TOXICITY AND ANTITERMITE ACTIVITIES OF THE ESSENTIAL OILS FROM *PIPER SARMENTOSUM*

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Abstract

The leaves of *Piper sarmentosum* were hydrodistilled using the modified Clevenger-type apparatus, and an average yield of essential oil of 1.10% (v/dry weight) was obtained. The leaf oils were analyzed by GC and GC-MS. A total of 31 components were identified. Spathulenol (21.0%), myristicin (18.8%), β-caryophyllene (18.2%) and (*E*,*E*)-farnesol (10.5%) were the major compounds found in the leaf oil. The leaf oil showed inhibitory activity against the larvae of *Artemia salina* with LC_{50} value of 35.2 μg/mL, and 100% mortality within two days at 1% concentration against the subterranean termite (*Coptotermes* sp.). The crude extract was then subjected to bioassay-guided isolation using silica gel column chromatography, and eluted with hexane containing increasing volumes of ethyl acetate and yielded three pure compounds. Their toxicity and antitermite activities of the three compounds were determined. Compound **2** showed the most potent activity against the larvae of *A. salina* with LC_{50} value of 7.5 μg/mL, while the LC_{50} values for compound **3** and compound **1** were 17.2 μg/mL and 22.5 μg/mL respectively. Compound **3** showed the strongest inhibitory activity against the subterranean termite (*Coptotermes* sp.) with 100% mortality after 3 days at 0.1% concentration followed by compound **2** with the same mortality rate at 0.5% concentration. Compound **1** showed the weakest inhibitory activity with 80% mortality after 3 days at 2% concentration. Based on spectroscopic data and comparison with published information, compound **1** and **2** have been identified as caryophyllene and myristicin respectively. Compound **3** is still being studied in order to elucidate its structure.

Introduction

The genus *Piper* belongs to the family Piperaceae, comprising more than 700 species distributed throughout the tropical and subtropical regions of the world [1]. Most of the species in this genus are aromatic, woody perennial climbers and rarely shrub. The *Piper* species have high commercial, economical and medicinal importance. Many species have been shown to possess antimicrobial, antifungal, antioxidant, insecticidal, allelopathic and antitumour activities [1]. Various compounds, including alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, chalcones, flavones and flavanones have been isolated from different *Piper* species. The chemistry of *Piper* species has been reviewed by several researchers [2, 3].

Piper sarmentosum Roxb., locally known as "kaduk", is a creeping herb with erect, slender branchlets about 30 cm tall. It is commonly found in damp open spaces, riverbanks cleared and cultivated lands in Sarawak [4]. The leaves are used in folk medicine as counter-irritants in poultices for headaches and pains in bones. A decoction of the boiled leaves may be utilized to treat coughs, influenza, toothaches and rheumatism. The root is also a remedy for toothache and may be made into a wash for fungoid dermititis on the feet [5]. A bioactive compound, isoasarone has been isolated from the roots of *P. sarmentosum* and showed insecticidal property similar to DDT [6]. Four phenylpropanoids isolated from the benzene-soluble fraction of the methanolic leaf extract showed antimicrobial activity against *Escherichia coli* and *Bacillus subtilis* [7], and the methanol extract of the leaves was found to possess a profound neuromuscular blocking activity in rat nerve-hemidiaphragm preparation [8]. The chloroform extract of the plant showed considerable antimalarial activity against *Plasmodium falciparum* and *Plasmodium berghei* parasites [9], while the water extract of the whole plant had a hypoglycaemic effect in rats [10]. In addition, some amides isolated from the hexane and methanol extracts of fruits exhibited antituberculosis and antiplasmodial activities [11]. A resent study showed that this plant is a good source of natural antioxidants as the methanol extracts were found to possess high antioxidant activity, which may be attributed to the high contents of vitamin E and xanthophylls [12].

This paper reported three compounds isolated by means of bioassay-guided chromatographic separation from the essential oil extracted from the leaves of *P. sarmentosum*. The bioactivity of these compounds against brine shrimp larvae and subterranean termites were also studied.

Experimental

Plant materials

The leave samples of *Piper sarmentosum* was collected around Kuching area. Sample was identified and authenticated by a plant taxonomist. A voucher specimen was deposited at the Herbarium of Universiti Malaysia Sarawak.

Extraction and analysis

The randomly picked leaves were air dried and ground. About 100 g of the ground samples were hydrodistilled using the modified Clevenger-type apparatus for 6 hours, and the oily layers obtained were separated and dried over anhydrous sodium sulphate and stored in vial at 4 – 5^{o}C. The yield was calculated based on dry weight of the plant material.

