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Fumigant Components from the Essential Oil of *Evodia Rutaecarpa* Hort Unripe Fruits

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Abstract: Essential oil of chinese medicinal herb, *Evodia rutaecarpa* unripe fruits was found to possess insecticidal activity against maize weevils, *Sitophilus zeamais* and red flour beetles *Tribolium castaneum*. The essential oil of *E. rutaecarpa* was obtained by hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC-MS). A total of 38 components of the essential oil were identified. The principal compounds in *E. rutaecarpa* essential oil were β-myrcene (17.7%), (Z)-β-ocimene (14.8%), α-phellandrene (14.7%), γ-terpinene (6.4%), linalool (5.7%) and β-thujene (5.1%). Bioactivity-guided chromatographic separation of the essential oil on repeated silica gel columns led to isolate three volatile components (β-myrcene, β-ocimene and α-phellandrene) from the essential oil. α-Phellandrene was strongest fumigant against *S. zeamais* adults, *T. castaneum* adults and *T. castaneum* larvae with LC_{50} values of 15.61, 19.78 and 47.96 mg/L air, respectively. β-Myrcene and β-ocimene also possess fumigant activity against the two species of insects but weaker fumigant activity than the crude essential oil.

Keywords: *Evodia rutaecarpa*, *Sitophilus zeamais*, *Tribolium castaneum*, Fumigant, β-Myrcene, β-Ocimene, α-Phellandrene

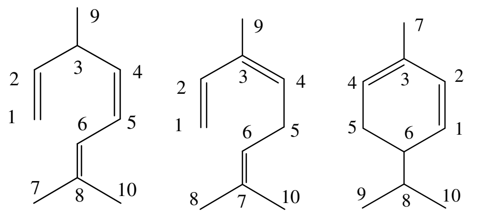
Introduction

During our screening program for new agrochemicals from local wild plants and Chinese medicinal herbs, essential oil derived from unripe fruits of *Evodia rutaecarpa* (Juss.) Benth. (Family: Rutaceae) was found to possess strong insecticidal activity against maize weevils, *Sitophilus zeamais* Motsch and red flour beetles, *Tribolium castaneum* Herbst. The maize weevils and red flour beetles are two of the most widespread and destructive primary insect pests of stored cereals[1]. 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[2]. Fumigation is still one of the most effective methods for the protection of stored food, feedstuffs and other agricultural commodities from insect infestation not only because of their ability to kill a broad spectrum of pests but because of their easy penetration into the commodity while leaving minimal residues[3]. However, repeated use of those fumigants (phosphine and methyl bromide) for decades has disrupted biological control by natural enemies and led to resurgence of stored-product insect pests, sometimes resulted in the development of resistance and had undesirable effects on non-target organisms[3]. 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 conventional fumigants in terms of low mammalian toxicity, rapid degradation and local availability[4,5]. The toxicity of a large number of essential oils and their constituents has been also evaluated against a number of stored-product insects[6].

The dried unripe fruit of *E. rutaecarpa* is a commonly used Chinese medicinal herb and has been recommended for the treatment of abdominal pain, acid regurgitation, nausea, diarrhea, hernia and dysmenorrhea and is also used externally for treatment of aphthous stomatitis[7]. The herb is also documented as insecticidal/anti-parasitic in the literature[8]. Chemical composition of essential oil of *E. rutaecarpa* has been widely investigated[9-11]. The essential oil of *E. rutaecarpa* showed strong fumigant toxicity against the adults of the red flour beetles and maize weevils[1]. Moreover, the essential oils also possessed fumigant toxicity against other stored products, rice weevil (*S. oryzae*) and bean weevil (*Callosobruchus chinensis*) as well as cigarette beetle (*Lasioderma serricorne*)[12,13]. But as far as we know, there are no reports about isolation of active (fumigant) components against stored product insects from this essential oil. In this report, three active compounds were isolated and identified from the essential oil of *E. rutaecarpa* by bioassay-directed fractionation.

