Research Article

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Larvicidal activities and chemical composition of essential oils from *Piper klotzschianum* (Kunth) C. DC. (Piperaceae)

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Abstract

BACKGROUND: Volatile oils from fresh roots, stems, leaves and seeds of *Piper klotzschianum* (Piperaceae) were obtained by hydrodistillation and analysed by GC-FID and GC-MS. In total, 25 components, representing more than 95% of the examined oils, were identified. The essential oils were evaluated against *Artemia salina* Leach nauplii and fourth-instar *Aedes aegypti* larvae.

RESULTS: The major chemical constituents that were identified from various parts of this plant were 1-butyl-3,4-methylenedioxybenzene and 2,4,5-trimethoxy-1-propenylbenzene in the root, 1-butyl-3,4-methylenedioxybenzene in the stems and leaves and 1-butyl-3,4-methylenedioxybenzene, limonene and α-phellandrene in the seeds. The biological activities of these essential oils generally exhibited high toxicity against *A. salina*, with LC_{50} values that ranged from 7.06 to 15.43 $\mu g\ mL^{-1}$, and significant larvicidal activity against fourth-instar *A. aegypti* larvae was observed in the essential oils from the seeds (LC_{50} of 13.27 $\mu g\ mL^{-1}$) and roots (LC_{50} of 10.0 $\mu g\ mL^{-1}$) of the plant.

CONCLUSION: The present study indicates that both essential oil of *P. klotzsdhianum* and the isolate 1-butyl-3,4-methylenedioxybenzene are potential resources for *A. aegypti* larva control. This is the first report of the biological activities of the oil and isolated compound.

Keywords: *Piper klotzschianum*; Piperaceae; *Aedes aegypti*; larvicidal activity; cytotoxic activities

1 INTRODUCTION

Dengue is a major public health problem in the world. According to World Health Organisation (WHO) estimates, 0.5 million people with dengue haemorrhagic fever (DHF) require hospitalisation each year; a very large proportion of these patients are children, and approximately 12 500 (2.5%) of these individuals die from DHF.[1]

Piperaceae is one of the largest basal angiosperm families and includes approximately 3000 species.[2] This family is currently divided into five genera: *Macropiper*, *Zippelia*, *Piper*, *Peperomia* and *Manekia*.[3] A wide variety of species, including plants that are commonly found throughout the world, are members of Piperaceae. Of the five genera that are considered to be part of this family, *Piper*, *Peperomia* and *Manekia* species are found in Brazil, primarily in the Amazonian and Atlantic forests.[4]

The largest genus in the family Piperaceae is *Piper*, which includes more than 1000 species.[5] *Piper* species have attracted interest owing to their insecticidal properties, including their resistance to the *Aedes* mosquito.[6,7] However, several other biological activities are also attributed to extracts or compounds that have been isolated from species of this genus.[8]

The essential oils present in the genus *Piper* are primarily composed of phenylpropanoids (particularly *trans*-anethole, elemicine and chavicol), common hydrocarbons and oxygenated monoterpenes and sesquiterpenes.[9–12]

To the best of the authors' knowledge, there has not been any phytochemical or biological study of the volatile components of *P. klotzschianum* Kunth. However, their leaves are used by the local population as poultice and infusions in treatment of rheumatism, flu and cough.[13] This paper describes the results of an investigation

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of the chemical composition of the volatile oils of this species and the remarkable toxicities of these oils against *Aedes aegypti* and *Artemia salina*.

2 EXPERIMENTAL METHODS

2.1 Plant material

Leaves, stems, roots and seeds of *P. klotzschianum* were collected in January 2009 from the Moraes farm, which is located in the Vila do Riacho (Forest of Gimuna) of the municipality of Aracruz (ES), Brazil. Botanical identification was achieved in the herbarium of the Botanical Garden of Rio de Janeiro (RJ, Brazil), and vouchers (numbers 480408, 480409 and 480410) were deposited.

