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Evaluation of larvicidal activity of the essential oil of *Ageratum conyzoides* L. aerial parts and its major constituents against *Aedes albopictus*

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Abstract
During our mass screening program for new agrochemicals from the wild plants and Chinese medicinal herbs, essential oil of *Ageratum conyzoides* L. (Compositae) aerial parts at flowering stage exhibited strong larvicidal activity against the Asian tiger mosquito, *Aedes albopictus*. Essential oil of *A. conyzoides* aerial parts was obtained by hydrodistillation and analyzed by GC and GC-MS. A total of 32 components of the essential oil were identified and the principal compounds in the essential oil were precocene II (45.75%), precocene I (14.09%), and caryophyllene (12.13%) followed by germacrene D (4.18%) and caryophyllene oxide (4.06%). The essential oil had higher content (59.84%) of phenylpropanoids than monoterpenoids (3.70%) and sesquiternoids (33.01%). The essential oil of *A. conyzoides* aerial parts exhibited larvicidal activity against *Ae. albopictus* with an LC_{50} value of 61.22 µg/ml while the two major constituents, precocene I and precocene II had LC_{50} values of 43.55 µg/ml and 41.63 µg/ml, respectively. The present findings indicated that the essential oil of *A. conyzoides* aerial parts and two major constituents have potential for use in control of *Ae. albopictus* larvae and could be useful in search of newer, safer and more effective natural compounds as larvicides.

Keywords: *Ageratum conyzoides*; *Aedes albopictus*; larvicidal activity; essential oil.

1. Introduction
Botanical pesticides have the advantage of providing novel modes of action against insects that can reduce the risk of cross-resistance as well as offering new leads for design of target-specific molecules. During the screening program for new agrochemicals from Chinese medicinal herbs and wild plants, essential oil of *Ageratum conyzoides* L. (Family: Compositae) aerial parts at flowering stage was found to possess strong insecticidal toxicity against the Asian tiger mosquito (*Aedes albopictus* Skuse). Goatweed (*A. conyzoides*) is an annual herbaceous plant (50-10 cm height, sometimes less than 10 cm) with a long history of traditional medicinal uses in several countries of the world and also has bioactivity with insecticidal and nematicidal acitivity [1]. In China, it is used medicinally to treat a variety of conditions, including common colds, headaches, boils, eczema, bleeding wounds, and burns [3]. It is native to tropical America, especially Brazil and an invasive weed in Africa, Australia, Southeast Asia and the USA. Now it is cultured and naturalized in Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hainan, Henan, Jiangsu, Jiangxi, Nanhai Zhudao, Shaanxi, Sichuan, Taiwan, and Yunnan province, China [2]. In the previous reports, various flavonoids, coumarins, triterpenoids, steroids, pyrrolizidine alkaloids, and benzofuran derivatives (chromenes) have been isolated and identified in the plant [3-16]. The chemical composition of the essential oil and extracts of *A. conyzoides* has been studied previously [17-25]. It is reported that essential oil derived from the aerial parts (leaves) of *A. conyzoides* exhibit insecticidal and well as repellency against several insects [26-32]. The insecticidal constituents were also isolated from the essential oil and n-hexane extract of *A. conyzoides* as (Z)-6-methyl-12-heptadecenoic acid, coumarin, 5, 6, 7, 8, 3'-pentamethoxy-4',5'-methylenedioxyflavone and 5, 6, 7, 8, 3' ,4' ,5'-heptamethoxyflavone [33, 34].
Ae. albopictus is an epidemiologically important vector for the transmission of many viral pathogens, including the Yellow fever virus, dengue fever and Chikungunya fever as well as several filarial nematodes such as *Dirofilaria immitis* [33]. Currently application of synthetic insecticides including organophosphates such as temephos and fenthion, and insect growth

regulators such as diflubenzuron and methoprene are effective control method of larval mosquitoes [36]. However, heavy and wide use of these synthetic insecticides has caused several environmental and health concerns [37]. 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, environmental friendly, easily biodegradable, and often inexpensive [38]. Many essential oils and constituent compounds derived from various essential oils can exert toxic activity against mosquito species [39-43]. In light of the above facts, the present research was undertaken to investigate larvicidal activity of the essential oil and its major constituents against the Asian tiger mosquito, *Ae. albopictus*.

2. Materials and Methods

2.1. Plant material

Fresh aerial parts of *A. conyzoides* (15 kg) at flowering stage were harvested from Fuzhou City (26.08° N and 119.28° E, Fujian Province, China) in August 2011. The plant was identified, and a voucher specimen (CMH-Shenghongji-Fujian-2011-07) was deposited at the herbarium of Department of Entomology, China Agricultural University, Beijing, China.

2.2. Essential oil extraction

The dried samples were ground to a powder using a grinding mill. Each 600 g portion of powder was soaked in 1,800 ml of distilled water for 3 h. The mixture was then boiled in a round-bottom flask, and steam distilled for 6-8 h. Volatile essential oil from distillation was collected in a flask. Separation of the essential oil from the aqueous layer was done in a separatory funnel, using the non-polar solvent, *n*-hexane. The solvent was evaporated using a vacuum rotary evaporator (BUCHI Rotavapor R-124, Switzerland). The sample was dried over anhydrous Na_2SO_4 and kept in a refrigerator (4 °C) for subsequent experiments.