The oils were analyzed on a Shimadzu GC-17A chromatograph equipped with a flame ionization detector using fused silica capillary column DB-5 (25 m × 0.22 mm ID and film thickness 0.25μm). The operation parameters were: N_2 as carrier gas at flow rate of 20 cm^3 min^{-1}, splitless, injector temperature 280^{o}C, and detector temperature 320^{o}C. The column was programmed initially at 50^{o}C for 2 minutes, and ramped to 300^{o}C at a rate of 6.5^{o}C min^{-1} and held for 7 minutes.

The oils were also analyzed using GC-MS (Shimadzu GC-17A) fixed with the same type of capillary column as GC and under ionization energy of 70 eV. The operation parameters were: He as carrier gas at flow rate of 20 cm^3 min^{-1}, splitless, injector temperature 250^{o}C, and detector temperature 280^{o}C. The column was programmed initially at 50^{o}C for 2 minutes, and then ramped to 300^{o}C at a rate of 6.5^{o}C min^{-1} and held for 7 minutes.

The components were identified tentatively by comparing their Kovat's retention indices with literature values and their mass spectral data with those from NIST mass spectral database. The retention indices were calculated for all components using a homologous series of n-alkanes as standards [13]. For mass spectral analysis, the components were identified by matching the MS with those of authentic standards held in the NIST library. Only similarity indices of 85 or higher were taken as proof of identity [14].

Bioassay-guided fractionation

The biological activity of the extract was performed against brine shrimp larvae (*A. salina*) and termites (*Coptotermes* sp.) using the methods described below.

About 2.0 mL of the essential oil was applied to a silica gel column, and eluted with hexane and a gradient mixture of hexane:EtOAc (19:1, 9:1, 4:1, 1:1). A total of 70 fractions (25 mL each) were collected and combined into 6 groups (PS1 to PS6) based on the similarities in TLC profiles. The activity against larvae of *A. salina* was found in fractions PS1, PS4 and PS6. These 3 fractions were then submitted to further fractionation and purification. Fraction PS1 (352 mg) was subjected to CC on silica gel and eluted with gradient mixture of hexane:EtOAc (9:1, 4:1) yielding 30 fractions, which were pooled into 3 groups. Preparative TLC of group 1[hexane:EtOAc (9:1)] yielded 252 mg of compound (**1**). Fraction PS4 (212 mg) was subjected to CC on silica gel and eluted with a mixture of hexane:benzene:chloroform (3:1:2) yielding 15 fractions, which were pooled into 3 groups. Preparative TLC of group 2 [hexane:benzene:chloroform (3:1:2)] yielded 157 mg of compound (**2**). Fraction PS6 (272 mg) was subjected to CC on silica gel and eluted with gradient mixture of hexane:EtOAc (4:1, 1:1) yielding 15 fractions, which were pooled into 3 groups. Preparative TLC of group 2 [hexane:EtOAc (4:1)] yielded 136 mg of compound (**3**). The bioactivities of these three compounds were determined against larvae of *A.salina* and termites (*Coptotermes* sp.)

Toxicity assay

The protocol established by Mclaughlin [15] was adopted for the toxicity assay against the larvae of *A. salina*. The test samples were prepared by dissolving separately 2 mg of each extract in 2 mL of methanol. From these solutions, 500, 50, and 5 μL were transferred to vials. The solvent was removed under vacuum and 5 mL of artificial seawater was added to each vial, resulting in final concentrations of 100, 10 and 1 μg mL^{-1}

respectively. Then 2 mL of these diluted solutions were transferred to 24 well multidish and second instar larvae of *A. salina* (10 per well) were added. After 24 hours contact, the number of dead larvae in each well was counted and the percentage of death was plotted against the concentrations (on a log scale). The LC_{50} values were determined graphically. The LC_{50} is defined as the lethal concentration of the sample at which 50% of the larvae do not show visible mobility. Controls with and without thymol (10 μg mL^{-1}) were run simultaneously. All experiments were run in triplicate.

