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Original Research Article

Essential Oil Composition and Larvicidal Activity of *Clinopodium gracile* (Benth) Matsum (Labiatae) Aerial Parts against the *Aedes albopictus* Mosquito

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Abstract

Purpose: To determine larvicidal activity of essential oil derived from *Clinopodium gracile* (Benth.) Matsum. (Labiatae) aerial parts against the larvae of *Aedes albopictus* Skuse.
Methods: Essential oil of C. gracile aerial parts was obtained by hydrodistillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The activity of the essential oil was evaluated, using World Health Organization (WHO) procedures, against the fourth larvae of *A. albopictus* for 24 h and larval mortality recorded at a various essential oil concentrations ranging from 12.5 – 200.0 µg/mL.
Results: A total of 34 components of the essential oil of *C. gracile* were identified. The essential oil has higher content of sesquiterpenoids (70.49 %) than monoterpenoids (12.21 %). The other principal compounds of the essential oil were germacrene D (20.59 %), nootkatone (8.22 %), morillol (7.74 %), β-elemene (7.38 %), α-bergamotene (6.08 %), *cis*-β-farnesene (5.47 %) and caryophyllene (5.17 %). The essential oil exhibited larvicidal activity against *A. albopictus* with a median lethal concentration (LC_{50}) of 42.56 µg/mL.
Conclusion: The findings obtained indicate that the essential oil of *C. gracile* has potentials for use in the control of *A. albopictus* larvae and could be useful in the search for new, safer and more effective natural compounds as larvicides.

Keywords: *Clinopodium gracile*, *Aedes albopictus*, Larvicidal activity, Mosquito, Essential oil

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INTRODUCTION

Females of many species of mosquitoes consume blood from living vertebrates, including humans. In the process of feeding on blood, some of them transmit extremely harmful human diseases, such as yellow fever, dengue fever, malaria, several forms of encephalitis and filariasis. The Asian tiger mosquito (*Aedes albopictus* Skuse) and the yellow fever mosquito (*A. aegypti* L.) are two main species of mosquito responsible for dengue fever and malaria in China.

The control of mosquito larvae worldwide depends primarily on continued applications of synthetic insecticides including organophosphates such as temephos and fenthion, and insect growth regulators such as diflubenzuron and methoprene [1]. However, heavy and wide use of these synthetic insecticides has caused several environmental and health concerns,

including disruption of natural biological control systems, outbreaks of other insect species, widespread development of resistance and undesirable effects on non-target organism [2]. Thus, there is urgent need to look for new strategies for mosquito control.

From this point of view, botanical pesticides, including essential oils, are promising since they are effective, environmentally friendly, easily biodegradable, and often inexpensive [2]. It is suggested that many essential oils and constituent compounds derived from various essential oils can exert toxic activity against mosquito species [3-10]. During our mass screening program for new agrochemicals from wild plants and Chinese medicinal herbs, the essential oil of slender wild basil, *Clinopodium gracile* (Benth.) Matsum. (Family: Labiatae) aerial parts, was found to possess larvicidal activity against the Asian tiger mosquito, *A. albopictus*.

Slender wild basil (*C. gracile*) is a traditional Chinese medicinal herb used to expel wind and clear away heat, as antienotic and analgesic [11]. It grows in fields, on mountain slopes or grasslands and is mainly distributed in Zhejang, Jiangsu, Anhui, Henan, Jiangxi, Fujian, Guangdong, Guangxi, Hunan, Guizhou, and Sichuan of China as well as India, Indonesia, Japan, Laos, Malaysia, Myanmar, Thailand, Vietnam [12]. The chemical constituents of this medicinal herb have been studied and flavonoids, saponins, phenols, steroids, triterpenoids, lignolic acids, clinopodic acids and hesperidin have been isolated from this medicinal herb [13-15]. Chemical composition of the essential oil of *C. gracile* aerial parts has also been determined [16]. However, a literature survey has shown that there is no report on larvicidal activity of *C. gracile* essential oil against mosquitoes, thus we decided to investigate the chemical constituents and larvicidal activity of the essential oil against the Asian tiger mosquito.

EXPERIMENTAL

Plant collection and identification

Fresh aerial parts of *C. gracile* (15 kg) at flowering stage were harvested from Lishui City (27.54° N latitude and 119.20° E longitude, Zhejiang Province, China) in October 2011. The herb was identified, and a voucher specimen (no. ENTCAU- Labiatae-10032) was deposited at the herbarium of Department of Entomology, China Agricultural University.

Extraction and isolation of essential oil

The samples was air-dired for two weeks, ground to powder using a grinding mill (Retsch Muhle, Germany), subjected to hydrodistillation using a modified Clevenger-type apparatus for 6 h and then extracted with *n*-hexane. The oil was dried over anhydrous Na_2SO_4 and kept in a refrigerator (4 °C) pending subsequent experiments.

