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Original Research Article

Larvicidal activity of *Illicium difengpi* BN Chang (Schisandraceae) Stem Bark and its Constituent Compounds against *Aedes aegypti* L

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Abstract

Purpose: *To determine the larvicidal activity of the essential oil derived from Illicium difengpi B.N. Chang stem bark (Schisandraceae) and its major constituents against the larvae of Aedes aegypti L.*
Methods: *Essential oil of I. difengpi stem bark was obtained by hydrodistillation and analyzed by gas chromatography (GC) and gas chromaotography-mas spectrometry (GC-MS). The activity of the essential oil and its major constituents was evaluated, using World Health Organization (WHO) procedures, against the fourth instar larvae of A. aegypti for 24 h, and larval mortalities recorded at essential oil/compound concentrations ranging from 6.0 - 200 µg/mL.*
Results: *A total of 36 components of the essential oil of I. difengpi were identified. The principal compounds are safrole (18.21 %), linalool (13.47 %), 1,8-cineole (12.84 %), and myristicin (8.06 %) followed by α-terpineol (4.77 %), β-pinene (4.45 %) and 4-terpineol (4.38 %). The essential oil exhibited larvicidal activity against A. aegypti with LC_{50} (median lethal concentration) of 31.68 µg/mL. The major constituents, myristicin, safrole, and 1, 8-cineole, exhibited LC_{50} of 15.26, 39.45, and 72.18 µg/mL, respectively.*
Conclusion: *The findings obtained indicate that the essential oil of I. difengpi and its major constituents have potentials for use in the control of A. aegypti larvae and may therefore be useful in the search for newer, safer and more effective natural compounds as larvicides.*

Keywords: *Illicium difengpi, Aedes aegypti, Larvicidal activity, Myristicin, Safrole, 1,8-Cineole, Linalool*

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INTRODUCTION

Mosquitoes transmit pathogens that cause serious human diseases including malaria, dengue fever, yellow fever, chikungunya, filariasis, several forms of encephalitis and filariasis. The yellow fever mosquito (*Aedes aegypti* L.) and the Asian tiger mosquito (*A. albopictus* Skuse) are two main species of mosquito responsible for dengue fever and malaria in China [1]. Currently, the control of mosquito larvae is reliance on synthetic insecticides (organophosphates such as dichlorvos, temephos and fenthion) and insect growth regulators (such as diflubenzuron and methoprene) [2]. However, the widespread usage of these synthetic insecticides has caused several environmental and health concerns, including disruption of natural biological control systems, development of resistance and

undesirable effects on non-target organism [2]. Hence, natural product based products including essential oils have gained special importance as potential new pesticides. Many essential oils and constituent compounds derived from various essential oils have been demonstrated to possess larvicidal activity against mosquito species [3-8]. During our mass screening program for new agrochemicals from wild plants and Chinese medicinal herbs, the essential oil of *Illicium difengpi* B. N. Chang (Schisandraceae) stem bark, was found to possess larvicidal activity against the yellow fever mosquito, *A. aegypti*.

Illicium difengpi, indigenous to China, is a toxic shrub that grows in the mountainous areas of Guangxi Zhuang Nationalty Autonomous Region. The stem bark is listed in Chinese Pharmacopoeia and has been applied as a traditional Chinese medicine to treat rheumatic arthritis by relieving lumbago and pain in the knees [9]. To date, around 40 compounds, including sesquiterpene lactones; steroids; triterpene acids, carboxylic acids, flavonoids, phenylpropanoids, neolignans, and their glycosides have been isolated from the stem bark of this plant [10-15]. The chemical composition of *I. difengpi* essential oil has also been studied previously [16-20]. However, a literature survey has shown that there is no report on the larvicidal activity of *I. difengpi* essential oil against mosquitoes. Hence, the objective of this study was to investigate the chemical constituents and larvicidal activity of the essential oil and its major constituents against the Asian tiger mosquito.

EXPERIMENTAL

Plant collection and identification

Fifteen kilograms of stem bark of *I. difengpi* was collected in September 2012 from Nanning City, Guangxi Autonomous Region (Guangxi, China). The plant was identified by Dr. Liu Quan Ru (College of Life Science, Beijing Normal University, Beijing, China), and a voucher specimen (CAU-Zhongcaoyao-Difengpi-00201) was deposited in the Department of Entomology, China Agricultural University, Beijing, China.