Antitermite assay

The modified method established by Sakasegawa *et al* [16] was used for the antitermite assay against a *Coptotermes* sp. collected around Kuching area. The termites were cultured for 2 – 3 days in a plaster container at room temperature. In the bioassay for termiticidal activity, cut filter paper (diameter 35 mm) were placed in each well of a 6-well multidish (3 rows × 2 lines, hole diameter 35 mm). The samples were diluted to 10.0%, 1.0% and 0.1% with methanol. Exactly 80 μL of these diluted samples were pipetted onto the filter paper in each well of one line. Exactly 80 μL of methanol were placed on the filter paper in each well of the other line, which acted as control. The methanol was allowed to evaporate from the filter paper for several hours. 6 termites (5 undifferentiated workers and a soldier) were placed on each well. The absence of soldier causes the initiation of physiological process in which a certain number of workers become soldiers [17]. The multidishes were closed tightly and kept at 25°C in an incubator. The numbers of living termites were counted each day. Each treatment was performed 6 times (3 wells/multidish × 2 times).

Results and Discussion

Hydrodistillation of the air dried leaves of *P. sarmentosum* yielded 1.10% (v/dry weight) of essential oil. There were 31 components (greater than 0.1%) identified in the leaf oil of *P. sarmentosum* (Table 1). Sesquiterpenoids were the main constituents with sesquiterpene hydrocarbons representing 30.3% and oxygenated sesquiterpenes 61.0% of the oil. The major component of the sesquiterpenes hydrocarbons was β-caryophyllene (18.2%) while spathulenol (21.0%), myristicin (18.8%) and (*E*,*E*)-farnesol (10.5%) were the main oxygenated sesquiterpenes. Monoterpenoids only made up of 5.1% of the oils with α-phellandrene the only monoterpene hydrocarbon present.

This leaf oil showed inhibitory activity against the larvae of *A. salina* with LC_{50} value of 35.2 μg/mL, and 100% mortality within two days at 1% concentration against the subterranean termite (*Coptotermes* sp.). After first fractionation, the fractions obtained were subjected to biological activity. The biological activity against larvae of *A. salina* was found in fractions PS1, PS4 and PS6 with all showing LC_{50} value of less than 30 μg/mL after 24 hours of contact (Table 2). The result showed that the three fractions might contain bioactive components.

Bioassay-guided chromatographic separation of the three active fractions afforded three compounds. All the three compounds were subsequently tested against *A. salina* and subterranean termite (*Coptotermes* sp.). Compound **2** and **3** showed stronger biological activity against the larvae of *A. salina* and termites compared to compound **1**. Compound **2** showed the most potent activity against the larvae of *A. salina* with LC_{50} value of 7.5 μg/mL, while the LC_{50} values for compound **3** and compound **1** were 17.2 μg/mL and 22.5 μg/mL respectively.

Compound **3** possess the strongest inhibitory activity against the subterranean termite with 100% mortality after 3 days at 0.1% concentration (Figure 1) followed by compound **2** with the similar mortality rate at 0.5% concentration (Figure 2). Compound **1** showed the weakest inhibitory activity with 80% mortality after 3 days at 2% concentration (Figure 3). However, detailed structure-activity relationship should be further investigated.
Based on spectroscopic data and comparison with published information, compound **1** and **2** has been identified as caryophyllene and myristicin respectively. Compound **3** is still being studied in order to elucidate its structure.

As there is no previous report on the activity of these three volatile oil components on termites, the data obtained will be used for further study to develop environmental friendly termiticital compounds.

Table 1: Chemical composition of the essential oils from the leaves of *P. sarmentosum*

Compound	Kovat's Index		% RA
	KI^1	KI^2	
α-Phellandrene	1005	1007	0.78
Piperitone	1245	1245	0.67
Cinnamyl alcohol	1314	1312	0.18
Eugenol	1366	1364	1.80
α-Copaene	1377	1377	0.29
Methyl eugenol	1407	1407	1.63
α-Ionone	1422	1422	2.96
γ-Elemene	1426	1425	2.48
β-Caryophyllene	1465	1467	18.19
α-Humulene	1469	1467	0.86
β-Guaiene	1481	1483	0.43
Germacrene D	1488	1487	1.26
Ethyl laurate	1494	1494	1.09
α-Farnesene	1499	1500	0.21
Elemicin	1512	1514	0.88
Bicylogermacrene	1516	1517	0.34
δ-Cadinene	1519	1519	0.72
Cadinadiene	1526	1527	0.96
Myristicin	1533	1532	18.77
γ-Cadinene	1543	1543	2.26
Germacrene B	1562	1562	2.26
Guaiol	1587	1589	0.69
Dehydrocarveol	1591	1593	0.85
Spathulenol	1617	1619	20.98
T-Muurolol	1636	1635	0.21
β-Eudesmol	1648	1648	0.58
β-Bisabolol	1668	1666	0.26
δ-Cadinol	1673	1674	0.29
α-Cadinol	1677	1676	0.70
E,Z-Farnesol	1696	1696	2.19
E,E-Farnesol	1720	1722	10.50

KI^1 = Kovat's retention indices obtained on a DB-5 column using the series of n-alkanes.
KI^2 = Kovat's retention indices from literature. [13]
RA = Relative area (peak area relative to total peak area).

