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Research Article

Nematocidal Constituents from the Ethanol Extract of *Evodia rutaecarpa* Hort Unripe Fruits

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The ethanol extract of Chinese medicinal herb, *Evodia rutaecarpa* Hort unripe fruits, was found to possess nematocidal activity against the root-knot nematodes, *Meloidogyne incognita*, during the screening program for new agrochemicals from local wild plants and Chinese medicinal herbs. Bioactivity-guided chromatographic separation of the ethanol extract of *E. rutaecarpa* on repeated silica gel columns led to isolate five constituent components (two limonoids, evodol and limonin; three alkaloids, evodiamine, rutaecarpine, and wuchuyuamide I). Evodiamine (LC_{50} = 73.55 μg/mL) and rutaecarpine (LC_{50} = 120.85 μg/mL) exhibited stronger nematocidal activity against *M. incognita* than the crude ethanol extract of *E. rutaecarpa* (LC_{50} = 131.54 μg/mL). Wuchuyuamide I, evodol, and limonin also possessed nematocidal activity against *M. incognita* with LC_{50} values of 147.87 μg/mL, 155.02 μg/mL, and 197.37 μg/mL, respectively, but weaker than the crude ethanol extract of *E. rutaecarpa*.

1. Introduction

Root-knot nematodes are important agricultural pests worldwide. Possible control strategies include chemical nematocides, crop rotation, and use of resistant cultivars, when available. The broad host range of *Meloidogyne* species, however, makes crop rotation difficult, and the use of nematocides, in spite of their efficacy for controlling nematodes, has negative side effects that have led to banning or restricting their use [1]. On the other hand, resistance-breaking populations of *Meloidogyne* are challenging the use of resistant cultivars [2]. Those are now resulting in a strong interest in nematicides of natural origin [3]. One alternative is to screen naturally occurring compounds in plants, which are known as plant secondary compounds. Many plant constituents and metabolites have been investigated for activity against plant-parasitic nematodes [4–7]. A series of nematicidal substances of plant origin such as triglycerides, sesquiterpenes, alkaloids, steroids, diterpenes, and flavonoids have been identified [3]. During our screening program for new agrochemicals from local wild plants and Chinese medicinal herbs, ethanol extract of *Evodia rutaecarpa* Hort unripe fruits (Family: Rutaceae) was found to possess strong nematocidal activity against the root-knot nematodes, *Meloidogyne incognita* (Kofoid and White) Chitwood. *M. incognita* is the most economically important and widely distributed nematode throughout China, and a considerable crop loss is caused by this nematode [7].

E. rutaecarpa unripe fruit 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 [8]. *E. rutaecarpa* is also documented as insecticidal/antiparasitic in the literature [9]. The essential oil of *E. rutaecarpa* unripe fruits possessed strong fumigant toxicity against two grain storage insects, *Tribolium castaneum* and *Sitophilus zeamais*, and three active constituent compounds were isolated from the essential oil of *E. rutaecarpa* [10, 11]. Two quinolone alkaloids isolated from the herb exhibited toxicity to brine shrimp (*Artemia salina*) [12]. Moreover, three alkaloids, evodiamine, rutaecarpine, and rhetsinine, isolated from *E. rutaecarpa* by bioassay-guided fractionation showed insecticidal activity against fruit