Extraction and isolation of essential oil

Stem bark samples of the plant were air-dried and ground to a powder using a grinding mill (Retsch Muhle, Haan, Germany). Each portion of the powder (600 g) was soaked in water at a ratio of 1:3 (w/v) for 3 h, prior to hydrodistillation using a round bottom flask over a period of 6 h. The volatile essential oil was collected in a flask. Separation of the essential oil from the aqueous layer was done in a separating funnel using a non-polar solvent, n-hexane and the n-hexane layer was then dried over anhydrous sodium sulfate. The solvent was evaporated at 40 ^{o}C using a Buchi Rotavapor R-124 vacuum rotary evaporator. The oil was kept in a refrigerator at 4 ^{o}C pending subsequent experiments. The constituents, myristicin (85 %), safrole (98 %), and 1, 8-cineole (98 %) and linalool (99 %) were purchased from Aladdin Industrial Inc (Shanghai, China).

Analysis of the essential oils

Gas chromatography was performed using Hewlett–Packard 5890 gas chromatograph equipped with a flame ionization detector and fused silica capillary column HP-5 (5 % diphenyl and 95 % dimethylpolysyloxane, 30 m × 0.25 mm, 0.25 µm film thickness), at a flow rate of 1 mL min^{-1}. Temperature was programmed from 60 to 280 °C (at a rate of 2 °C min^{-1}); injector and detector temperatures were 270 and 300 °C, respectively. The components of the essential oil were separated and identified by gas chromatography–mass spectrometry (GC-MS) using Agilent 6890N gas chromatography coupled to Agilent 5973N mass selective detector. The system was equipped with a flame ionization detector and capillary column with HP-5MS (30 m × 0.25 mm × 0.25 µm). GC settings were as follows: the initial oven temperature was held at 60 ^{o}C for 1 min and ramped at 10 °C min^{-1} to 180 °C where it was held for 1 min, and then ramped at 20 °C min^{-1} to 280 °C and held there for 15 min. The injector temperature was maintained at 270 °C. The samples (1 µL, diluted to 100:1 with acetone) were injected, with a split ratio of 1:10. The carrier gas was helium used at a flow rate of 1.0 ml min^{-1}. Spectra were obtained over the scan range 20 to 550 m/z at 2 scans s^{-1}. Most constituents were identified by gas chromatography by comparison of their retention indices with those published in the literature or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of n-alkanes (C8–C24) under the same operating conditions. Further identification was made by comparison of their mass spectra with those stored in NIST 05 and Wiley 275 libraries or with mass spectra from literature [21]. Relative contents of the oil components were calculated based on the GC peak area normalization method without applying correction factors.

Insect cultures and rearing conditions

Mosquito eggs of *A. aegypti* utilized in bioassays were obtained from a laboratory colony maintained in the Department of Vector Biology and Control, Institute for Infectious Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The original eggs of *A. aegypti* were collected from Haikou, Hainan province, China in 1999. Adults were maintained in a cage (60 × 30 × 30 cm) at 28 - 30 °C and 75 - 85 % RH. The females were fed with blood every alternate day whereas the males were fed with 10 % glucose solution soaked on cotton pad, which were hung in the middle of the cage.

A beaker with strips of moistened filter paper was kept for oviposition. The eggs laid on paper strips were kept wet for 24 h and then dehydrated at room temperature. The dehydrated eggs were placed on a plastic tray containing tap water in our laboratory at 26 – 28 °C and natural summer photoperiod for hatching and yeast pellets served as food for the emerging larvae.

The newly emerged larvae were then isolated in groups of ten specimens in 100 ml tubes with tap water and a small amount of dog food (Pedigree Adult Dry Dog Food, Mars China, Beijing). Larvae were daily controlled until they reached the fourth instar (within 12 h), when they were utilized for bioassays.

Larvicidal bioassay

Range finding studies were run to determine the appropriate testing concentrations. Concentrations of 6, 12.5, 25, 50, 100, and 200 µg/mL of essential oil and its constituents were tested. The larval mortality bioassay was carried out according to the test method for larval susceptibility recommended by the World Health Organization (WHO) [22]. Twenty larvae were placed in glass beaker with 250 ml of aqueous suspension of tested material at various concentrations, and an emulsifier dimethyl sulfoxide (DMSO) was added in the final test solution (< 0.05 %).

Five replicates per concentration were run simultaneously and with each experiment, a set of controls using 0.05 % DMSO and untreated sets of larvae in tap water, were also run for comparison. For comparison, commercial chlorpyrifos (purchased from National Center of Pesticide Standards, Tiexi District, Shenyang 110021, China) was used as positive control. The toxicity of chlorpyrifos was determined at concentrations of 5, 2.5, 1.25, 0.6, and 0.3 µg/mL. The assay was carried out in a growth chamber (Ningbo Jiangnan Instrument Factory, Ningbo 315012, China. http://www.nb-jn.com) (L16:D9, 26 - 27 oC, 78 - 80 % relative humidity). Mortality was recorded after 24 h of exposure.