Experimental

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Bruker ACF300 [300MHz (^{1}H)] and AMX500 [500MHz (^{1}H)] instruments using deuterochloroform ($CDCl_3$) as the solvent with tetramethylsilane (TMS) as the internal standard. Electron impact ionone mass spectra (EIMS) were determined on a micromass VG7035 mass spectrometer at 70 eV (probe). The crude essential oil (25 mL) was chromatographed on a silica gel (Merck 9385, 1,000 g) column (85 mm i.d., 850 mm length) by gradient elution with a mixture of solvents (*n*-hexane, *n*-hexane-ethyl acetate and acetone). Fractions of 500 mL were collected and concentrated at 40 °C and similar fractions according to TLC profiles were combined to yield 18 fractions. Each fraction was tested with fumigant toxicity bioassay (see below) to identify the bioactive fractions (Fraction 4, 7 and 11). Fractions that possessed fumigant toxicity, with similar TLC profiles, were pooled and further purified by preparative silica gel column chromatography (PTLC) until to obtain three pure compounds for determining structure (Figure 1). The spectral data of β-myrcene (**1**) (1.2 g) matched with the previous report[14,15]. The data of β-ocimene (**2**) (1.7 g) matched with the previous report[14,16]. The spectral data were identical to the published data of α-phellandrene (**3**) (0.8 g)[14,16].



beta-Myrcene (1) beta-Ocimene (2) ¦ÁPhellandrene (3)

Figure 1. Compounds isolated from *Evodia rutaecarpa* essential oil

Chinese medicinal herb and hydrodistillation

Five kilograms of *E. rutaecarpa* unripe fruits were purchased from Anguo Chinese Herbs Market (Hebei Province 071200, China). A voucher specimen (CMH-WuZhuYu-ZheJiang-2009-09) was deposited in the department of entomology, China Agricultural University. To obtain volatile essential oil, the medicinal herb was first ground to a powder then soaked in water at a ratio of 1:4 (w/v) for 1 h, prior to hydrodistillation using a round bottom container over a period of 6 h. The volatile essential oil was collected in a specific receiver, measured, dried over anhydrous sulfate, weighed and stored in airtight containers.

Analysis of the essential oil

Components of the essential oil were separated and identified by gas chromatography–mass spectrometry (GC–MS) agilent 6890N gas chromatography hooked to agilent 5973N mass selective detector. They equipped with a flame ionization detector and capillary column with HP-5MS (30 m×0.25 mm×0.25 μm). 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 for 1 min and then ramped at 20 °C min^{-1} to 280 °C for 15 min. The injector temperature was maintained at 270 °C. The samples (1 μL) were 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 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]. Component relative percentages were calculated based on GC peak areas without using correction factors.

Fumigant toxicity assay

The insects were obtained from laboratory cultures maintained for the last 15 years in the dark in incubators at 29-30 °C and 65-75% r.h. The red flour beetles were reared on wheat flour mixed with yeast (10:1, w:w) while the maize weevils were reared on whole wheat at 12-13% moisture content. The unsexed adults of both species used in the experiments were 2-4 weeks post-eclosion. The larvae of *T. castaneum* used in the experiments were 16-day old. A Whatman filter paper (diameter 2.0 cm) was placed on the underside of the screw cap of a glass vial (diameter 2.5 cm, height 5.5 cm, volume 24 mL). Range-finding studies were run to determine the appropriate testing concentrations. A serial dilution of the essential oil/compound (six concentrations) was prepared in *n*-hexane. Ten microliters of an appropriate concentration of oil and compounds was added to the filter paper. The solvent was allowed to evaporate for 15 s before the cap was placed tightly on the glass vial (with 10 insects) to form a sealed chamber. *n*-Hexane was used as controls. Six replicates were used in all treatments and controls and they were incubated at 29-30 °C and 65-75% r.h. for 24 h. The insects were then transferred to clean vials with some culture media and kept in an incubator for determination of end-point mortality, which was reached after one week. The experiments were repeated in three times. Results from all replicates for the pure compounds and essential oil were subjected to probit analysis using the priprobit program V1.6.3 to determine LC_{50} values[18].