Analysis of the essential oils

Capillary 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 °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 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 (C_8–C_{24}) 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 [17]. Relative contents of the oil components were calculated based on GC peak areas without applying correction factors.

Insect cultures and rearing conditions

Mosquito eggs of *A. albopictus* 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 dehydrated eggs were placed on a plastic tray containing tap water to hatch and yeast pellets served as food for the emerging larvae. The eggs batches, collected daily, were kept wet for 24 h and then placed in distilled water in the laboratory at 24 – 26 °C and natural summer photoperiod for hatching. The newly emerged larvae were then isolated in groups of ten specimens in 100 ml tubes with mineral water and a small amount of dog or cat food. Larvae were daily controlled until they reached the fourth instar stage, when they were utilized for bioassay (within 12 h).

Larvicidal bioassay

Range-finding studies were run to determine the appropriate testing concentrations. Concentrations of 200, 100, 50, 25, and 12.5 µg/mL of essential oil were tested. The larval mortality bioassay was carried out according to the test method for larval susceptibility proposed by the World Health Organization (WHO) [18]. 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 (L16:D9, Ningbo Jiangnan Instrument Factory, Ningbo 315012, China (http://www.nb-jn.com/n2/) set at 26 - 27 ^{o}C and 78 – 80 % relative humidity. Mortality was recorded after 24 h of exposure and the larvae were starved of food over this period.

Statistical analysis

Percent mortality was corrected for control mortality using Abbott's formula [19]. 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 [20]. Samples for which the 95 % fiducial limits did not overlap were considered to be significantly different.

RESULTS

The yield of *C. gracile* essential oil was 0.09 %v/w while its density was determined to be 0.83 g/mL. A total of 34 components of the essential oil of *C. gracile* flowering aerial parts were identified (Table 1). The principal compounds in *C. gracile* essential oil were germacrene D (20.59 %), nootkatone (8.22 %), morillol (7.74 %), β-elemene (7.38 %), α-bergamotene (6.08 %), *cis*-β-farnesene (5.47 %) and caryophyllene (5.17 %). Sesquiterpenoids represented 17 of the 34 compounds, corresponding to 70.49 % of the whole essential oil while 11 of the 34 constituents were monoterpenoids, corresponding to 12.21 % of the essential oil of *C. gracile*.

Table 1a: Main compounds of the essential oil of *Clinopodium gracile* aerial parts.

Peak no.	Compound	Retention index	(%)
1	**Morillol**	**976**	**7.74**
2	Amyl ethyl ketone	987	0.65
3	3-Octanol	993	3.42
4	1,8-Cineol	1032	1.59
5	*cis*-β-Ocimene	1038	0.34
6	Linalool	1094	1.18
7	Octen-1-ol acetate	1105	2.62
8	Octan-3-ol acetate	1129	1.39
9	Camphor	1146	0.63
10	(-)-Borneol	1167	1.33
11	4-Terpineol	1175	0.72
12	α-Terpineol	1191	0.81
13	Piperitone	1253	0.64
14	Bornyl acetate	1289	0.96
15	4-Hydroxy-3-methylacetophenone	1323	0.97
16	**β-Elemene**	**1391**	**7.38**
17	Methyleugenol	1403	0.53
18	**Caryophyllene**	**1420**	**5.17**
19	**α-Bergamotene**	**1434**	**6.08**
20	***cis*-β-Farnesene**	**1438**	**5.47**

Table 1(b): Main compounds of the essential oil of *Artemisia frigida* aerial parts (*contd*)

Peak no.	Compound	Retention index	(%)
21	Geranyl acetone	1453	1.25
22	**Germacrene D**	**1485**	**20.59**
23	δ-Cadinene	1523	0.89
24	Eugenol acetate	1529	1.36
25	Elemol	1551	1.06
26	Germacrene B	1561	2.17
27	*trans*-Nerodilol	1563	1.39
28	Spathulenol	1572	0.95
29	Caryophylene oxide	1578	1.77
30	Globulol	1582	1.45
31	β-Eudesmol	1648	2.28
32	α-Cadinol	1653	4.76
33	*cis*-Lanceol	1765	0.11
34	**Nootkatone**	**1820**	**8.22**
	Total identified		***97.37***
	Monoterpenoids		12.21
	Sesquiterpenoids		70.49
	Others		15.67

**RI = retention index*

Table 2: Larvicidal activity of *Clinopodium gracile* essential oil against fourth-instar larvae of *Aedes albopictus*

Treatment	LC_{50} (μg/mL) (95% CL)	LC_{95} (μg/mL) (95% CL)	Slope ± SD	Chi-square value (χ^2)
C. gracile	42.56	134.19	0.56 ± 0.06	14.87*
Mean Range	(39.52-45.76)	(123.58-147.16)		
Chlorpyrifos	1.86	6.65	0.87 ± 0.01	3.13*
Mean Range	(1.71-2.05)	(6.21-7.48)		

** Significant at p < 0.05 level.*

The essential oil possessed strong larvicidal activity against the 4th instar larvae of *A. albopictus* with LC_{50} value of 42.56 μg/mL (Table 2).