2.2 Extraction of the essential oils

The oils from the fresh leaves, stems, roots (25 g from each of these plant parts) and seeds (6 g) of *P. klotzschianum* were obtained by hydrodistillation in a Clevenger-type apparatus that consisted of a 250 mL distillation bottle, a jacketed coil condenser and a graduated receiver. The plant material was extracted by 2 h of hydrodistillation, and all of the hydrodistillations were performed in triplicate. The condensation of the steam led to the accumulation of the essential oils in the receiver, and a micropipette was used to separate these oils from the water. The extracted oils were dried over anhydrous sodium sulfate (Merck), weighed and stored at approximately 0 °C under a nitrogen atmosphere until analyses were performed. The oil yields were calculated on the basis of the masses of fresh material that were used.

2.3 Chemical analysis of the essential oils

The essential oils that were obtained by hydrodistillation were analysed by GC analyses; these analyses were performed with a GC-17A series instrument (Shimadzu, Japan) equipped with a flame ionisation detector (FID). The following chromatographic conditions were used: a fused silica capillary column (30 m × 0.22 mm) with a DB-5 bonded phase (0.25 μm film thickness); carrier gas N_2 at a flow rate of 1.8 mL min^{-1}; injector temperature 220 °C; detector temperature 240 °C; column temperature programmed to begin at 40 °C (remaining isothermal for 2 min) and then to increase at 3 °C min^{-1} to 240 °C (remaining isothermal at 240 °C for 15 min); injection volume 1.0 μL (1% w/v in CH_2Cl_2); split ratio 1:10; column pressure 115 kPa.

The compounds were identified using a Shimadzu GC-MS, model GCMS-QP5050A, and a fused silica capillary column (30 m × 0.22 mm) with a DB-5 bonded phase (0.25 μm film thickness), interfaced with an ion trap detector. The following conditions were used: oven and injector temperatures as described above; transfer line temperature 240 °C; ion trap temperature 220 °C; carrier gas He at a flow rate of 1.8 mL min^{-1}; split ratio 1:10; column pressure 100 kPa. The ionisation energy of the mass detector was 70 eV, the scan range was 29–450 amu and the scan time was 1 s. The components of the examined essential oils were characterised by comparing their retention indices (RIs) relative to a standard alkane series (C_9–C_{24}) and by comparing their mass spectra with reference data from either the equipment database (Wiley 330.000) or the extant literature.[14]

The ^1H and ^{13}C NMR spectra, including DEPT experiments, of the essential oils of the roots were performed on a Varian spectrometer, model Gemini 2000, that was operated at 300 MHz for ^1H NMR and at 75 MHz for ^{13}C NMR. Chemical shifts were recorded in δ (ppm) relative to the reference of TMS.

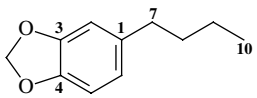
2.4 Tests of lethality against *Artemia salina*

The brine shrimp lethality assay was performed using methods that had been previously described in the literature, with few modifications.[15,16] Brine shrimp (*A. salina*) eggs were hatched in natural sea water that was obtained from the local region known as Ondina's beach in Salvador (BA), Brazil. The essential oils and 1-butyl-3,4-methylenedioxybenzene, the main compound that was isolated from these oils, were tested at concentrations of 5, 10, 20, 30 and 50 μg mL^{-1}. After 24 h of incubation in these essential oil concentrations at 25 °C, the nauplii that remained alive were evaluated. The collected data were computerised, and LC_{50} values were determined by probit analysis.