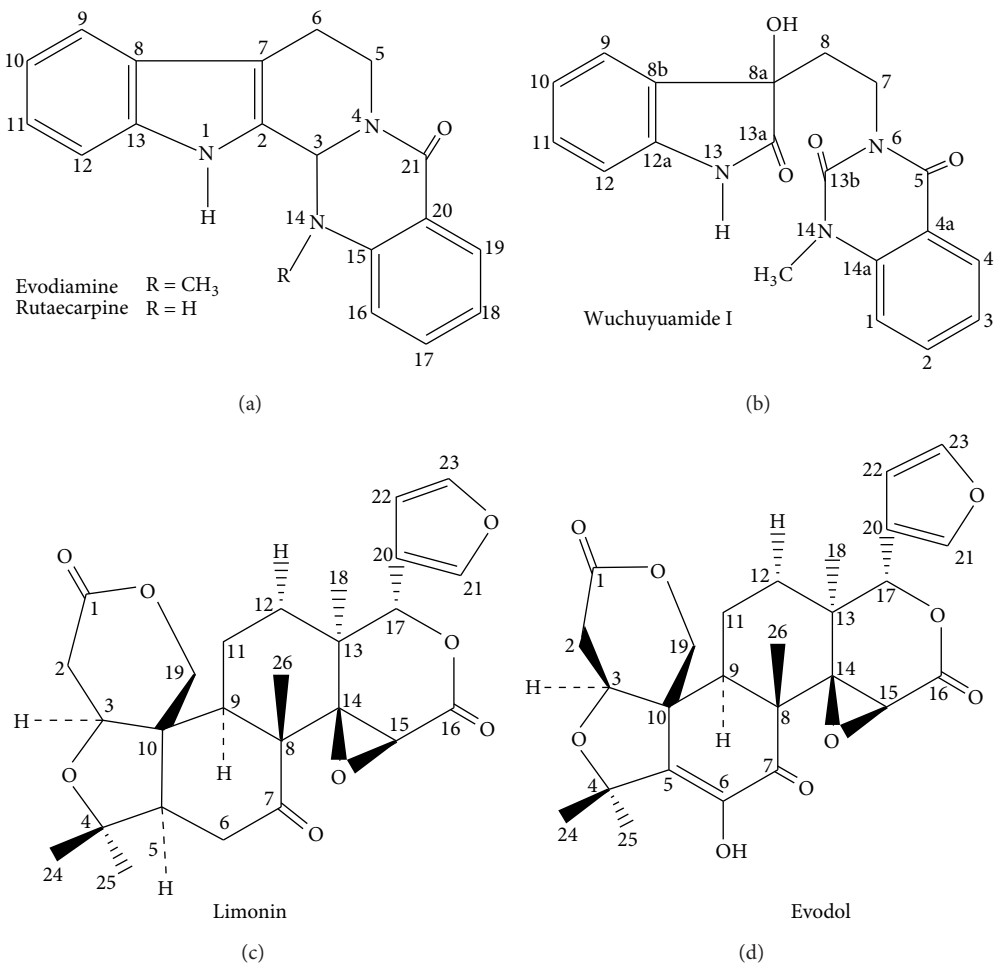

Figure 1: Structures of feeding deterrents isolated from *Evodia rutaecarpa* unripe fruits.

flies (*Drosophila melanogaster*) [13]. The ethanol extract of *E. rutaecarpa* also possessed strong antifeedant activity against the two stored product insect pests (*T. castaneum* and *S. zeamais*) [14]. However, as far as we know, there are no reports about isolation of active components against nematodes from this Chinese medicinal herb. In this paper, the ethanol extract of *E. rutaecarpa* was evaluated for toxicity against *M. incognita* and five active constituent compounds (Figure 1) were isolated and identified from the ethanol extract of *E. rutaecarpa* by bioassay-directed fractionation.

2. Experimental

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Bruker ACF300 (300 MHz (^{1}H)) and AMX500 (500 MHz (^{1}H)) instruments using deuterochloroform ($CDCl_3$) as the solvent with tetramethylsilane (TMS) as the internal standard. Electron impact mass spectra (EIMS) were determined on a Micromass VG7035 mass spectrometer at 70 eV (probe).

2.1. Chinese Medicinal Herb and Extraction. *E. rutaecarpa* (5 kg, dried unripe fruits), purchased from Anguo Chinese Herbs Market, Hebei province, China, were ground to a powder and extracted with 95% ethanol (3 × 10 L) at room temperature over a period of three weeks. The extracts were concentrated using a vacuum rotary evaporator to afford a syrupy gum (454 g). This syrup was partitioned between methanol water and *n*-hexane (3 × 1,000 mL). The *n*-hexane extracts were evaporated off to give a residue (232 g). The aqueous layer was repartitioned with chloroform (3 × 1,000 mL) to provide a residue (44 g) after evaporation of chloroform. Further partitioning with ethyl acetate (3 × 1,000 mL) gave a residue (65 g) after evaporation of the solvent.

