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Mosquitocidal Activity of Extracts from *Ammi visnaga* (Apiaceae) Seeds

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Abstract

Aedes aegypti mosquitoes are responsible for transmission of many viral diseases, such as Zika fever, dengue fever, yellow fever, and chikungunya. Emergence of resistance to currently used pesticides among mosquitoes has increased the importance for the search for novel mosquito control agents. Natural products, particularly plant and microbe derived secondary metabolites, are good sources in the search for such compounds. *Ammi visnaga* (Lam.) Lamarck is a plant in the Apiaceae family native to North Africa, Europe, and Asia. In the search for environmentally benign and effective insecticides as part of an ongoing joint effort between the USDA (US Department of Agriculture) and the DWFP (Deployed War Fighter Protection) program sponsored by the Department of Defense, we have investigated ethyl acetate extract of *A. visnaga seeds*. Two furanochromones, khellin and visnagin that exhibited larvicide activity against *Aedes aegypti* mosquito larvae were isolated from the ethyl acetate extract of the seeds by bioassay-guided fractionation. This is the first report of mosquito larvicidal activity of khellin and visnagin.

Keywords

Aedes aegypti, *Ammi visnaga*, Mosquito Larvicide, Apiaceae, Khellin, Visnagin

1. Introduction

Ammi visnaga (Lam.) Lamarck is a plant in the Apiaceae family that is native to North Africa, Europe and Asia. The flowers of this plant are white and composed of umbel, which is characteristic for the Apiaceae family. The seeds are tiny (about 2 mm long) and oval-shaped and have been used in traditional medicine to make herbal remedies to treat kidney stones [1]. Recent laboratory studies have shown that an aqueous extract of

the seeds acts as a diuretic and inhibits the formation of calcium oxalate crystals in the kidneys and prevents cell damage caused by oxalate in renal epithelial cells [2]. Plants in the apiaceae family are good sources of biologically active compounds [3]-[5]. Natural products are a good source of diverse molecular structures as leads for agrochemicals and insecticides [6]-[8]. As part of an ongoing joint effort between the USDA (US Department of Agriculture) and the DWFP (Deployed War Fighter Protection) program sponsored by the Department of Defense, we have investigated an ethyl acetate extract of *A. visnaga seeds*. In this paper, we describe the bioassay guided isolation of mosquito larvicide active constituents of ethyl acetate extract of *A. visnaga* seeds.

2. Materials and Methods

2.1. Instrumentation

^{1}H, and ^{13}C NMR spectra were recorded in CDCl$_3$ on a Bruker a Bruker AMX NMR spectrometer (Billerica, MA) operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. Column chromatography was performed using Biotage, Inc. (Charlottesville, VA) with Isolera pump equipped with a Horizon fraction collector and a dual wave length (254 and 280 nm) UV detector. HPLC analyses were carried out on Agilent 1290 infinity series liquid chromatography system with a vacuum degasser, quaternary pump, diode array detector, and auto-sampler with data acquisition by Chemstation software (Agilent Corporation, USA).

Crude extracts and Biotage column chromatography fractions of the extracts and sub-fractions were analyzed on silica gel TLC plates GF with fluorescent indicator (250 μm, Analtech, Newark, DE, USA). UV light (at 254 nm and 365 nm), Iodine vapor, and anisaldehyde spray reagent were used for the detection of compounds on TLC plates after elution.

2.2. Plant Material

Seeds of *A. visnaga* were purchased from Swallowtail Garden Seeds (Santa Rosa, CA USA) in September 2014. Seeds (250 g) were crushed in a grinder to a fine powder and transferred to an Erlenmeyer flask containing ethyl acetate (1 L) and stirred vigorously for 4 h at room temperature. The ethyl acetate extract was filtered through filter paper (Whatmann #1). This process was repeated twice and the combined extracts were concentrated at 40°C under reduced pressure. An off-white precipitate was produced upon concentration. This precipitate was filtered (0.9 g) and the supernatant was dried to afford 14.88 g of greenish brown extract.

2.3. Isolation of Compounds

TLC of the precipitate eluted in 70% ethyl acetate in hexane indicated the presence of two major compounds. This was purified using 25 g SNAP silica gel column using gradient elution from 30% - 80% ethyl acetate in hexane (600 mL, 20 column volume).

The residual extract (12 g) was loaded on to a Biotage 340 g SNAP silica gel column and eluted with ethyl acetate in hexane (0% - 100%) and 50 mL fractions were collected.