2.5 The mosquito larvicidal activity of essential oils against *Aedes aegypti*

The larvicidal activity of the essential oils from the seeds and roots of *P. klotzschianum* was evaluated using a modified version of a protocol recommended by the World Health Organisation.[17,18] Fourth-instar *A. aegypti* larvae aged 4–6 days (all white head) were collected from a mosquito colony maintained at the insectaria of the Instituto de Química e Biotecnologia da Universidade Federal de Alagoas. The mosquitos were maintained at a temperature of 27.1 ± 4 °C and a relative humidity of 69.9 ± 7.8% and experienced a photoperiod of approximately 12 h each day. The hatching of larvae occurred in distilled water, and adult insects were fed with 10% glucose aqueous solution in cotton balls that were changed daily.[18] Initially, all of the essential oil samples (250, 100 , 20, 15, 10, 7.5 and 5 μg mL^{-1}) were dissolved in distilled water containing 0.33% dimethylsulfoxide (DMSO) and placed in beakers (100 mL). A total of 45 larvae in three replicates of 15 larvae each were exposed to each essential oil concentration that was tested. In these assays, negative (H_2O with 0.33% DMSO) and positive (reformulated synthetic Temephos® in distilled water) controls were performed in parallel for comparison purposes. The

Table 1. The yields (%, w/w) of essential oils that were extracted from *Piper klotzschianum* and the structure of the major component of these oils

Plant parts				Major component
Roots[a]	Stems[a]	Leaves[a]	Seeds[b]	
0.77 ± 0.014	0.13 ± 0.017	1.81 ± 0.063	13	

[a] Mean ± SD from three replications.
[b] One extraction.

mortality of the larvae was determined after 48 h of incubation with the essential oil at 28 ± 2 °C. Larvae were considered to be dead if they did not respond to a stimulus or did not rise to the surface of the solution. The lethal concentration values LC_{50} were calculated by probit analysis.

1-Butyl-3,4-methylenedioxybenzene. Colourless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.93 (3H, t, J + 7.2 Hz, H-10), 1.28–1.42 (2H, m, H-9), 1.52–1.62 (2H, m, H-8), 2.54 (2H, t, J + 7.5 Hz, H-7), 5.92 (2H, s, OCH_2O), 6.63 (1H, dd, J + 8.1 and 1.8 Hz, H-6), 6.67 (1H, d, J + 1.8 Hz, H-2), 6.73 (1H, d, J + 8.1 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.90 (CH_3), 22.19 (C9), 33.88 (C8), 35.34 (C7), 100.61 (OCH_2O), 107.94 (C5), 108.80 (C2), 120.96 (C6), 136.71 (C1), 145.32 (C4), 147.39 (C3); EIMS *m/z* (rel. int.): 178 $[M]^+$ (46), 136 (39), 135 (100), 105 (8), 91 (7), 79 (11), 77 (41), 65 (9), 51 (21), 41 (5), 39 (12).

3 RESULTS AND DISCUSSION

3.1 The analysis of essential oils

The essential oils from the leaves, stems, roots and seeds of *P. klotzschianum* presented clear aspects and pleasant aromas. Table 1 summarises the yields of these oils. From this table it can be seen that the essential oil quantities that were extracted from the leaves of *P. klotzschianum* were more than twice the essential oil quantities that were extracted from the roots of *P. klotzschianum*, and the stems of this species had a lower essential oil content than the other plant components that were examined. Using GC-FID, a total of 23 compounds were detected, identified and quantified in terms of relative percentages. This process permitted the identification of 99.3–99.9% of the compounds that were present in the oils. In particular, 1-butyl-3,4-methylenedioxybenzene and 2,4,5-trimethoxy-1-propenylbenzene were the main compounds that were detected in the roots, stems and leaves of *P. klotzschianum*, and 1-butyl-3,4-methylenedioxybenzene, limonene and α-phellandrene were the major compounds in the essential oils of the seeds of this plant (Table 2). Among the different parts of the plant that were examined, the roots had the greatest relative quantity of 1-butyl-3,4-methylenedioxybenzene, whereas the stems and leaves had significant concentrations of 2,4,5-trimethoxy-1-propenylbenzene. In this study, these reported compounds were identified and quantified for the first time in this species. The presence of 1-butyl-3,4-methylenedioxybenzene was also confirmed by ^{1}H and ^{13}C NMR techniques (as described in Section 2). The ^{1}H NMR spectrum of the essential oils allowed identification of the AMX substitution pattern of the aromatic ring and the methylenedioxy group by the characteristic signals. In addition, two benzylic hydrogen signals were observed. The analysis of ^{13}C NMR through a DEPT 135° spectrum confirmed the ^{1}H NMR findings. The mass spectrum that was obtained in the GC-MS (as described in Section 2) appeared to show a molecular ion

