Agricultural Sciences, 2015, 6, 1195-1201
Published Online October 2015 in SciRes. http://www.scirp.org/journal/as
http://dx.doi.org/10.4236/as.2015.610114

Scientific Research Publishing

Phomalactone as the Active Constituent against Mosquitoes from *Nigrospora spherica*

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Received 16 July 2015; accepted 18 October 2015; published 22 October 2015

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Abstract

The culture filtrate of a plant pathogenic fungus that infected *Zinnia elegans* and *Hydrangea macrophylla* was investigated for mosquitocidal constituents by bioassay guided isolation. The fungus responsible for the pathogenic effects on *Zinnia elegans* and *Hydrangea macrophylla* plants had been identified as *Nigrospora spherica* by molecular techniques. The mosquito adulticidal constituent in the culture filtrate was identified as phomalactone by spectroscopic techniques. Laboratory bioassays showed that phomalactone had larvicidal activity against permethrin susceptible and resistant *Aedes aegypti* larvae and topical adulticide activities on permethrin susceptible and resistant *Aedes aegypti* and *Anopheles quadrimaculatus* mosquitoes. Phomalactone was effective as a topical adulticide against the standard Orlando reference strain of *Ae. aegypti* with an LD_{50} of 0.64 μg/org. Activity against *An. quadrimaculatus* was 0.20 μg/org.

Keywords

***Nigrospora spherica*, Phomalactone, *Anopheles quadrimaculatus*, *Aedes aegypti*, Permethrin Resistance, Adulticide, Larvicide**

1. Introduction

Many mosquito species have developed resistance due to continuous use of currently available insecticides in

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How to cite this paper: Meepagala, K.M., Becnel, J.J. and Estep, A.S. (2015) Phomalactone as the Active Constituent against Mosquitoes from *Nigrospora spherica*. *Agricultural Sciences*, **6**, 1195-1201. http://dx.doi.org/10.4236/as.2015.610114

the market and therefore there is an urgent need to identify and develop compounds that have different modes of action to those that are currently in use [1]. The mosquito species *Aedes aegypti* (L.) (Diptera: Culicidae) transmits viral pathogens that are responsible for yellow fever, dengue fever and chikunguya, each of which can cause severe human morbidity and mortality. *Ae. aegypti* mosquitoes and several other mosquito species have developed resistance to currently used pyrethroids. Natural products, particularly those derived from microbes, can be a good source of novel insecticides as these organisms have evolved to produce compounds that play an important role in protection from other predators [2] [3].

There have been few investigations on mosquitocidal activity of metabolites of plant pathogenic fungi. Plant pathogenic fungi may possess not only herbicidal or phytototoxic secondary metabolites but also may have evolved to produce insecticides and fungicides to compete in the biosphere. As a part of the ongoing investigation for environmentally benign mosquito control agents under the Deployed War Fighter Protection (DWFP) program of the Department of Defense (DoD), a plant pathogenic fungus infecting leaves of *Zinnia elegans* and *Hydrangea macrophylla* was investigated as a source for insecticides. The identity of the fungus was confirmed as *Nigrospora spherica* by molecular techniques. This fungus infects *Z. elegans* and *H. macrophylla* plants and causes necrosis and wilting of the host plants.

2. Materials and Methods

2.1. General Chemical Methods*.*

Extracts were analyzed on 250-micron silica gel TLC plates GF with fluorescent indicator (Analtech, Newark, DE, USA). Iodine vapor, UV light (at 254 nm and 365 nm), and anisaldehyde spray reagents were used for the detection of compounds. Column chromatography was carried out by a Biotage flash chromatography system. All solvents were reagent grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury AS400 spectrometer operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The HR-ESIMS was measured by using a JEOL ACCU TOF JMS-T1000 mass spectrometer.

