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Full Length Research Paper

Chemical composition and toxicities of the essential oil derived from *Hyssopus cuspidatus* flowering aerial parts against *Sitophilus zeamais* and *Heterodera avenae*

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Water-distilled essential oil from *Hyssopus cuspidatus* Boriss (Family: Labiatae) from the aerial parts at the flowering stage was analyzed by gas chromatography-mass spectrometry (GC-MS). Thirty-nine compounds, accounting for 96.78% of the total oil, were identified and the main components of the essential oil of *H. cuspidatus* flowering aerial parts were thymol (19.65%), *trans*-pinocamphone (15.30%), γ-terpinene (14.63%), ρ-Cymene **(7.49%), and β-pinene (6.57%) followed by caryophyllene (4.33%), 1,8-cineol (4.23%) and germacrene D (3.53%). The essential oil of *H. cuspidatus* possessed contact toxicity against the maize weevil, *Sitophilus zeamais* adults with an LD_{50} value of 24.44 µg/adult and also showed pronounced fumigant toxicity against *S. zeamais* (LC_{50} = 16.72 mg/L air). The essential oil of *H. cuspidatus* exhibited strong nematicidal activity against the cereal cyst nematode, *Heterodera avenae* with an LC_{50} value of 338.70 µg/mL. The essential oil of *H. cuspidatus* shows potential to be developed as a possible natural insecticide/nematicide for control of stored product insects/nematodes.**

Key Words: *Hyssopus cuspidatus*, *Sitophilus zeamais*, *Heterodera avenae*, nematicidal activity, contact toxicity, fumigant, essential oil composition

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 (Isman, 2006). During our mass screening program for new agrochemicals from Chinese medicinal herbs and wild plants (Liu et al., 2007), essential oil of *Hyssopus cuspidatus* Boriss (Family: Labiatae) flowering aerial parts was found to possess strong toxicities against the maize weevil, *Sitophilus zeamais* (Motsch) and the cereal cyst nematode, *Heterodera avenae* Wollenweber. *S. zeamais* is one of the most widespread and destructive primary insect pests of stored cereals (Liu and Ho, 1999). Infestations not only cause significant losses due to the consumption of grains; they also result in elevated temperature and moisture conditions that lead to an accelerated growth of molds, including toxigenic species (Magan et al., 2003). Currently, control of stored product insects relies heavily on the use of synthetic insecticides and fumigants. However, repeated use of synthetic fumigants for decades has led to resurgence of stored-product insect pests, sometimes resulted in the development of resistance, and had undesirable effects on non-target organisms (Zettler and Arthur, 2000). These problems have highlighted the need to develop new types of selective insect-control alternatives with fumigant action. Plant essential oils and their components have been shown to possess potential to be developed as new fumigants and they may have the advantage over

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conventional fumigants in terms of low mammalian toxicity, rapid degradation and local availability (Isman, 2006; Rajendran and Srianjini, 2008).

H. avenae is one of the most important parasitic nematodes of cereals (barley, oats, rye, wheat). It is cosmopolitan in distribution and has been reported in different parts of the world. It was first found occurring from Hubei province in the centre of China in 1989 (Chen et al., 1991) and now has been found in more than 300 countries of 13 provinces of China. Nematode management is generally based upon chemical treatments (soil fumigation), but environmental concerns and governmental regulations (United Nations Environment Programme, 2000) are now resulting in a strong interest in nematicides of natural origin (Chitwood, 2002). Many plant constituents and metabolites including essential oils and monoterpenoids have been investigated for activity against plant-parasitic nematodes (Sangwan et al., 1985; Kim et al., 2008; Ntalli et al., 2010; Bai et al., 2011; Du et al., 2011; Li et al., 2011; Zhang et al., 2011). The results suggest that some of the essential oils tested and selected monoterpenoids are potential natural pesticides in the control of nematodes.

H. cuspidatus usually grows on stony, dry hillside grasslands and is mainly distributed in Xinjiang Uyghur Autonomous Region, China and also in Kazakhstan, Mongolia, and Russia (Committee of Flora of China, 1977). It has also been used in traditional Uyghur medicine (Xinjiang, China) in the treatment of cough, asthma, bronchitis, trauma, and rheumatism (Committee of Flora of China, 1977; Ablizl et al., 2009). Previous phytochemical studies on *H. cuspidatus* resulted in the identification of several monoterpenoids, diterpenoids, sesquiterpenoids, triterpenoids, flavonoids, and phenethyl glucosides (Ablizl et al., 2011; Furukawa et al., 2011). The chemical composition of *H. cuspidatus* essential oil was also studied previously (Xue et al., 1990; Fu and Zhang, 2008; Ablizl et al., 2009; Zhou et al., 2010). However, a literature survey has shown that there is no report on toxicities of *H. cuspidatus* essential oil against insects and nematodes; thus we decided to determined chemical composition and toxicities of the essential oil against nematodes and insects.