2.2. Chromatography. Based on bioassay, the strongest nematocidal extract, the $CHCl_3$ residue (20 g) was applied to a silica gel column (600 mm in height and 100 mm in diameter, 160–200 mesh, Qingdao Marine Chemical Plant, Shandong province, China), eluting with petroleum ether containing increasing accounts of ethyl acetate (from 100 : 1 to 0 : 100) to give 27 combined fractions according to thin-layer chromatography (TLC) detection. Based on bioassay, fractions 2 (232 mg), 3 (283 mg), 6 (194 mg), 13-14 (503 mg), and 22–24 (632 mg) were chosen for further

Table 1: Nematocidal toxicity of the ethanol extract of *E. rutaecarpa* and isolated compounds against *Meloidogyne incognita*.

Compounds	Concentration	LC_{50} (μg/mL) (95% FL)	Slope ± SD	Chi square (χ^2)
Evodiamine	12.5–200.0	73.55 (45.62–140.36)	1.08 ± 0.08	20.34
Evodol	25.0–400.0	155.02 (82.29–273.23)	0.85 ± 0.07	16.81
Limonin	25.0–400.0	197.35 (91.82–381.60)	0.81 ± 0.06	18.44
Rutaecarpine	12.5–200.0	120.85 (76.16–203.07)	0.67 ± 0.05	16.25
Wuchuyuamide I	25.0–400.0	147.87 (47.95–458.70)	0.58 ± 0.09	15.89
Ethanol extract	25.0–400.0	131.54 (51.12–238.98)	0.69 ± 0.11	19.12
Carbofuran	25.0–400.0	72.29 (37.86–117.97)	1.21 ± 0.14	13.57

fractionation. Evodol (14.3 mg) was isolated from fraction 2 after being repeatedly purified on silica and PTLC (precoated GF254 plates, Qingdao Marine Chemical Plant). Fraction 3 was further chromatographed on silica gel column and repeated PTLC to provide the bioactive compound, which was recrystallized and determined to be 1imonin (21.0 mg). Wuchuyuamide I (10.4 mg) was obtained from further chromatographed on silica gel TLC and recrystallized from fraction 6. Fraction 13-14 were further chromatographed on silica gel column and silica gel TLC to obtain rutaecarpine (26.4 mg) after recrystallization. Evodiamine (27.9 mg) was obtained from further chromatographed on silica gel TLC and recrystallized from fraction 22–24. The spectral data of evodol and limonin matched with the previous reports [15, 16]. The data of wuchuyuamide, rutaecarpine, and evodiamine matched with the previous reports [16–18].

2.3. Nematocidal Assay. Egg masses of *M. incognita* obtained from tomato roots with aid of a stereomicroscope were maintained in Petri dishes during 24 h in distilled H_2O for the juvenile eclosion. Range-finding studies were run to determine the appropriate testing concentrations. A serial dilution of the crude extract and pure compounds (firstly prepared in 500 μL of acetone as a stock solution) was prepared in H_2O solution with 1% DMSO. 20 μL portions of H_2O containing ca. 30 juveniles (J_2) were transferred to vials to which 980 μL of the solution containing the ethanol extract or pure compounds was added and the final concentration of acetone was 0.5%. The vials were kept on a hood at 25°C. The counting of the inactive nematodes was performed at every 24 h for 72 h. After the last counting, the inactive juveniles were maintained in distilled H_2O for 24 h to observe their revival. Six repetitions for each treatment were performed using H_2O and a 1% DMSO in H_2O solution (containing 5 μL of acetone) as control. 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} (median lethal concentration) values and their 95% confidence intervals (CI 95%) [19]. Carbofuran was purchased from National Center of Pesticide Standards (8 Shenliao West Road, Tiexi District, Shenyang 110021, China) and used as a positive control.