2.2. Fungal Material

PDA (potato dextrose agar) and Potato dextrose broth (PDB) were from Difco™ (Becton, Dickinson and Company, Sparks, MD, USA). Infected *H. macrophylla* and *Z. elegana* leaves that are showing necrosis were collected in Oxford, Mississippi during August 2013. Each leaf was surface sterilized by dipping in 0.5% sodium hypochlorite in deionized water for 1 min and then rinsing with sterile deionized water three times. Half strength PDA (potato dextrose agar) plates were inoculated with the fungus by placing a small piece (about 0.5 mm width ×3 mm length) of the leaf tissue from an infection site from the surface-sterilized leaf. The plates were allowed to grow for 10 days at 27˚C with a 12 h scotophase. Single colonies from each plate were cultured on PDA plates and allowed to grow for 10 days at 27˚C with a 12 h scotophase in an incubator. In two separate 250 mL Erlemmayer flasks potato dextrose broth (PDB) (100 mL) was inoculated with a single fungal colony obtained on PDA plates that originated from each individual leaf tissue sample by adding a piece of agar with mycelia using a sterile drinking straw (approx. 0.5 cm diameter) and allowed to grow for 14 days in a shaker (90 rpm) under the same temperature and light conditions as noted previously. This PDB liquid medium was used to inoculate eight flasks of 500 mL PDB (5 mL in each flask) and allowed to grow for 14 days in a shaker (90 rpm) at 27˚C with a 12 h scotophase.

2.3. Molecular Identification of the Fungus

Fungi were grown in PDB as described above. Isolated fungi were identified by Accugenix® (Charles River, Newark, DE) by sequencing the internal transcribed spacer (ITS) region of the ribosomal RNA and also by molecular techniques described by Meepagala *et al.* [4].

2.4. Extraction and Isolation

Following incubation for 14 days, mycelia were separated from 4 L of PDB by filtration through miracloth (EMD Millipore, Billerica, MA) followed by filter paper (Whatman #1), and each 500 mL filtrate was extracted twice with (500 mL) ethyl acetate. The mycelia were crushed separately in a blender and also extracted twice each with ethyl acetate (200 mL). The ethyl acetate extracts of the filtrate and the mycelia were dried over an-

hydrous Na_2SO_4 and the solvent was evaporated under reduced pressure at 40˚C to obtain light brown viscous extracts of 1.6 and 0.7 g, respectively. TLC of the two extracts indicated that the ethyl acetate extract of filtered culture medium was rich in two major compounds. Therefore the fungal broth ethyl acetate extract was subjected to Biotage flash column chromatography using a 50 g silica gel column eluted with 0% - 40% (700 mL) isopropanol in hexane. Fractions of 15 mL were collected and fractions with similar TLC profiles were combined to obtain six fractions. Fractions 2 and 3 were combined (930 mg) and further purified by flash column chromatography with 1% - 5% isopropanol in CH_2Cl_2 to obtain (**1**) (507 mg) and (**2**) (270 mg) as colorless viscous oils (**Figure 1**).

Compound (**1**)—^{1}H NMR($CDCl_3$): δ 5.85 (1H, *m*, H-8), 5.53 (1H, *dd*, *J* = 8, 14 Hz, H-7), 4.44 (1H, *q*, *J* = 8, H-6), 4.08 (1H, *dd*, *J* = 1.2, 14 Hz, H-5), 2.21 (2H, *m*), 2.08 (2H, *m*, H-4), 3.08 (1H, *d*, *J* = 4 Hz, OH), 1.8 (3H, *d*, *J* = 6.4 Hz, CH_3); ^{13}C NMR($CDCl_3$): δ 18.25 (C-9), 63.27 (C-5), 81.8 (C-6), 122.6 (C-3), 124.3 (C-7), 133.1 (C-8), 145.33 (C-4), 164.16 (C-2) ; HRMS (ESI-TOF) m/z 157.08632 [M + H], (calcd for $C_8H_{13}O_3$, 157.08647).

Compound (**2**)—^{1}H NMR($CDCl_3$): δ 6.99 (1H, *dd*, *J* = 5, 10 Hz, H-4), 6.08 (1H, *d*, *J* = 10 Hz, H-3), 5.91 - 6.0 (1H, *m*, H-8), 5.80 - 5.73 (1H, *m*, H-7), 4.79 (1H, *dd*, *J* = 4, 8, H-6), 4.18 (1H, *d*, *J* = 4 Hz, H-5), 3.08 (1H, *d*, *J* = 4 Hz, OH), 1.8 (3H, *dd*, *J* = 4, 6.4 Hz, CH_3); ^{13}C NMR($CDCl_3$): δ 18.25 (C-9), 63.27 (C-5), 81.8 (C-6), 122.6 (C-3), 124.3 (C-7), 133.1 (C-8), 145.3 (C-4), 164.2 (C-2); HRMS (ESI-TOF) m/z 155.07081 [M + H], (calcd for $C_8H_{11}O_3$, 155.07082).