Table 2. Chemical composition of the essential oils of *Piper klotzschianum*

Compound	$RI_{Cal.}$	$RI_{Lit.}$	Percentage of area[a]			
			Roots	Stems	Leaves	Seeds[b]
α-Pinene	935	939	—	—	—	1.37
β-Pinene	978	980	—	—	—	0.47
β-Myrcene	990	991	—	—	—	0.40
α-Phellandrene	**1004**	**1005**	—	—	—	**16.96**
p-Cymene	1028	1026	—	—	—	7.37
Limonene	**1031**	**1031**	—	—	—	**17.75**
NI	1348	Ne	—	0.41 ± 0.05	—	—
α-Cubebene	1352	1351	—	—	tr	0.47
α-Copaene	1377	1376	tr	tr	tr	0.70
β-Cubebene	1390	1390	tr	0.20 ± 0.09	1.44 ± 0.23	1.66
β-Elemene	1393	1391	—	0.24 ± 0.09	tr	tr
1-Butyl-3,4-methylenedioxybenzene	**1408**	**Ne**	**96.19 ± 0.01**	**84.75 ± 1.08**	**81.04 ± 3.89**	**36.92**
NI	1419	Ne	—	1.95 ± 0.36	1.56 ± 0.19	tr
3,4-Methylenedioxybenzyl methyl ketone	1423	Ne	0.47 ± 0.06	0.36 ± 0.11	tr	0.81
α-*trans*-Bergamotene	1436	1436	—	1.44 ± 0.26	—	8.84
β-Farnesene	1459	1458	tr	0.36 ± 0.02	—	—
Germacrene D	1481	1480	0.21 ± 0.01	0.39 ± 0.07	1.05 ± 0.18	0.78
β-Sesquiphellandrene	1486	—	—	0.71 ± 0.11	—	1.62
Eremophilene	1488	Ne	—	tr	0.76 ± 0.20	tr
Bicyclogermacrene	1495	1494	tr	0.96 ± 0.15	2.68 ± 0.60	1.16
Torreyol	1503	—	0.47 ± 0.03	0.36 ± 0.03	tr	tr
β-Bisabolone	1509	1509	0.28 ± 0.01	1.20 ± 0.30	—	—
δ-Cadinene	1525	1524	0.11 ± 0.01	0.38 ± 0.07	0.72 ± 0.20	0.45
(*Z*)-Isoelemicin	1574	1573	—	0.82 ± 0.13	1.23 ± 0.35	0.25
2,4,5-Trimethoxy-1-propenyl benzene	1589	Ne	1.84 ± 0.20	5.36 ± 0.51	9.10 ± 2.22	1.37
Total identified			**99.57**	**99.89**	**99.58**	**99.35**

[a] All of the values are reported as the mean ± SD of three replicates.
[b] Only one quantification attempt (no repetition); tr – trace compound (less than 0.10%); $RI_{Cal.}$ – calculated retention indices; $RI_{Lit.}$ = retention indices from Adams;[14] NI – unidentified; Ne – not found.