MATERIALS AND METHODS

Insect and nematode

The maize weevils (*S. zeamais*) were obtained from laboratory cultures maintained for the last 15 years in the dark in incubators at 29-30°C and 70-80% relative humidity. The maize weevils were reared on whole wheat at 12-13% moisture content in glass jars (diameter 85 mm, height 130 mm) at 29-30°C and 70-80% relative humidity. Unsexed adult weevils and beetles used in all the experiments were about 2 weeks old.

Cysts of *H. avenae* were extracted from rhizosphere soil of wheat roots collected in Zhengzhou city (34.44 °N latitude and 112.56 °E longitude), Henan province, China. Cyst masses were stored at -4°C for a month firstly and were maintained in Petri dishes at 15°C during 3-7 days for the juvenile eclosion. Only freshly hatched second stage juveniles (J_2) were used in the experiments.

Plant material

Air-dried flowering aerial parts (5 kg) of *H. cuspidatus* (cultivated and harvested from Taicheng, Xinjiang, 46.46 °N latitude and 82.59 °E longitude) were purchased from Anguo Chinese Herbs Market, Hebei province, China and were ground to a powder using a grinding mill (Retsch Mühle, Germany). The medicinal herb was identified by Dr. Liu, Q.R. (College of Life Sciences, Beijing Normal University, Beijing 100875, China) and a voucher specimen (CMH-XinjiangShenxiancao-2010-05) was deposited in the Department of Entomology, China Agricultural University. Each 600 g portion of powder ground was mixed in 1,800 ml of distilled water and soaked for 3 h. The mixture was then boiled in a round-bottom flask, and steam distilled for 6 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.

Gas chromatography-mass spectrometry

The essential oil of *H. cuspidatus* flowering aerial parts was subjected to gas chromatography-mass spectrometry (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 ramped at 10°C min^{-1} to 180°C held for 1 min, and then ramped at 20°C min^{-1} to 280°C and held for 15 min. The injector temperature was maintained at 270°C. The sample (1 µL) was injected neat, 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 (Fu and Zhang, 2008; Ablizl et al., 2009; Zhou et al., 2010) 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 08 and Wiley 275 libraries or with mass spectra from literature (Adams, 2007). Component relative percentages were calculated based on normalization method without using correction factors.

Topical application

Range-finding studies were run to determine the appropriate testing concentrations of the essential oil. A serial dilution (six concentrations, 5-50%) of the essential oil was prepared in *n*-hexane. Aliquots of 0.5 µL per insect were topically applied dorsally to the thorax of insects, using a Burkard Arnold microapplicator. Controls were determined using 0.5 µL *n*-hexane per insect. Ten insects were used for each concentration and control, and the experiment was replicated six times. Both treated and control insects were then transferred to glass vials (10 insects/vial) with culture media and kept in incubators at 29-30°C and 70-80% relative humidity. It was observed daily for determination of end-

Table 1. Chemical constituents of essential oil derived from *Hyssopus cuspidatus* flowering aerial parts.

Peak	Compounds	RI*	Peak area (%)
1	α-Pinene	939	1.44
2	β-Thujene	966	0.45
3	β-Pinene	981	6.57
4	δ-4-carene	1002	0.37
5	ρ-Cymene	1025	7.49
6	1,8-Cineol	1031	4.23
7	γ-Terpinene	1057	14.63
8	α-Terpinolene	1089	2.04
9	Linalool	1097	0.80
10	*trans*-Pinocarveol	1139	1.71
11	cis-β-Terpineol	1145	0.07
12	*trans*-Pinocamphone	1159	15.30
13	α-Terpineol	1188	0.50
14	Myrtenol	1196	1.85
15	Thymol	1292	19.65
16	4-Vinylguaiacol	1311	1.32
17	Chavibetol	1362	0.74
18	α-Copaene	1375	0.28
19	β-Bourbonene	1382	0.55
20	Methyleugenol	1403	0.47
21	α-Gurjunene	1411	0.23
22	Caryophyllene	1420	4.33
23	α-Caryophyllene	1452	0.28
24	*allo*-Aromadendren	1456	0.46
25	Germacrene D	1480	3.53
26	Bicyclogermacrene	1500	1.56
27	1ξ,6ξ,7ξ-Cadina-4,9-diene	1502	0.70
28	β-Bisabolene	1506	1.11
29	γ-Cadinene	1513	0.33
30	δ-Cadinene	1525	0.13
31	Dihydroactinolide	1538	0.18
32	Elemol	1547	0.51
33	Ledol	1562	0.31
34	Spathulenol	1572	0.39
35	Caryophyllene oxide	1583	1.13
36	Isoaromadendrene epoxide	1594	0.28
37	γ-Eudesmol	1631	0.46
38	β-Eudesmol	1648	0.26
39	Valerenol	1655	0.14
	Total		96.78
	Monoterpenoids		55.22
	Sesquiterpenoids		17.15
	Others		24.41