Results and Discussion

Chemical constituents of the essential oil

Hydrodistillation of dried unripe fruits of *E. rutaecarpa* yielded 2.45% essential oil (v/w). A total of 38 components were identified in the essential oil, accounting for 97.5% of the total oil (Table 1). The main constituents of the essential oil were *β*-myrcene (17.7%), (*Z*)-*β*-ocimene (14.8%), *α*-phellandrene (14.7%), *γ*-terpinene (6.4%), linalool (5.7%) and *β*-thujene (5.1%). The essential oil of *E. rutaecarpa* is characterized by a high content of monoterpenoids (80.2%) and sesquiterpenoids comprise only 15.7% of the essential oil. The results of chemical composition of the essential oil were quite different from the previous reports. For example, *trans*-*β*-ocimene (66.1%) was the major compound followed by *cis*-*β*-ocimene (8.6%) and *β*-myrcene (6.2%) in the essential oil of *E. rutaecarpa* unripe fruits harvested from sichuan province (Southwest China)[19]. However, *β*-myrcene (39.3%) and *β*-phellandrene (12.9%) were two main components of the essential oil of *E. rutaecarpa* fruits collected from hunan province (Central China)[20]. *β*-Caryophyllene oxide (14.7%), *Z*-11-pentadencenal (13.6%) and globulol (7.2%) were the main constitutes of the essential oil of *E. rutaecarpa* leaves harvested from shanxi province (Northwest China)[21]. while Jiang *et al.*[22] found that the essential oil of *E. rutaecarpa* leaves contained *trans*-nerolidol (18.9%), *γ*-eudesmol (15.8%), *β*-caryophyllene (11.1%) and *β*-eudesmol (10.1%). The above findings suggest that further studies on plant cultivation and essential oil standardization are needed because chemical composition of the essential oil varies greatly with the plant population.

Table 1. Chemical constituents of essential oil from *Ligusticum chuanxiong*

Peak	Compounds	RI*	Formula	RA**, %
1	*α*-Pinene	931	$C_{10}H_{16}$	0.31
2	*β*-Thujene	967	$C_{10}H_{16}$	5.13
3	Sabinene	977	$C_{10}H_{16}$	0.08
4	*β*-Pinene	981	$C_{10}H_{16}$	0.28
5	*β*-Myrcene	991	$C_{10}H_{16}$	17.70
6	*α*-Phellandrene	1005	$C_{10}H_{16}$	14.74
7	4-Carene	1022	$C_{10}H_{16}$	1.44
8	*β*-Phellandrene	1029	$C_{10}H_{16}$	4.70
9	(*Z*)-*β*-Ocimene	1038	$C_{10}H_{16}$	14.83
10	*γ*-Terpinene	1057	$C_{10}H_{16}$	6.43
11	*cis*-Linaloloxide	1067	$C_{10}H_{18}O_2$	0.61
12	Linalool	1094	$C_{10}H_{18}O$	5.70
13	*cis*-*ρ*-Menth-2-en-1-ol	1126	$C_{10}H_{18}O$	0.76

Contd...

14	*neo*-Alloocimene	1139	$C_{10}H_{16}$	2.77
15	*trans*-ρ-Menth-2-en-1-ol	1145	$C_{10}H_{18}O$	0.70
16	4-Terpineol	1179	$C_{10}H_{18}O$	0.61
17	Cryptone	1180	$C_9H_{14}O$	1.15
18	α-Terpineol	1191	$C_{10}H_{18}O$	1.21
19	Safranal	1197	$C_{10}H_{14}O$	0.27
20	(*R*)-Carvone	1216	$C_{10}H_{14}O$	0.18
21	Phellandral	1281	$C_{10}H_{16}O$	1.54
22	*trans*-Anethole	1285	$C_{10}H_{12}O$	0.31
23	Eugenol	1356	$C_{10}H_{12}O_2$	0.35
24	β-Geranyl acetate	1381	$C_{10}H_{20}O_2$	0.87
25	β-Cubebene	1390	$C_{15}H_{24}$	0.76
26	β-Elemene	1391	$C_{15}H_{24}$	0.64
27	(*E*)-α-Ionone	1399	$C_{15}H_{24}$	1.02
28	Caryophyllene	1420	$C_{15}H_{24}$	2.29
29	γ-Elemene	1437	$C_{15}H_{24}$	0.51
30	γ-Selinene	1455	$C_{15}H_{24}$	0.82
31	β-Selinene	1473	$C_{15}H_{24}$	0.51
32	α-Selinene	1492	$C_{15}H_{24}$	1.10
33	Muurolene	1498	$C_{15}H_{24}$	1.19
34	δ-Cadinene	1523	$C_{15}H_{24}$	0.54
35	Nerolidol	1576	$C_{15}H_{26}O$	0.91
36	Spathulenol	1578	$C_{15}H_{24}O$	1.18
37	Caryophyllene oxide	1583	$C_{15}H_{24}O$	1.93
38	α-Cadinol	1596	$C_{15}H_{26}O$	1.45
	Total identified			98.25
	Monoterpenoids			80.80
	Sesquiterpenoids			13.50
	Other			2.95