Similar fractions were combined according to the TLC profile to obtain 25 fractions. Each fraction was submitted to mosquito larvicide assay.

Khellin—^{1}H NMR 500 mHZ: δ 2.39 (3H, *s*), 4.06 (3H, *s*, OMe), 4.19 (3H, *s*), 6.05 (1H, *s*, H-3), 7.01 (1H, *d*, *J* = 4 Hz), 7.64 (1H, *d*, *J* = 4 Hz), ^{13}C NMR: δ 20.21,61.59, 62.42, 105.25, 110.72, 113.79, 119.47, 129.97, 145.61, 147.18, 147.4, 148.90, 164.07, 178.27.

Visnagin—^{1}H NMR: δ 2.32 (3H, *s*), 4.17(3H, *s*), 6.04 (1H, *s*), 7.03(1H, *d*, *J* = 4 Hz), 7.22 (1H, *s*), 7.59 (1H, *d*, *J* = 4 Hz),; ^{13}C NMR: δ 19.98, 61.78, 95.15, 105.31, 110.81, 112.40, 116.67, 145.13, 153.52, 155.94, 157.76, 163.87, 178.23.

2.4. HPLC Analyses

Chromatography was performed on a Luna silica column (250 × 4.6 mm, 5 μm; Phenomenex, USA) fitted with a silica guard column (10 × 3.0 mm). Mobile phase used was 7:3 ethyl acetate in hexane. Elution of the mobile phase was carried out at 25°C and at a flow rate of 1.0 mL/min. UV wavelength of 254 nm was used for detection of the chromatography peaks. All samples were analyzed by HPLC using an injection volume of 5 μL. Five standard solutions of purified khellin and visnagin were prepared by serial dilution of stock solutions with ethyl acetate. The calibration curves were generated by plotting the absorbance peak area against each concentration of the two compounds. Slope of the curves, Y-intercept and linearity of the curves were determined by linear regression.

2.5. Mosquitocidal Activity Assays

2.5.1. Mosquito Strains

Permethrin-susceptible and resistant strains of *Ae. aegypti* were used for these studies. The Orlando (ORL) strain of permethrin-susceptible *Ae. aegypti* has been in continuous colony at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) since originally collected near Orlando, Florida in 1952. The pyrethroid-resistant Puerto Rican strain (PR) of *Ae. aegypti* (NR-48830, BEIResources, Atlanta, GA) was originally started from eggs collected in San Juan Puerto Rico in June 2012 and this colony is maintained at CMAVE in a containment insectary. Rearing procedures for the mosquitoes have been described previously and are standardized to produce a consistent body size and mass (2.3 - 2.6 mg/female) [9]. Mosquitoes used were 3 - 6 days post-emergence and maintained as adults with free access to 10% sucrose in water.

2.5.2. Adult Topical Bioassays

In order to determine the toxicity of isolated pure compounds against female adult *Ae. aegypti* mosquitoes, stock solutions of test compounds were prepared as 10% w/v in DMSO. The stock solutions were then serially diluted 1:10 in acetone. The assay was performed according to published methods on cold anesthetized female mosquitoes [10]. Relative humidity and temperature were maintained at 80% and 26°C - 27°C respectively. Bioassays were replicated 3 times at each dose unless noted otherwise with cohorts of 10 - 20 mosquitoes. Technical-grade permethrin (Chemservice, West Ches-

ter, PA), a mixture of 46.1% cis and 53.2% trans isomers was used as the positive control and diluted in the same manner as for the test compounds. This method results in an LD_{50} for permethrin reliably around 0.1 - 0.2 ng per mosquito for the ORL strain.

2.5.3. Larvicide Bioassay

The larvicide assay was performed as described previously with some modification for use with smaller volumes to conserve limited stocks of test compounds [10] [11]. Briefly, eggs for use were hatched in 100 mL of deionized water with approximately 40 mg of ground larval diet (1:1 alfalfa powder: pig chow). The cup was briefly deoxygenated under vacuum to synchronize hatching. The following day, five first instar larvae were transferred in 188 μL of solution into a well of a 96-well polycarbonate tissue culture plates (Becton Dickinson). Ten microliters of the larval diet was added to each well. Finally, two μL of diluted test compounds were added and the wells were mixed gently. A range of dilutions for the test compounds were used to determine activity. Permethrin was used as a positive control and DMSO as a negative control. Mortality was assessed at 24 hours after treatment. Loss of movement in individual larvae after agitation with a pipette tip was scored as dead. Assays were repeated 3 times on different days unless noted.