*RI, retention index as determined on a HP-5MS column using the homologous series of *n*-hydrocarbons.

point mortality, which was reached after one week. The insects were considered dead if appendages did not move when probed with a camel brush. Results from all replicates were subjected to Probit analysis using the PriProbit Program V1.6.3 to determine LD_{50} values (Sakuma, 1998).

Fumigant bioassay

Range-finding studies were run to determine the appropriate testing concentrations of the essential oil. A serial dilution of the essential oil (six concentrations, 5-30%) was prepared in *n*-hexane. A Whatman filter paper (CAT no. 1001020, diameter 2.0 cm) were each impregnated with 10 µL dilution, and then placed on the underside of the screw cap of a glass vial (diameter 2.5 cm, height 5.5 cm, volume 25 mL). The solvent was allowed to evaporate for 20 s before the cap was placed tightly on the glass vial, each of which contained 10 insects inside to form a sealed chamber. Preliminary experiments demonstrated that 20 s was sufficient for the evaporation of solvents. The vials were upright and the Fluon (ICI America Inc) coating restricted the insects to the lower portion of the vial to prevent them from the treated filter paper. *n*-Hexane was used as a control. Six replicates were carried out for all treatments and controls, and they were incubated for 24 h. The insects were then transferred to clean vials with some culture media and returned to the incubator and observed daily for determination of end-point mortality, which was reached after one week. The insects were considered dead if appendages did not move when probed with a camel brush. The experiments were repeated three times. The LC_{50} values were calculated by using Probit analysis (Sakuma, 1998).

Nematicidal toxicity bioassay

Range-finding studies were run to determine the appropriate testing concentrations. A serial dilution of the essential oil (five concentrations, 100-1,600 ppm) was prepared in H_2O solution with 2% DMSO. Aliquots of H_2O (20 µL) containing ca. 100 juveniles (J2) were transferred to vials to which 980 µL of the solution containing the essential oil was added. The vials were kept on a hood at 20°C. The nematodes were counted every 24 h for 48 h. The nematodes were considered dead if the nematodes kept not moving and stiff after added 1-2 drops of 1% NaOH solution. Six repetitions for each treatment were performed using H_2O and a 2% DMSO in H_2O solution as a control. The experiments were repeated three times. Results from all replicates were subjected to Probit analysis using the PriProbit Program V1.6.3 to determine LC_{50} values (Sakuma, 1998). Carbofuran was purchased from National Center of Pesticide Standards (8 Shenliao West Road, Tiexi District, Shenyang 110021, China) and used as a positive control because of one of comerically used nematicides.

RESULTS AND DISCUSSION

The steam distillation for 6 h of *H. cuspidatus* aerial parts afforded essential oils with a yield of 0.62% (V:W) and the density of the concentrated essential oil was determined to be 0.85 g/mL. GC–MS analysis of the essential oil of *H. cuspidatus* led to the identification and quantification of a total of 39 major components accounting for 96.78% of the total components present (Table 1). The main components of the essential oil of *H. cuspidatus* flowering aerial parts were thymol (19.65%), *trans*-pinocamphone (15.30%), γ-terpinene (14.63%), ρ-Cymene (7.49%), and β-pinene (6.57%) followed by caryophyllene (4.33%), 1,8-cineol (4.23%) and germa-

Table 2. Toxicities of *Hyssopus cuspidatus* essential oil against *Sitophilus zeamais* adults and *Heterodera avenae* larvae.

Insects	Toxicities	Treatment	LC_{50} (mg/L air) (μg/mL) (95%FL)	LD_{50} (μg /adult) (95%FL)	Slope ± SE	Chi square (χ^2)
S. zeamais	Fumigant	Essential oil	16.72 (15.58-17.45)	-	4.52 ± 0.45	13.57
		MeBr[a]	0.67	-	-	-
	Topical application	Essential oil	-	24.44 (22.58-26.77)	5.71 ± 0.55	17.36
		Pyrethrum extract[b]	-	4.29 (3.86-4.72)	0.73 ± 0.02	13.51
H. avenae	Nematicidal	Essential oil	338.70[c] (305.89-373.23)	-	2.58 ± 0.26	13.59
		Carbofuran	137.12[c] (125.96-151.16)	-	1.67 ± 0.14	1.78