**RI, retention index as determined on a HP-5MS column using the homologous series of* *n*-hydrocarbons; **RA, relative area (peak area relative to total peak area)

Fumigant toxicity of isolated compounds

Three isolated compounds possess fumigant activity against the two grain storage product insects (Table 2). α-Phellandrene showed stronger fumigant toxicity to *S. zeamais* adults (LC_{50} = 15.61 mg/L air), *T. castaneum* larvae (LC_{50} = 47.96 mg/L air) and adults (LC_{50} = 19.78 mg/L air) than β-myrcene against *S. zeamais* adults (LC_{50} = 53.33 mg/L air), *T. castaneum* larvae (LC_{50} = 87.09 mg/L air) and adults (LC_{50} = 43.59 mg/L air). The LC_{50} values of β-ocimene against *S. zeamais* adults, *T. castaneum* larvae and adults were 28.66, 67.75 and 35.18 mg/L air, respectively meanwhile the LC_{50} values of essential oils were 36.89, 57.31 and 24.51 mg/L air, respectively. The 16-day old larvae of *T. castaneum* were twice as tolerant (based on LC_{50} values) to fumigant toxicity of the three compounds than the adults of *T. castaneum* (Table 2). In the previous reports, β-ocimene exhibited fumigant toxicity against *T. castanuem* adults with LC_{50} values (72 h) of 14.8 μL/L and against *S. oryzae* adults with LC_{50} values of 3.2 μL/L[23] while β-myrcene possessed fumigant activity to bean weevil (*A. obtectus*) with LC_{50} values of 29.4 (male) and 38.8 mg/L air (female)[24]. β-Myrcene was also demonstrated to have contact and fumigant toxicity against other stored product insects, *e.g. S. oryzae* and

T. castaneum[25]. However, compared with the current used fumigant methyl bromide (MeBr), all the three compounds possessed 23-77 times less toxic (fumigant) against adults of the maize weevils while 11-25 times less toxic to the red flour beetles because MeBr exhibited fumigant toxicity against adults of *S. zeamais* and *T. castaneum* with LC_{50} values of 0.67 and 1.75 mg/L air, respectively[1]. The isolated compounds have low mammalian toxicity[26,27]. *E. rutaecarpa* unripe fruits have been used as a common Chinese medicinal herb[7]. However, there are no toxicity data for this herb available on human consumption.

Table 2. Fumigant toxicity of the essential oil of *Evodia rutaecarpa* and isolated compounds against *S. zeamais* and *T. castaneum*

Compounds	$LC_{50,}$ mg/ L air, 95% fiducial limits		
	S. zeamais adults	*T. castaneum* adults	*T. castaneum* larvae
β-Myrcene	51.33 (49.62-52.15)	43.59 (40.22-45.66)	87.09 (83.14-93.75)
β-Ocimene	28.66 (26.84-30.73)	35.18 (33.44-37.65)	67.75 (61.81-72.46)
α-Phellandrene	15.61 (13.31-17.53)	19.78 (17.65-20.56)	47.96 (44.78-51.23)
Essential oil	36.89 (32.45-38.29)	24.57 (23.05-25.83)	57.31 (54.67-61.26)
MeBr*	0.67	1.75	-

**data from Liu & Ho (1999)*

Considering the currently used fumigants are synthetic insecticides, fumigant activity of the crude essential oil and the three isolated compounds are quite promising and they show potential to be developed as possible natural fumigants for control of stored product insects with low toxicity to humans. However, little has been done on mechanisms of action of these three monoterpenes against insects. In addition, further testing is necessary to evaluate the spectrum of fumigant activity against other stored-product insects (as well as other life stages of these stored-product insects, especially eggs) and their phytotoxicity to crop seeds, and to develop formulations to improve the efficacy and stability and to reduce cost.

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