DISCUSSION

The main constituents of *C. gracile* essential oil were germacrene D, nootkatone, morillol, β-elemene, α-bergamotene, *cis*-β-farnesene and caryophyllene. Its chemical composition was quite different from that reported in other study [17]. The essential oil of *C. gracile* aerial parts harvested from Guilin City (24.18° N latitude and 119.45° E longitude, Guangxi Zhuang Autonomous Region, China) contained 31 constituent compounds and the main constituents were *trans*-β-farnesene (21.81%), 1-octen-3-ol (16.99%), 3-hexen-1-ol (9.80%), propylene glycol (6.19%), and caryophyllene (6.18%). The above results suggest that there were some variations in chemical composition of essential oil of *C. gracile* aerial parts collected from different sites because it has been proved that there is variation in the chemical composition of essential oil of plants collected from different areas [21,22]. Studies on plant cultivation and essential oil standardization indicate that chemical composition of essential oil varies greatly with plant population.

The essential oil of *C. gracile* aerial parts possessed strong larvicidal activity against the 4th instar larvae of *A. albopictus*. The commercial insecticide, chlorpyrifos showed larvicidal activity against the mosquitoes with a LC_{50} value of 1.86 μg/mL, thus the essential oil of *C. gracile* was 23 times less toxic to *A. albopictus* larvae compared with chlorpyrifos.

However, compared with the other essential oils/extracts in the literature, the essential oil of *C. gracile* exhibited the same level of or stronger larvicidal activity against *A. albopictus* larvae, e.g., essential oil of *E. urophylla* (LC_{50} = 95.5 μg/mL) [4]; essential oil of *Cinnamomum osmophloeum* of cinnamaldehyde type (LC_{50} = 40.8 μg/mL) [5]; leaf essential oil of *Cryptomeria japonica* (LC_{50} = 51.2 μg/mL) [6]; leaf and twig essential oils from *Clausena excavata* (LC_{50} = 41.1 μg/mL) [7]; essential oil of *Achillea millefolium* (LC_{50} = 211.3 μg/mL), *Helichrysum italicum* (LC_{50} = 178.1 μg/mL) and *Foeniculum vulgare* (LC_{50} = 142.9 μg/mL) [23]; essential oils of *Salvia elegans* and *S. splendens* (LC_{50} = 46.4 ppm and LC_{50} = 59.2 ppm, respectively) [25]; and ethanolic extractives of *Borassus flabellifer* (LC_{50}

= 60 µg/mL) [26]. However, the essential oil of *C. gracile* possessed weaker larvicidal activity against *A. albopictus* larvae than the essential oil of *Eucalyptus camaldulensis* (LC_{50}, 31.0 µg/mL) [4]; acetone extract of *Ricinus communis* seed (LC_{50}, 16.84 µg/mL) [3] and hexane extract of *Acorus calamus* (LC_{50}, 21.26 ppm) [24].

In previous reports, one of the main constituent compounds of the essential oil, germacrene D, exhibited strong larvicidal activity against three mosquitoes, *Anopheles stephensi* (LC_{50} = 16.95 ppm), *Ae. aegypti* (LC_{50} = 12.70 ppm), and *Culex quinquefasciatus* (LC_{50} = 21.28 ppm) [27] while in another report [28], germacrene D showed larvicidal activity against *Ae. aegypti* and *A. stephensi* with 24 h LC_{50} values of 63.6 and 59.5 µg/mL, respectively. Another constituent compound, nootkatone has been shown to exert insecticidal activity and repellency against several insects/ticks, e.g., *Drosophila melanogaster* [29], *Sitophilus zeamais* and *S. oryzae* [30], *Coptotermes formosanus* [31] and four species of ticks, *Amblyomma americanum*, *Dermacentor variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* [32].

Nootkatone was reported to exhibit inhibitory effect on acetylcholinesterase of arthropods [33]. However, nootkatone has not been evaluated for larvicidal activity against mosquitoes so far. It seems that the two main constituent compounds may be active larvicidal agents against the Asian tiger mosquito in the essential oil of *C. gracile*. The isolation and identification of the bioactive compounds in the essential oil of *C. gracile* flowering aerial parts are of utmost importance to determine if their potential application in controlling mosquito pests can be fully exploited.

Considering that the currently used larvicides are synthetic insecticides, larvicidal activity of the crude essential oil is quite promising and it shows its potential for use in the control of *A. albopictus* larvae and could be useful in the search for newer, safer and more effective natural compounds as larvicides.

For the actual use of *C. gracile* aerial parts 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 aerial parts are used as antienotic and analgesic [11] and appear to be safe for human consumption. However, no experimental data on its toxicity in humans 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 *C. gracile* aerial parts demonstrates some activity against *Aedes albopictus* mosquito larva but needs to be further evaluated for safety in humans and to enhance its activity.

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