit showing free branch, E. female spike, F. wilted female spike), *P. rubroglandulosum* ♀ (G. habit showing free branch, H. female spike, I. fruiting spike), *P. pendulispicum* (J. habit showing leaves on epiphytic branch, K. fruiting spike), and *Piper* sp. (L. leaves with male spike).

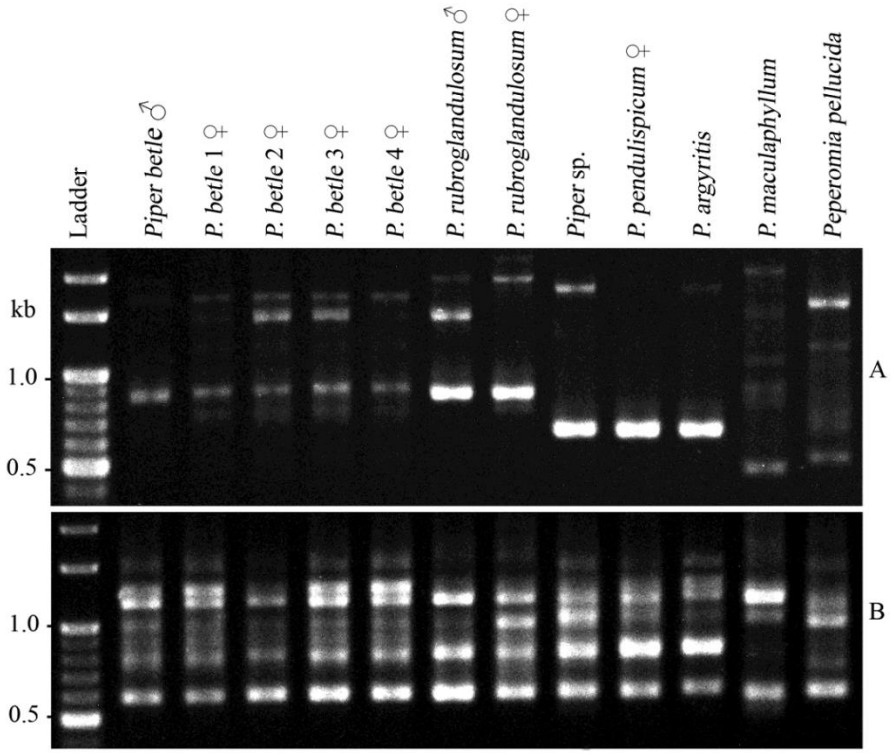

Figure 3. Two examples of the RAPD banding patterns of the *Piper* species obtained using the primer sequences GTCCACACGG (A) and CAATCGCCGT (B).

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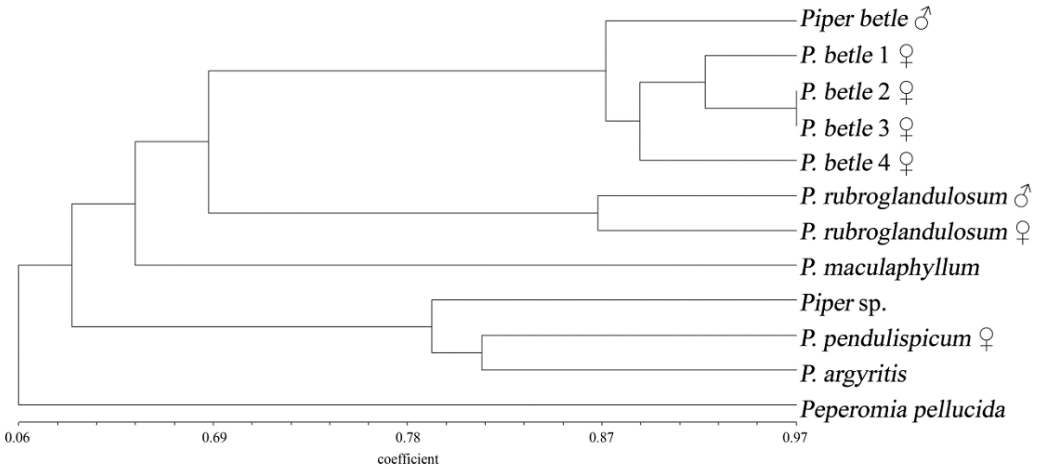

Figure 4. Dendrogram constructed from the RAPD analysis obtained from six *Piper* species and *Peperomia pellucida* as an outgroup using 22 primers and the NTSYS-pc 2.1 program.