3. Results and Discussion

The TLC of fractions 19 and 20 indicated the presence of the same two major compounds in the precipitate obtained upon concentration of the ethyl acetate extract of the seeds. Therefore fractions 19 and 20 were combined and 2.5 g of the combined fraction was purified further using a 50 g silica gel biotage column using ethyl acetate in hexane (10% - 50%) to obtain khellin (1.2 g) and visnagin (640 mg) upon recrystallization using ethyl acetate in hexane (Figure 1).
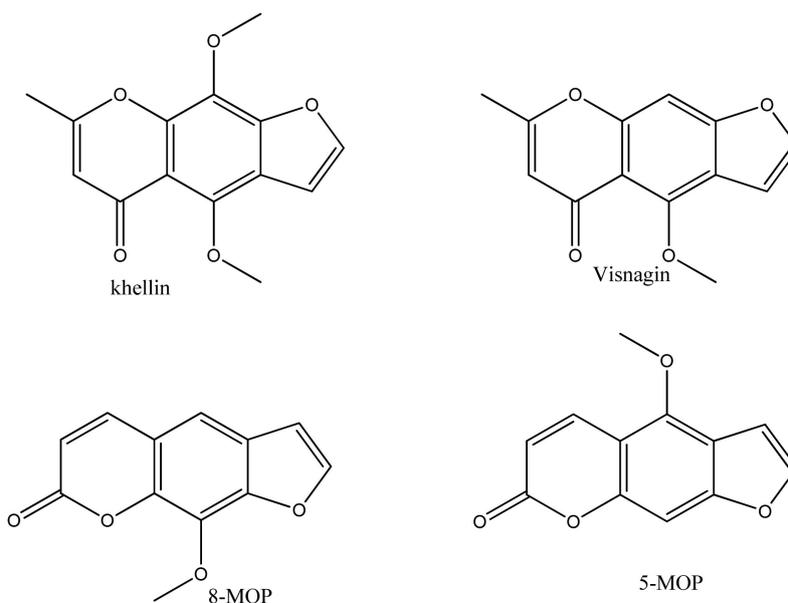

Figure 1. Compounds tested for topical adult mosquitocidal activity and lavicide activity.

HPLC analysis of the ethyl acetate extract (**Figure 2**) of the seeds of *A. visnaga* also indicated the presence of two major compounds in the extract. These two compounds were isolated and identified as khellin and visnagin. Khellin is the major constituent and visnagin is the minor constituent. Concentrations of khellin and visnagin in the ethyl acetate extract of the seeds were determined using the standard curves ($R^2 = 0.99$) constructed (**Table 1**) using purified khellin and visnagin. According to HPLC analysis the concentrations of khellin and visnagin are 57% and 0.45% by weight of the crude extract.