[a]data from Liu and Ho (1999); [b]data from Liu et al. (2010a); [c]Significant difference (no overlaps in 95% fiducial limit).

crene D (3.53%). Monoterpenoids represented 14 of the 39 compounds, corresponding to 55.22% of the whole oil while 21 of the 39 constituents were sesquiterpenoids (17.15% of the crude essential oil). The results were different from that of the previous reports. For example, Xue et al. (1990) demonstrated that the essential oil of *H. cuspidatus* contained d-pinocamphone (44.3%), l-pinocamphone (12.2%), β-pinene (8.2%) and 1,8-cineole (7.1%). Moreover, berbenone (23.84%), β-pinene (19.76%), pinocamphone (17.95%), eucalyptol (7.16%), myrtenol (7.06%), methane (3.56%), and *trans*-pinocarveol (3.00%) were the main compounds of the essential oil of *H. cuspidatus* aerial parts (Zhou et al., 2010). It suggested that there are some variations in chemical composition of the essential oil of *H. cuspidatus* and These differences might have been due to harvest time and local, climatic and seasonal factors as well as storage duration of medicinal herbs. Further studies on plant cultivation and essential oil standardization are needed.

The essential oil of *H. cuspidatus* flowering aerial parts exhibited contact toxicity against *S. zeamais* adults with an LD_{50} value of 24.44 μg/adult (Table 2). However, compared with the pyrethrum extract (25% pyrethrine I and pyrethrine II), the essential oil demonstrated one-fifth as acute toxic as the pyrethrum extract against the insects because of pyrethrum extract (LD_{50} = 4.29 μg/adult against *S. zeamais* adults) (Liu et al., 2010a). The essential oil of *H. cuspidatus* also possessed pronounced fumigant toxicity against *S. zeamais* adults (LC_{50} = 16.72 mg/L air). No death of insects was observed in the control under current concentration. The currently used grain fumigant, methyl bromide (MeBr) was reported to have fumigant activity against *S. zeamais* adults with a LC_{50} value of 0.67 mg/L air (Liu and Ho, 1999). Compared with MeBr, the essential oil was 25 times less active against the insects. However, compared with the other essential oils in the previous studies that were tested using a similar bioassay, the essential oil of *H. cuspidatus* flowering aerial parts exhibited the same level or stronger fumigant toxicity against *S. zeamais* adults than the essential oils of *Caryopteris incana* (Chu et al., 2011), *Kadsura heteroclite* (Li et al., 2011), *Illicium fragesii* (Wang et al., 2011), *I. simonsii* (Chu et al., 2010), *Murraya exotica* (Li et al., 2010) and several essential oils from Genus *Artemisa* (Liu et al. 2010a; 2010b; Jiang et al. 2012). Considering the currently used fumigants are synthetic insecticides, fumigant activity of the essential oil of *H. cuspidatus* is quite promising and it shows potential to be developed as a possible natural fumigant for control of stored product insects. However, for the practical application of the essential oil as novel fumigant, further studies on the safety of the essential oil to humans and on development of formulations are necessary to improve the efficacy and stability and to reduce cost.

The essential oil of *H. cuspidatus* possessed strong nematicidal activity against *H. avenae* with an LC_{50} value of 338.70 μg/mL (Table 2). No death of nematodes was observed in the control under current concentration. Compared with a synthetic insecticide, carbofuran (LC_{50} = 137.12 μg/mL), the essential oil exhibited half level of toxicity against *H. avenae* and shows potential to be developed as a possible natural nematicide for control of the cereal cyst nematodes.

In previous studies, some of the main components of the essential oil of *H. cuspidatus* were found to possess fumigant activity against insects and mites. For example, thymol exhibited fumigant toxicity against the German

cockroach (*Blattella germanica*) (Phillips and Appel, 2010), Mediterranean flour moth (*Ephestia kuehniella*) (Karaborklu et al., 2011), and tracheal mites (*Acarapis woodi*) (Ellis and Baxendale, 1997) as well as twospotted spider mite (*Tetranychus urticae*) (Lim et al., 2011). γ-Terpinene possessed fumigant toxicity against Japanese termite (*Reticulitermes speratus*) (Seo et al., 2009), head lice (*Pediculus humanus capitis*) (Toloza et al., 2006) and German cockroaches (*Blattella germanica*) (Alzogaray et al., 2011). However, there is no report on fumigant toxicity of *trans*-pinocamphone against insects/mites. The isolation and identification of the bioactive compounds in the essential oil of *H. cuspidatus* are of utmost importance so that their potential application in controlling stored-product pests/nematodes can be fully exploited.

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