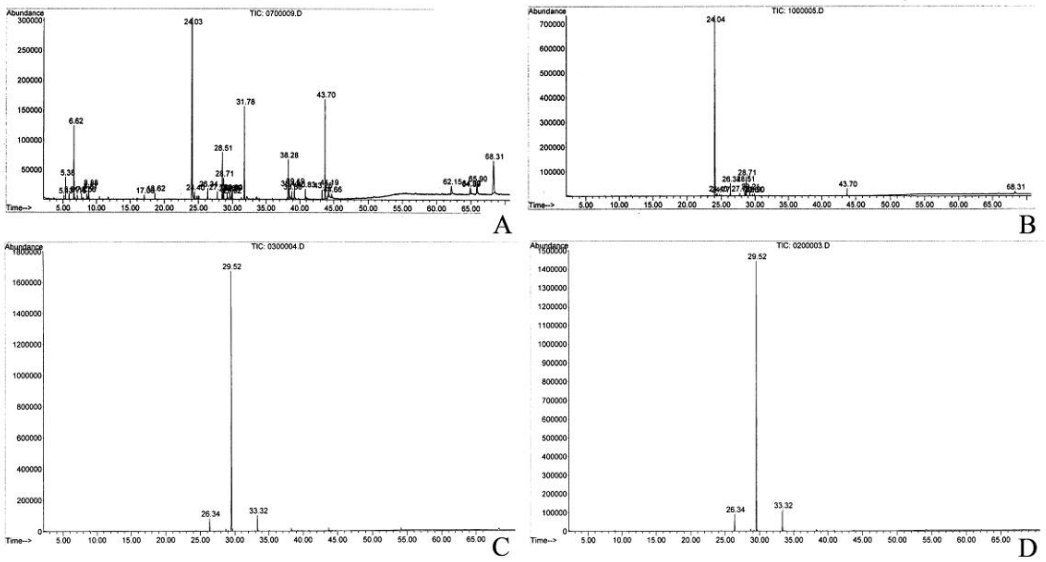

Figure 5. Chromatograms of crude hexane extracts of the leaves of the four examined *Piper* samples; *P. betle* ♂ (A), *P. betle* ♀ (B), *P. rubroglandulosum* ♂ (C), and *P. rubroglandulosum* ♀ (D).

Article 3: Submitted

Sanubol, A., Chaveerach, A.*, Sudmoon, R., Tanee, T., and Liehr, T. Chemical activities of nine betel-like-scented *Piper* species against pathogenic microorganisms. Submitted to Acta Biochimica Polonica (ISI impact factor 1.395).

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.

Acknowledgments

The authors are thankful for the financial support provided by the Thailand Research Fund and Khon Kaen University through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0027/2553) to Arisa Sanubol and Arunrat Chaveerach and the Research Career Development Grant (Grant No. RSA5580054). This research was also supported by the Genetics and Environmental Toxicology (GET) Research Group, Khon Kaen University.

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).