2.5. Acetylphomalactone (Compound 3)

Phomalactone (40 mg, 0.25 mmol) was dissolved in pyridine (4 mL). Acetic anhydride (0.1 mL, 0.9 mmol) was added and stirred under ambient temperature for 12 hr. The reaction mixture was cooled on ice, acidified with 1N HCl (20 mL) and extracted with ethylacetate (30 mL ×2) and the ethylacetate layer was dried over anhy. Na_2SO_4 and evaporated to obtain a pale yellow viscous oil. Phomalactone acetate (**3**) (**Figure 1**). was isolated by preparative thin layer chromatography (20 cm × 20 cm; 250 micron silica gel plates eluting with 5% isopropanol in CH_2Cl_2 to obtain a white cryatalline solid (32 mg). ^{1}H NMR($CDCl_3$): δ 6.96 (1H, dd, *J* = 5.2, 9.6 Hz), 6.20 (1H, *d*, *J* = 9.6 Hz), 5.94 (1H, *dq*, *J* = 8, 14 Hz), 5.59 (1H, *ddq*, *J* = 1.6, 6.8, 15.1 Hz), 5.24 (1H, *dd*, *J* = 3.2, 5.6 Hz), 4.87 (1H, *dd*, *J* = 2.8, 7.6), 2.04 (3H, *s*, OAc), 1.73 (3H, *dd*, *J* = 1.2, 6.4 CH_3); ^{13}C NMR($CDCl_3$): δ 17.93, 20.61, 64.15, 79.44, 123.49, 124.69, 133.01, 140.78, 162.65, 170.01, HRMS (ESI-TOF) m/z 196.08136 [M + H], (calcd for $C_{10}H_{13}O_4$, 197.081385).

2.6. Biological Activity Assays

2.6.1. Mosquito Strains

Two strains of *Ae. aegypti* and one strain of *An. quadrimaculatus* 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. An *Ae. aegypti* pyrethroid-resistant (PR) strain was started from eggs collected in San Juan, Puerto Rico in June of 2012 and is also maintained at CMAVE in a containment insectary. Rearing procedures for the ORL strain have been described previously [5] and are standardized to produce a consistent body size and mass (2.3 - 2.6 mg/female). The PR strain is reared using the same procedure but is pressured as third instar larvae with 100 µg/mL permethrin every third generation to maintain resistance. The resistance ratio of the PR strain (F5 generation) to permethrin is greater than 50-fold when compared to the ORL strain. The *An. quadrimaculatus* strain used in these studies was also collected near Orlando, Florida and has been maintained in colony since 1952. Rearing has also been described previously [6]. Mosquitoes used were 3 - 6 days post-emergence and main-

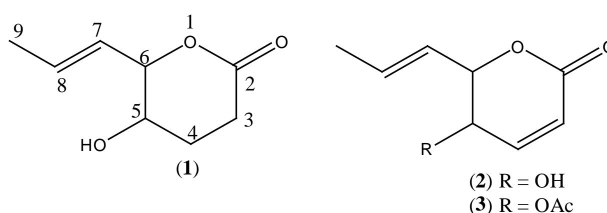

Figure 1. Structures of compounds.

tained as adults with free access to 10% sucrose in water.

2.6.2. Adult Topical Bioassays

To determine the toxicity of each compound against female mosquitoes, test samples were initially diluted to a 10% solution in DMSO. This solution was then serially diluted 1:10 in acetone. Each addition of diluent and stock solution was weighed to allow for accurate concentration calculations and to account for pipetting variability because of the volatility of acetone. Prior to application, 3 - 6-day-old female mosquitoes were cold-anaesthetized and placed on a 4˚C chill table (BioQuip Products, Rancho Dominguez, CA). A 0.5 µL droplet of chemical solution was applied to the thorax using a 700 series gastight syringe and a PB 600 repeating dispenser (Hamilton, Reno, NV). Control treatments with 0.5 µL of acetone alone give control mortality of less than 10%. After treatment, mosquitoes were kept in plastic cups and supplied with 10% sucrose solution in water for 24 h before mortality was recorded. Temperature and relative humidity were maintained at 26˚C - 27˚C and 80%, respectively. Cohorts of 10 - 20 mosquitoes were treated at each dose. Bioassays were replicated 3 times unless noted otherwise. In these studies, the testing of *An. quadrimaculatus* was performed twice. Technical-grade permethrin (Chemservice, West Chester, PA), a combination of 46.1% cis and 53.2% trans isomers, was used as the positive control in all assays and diluted in the same manner as the test compounds. This system reliably results in a LD_{50} for permethrin of around 0.1 - 0.2 ng per mosquito for the ORL strain.