Table 2: LC_{50} values against larvae of *A.salina*.

Fraction	PS1	PS2	PS3	PS4	PS5	PS6
LC_{50} (μg/mL)	29.1	>100	nd*	21.6	82.0	23.5

nd* – not done due to inadequate amount of sample

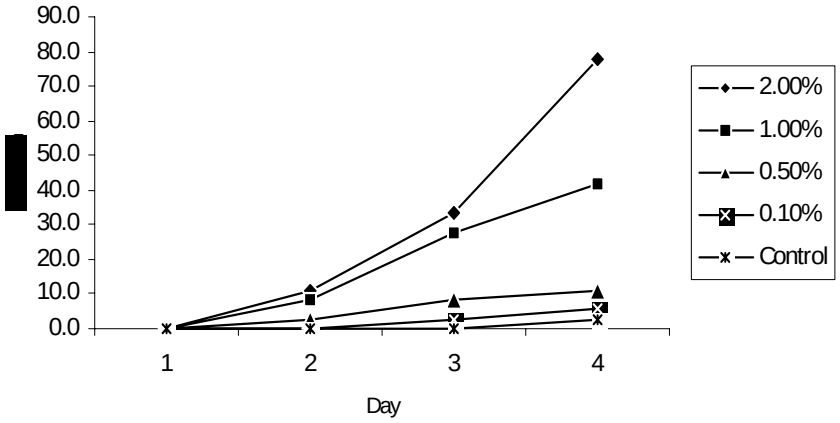

Figure 1: Antitermite activity of Compound (**1**) at different concentrations

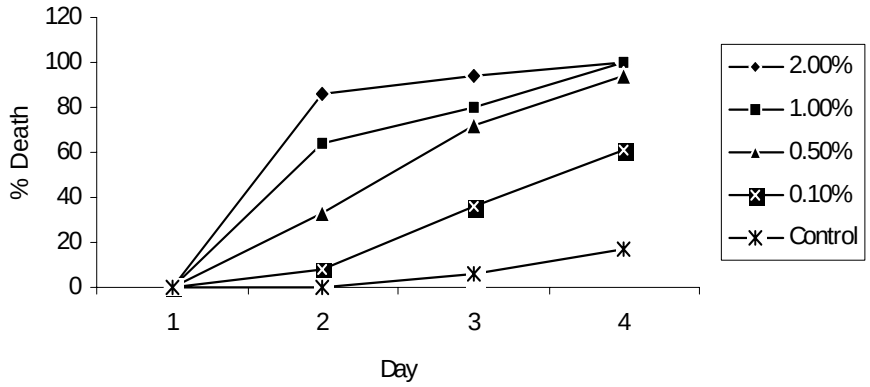

Figure 2: Antitermite activity of Compound (**2**) at different concentrations

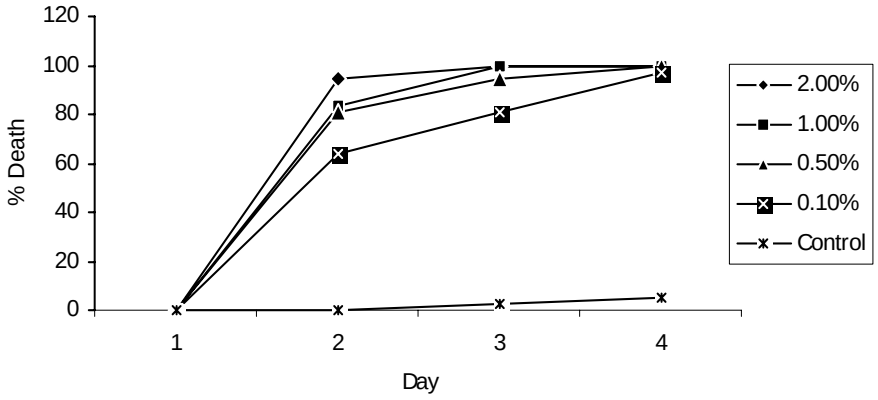

Figure 3: Antitermite activity of Compound (**3**) at different concentrations

Conclusion

The finding of this study showed that essential oil from leaves of *P. samentosum* contains larvicidal and termiticidal components. Further studies are required to increase the lethality of these components due to the synergy achieved by the presence of other minor components of the oil.