Table 3. Biological activities of the essential oils from different parts of *Piper klotzschianum*, determined by *in vitro* short-term toxicity assay against *Artemia salina* and *Aedes aegypti*

Samples	*A. salina* LC_{50} ($\mu g\ mL^{-1}$)	*A. aegypti* LC_{50} ($\mu g\ mL^{-1}$)
Roots	15.43	10.00
Seeds	14.01	13.27
Leaves	7.62	—
Stems	7.06	—
1-Butyl-3,4-methylenedioxybenzene	7.06	—

at *m/z* 178 and thus suggested the predominance of a compound with a molecular formula of $C_{11}H_{14}O_2$. By these data, it was possible to confirm that the major component present in this oil was the phenylpropanoid 1-butyl-3,4-methylenedioxybenzene. These spectroscopic data are in agreement with data in the literature with respect to this substance.[19] This compound was previously found in species of *P. vahlii* and *P. anisum*.[20]

3.2 Larvicidal activity and brine shrimp test evaluations

In the assays of the mosquito larvicidal activity of the seed and root essential oils of *P. klotzschianum* against fourth-instar *A. aegypti* larvae, LC_{50} values of 13.27 and 10 $\mu g\ mL^{-1}$ respectively (Table 3) were observed. In all of the experiments, 100% mortality was found for the positive control, whereas the larvae remained alive in the negative control. The results that were obtained in this study suggested that these essential oils may be regarded as a promising natural source of mosquito larvicidal agents, as the mosquito larvicidal activity of these oils is often attributed to the complex mixture of compounds that they represent. Because the essential oils from the stems and leaves of *P. klotzschianum* had similar compositions (although much greater quantities of oil were present in the leaves than in the stems), the brine shrimp test was used to assess the toxic activities of these oils. This assessment revealed that the essential oils of *P. klotzschianum* and the main compound that was isolated from the stem and leaf essential oils of *P. klotzschianum* displayed relatively strong toxicity against the nauplii of brine shrimp (*A. salina*). In particular, the observed LC_{50} values of the essential oils from the roots, stems, leaves and seeds of *P. klotzschianum* were 15.43, 7.06, 7.62 and 14.01 $\mu g\ mL^{-1}$ respectively. The LC_{50} value for 1-butyl-3,4-methylenedioxybenzene was 7.06 $\mu g\ mL^{-1}$, demonstrating the high toxicity of this compound (Table 3). The low LC_{50} values that were observed for the essential oils of *P. klotzschianum* leaves and stems suggest that these components play a role in protecting the plant against insect attack. In a previous study employing essential oil of *P. marginatum* against *A. aegypti*, it was also established that the phenyl asarone {1,2,4-trimethoxy-5-[(*E*)-prop-1-enyl]benzene} is the compound that is responsible for activity.[6] Other phenylpropanoids such as eugenol and its synthetic derivatives also exhibit activity.[21] Compared with the activities that were observed in these previous studies, the mosquito larvicidal activities that were observed in the present work were more potent; the main component of *P. klotzschianum* essential oils (1-butyl-3,4-methylenedioxybenzene), among other phenylpropanoids, appears to be responsible for this activity.

4 CONCLUSIONS

This study demonstrates the high toxicity of these oils against *A. aegypti* and *A. salina*. These results indicate that the essential oils of this plant contain compounds that could lead to the replacement of synthetic insecticides, such as Temephos®. The mosquito larvicidal activities that were observed are more potent than the previously reported activities of the essential oil of *P. marginatum*, but the active components of both these oils appear to be phenylpropanoid derivatives.