Column chromatography fractions and isolated pure compounds were tested with the topical mosquito adulticide assay and the mosquito larvicidal assay (**Table 2** and **Table 3**). Visnagin and khellin showed moderate activity as topical adulticides with mortality around 75% and 67% against the ORL strain of mosquitoes and 52% and 57% against the PR strain of mosquitoes respectively at 5 µg/mosquito, the highest concentration tested (**Table 2**). The two furanocoumarins 5-MOP and 8-MOP (**Figure 2**) (that have been isolated by us previously from *Ruta graveolens*) [12] also showed weaker activities in the adulticide assay against both strains of mosquitoes comparable to khellin and visnagin. The positive control permethrin showed 100% mortality of ORL strain
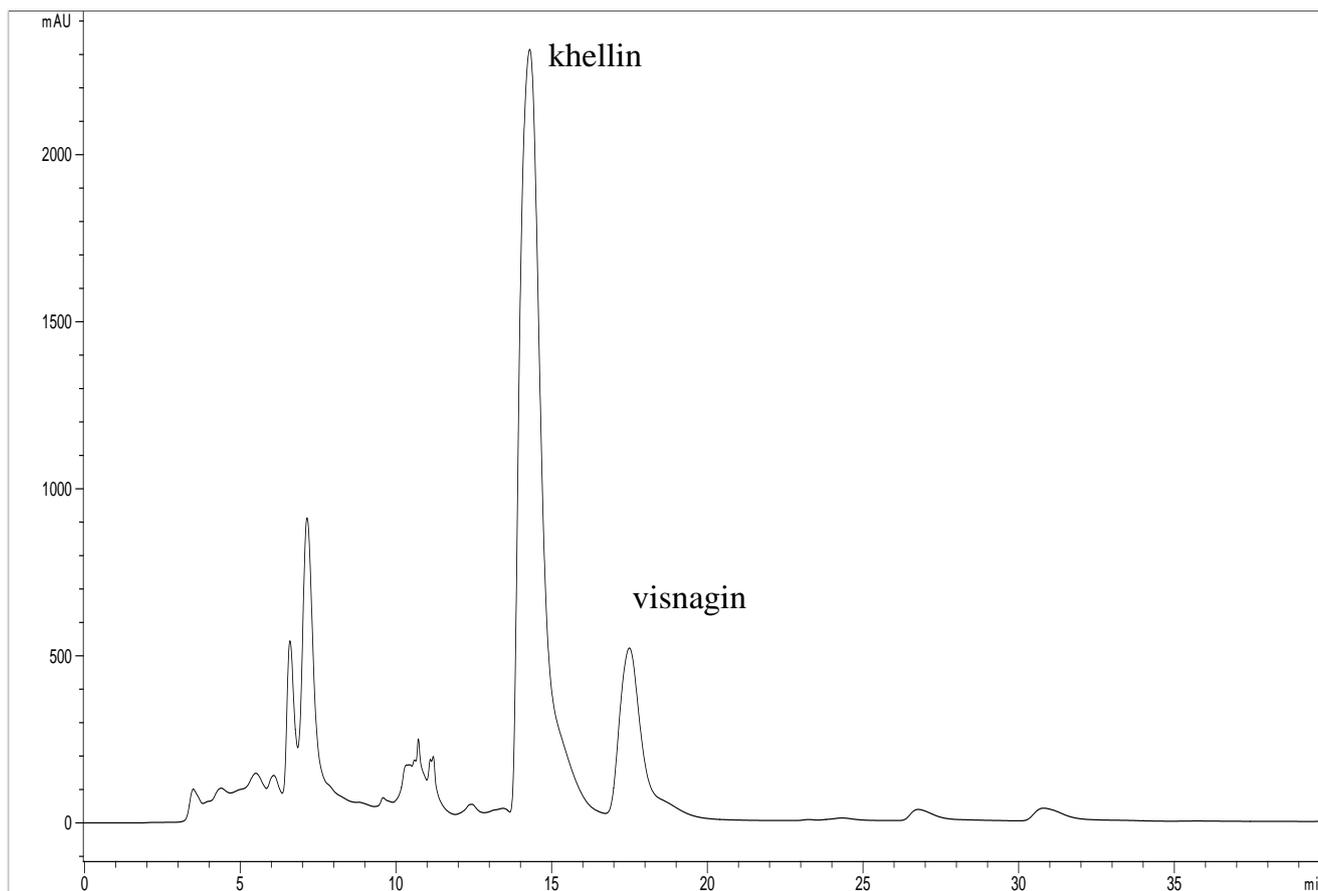

Figure 2. HPLC profile of ethyl acetate extract of *A. visnaga* seeds at 1.58 mg/mL monitored at 254 nm. The injection volume was 5 µL and the flow rate was 1.0 mL/min.

Table 1. Calibration curves of khellin and visnagin (n = 3).

Compound	Concentration range mg/mL	Equation	Correlation coefficient (r^2)
Khellin	0.13 - 2	y = 7338.4x + 361.19	0.99
Visnagin	0.13 - 2.1	y = 8546.4x + 560.45	0.99

Table 2. Adult topical bioassay on ORL strain and PR strain of *Aedes aegypti* (n = 3).

Name	ORL (µg/mosq)				PR (µg/mosq)			
	5	2.5	1.25	0.625	5	2.5	1.25	0.625
5-MOP	55 ± 23.8	23.3 ± 40.4	6.7 ± 5.8	3.3 ± 5.8	35 ± 12.9	30 ± 17.3	16.7 ± 5.8	16.7 ± 11.5
8-MOP	67.5 ± 5	36.7 ± 15.3	20 ± 10	6.7 ± 5.8	62.5 ± 17.1	40 ± 17.3	16.7 ± 5.8	13.3 ± 5.8
Khellin	75 ± 19.1	60 ± 17.3	16.7 ± 15.3	16.7 ± 15.3	52.5 ± 18.9	46.7 ± 11.5	23.3 ± 25.2	6.7 ± 5.8
Visnagin	67.5 ± 15	43.3 ± 15.3	16.7 ± 5.8	3.3 ± 5.8	57.5 ± 27.5	43.3 ± 15.3	16.7 ± 5.8	10 ± 17.3
Acetone	0				7.5 ± 5			
Perm (462 ng/mosq.)					100			
Perm (17.8 ng/mosq.)					60 ± 14.1			
Perm (1.8 ng/mosq.)	100							
Perm (0.23 ng/mosq.)	82.5 ± 20.6							
Untreated	0				0			

Table 3. Larvicide bioassay on ORL strain and PR strain of *Aedes aegypti* (n = 3).