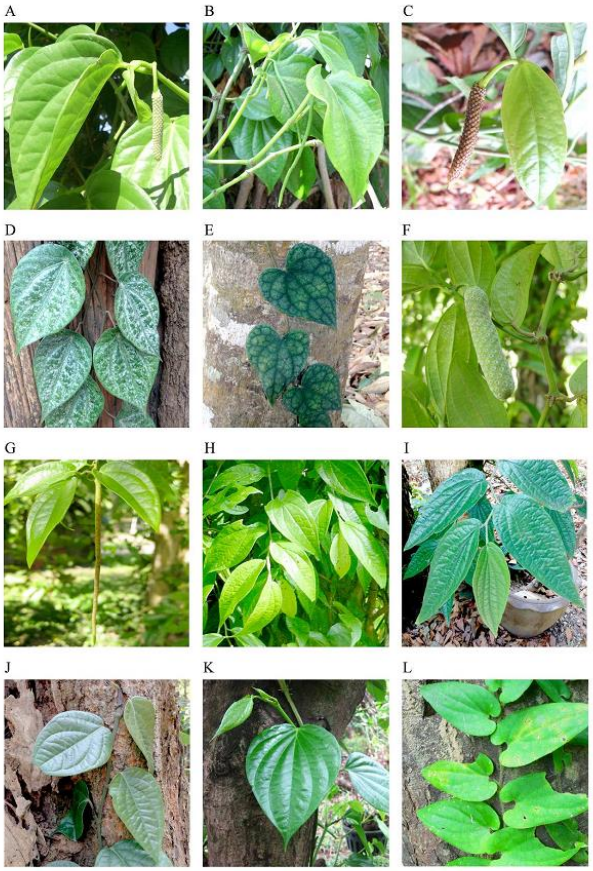

Figure 1. The nine Piper species investigated: ♀ Piper betle (A), ♂ P. betle (B), P. betloides (C), P. crocatum (D), P. maculaphyllum (E), ♀ P. rubroglandulosum (F), ♂ P. rubroglandulosum (G), P. semiimmersum (H), P. submultinerve (I), P. tricolor (J-K) and P. yinkiangense (L).
284x414mm (300 x 300 DPI)

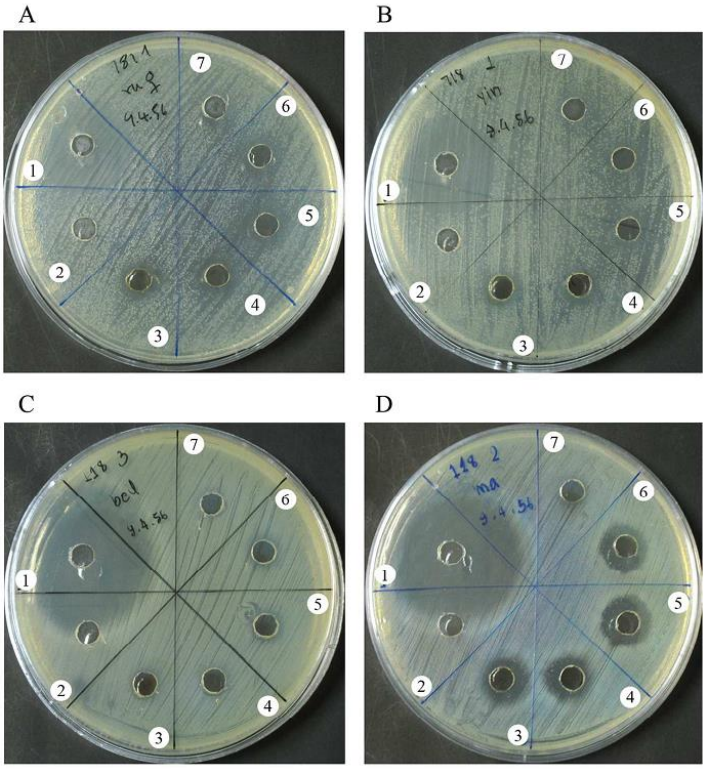

Figure 2. Examples of different dilution levels and clear zones of crude hexane extracts from the leaves of Piper species against Pseudomonas aeruginosa (♀ Piper rubroglandulosum (A) and P. yinkiangense (B)) and Staphylococcus aureus (Piper betloides (C) and P. maculaphyllum (D)). Treatments: antibiotic (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)

Article 4: Submitted

Sanubol, A., Sudmoon, R., Tanee, T., and Liehr, T. and Chaveerach, A.* Quantity and chemical components of the volatile oils of nine betel-like-scented *Piper* plants. Submitted to Journal of Chromatography (ISI impact factor 4.258).