2.3. GC-MS analysis

The essential oil of *A. conyzoides* aerial parts was subjected to GC-MS analysis on an Agilent system consisting of a model 6890N gas chromatograph, a model 5973N mass selective detector (EIMS, electron energy, 70 eV), and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a 5% phenyl-methylpolysiloxane stationary phase, film thickness of 0.25 µm, a length of 30 m, and an internal diameter of 0.25 mm. The GC settings were as follows: the initial oven temperature was held at 60 °C for 1 min and increased at 10 °C min^{-1} to 180 °C for 1 min, and then increased at 20 °C min^{-1} to 280°C for 15 min. The injector temperature was maintained at 270 °C. The samples (1 µl, diluted to 1:100 with acetone) were injected, with a split ratio of 1: 10. The carrier gas was helium at flow rate of 1.0 ml min^{-1}. Spectra were scanned from 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 of 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 [44]. Component relative percentages were calculated based on GC peak areas without using correction factors.

2.4. Insect cultures and rearing conditions

Eggs of *Ae. 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 at 24-26 °C and natural summer photoperiod 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 mineral water and a small amount of dog food. Larvae were daily controlled until they reached the fourth instar stage, when they were utilized for bioassay (within 12 h).

2.5. 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) [45]. 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, commercial chlorpyrifos (purchased from National Center of Pesticide Standards, Tiexi District, Shenyang, 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, 26-27 °C, 78-80% relative humidity). Mortality was recorded after 24 h of exposure and the larvae were starved of food over this period.

2.6. Statistical analysis

Percent mortality was corrected for control mortality using Abbott's formula [46]. 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 [47]. Samples for which the 95% fiducial limits did not overlap were considered to be significantly different.

3. Results and Discussion

3.1. Chemical composition of the essential oil

The hydrodistillation of aerial parts of *A. conyzoides* at flowering stage of afforded essential oil (yellow) with a yield of 0.11% (v/w) and the density of the concentrated essential oil was determined to be 0.91 g/ml. The GC and GC–MS analysis of the essential oils of the aerial parts of *A. conyzoides* led to the identification and quantification of a total of 32 major components accounting for 98.19% of the total components present (Table 1). The main components found in the essential oil were precocene II (45.75%), precocene I (14.09%), and caryophyllene (12.13%) followed by germacrene D (4.18%) and caryophyllene oxide (4.06%) (Table 1). The essential oil of *A. conyzoides* had higher content (59.84%) of phenylpropanoids (precocenes) than monoterpenoids and sesquiternoids. Sesquiterpenoids represented 19 of the 32 compounds, corresponding to 33.01% of the whole oil while 9 of the 32 constituents were monoterpenoids (3.07% of the crude essential oil). In previous reports, precocene II,

precocene I and caryophyllene were major constituents of the essential oil of *A. conyzoides* although there are some variations [17-25]. For example, the essential oil of *A. conyzoides* aerial parts harvested from Guangzhou, China contained precocene II (25.89%), β-caryophyllene (23.78%), precocene I (14.76%), α-farnesene (5.42%) and coumarin (5.18%) [21] while the essential oil of *A. conyzoides* from the Guinea Gulf was characterized by the presence of high percentages of precocene I (34.4%), β-caryophyllene (24.6%) and caryophyllene oxide (12.2%) as well as small amounts of precocene II (0.8%) [23]. Moreover, the leaf essential oil of *A. conyzoides* harvested from the northeast of Brazil consisted exclusively of the chromenes precocene I (95.4%) and II (4.5%) [22] while the essential oil of fresh flowering aerial parts of *A. conyzoides* collected from Kumaun Himalaya, India mainly contained precocene II (42.5%), β-caryophyllene (20.7%), precocene I (16.7%), α-humulene (6.6%) and ρ-cymene (3.3%) [25].

Table 1: GC-MS analysis of essential oil of *Ageratum conyzoides* aerial parts.