Name	ORL (µg/µl)				PR (µg/µl)			
	1	0.5	0.25	0.125	1	0.5	0.25	0.125
5-MOP	0	0	0	0	0	0	0	0
8-MOP	53.3 ± 11.5	33.3 ± 30.6	26.7 ± 23.1	20 ± 20	80 ± 34.6	80 ± 34.6	80 ± 20	60 ± 20
Khellin	100	93.3 ± 11.5	60 ± 20	13.3 ± 11.5	100	100	93.3 ± 11.5	20 ± 20
Visnagin	93.3 ± 11.5	80	33.3 ± 30.6	6.7 ± 11.5	93.3 ± 11.5	86.7 ± 11.5	60 ± 34.6	13.3 ± 11.5
DMSO	0				0			
Perm (63.5 pg/ul)	100							
Perm (16.2 pg/ul)	26.7 ± 11.5							
Perm (3.7 ng/ul)					93.3 ± 11.5			
Perm (0.5 ng/ul)					46.7 ± 23.1			

adult mosquitoes at 1.8 ng/mosquito whereas the PR strain adult mosquitoes showed 60% mortality at 17.8 ng/mosquito.

In the larvicidal assay (Table 3) khellin showed activity with 100% and 93% mortality at 1 µg/µL and at 0.5 µg/µL respectively for the ORL strain whereas visnagin showed 93% and 80% mortality at the same concentrations for the same mosquito strain. For the PR strain, khellin showed slightly better larvicidal activity with 100% mortality at 1

and 0.5 μg/μL and 93% mortality at 0.25 μg/μL. Visnagin was less active with 93% mortality at 1 μg/μL. Permethrin showed 100% mortality of the ORL strain larvae at 63 pg/μL and the PR strain larvae showed 93% mortality at 3.7 ng/μL.

Khellin was slightly more effective than visnagin as a larvicide but both khellin and visnagin were less effective as an adulticide. Between the two furanocoumarins, 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP), 5-MOP was ineffective as a larvicide in both strains of mosquitoes but it was active against the PR strain with 80% mortality at 1, 0.5, 0.25 μg/μL but showed less activity for the ORL strain. Both 8-MOP and 5-MOP showed weaker activity as an adulticide with around 35% - 67% mortality at the highest concentration tested. Previous work shown that khellin forms monofunctional adducts to DNA in the presence of near UV light [13] [14].

The difference in activity between the adults and larvae could be due to poor absorption of the compounds in adults compared to larvae or due to different mechanisms and rates of detoxification of these compounds. Khellin and visnagin are closely related furanochromones. 5-MOP and 8-MOP are structurally closely related furocoumarins that have been previously isolated in our laboratory from the ethyl acetate extract of *Ruta graveoleans* leaves and roots [12].

Even though khellin has been shown to be phototoxic, Kagen *et al*. [15] have shown that khellin had no toxicity towards *Ae. egypti* larvae up to 7.7 ppm under UV light or in the absence of UV light. Our studies showed khellin and visnagin to have moderate to weak larvicidal activity with 100% mortality at 500 ppm (0.5 μg/μL) or higher for visnagin and 86% and 60% mortality for khellin at the same concentrations respectively. It has also been shown that khellin showed a markedly reduced adult emergence under near UV irradiation on the mosquito strain *Aedes atropalpus* [16].

4. Conclusion

In summary, bioactive compounds of ethyl acetate extract of *A. visnaga* seeds were isolated by bioassay guided fractionation followed by spectroscopic techniques. Khellin and visnagin were identified as mosquito larvicide constituents in the ethyl acetate extract. These two compounds were only moderately active as mosquito adulticides. This is the first report of mosquito larvicidal activity of khellin and visnagin. Between the two furanocoumarins 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP), 5-MOP was ineffective as a larvicide against both strains of mosquitoes.

Acknowledgements

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