Statistical analysis

Percent mortality was corrected for control mortality using Abbott's formula [23]. Results from all replicates for the pure compounds/oil were subjected to probit analysis using PriProbit Program V1.6.3 to determine LC_{50} values and their 95 % confidence intervals [24]. Samples for which the 95 % fiducial limits did not overlap were considered to be significantly different.

RESULTS

The six-hour steam distillation of *I. difengpi* stem bark afforded essential oil (yellow) with a yield of 0.11 % (v/w); the density of the concentrated essential oil was determined to be 0.86 g/mL. The GC–MS analysis of the essential oil led to the identification of a total of 36 major components accounting for 99.93 % of the total components present (Table 1).

The principal compounds of the essential oil were safrole (18.21 %), linalool (13.47 %), 1,8-cineole (12.84 %), and myristicin (8.06 %) followed by α-terpineol (4.77 %), β-pinene (4.45 %) and 4-terpineol (4.38 %). Monoterpenoids represented 16 of the 36 compounds, corresponding to 56.97 % of the whole essential oil while only 4 of 36 constituents were phenylpropanoids, corresponding to 28.97 % of the essential oil. Sesquiterpenoids represented 16 of the 36 compounds, corresponding to 13.99 % of the essential oil of *I. difengpi* stem bark.

The essential oil exhibited larvicidal activity against the 4th instar larvae of *A. aegypti* with a LC_{50} of 31.68 µg/mL (Table 2). The major constituents, myristicin, safrole, and 1,8-cineole had LC_{50} of 15.26, 39.45 and 72.18 µg/mL, respectively. However, linalool did not exhibit larvicidal activity against the 4th instar larvae of *A. aegypti* at the concentrations tested in this study.

Table 1: Identified constituents of essential oil of *Illicium difengpi* stem bark

Peak no.	Compound	RI*	Content (%)
	Monoterpenoids		*56.97*
1	α-Pinene	939	2.51
2	β-Pinene	974	4.45
3	δ-3-Carene	1008	0.69
4	1,8-Cineole	1032	12.84
5	γ-Terpinene	1059	1.47
6	*cis*-Linalool oxide	1069	2.49
7	δ-Terpinene	1079	0.56
8	Linalool	1094	13.47
9	Fenchol	1117	1.27
10	Pinocarveol	1139	2.58
11	Camphor	1146	1.94
12	Borneol	1174	2.84
13	4-Terpineol	1177	4.38
14	α-Terpineol	1189	4.77
15	Piperitone	1250	0.19
16	Bornyl acetate	1287	0.52
	Sesquiterpenoids		*13.99*
17	α-Cubebene	1345	0.24
18	Copaene	1375	2.13
19	β-Elemene	1389	0.54
20	*iso*-Caryophyllene	1409	0.89
21	*trans*-α-Bergamotene	1438	0.22
22	Alloaromadendren	1458	0.33
23	α-Amorphene	1479	0.50
24	α-Muurolene	1503	0.91
25	γ-Cadinene	1515	0.67
26	Calamenene	1520	0.66
27	δ-Cadinene	1523	1.85
28	Cadine-1,4-diene	1531	0.30
29	Spathulenol	1578	0.52
30	Caryophyllene oxide	1583	0.38
31	τ-Muurolol	1645	1.63
32	α-Cadinol	1654	2.22
	Phenylpropanoids		*28.97*
33	ρ-Ethylguaiacol	1280	0.28
34	Safrole	1287	18.21
35	Methyleugenol	1402	2.32
36	Myristicin	1517	8.16
	Total identified		99.93

**RI = retention index*

Table 2: Larvicidal activity of the essential oil of *Illicium difengpi* and its major constituents against fourth-instar larvae of *Aedes aegypti*

Treatment	LC_{50} (µg/ml) (95% CL)	LC_{95} (µg/ml) (95% CL)	Slope ± SD	Chi-square value
Essential oil	31.68	123.12	5.97 ± 0.54	11.31*
Mean range	(35.78-55.23)	(114.63-137.87)		
1,8-Cineole	72.18	147.23	2.51 ± 0.14	7.86*
Mean Range	(65.59-79.12)	(133.64-161.78)		
Linalool	> 200.00	-	-	-
Myristicin	15.26	55.40	5.02 ± 0.41	8.22*
Mean Range	(13.93-16.78)	(49.72-61.29)		
Safrole	39.45	118.65	4.63 ± 0.43	7.84*
Mean Range	(36.71-42.32)	(107.21-137.92)		
Chlorpyrifos	1.53	5.34	0.93 ± 0.04	4.24*
Mean Range	(1.36-1.75)	(4.78-5.88)		