2.6.3. Larval Bioassay

The larval bioassay has been described previously [7] but has been modified for use of smaller volumes to conserve limited stocks of test compounds. 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, Bedford MA). Two microliters of the larval diet was added to each well. Finally, 10 µL of a diluted test compound was added and the well was gently mixed. 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. Lack of movement in individual larvae after agitation with a pipette tip was scored as dead. Assays were repeated 3 times unless noted.

3. Results and Discussion

Preliminary bioassay of **1** and **2** conducted on the Orlando (ORL) strain of permethrin-susceptible 1st instar *Ae. aegypti* larvae indicated that only **2** had larvicidal activity (**Table 1**).

When **1** and **2** were tested on adult *Ae. aegypti* female mosquitoes (ORL) by topical application to evaluate adulticidal activity, only **2** showed activity with 100% mortality at 3.1 and 1.6 µg/mosquito and 96% mortality at 0.78 µg/mosquito (**Table 2**).Compound **1** had 16%, 10%, and 0% mortality at the same concentrations respectively in the same bioassay (**Table 2**).

Based on these preliminary results and to gain insight into structural aspects of activity, compound **2** and an analog (compound **3**), prepared by acetylation of **2**, were further evaluated against larvae of *Ae. aegypti* using Puerto Rico (PR) strain that is permethrin resistant, and Orlando strain that is permethrin susceptible (**Table 3**). Compound **2** showed 100% and 93% mortality at 1 and 0.5 µg/µL whereas **3** showed 100% mortality at the same concentrations on permethrin susceptible Orlando mosquito larvae. Under the same conditions, **2** showed a slightly better activity against permethrin-resistant Puerto Rico strain. Permethrin exhibited 100% mortality on

Table 1. Larvicide activity of Orlando (ORL) strain of permethrin-susceptible 1st instar *Ae. aegypti* larvae.

Compound	Dose (ppm) % mortality				
	250	125	62.5	31.25	15.63
1	<10				
2	100	100	53.3	40.0	0.0

All samples were initially tested at 250 mM (n = 3). Samples that produced >40% mortality were tested at further dilutions (n = 3). Positive controls resulted in 100% mortality and negative controls had mortality <5%.

Table 2. Adulticide activity of Orlando (ORL) strain *Ae. aegypti* adult female mosquitoes.

Compound	Dose (μg/mosquito) % mortality		
	3.125	1.5625	0.78125
1	16.67	10.00	0.00
2	100	100	96.67

All samples were initially tested at 250 mM (n = 3). Samples that produced >40% mortality were tested at further dilutions (n = 3). Positive controls resulted in 100% mortality and negative controls had mortality <5%.

Table 3. Larvicidal activity of 2, 3 and permethrin against different strains of mosquitoes.

compound	*Aedes aegypti* strain	Dose (μg/μL) % mortality			
		1	0.5	0.25	0.1
2	Orlando strain	100	93.3 ± 6.7	53.3 ± 13.3	53.3 ± 29.1
	Puerto Rico strain	100	100	93.3 ± 6.7	60.0 ± 20.0
3	Orlando strain	100	100	53.3 ± 29.0	26.6 ± 13.3
	Puerto Rico strain	100	80.0 ± 20.0	73.3 ± 17.6	53.3 ± 6.7
Permethrin	Orlando strain	100			
	Puerto Rico strain	26.7 ± 17.6			

the larvae of permethrin susceptible Orlando mosquitoes as expected, whereas the mortality of the larvae of permethrin-resistant Puerto Rico strain was 26% at 1 μg/μL (**Table 3**).

Compounds **2** and **3** were also tested for adulticidal activity by topical application on 3 - 6 day post-emergence adult female mosquitoes (**Table 4**). Testing of both compounds at a dose of 5 μg/ insect showed good activities. Compound **3** produced 100% mortality of the ORL strain as well as Puerto Rico strain. Compound **2** was less active as an adulticide with 61% ± 28.3% mortality in the permethrin-susceptible mosquitoes but with100% mortality in the PR strain. Permethrin control showed mortality of 100% and 90% on ORL and PR strains respectively and acetone treated mortality was at 3.3% ± 1.7%.