Elsevier Editorial System(tm) for Journal of Chromatography A
Manuscript Draft

Manuscript Number:

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

Article Type: Full Length Article

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

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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.

Acknowledgments

The authors are thankful for the financial support provided by the Thailand Research Fund and Khon Kaen University through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0027/2553) to Arisa Sanubol and Arunrat Chaveerach and the Research Career Development Grant (Grant No. RSA5580054). This research was also supported by the Genetics and Environmental Toxicology (GET) Research Group, Khon Kaen University.

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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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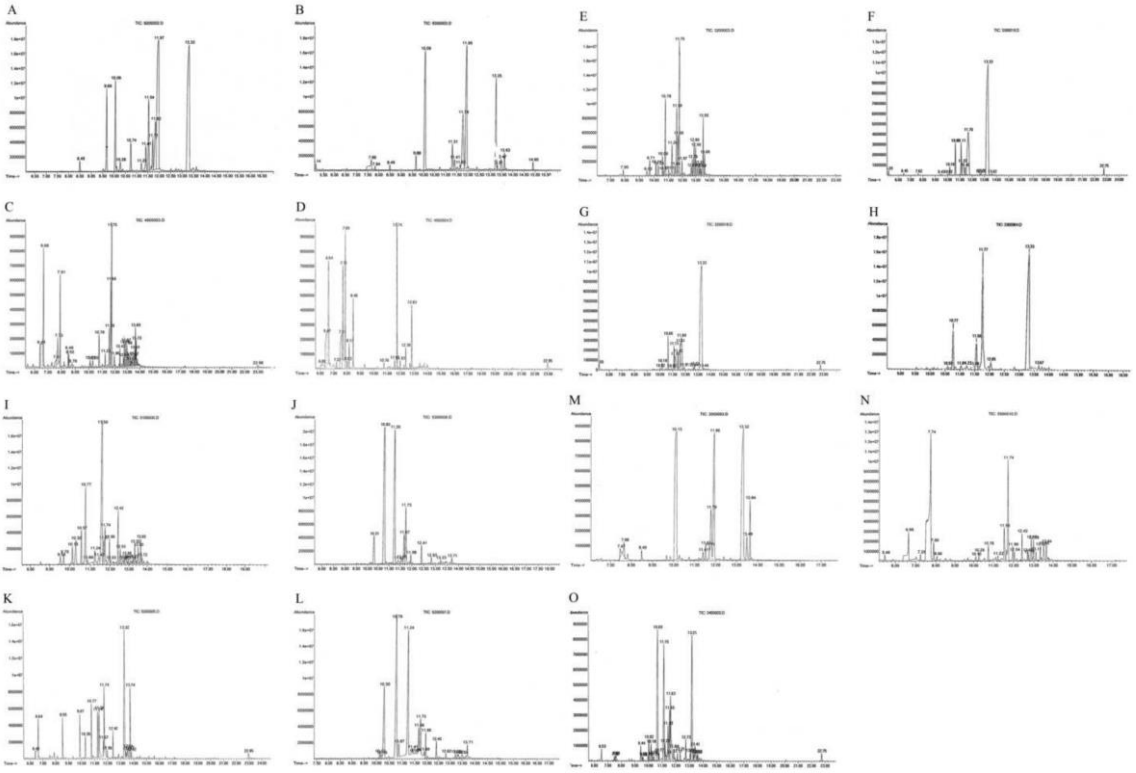
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A


สบู่สมุนไพร
น้ำมันหอมพลู
ลดสิวและผดผื่น 150.-
เหมาะสำหรับผิวธรรมดา-ผิวมัน

B


เจลอาบน้ำสมุนไพร
น้ำมันหอมพลู

C


สเปรย์สมุนไพร