Peak No	Compound	RI[a]	Percent Composition
1	α-Pinene [b]	939	1.44
2	β-Pinene [b]	974	0.09
3	β-Myrcene [b]	991	0.25
4	1,8-Cineole [b]	1032	0.55
5	Linalool [b]	1094	0.23
6	Borneol [b]	1174	0.38
7	4-Terpineol [b]	1177	0.45
8	α-Terpineol [b]	1189	0.19
9	Bornyl acetate [b]	1287	0.12
10	α-Cubebene	1345	0.11
11	α-Longipine	1350	0.67
12	Eugenol [b]	1356	0.26
13	Copaene	1375	0.36
14	β-Cubebene	1388	0.95
15	Methyleugenol [b]	1403	1.38
16	(Z)-Caryophyllene	1409	3.64
17	β-Caryophyllene [b]	1420	12.13
18	α-Bergamotene	1430	0.17
19	α-Caryophyllene	1454	0.11
20	(E)-β-Famesene	1457	0.69
21	Precocene I [b]	1467	14.09
22	Germacrene D	1485	4.18
23	Viridiflorene	1495	0.14
24	γ-Cadinene	1513	0.12
25	β-Sesquiphellandrene	1524	0.93
26	Dihydroactinidiolide	1525	0.25
27	*cis*-Nerolidol	1535	0.67
28	Spathulenol	1578	1.78
29	Caryophyllene oxide [b]	1583	4.06
30	Humulene oxide II	1606	0.37
31	Precocene II [b]	1656	45.75
32	β-Bisabolol	1673	1.68
	Total identified		98.19
	Monoterpenoids	9/32	3.70
	Sesquiterpenoids	19/32	33.01
	Precocenes	2	59.84
	Others	2	1.64

[a] RI, retention index as determined on a HP-5MS column using the homologous series of *n*-hydrocarbons; [b] Identification based on comparison of RI and spectra with authentic standards.

Table 2: Larvicidal activity of the essential oil of *Ageratum conyzoides* and its major constituents against the fourth-instar larvae of *Aedes albopictus*

Treatment	LC_{50} (µg/ml)	95% FL*	Slope ± SD	Chi-square value (χ^2)
Essential oil	61.22	56.68-66.51	8.96 ± 0.78	9.04
β-Caryophyllene	> 100	-	-	-
Precocene I	43.55	38.83-48.08	5.30 ± 0.51	8.48
Precocene II	41.63	37.42-45.91	6.89 ± 0.65	9.88
Chlorpyrifos	1.86	1.71-2.05	0.87 ± 0.01	3.13

* FL: Fiducial limits

3.2. Larvicidal activity of the essential oil

The essential oil of *A. conyzoides* aerial parts possessed strong larvicidal activity against the 4th instar larvae of *Ae. albopictus* with a LC_{50} value of 61.22 µg/ml (Table 2). The commercial insecticide, chlorpyrifos exhibited larvicidal activity against the mosquitoes with a LC_{50} value of 1.86 µg/ml, thus the essential oil of *A. conyzoides* aerial parts was 33 times less toxic to *Ae. albopictus* larvae compared with chlorpyrifos. However, compared with the other essential oils using the same bioassay in the literature, the essential oil of *C. pendulum* exhibited the same level of or stronger larvicidal activity against *Ae. albopictus* larvae, e.g., essential oils of *Salvia splendens* (LC_{50} = 59.2 ppm, respectively) [48]; *Toddalia asiatica* roots (LC_{50} = 69.09 µg/ml) [40]; *Allium macrostemon* (LC_{50} = 72.86 µg/ml) [35]; *Eucalyptus urophylla* (LC_{50} = 95.5 µg/ml) [49]; and essential oils 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) [50]. The essential oil of *A. conyzoides* was demonstrated to exhibit larvicidal activity against the 4th instar larvae of *Ae aegypti* with a 48 hr LC_{50} value of 148 µg/ml [51].

The major constituents of the essential oil, precocene II and precocene I exhibited similar larvicidal activity against the 4th instar larvae of *Ae. albopictus* with LC_{50} values of 41.63 µg/ml and 43.55 µg/ml, respectively (Table 2). However, another major constituent, β-caryophyllene did not show any toxicity to the larvae of *Ae. albopictus* at the tested concentrations. In previous report [52], β-caryophyllene showed weak larvicidal activity against *Ae. aegypti* (LC_{50} = 1038 ppm). It is suggested that the larvicidal activity of the essential oil may be attributed to precocene II and precocene I. In the previous studies, precocene II and precocene I were demonstrated to inhibit the synthesis of juvenile hormone in a number of insects. Consequently, this inhibition can disturb the embryonic development, induce premature metamorphosis, decrease the reproductive potential, and affect the insect behavior including the antifeedant and repellent effect [53-56]. Moreover, they exhibited larvicidal and growth-inhibiting activities against several mosquitoes, e.g. *Ae. aegypti*, *Anopheles sacharovi* and *An. stephensi* [57-60]. Considering that the currently used larvicides are synthetic insecticides, larvicidal activity of the crude essential oil of *A. conyzoides* is quite promising and it shows its potential for use in the control of *Ae. albopictus* larvae and could be useful in the search for newer, safer and more effective natural compounds as larvicides. For the actual use of *A. conyzoides* aerial parts essential oil and its constituents as a novel larvicide or insecticide to be realized, further research is needed to establish their human safety and environmental safety. Additionally, their larvicide modes of action have to be established, and formulations for improving larvicidal potency and stability need to be developed.

4. Conclusions

Chemical composition of the essential oil of *A. conyzoides* aerial parts at flowering stage was determined and the essential oil demonstrated larvicidal activity against the mosquito *Ae. albopictus*. It showed potential to be developed as a possible natural larvicide for mosquito control but needs to be further evaluated for safety to humans and modes to enhance efficiency against the target species.

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