Based on this initial activity testing data (**Table 4**), Compounds **2** and **3** were used for additional testing to obtain LD_{50} values as described in the methods (**Table 5**). At least three independent repetitions were performed for each compound using various mosquito strains. Both compounds were tested against a permethrin-susceptible ORL strain and permethrin-resistant PR strain. A pyrethroid-susceptible strain of *An. quadrimaculatus* (ORL strain) was also tested against Compound **2**. Control mortality in all assays averaged less than 10% so no correction for background mortality was necessary.

When compared with the other two compounds, Compound **2** was the most effective against the standard ORL reference strain of *Ae. aegypti* with an LD_{50} of 0.64 μg/org. Activity of Compound **2** against *An. quadrimaculatus* was slightly better at 0.20 μg/org in comparison to the ORL strain of *Ae. aegypti*, which may be accounted for by the sensitivity of the *Anopheles* strain.

Chemical modification of Compound **2** produced differing activity levels. Compound **3**, obtained by acetylation of **2** resulted in a small reduction in efficacy to 0.89 μg/org when tested on the ORL strain of *Ae. aegypti* (**Table 5**). However, confidence intervals overlap between compounds **2** and **3** indicating no significant difference in activity between these two compounds. Compound **1,** the analogof **2** with a reduced double bond, abolished activity (>10 μg/org) in this assay against the ORL strain. Therefore, compound **1** was not tested further.

Comparative testing of both the susceptible and resistant *Ae. aegypti* strains with permethrin gave the expected differences in efficacy. The LD_{50} of the ORL strain was 0.26 ng/org and the PR strain was 25 ng/org for a resistance ratio of 96:1. Permethrin caused leg autotomy as expected in insects exposed to pyrethroids [8]. By comparison, no significant differences in efficacy were observed between compound **2** and compound **3** at 0.64 μg/org and 0.66 μg/org respectively. Also, no leg dropping was observed with these compounds. When considering all these observations in activity of these compounds, the mechanism of action is likely not the same as that for pyrethroids thus making these compounds a potentially useful chemical class against resistant mosquitoes.

Table 4. Adult topical assay of compounds on *Ae. aegypti*tested at 5 µg/insect.

Sample	Orlando strain (n = 3)	Puerto Rico strain (n = 2)
2	61.7 ± 28.3	100
3	100	100
Permethrin	100	90.0

Table 5. LD_{50}values ofcompounds on adult topical assay.

Compound	Species (strain)	LD_{50} (µg/org)	95% CI	R^2	n
1	*Ae. aegypti* (ORL)	>10	--	--	--
2	*Ae. aegypti* (ORL)	0.69	0.55 - 0.83	0.993	170
	Ae. aegypti (PR)	0.64	0.43 - 0.84	0.985	160
	An. quad (ORL)	0.20	0.14 - 0.26	0.987	220
3	*Ae. aegypti* (ORL)	0.89	0.75 - 1.02	0.995	240
	Ae. aegypti (PR)	0.66	0.49 - 0.82	0.989	240
Permethrin	*Ae. aegypti* (ORL)	0.00026	0.00015 - 0.00026	0.995	170
	Ae. aegypti (PR)	0.025	0.0059 - 0.055	0.916	180

Phomalactone (**2**) has been isolated from various plant pathogenic fungi from varying genera belonging to *Nigrospora* sp. [7] [9] [10]-[12], *Phoma* sp. [13] [14], and Xylaria species [15]. It has also been isolated from entomopathogenic fungi *Hirsutella thompsonii* [16], *Paecilomyces cateniobliquus* [17], and *Verticilium chlamydosporium* [18]. It has been shown that phomalactone causes the toxicity of the entomopathogenic fungi *Hirsutella thompsonii* and *Paecilomyces cateniobliquus* to *Helicoverpa armigera* (cotton boll worms) and *Rhagoletis pomonella* (apple maggot). It is interesting to note the occurrence of phomalactone in these genetically unrelated fungi; plant pathogenic and entomopathogenic fungi, indicating that these organisms could have evolved independently. Synthesis of more analogs and in-depth investigations need to be carried out on the use of phomalactone, its analogs, or the fungal spores as biological control agents.

Acknowledgements

The authors would like to thank Jason Martin, Eric Briscoe and Jessica Louton for technical assistance.

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