**Significant at p < 0.05 level*

DISCUSSION

GC-MS results show that the main components of *I. difengpi* stem bark essential oil are safrole, linalool, 1,8-cineole, and myristicin. In previous reports, safrole and linalool were also demonstrated to be the two major constituents of the essential oil although their contents varied somewhat. For example, the major components of the essential oil of *I. difengpi* stem bark were safrole (21.74 %), linalool (15.51 %), 1,8-cineole (8.77 %), benzene 1,2-dimethoxy-4-(2-propenyl) (6.30 %), and α-terpineol (5.89 %) [17] while Liu *et al* [18] reported that the essential oil of *I. difengpi* stem bark contained safrole (28.64 %) and linalool (16.83 %), followed by 1,8-cineole (4.74 %) and camphor (4.52 %).

However, safrole (23.61 %), linalool (12.93 %), and germacrene D (5.35 %) were the main components in the essential oil of *I. difengpi* stem bark [20]. Moreover, there were some variations in the essential oils derived from different parts of *I. difengpi*. For example, the essential oil of *I. difengpi* fruits had limonene (9.54 %), 1, 8-cineole (9.00 %), and α-calacorene (8.29 %) [19] while the essential oil of *I. difengpi* leaves contained safrole (43.31 %), linalool (16.58 %), 1,8-cineole (8.77 %), β-pinene (7.40 %), and α-pinene (5.73 %) [16]. The above results suggest that studies on plant cultivation and essential oil standardization are needed because chemical composition of essential oil varies greatly with plant population.

The essential oil of *I. difengpi* stem bark possessed strong larvicidal activity against the 4th instar larvae of *A. aegypti*. Moreover, among 4 major constituents, only myristicin exhibited stronger (no overlaps in 95 % fiducial limit) larvicidal activity than the crude essential oil against *A. aegypti* larvae and safrole possessed the same level of larvicidal activity as the essential oil (Table 2). It is suggested that myristicin maybe a major contributor to larvicidal activity of the essential oil of *I. difengpi* stem bark. The commercial insecticide, chlorpyrifos showed larvicidal activity against the mosquitoes with a LC_{50} value of 1.53 µg/mL, thus the essential oil of *I. difengpi* was 21 times less toxic to *A. aegypti* larvae and myristicin was 10 times less toxic than chlorpyrifos. However, compared with the other essential oils reported in the literature, the essential oil of *I. difengpi* exhibited the same level of or stronger larvicidal activity against *A. aegypti* and *A. albopictus* larvae, e.g., essential oil of *Clinopodium gracile* (LC_{50} = 42.56 µg/mL) [5], *Zanthoxylum avicennae* (LC_{50} = 48.79 µg/mL) [6], *Cryptomeria japonica* (LC_{50} = 56.8 µg/mL) [25], *Toddalia asiatica* (LC_{50} = 69.09 µg/ml) [1], *Allium macrostemon* (LC_{50} = 72.86 µg/mL) [7] and *Eucalyptus urophylla* (LC_{50} = 95.5 µg/mL) [26].

In the previous studies, myristicin was shown to have contact and fumigant toxicity against several insects and to exhibit strong synergistic activity because it has been demonstrated to possess strong inhibitory effects on many mammalian P450s [10,27,28]. However, this is the first time to report larvicidal activity of myristicin against mosquitoes. As for another isolated compound, safrole was found to possess larvicidal activity against two mosquitoes, *A. aegypti* and *A. albopictus* [29,30]. Considering that the currently used larvicides are synthetic insecticides, larvicidal activity of the essential oil of *I. difengpi* stem bark and its constituents especially myristicin is quite promising and they show potential for use in the control of *A. aegypti* larvae and could be useful in the search for newer, safer and more effective natural compounds as larvicides.

For the actual use of *I. difengpi* stem bark essential oil and its constituents as novel larvicides or insecticides to be realized, further research is needed to establish their human safety and environmental safety. In traditional Chinese medicine, the plants are used to treat rheumatic arthritis [9] and appear to be safe for human consumption. However, no experimental data on its toxicity to human is available, to the best of our knowledge. Additionally, their larvicide modes of action have to be established, and formulations for improving larvicidal potency and stability need to be developed. Furthermore, field evaluation and further investigation of the effects of the essential oil on non-target organisms are necessary.

CONCLUSION

The essential oil of *I. difengpi* stem bark and its major constituents demonstrate some activity against *Aedes aegypti* mosquito larva but needs to be further evaluated for safety in humans and to enhance its activity.

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