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REFERENCES

1 World Health Organisation website. [Online]. WHO. Available: http://www.who.int/media [12 December 2009].
2 Bornstein AJ, Taxonomic studies in the Piperaceae I. The pedicellate *Pipers* of Mexico and Central America (*Piper* subg. *Arctottonia*). *J Arn Arbor* **70**:1–55 (1989).
3 Jaramillo MA, Manos PS and Zimmer EA, Phylogenetic relationship of the perianthless Piperales: reconstructing the evolution of floral development. *Int J Plant Sci* **165**:403–417 (2004).
4 Monteiro D and Guimarães EF, Flora do Parque Nacional do Itatiaia – Brasil: *Peperomia* (Piperaceae). *Rodriguésia* **59**:161–195 (2008).
5 Jaramillo MA and Manos PS, Phylogeny and patterns of floral diversity in the genus *Piper* (Piperaceae). *Amer J Bot* **88**:706–716 (2001).
6 Autran ES, Neves IA, Da Silva CSB, Santos GKN, Da Câmara CAG and Navarro DMAF, Chemical composition, oviposition deterrent and larvicidal activities against *Aedes aegypti* of essential oils from *Piper marginatum* Jacq. (Piperaceae). *Bioresour Technol* **100**:2284–2288 (2009).
7 Paula VF, Barbosa LC, Demuner AJ, Piló-Veloso D and Picanço MC, Synthesis and insecticidal activity of new amide derivates of piperine. *Pest Manag Sci* **56**:168–174 (2000).
8 Qin W, Huang S, Li C, Chen S and Peng Z, Biological activity of the essential oil from the leaves of *Piper sarmentosum* Roxb. (Piperaceae) and its chemical constituents on *Brontispa longissima* (Gestro) (Coleoptera: Hispidae). *Pestic Biochem Physiol* **96**:132–139 (2010).
9 Péres VF, Moura DJ, Sperotto ARM, Damasceno FC, Caramão EB, Zine CA *et al.*, Chemical composition and cytotoxic, mutagenic and genotoxic activities of the essential oil from *Piper gaudichaudianum* Kunth leaves. *Food Chem Toxicol* **47**:2389–2395 (2009).
10 Mundina M, Vila R, Tomi F, Tomás X, Cicció JF, Adzet T *et al.*, Composition and chemical polymorphism of the essential oils from *Piper lanceaefolium*. *Biochem Syst Ecol* **29**:739–741 (2001).
11 Santos PRD, Moreira DL, Guimarães EF and Kaplan MAC, Essential oil analysis of 10 Piperaceae species from the Brazilian Atlantic Forest. *Phytochemistry* **58**:547–551 (2001).
12 Evans PH, Bowers WS and Funk EJ, Identification of nematocidal components in the leaves of *Piper betle* (Piperaceae). *J Agric Food Chem* **32**:1254–1256 (1984).
13 do Nascimento JC, de Paula VF, David JM and David, JP, Occurrence, biological activities and ^{13}C NMR data of amides from *Piper* (Piperaceae). *Quim Nova* **35**:2288–2311 (2012).
14 Adams RP, *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*, 4th edition. Allured Publishing Corp., Carol Stream, IL, 804 pp. (2007).
15 Meyer BN, Ferrigini N, Putnam JE, Jacobsen LB, Nichols DE and McLaughlin JL, Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med* **45**:31–34 (1982).
16 David JP, Silva EF, Moura DL, Guedes MLDS, Assunção RDJ and David JM, Lignans and triterpenes from cytotoxic extract of *Eriope blanchetii*. *Quim Nova* **24**:730–733 (2001).
17 WHO Instructions for determining the susceptibility or resistance of mosquito larvae to insecticide, WHO/VBC/81.807:16 (1981).

18 Oliveira PV, Ferreira JC, Jr, Moura FS, Lima GS, Oliveira FM, Oliveira PES *et al.*, Larvicidal activity of 94 extracts from ten plant species of northeastern Brazil against *Aedes aegypti* L. (Diptera: Culicidae). *Parasitol Res* **107**:403–407 (2010).
19 Marques AM, Veloso SML, Guimarães EF and Kaplan MAC, Caracterização de derivado arilbutanoídico em folhas e raízes de *Ottonia anisum* Sprengel. *Rev Brasil Farmacogn* **18**: 709–718 (2008).
20 Moreira DL, Kaplan MAC and Guimarães EF, 1-Butyl-3,4-methylenedioxybenzene as the major constituent essential oil from *Ottonia anisum* Sprengel (Piperaceae). *J Essent Oil Res* **9**:565–568 (1997).
21 Barbosa JDF, Silva VB, Alves PB, Gumina G, Santos RLC, Sousa DP *et al.*, Structure–activity relationships of eugenol derivatives against *Aedes aegypti* (Diptera: Culicidae) larvae. *Pest Manag Sci* **68**:1478–1483 (2012).