3. Results and Discussion

Five isolated constituent compounds and the crude ethanol extract of *E. rutaecarpa* unripe fruits exhibited nematocidal toxicity against the root-knot nematodes, *M. incognita* (Table 1). The two alkaloids, evodiamine (LC_{50} = 73.55 μg/mL) and rutaecarpine (LC_{50} = 120.85 μg/mL), exhibited stronger nematocidal activity against *M. incognita* than the crude ethanol extract (LC_{50} = 131.54 μg/mL). It indicated that the nematocidal activity of ethanol extract of *E. rutaecarpa* unripe fruits may be attributed to the present of the two alkaloids. Moreover, evodiamine shows the same level of nematocidal activity against *M. incognita* as the positive control, carbofuran (LC_{50} = 72.29 μg/mL), while the ethanol extract of *E. rutaecarpa* unripe fruits and rutaecarpine exhibit only half level of nematocidal activity, compared with carbofuran. In the previous studies, evodiamine was demonstrated to possess anticancer activities both *in vitro* and *in vivo* by inhibiting proliferation, invasion, and metastasis, inducing apoptosis of a variety of tumor cell lines [20]. Moreover, evodiamine has been shown to reduce fat uptake in mouse studies. It is suspected that its mechanism of action is similar to that of capsaicin [21]. As such, it has been included in some dietary supplements. Rutaecarpine has shown a variety of intriguing biological properties such as antithrombotic, anticancer, anti-inflammatory and analgesic, antiobesity and thermoregulatory, vasorelaxing activity, as well as effects on the cardiovascular and endocrine systems [22]. Moreover, the two isolated alkaloids, evodiamine and rutaecarpine, exhibited insecticidal activity against the fruit flies (*D. melanogaster*) [13]. However, the two isolated constituent compounds were first time to be evaluated for nematocidal activity against the root-knot nematodes.

The other three isolated constituents, wuchuyuamide I, evodol, and limonin, also possessed nematocidal activity against *M. incognita* with LC_{50} values of 147.87 μg/mL, 155.02 μg/mL, and 197.37 μg/mL, respectively, but weaker than the ethanol extract of *E. rutaecarpa* (Table 1). In the previous reports, two limonoids, evodol and limonin, were demonstrated to possess antifeedant/feeding deterrent activity against Colorado potato beetle (*Leptinotarsa decemlineata*) and the fall armyworm larvae (*Spodoptera frugiperda*) [23–25] and also exhibited mosquito larvicidal

activity against *Aedes albopictus* and *A. aegypti* [26, 27]. Several tirucallane triterpenoids derived from *Melia azedarach* fruits, for example, 3-α-tigloylmelianol, melianone, 21-β-acetoxy-melianone, and methyl kulonate as well as limonin were evaluated for their nematocidal activity against *M. incognita*, and no significant effect on *M. incognita* was observed at a dose range of 31.2–500 μg/mL [28]. However, the two other isolated constituent compounds were first time to be evaluated for nematocidal activity against the root-knot nematodes.

Considering the currently used nematocides are synthetic and usually possess high toxic to nontarget organisms, nematocidal activity of the ethanol extract of *E. rutaecarpa* unripe fruits and the five isolated constituent compounds are quite promising and they show potential to be developed as possible natural nematocides for control of the root-knot nematodes. However, little has been done on mechanisms of action of these five compounds (two limonoids and three indole alkaloids) against nematodes. In addition, further testing is necessary to evaluate the spectrum of nematocidal activity against other plant parasitic and free-living nematodes and their phytotoxicity to crops and to develop formulations to improve the efficacy and stability and to reduce cost. Moreover, for the practical use of the ethanol extract of *E. rutaecarpa* unripe fruits and its constituents as novel nematocides to proceed, further research is needed to establish their human safety and environmental safety. However, in traditional Chinese medicine, the unripe fruits of *E. rutaecarpa* were commonly used for the treatment of abdominal pain, acid regurgitation, nausea, diarrhea, hernia, and dysmenorrhea and were also used externally for treatment of aphthous stomatitis [8]. However, no experimental data about the safety of ethanol extract of this medicinal herb and the four other isolated constituents is available so far. Therefore, any attempt to develop an alkaloid-derived agrochemical must be carefully evaluated for harmful effects.

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