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References

1. Sumathykutty, M.A., Rao, J.M., Padmakumari, K.P., & Narayanan, C.S., 1999. Essential oil constituents of some *Piper* species. Flavour Fragrance. J., **14**, 279 - 282.
2. Sengupta, S., & Ray, A.B., (1987). The chemistry of *Piper* species: a review. *Fitoterapia*, **58**, 147-165
3. Parmer, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., Tyagi, O.D., Prasad, A.K., Wengel, J., Olsen, C.E., & Boll, P.M., 1997. Phytochemistry of the Genus *Piper*. *Phytochemistry*, **46 (4),** 597 – 673.
4. Tawan, C.S. & Ipor, I.B., (1993). Some common wild *Piper* of Sarawak. In: Ibrahim, M.Y., Bong, C.E.J. & Ipor, I.B. (Eds). *The Pepper Industry: Problems and Prospect*. Universiti Pertanian Malaysia, Bintulu Campus, 72 – 78.
5. Muhammad, Z. & Mustafa, A.M., (1994). *Traditional Malay Medicinal Plants*. Fajar Bakti, Kuala Lumpur.
6. Satariah, H., Hapipah, M.A., Khalijah, A., Abdul Aziz, K., Habsah, A.K., Kamaliah, M. & Hadi, A.H., (1999). Chemical constituents and insecticidal activity of *Piper sarmentosum*. In: Manaf Ali, A., Khozirah, S & Zuriati, Z. (Eds.), *Phytochemical and Biopharmaceutins from the Malaysian Rain Forest*. FRIM, Kepong, 62-66.
7. Masuda, T., Inamuzi, A., Yamada, Y., Padolina, W.G., Kikuzaki, H. & Nakatani, N., (1991). Antimicrobial phenylpropanoids from *Piper sarmentosum*. *Phytochemistry* **30**, 3227–3228

8. Ridtitid, W., Rattanaprom, W., Thaina, P., Chittrakarn, S., & Sunbhanich, M. (1998). Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* leaves in the rat phrenic nerve–hemidiaphragm preparation. *J. Ethnopharmacol*., **61**, 135–142.
9. Najib Nik, A., Rahman, N., Furuta, T., Kojima, S., Takane, K., & Ali Mohd, M., (1999). Antimalarial activity of extracts of Malaysian medicinal plants. *J. Ethnopharmacol*. **64**, 249–254
10. Peungvicha, P., Thirawarapan, S., Temsiririrkkul, R., Watanabe, H., Prasain, J. K., & Kadota, S. (1998). Hypoglycemic effect of the water extract of *Piper sarmentosum* in rats. *J. Ethnopharmacol*., **60**, 27–32.
11. Rukachaisirikul, T., Siriwattanakit, P., Sukcharoenphol, K., Wongvein, C., Ruttanaweang, P., Wongwattanavuch, P. & Suksamrarn, A. (2004). Chemical constituents and bioactivity of *Piper sarmentosum*. *J. Ethnopharmacol.*, **93**, 173-176.
12. Chanwitheesuk, A., Teerawutgulrag, A. & Rakariyatham, N. (2005). Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chemistry*, **92**, 491-497.
13. Acree, T. and Arn, H. (2004). http://www.Flavornet.com. [online], retrieved on 7 March, 2005.
14. Adam, R.P., 1995. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured, Carol Stream, IL., USA.
15. McLaughlin, J.L., (1991). Grown gall tumours on potato disc and brine shrimp lethality: two simple bioassay for higher plants screening and fractionation. In: Hostettmann, K. (Ed), *Assays for Bioactivity*, Academic Press, San Diego, 2-32.
16. Sakasegawa, M., Hori, K. & Yatagai, M., (2003). Composition and antitermite activities of essential oils from *Melaleuca* species. *J. Wood Science*, **49**, 181-187.
17. Tellez, M.R., Khan, I.A., Kobaisy, M., Schrader, K.K., Dayan, F.E. and Osbrink, W., (2002). Composition of the essential oil of *Lepidium meyenii* Walp.. *Phytochemistry*, **61**, 149-155.