

Antifungal, mosquito deterrent, and larvicidal activity of *N*-(benzylidene)-3-cyclohexylpropionic acid hydrazide derivatives

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Abstract Hydrazone derivatives possess good antifungal and insecticidal activities and their structures are used in pesticide design. In the present study, for the first time, ten hydrazone derivatives (**1–10**) were evaluated for their antifungal activity against *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis* species and for their biting deterrent and larvicidal activity against *Aedes aegypti*, the yellow mosquito. The 96-well microbioassay revealed that compounds **3**, **5**, **7**, and **9** showed strong antifungal activity at 30 μ M against *Phomopsis obscurans*, whereas only two compounds (**5** and **9**) demonstrated strong antifungal activity against *Phomopsis viticola*. Compound **5** showed the highest biting deterrent activity against *Ae. aegypti* when compared with *N,N*-diethyl-*meta*-toluamide, the positive control at 25 nmol/cm². Compound **9** exhibited the highest larvicidal activity in toxicity bioassay with LD₅₀ values of 57.4 and

4.35 ppm and LD₉₀ values of 297.8 and 19.1 ppm, respectively, at 24 and 48 h post treatment. Compounds **5** bearing 4-fluoro substituent on benzene ring and **9** carrying a 4-isopropyl group on the benzene ring were found to be the most active compounds in both bioassays. These results could be useful information for development of new effective fungicides and insecticides in the near future.

Keywords Hydrazone · Fungicide · Plant pathogen · *Phomopsis obscurans* · *Phomopsis viticola* · Mosquito control · *Aedes aegypti*

Introduction

Plant pathogens and insect pests cause substantial damage to many economically important crops. Pesticides are used predominantly in agriculture and against insect vectors. In many cases, pesticides have become less effective as target organisms have developed resistance. In addition, these pesticides are threats to human health and threats to environmental through agriculture. Increased resistance to commercially available pesticides has led to a need for new agrochemicals and insecticides. In a program aimed at discovering new fungicides and insecticides as alternatives to conventional synthetic-based pesticides, we investigated ten hydrazone derivatives for their potential uses as disease control agents for plant pathogenic fungi and biopesticides as mosquito control agents (Dayan *et al.*, 2009; Duke *et al.*, 2010).

Filamentous fungi of the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis* species, are major plant pathogens worldwide. Anthracnose (caused by *Colletotrichum* sp.), *Phomopsis* and *Botrytis* diseases are serious problems of strawberry fruit in especially southeastern US

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(Crockett *et al.*, 2011; Sobolev *et al.*, 2011). The wilt-inducing strains of *Fusarium oxysporum* are widely distributed soil inhabiting fungi and responsible for severe damage to many economically important plant species (Fravel *et al.*, 2003). Several *Fusarium* species have been reported to infect orchidaceous plants worldwide (Latiffah *et al.*, 2009). Chemical control of plant diseases has become more difficult as several effective fungicides have lost their registration for use and some pathogens have evolved to be resistant to the most commonly used fungicides. Fungicides with new modes of action are needed to combat these resistant pathogens and to develop strategies for delaying evolution of resistance to fungicides in general (Duke *et al.*, 2003).

Mosquito borne diseases are the major health problems that have significant social and economical impact. The worldwide yellow fever, dengue fever, and its severe form dengue hemorrhagic fever, transmitted by *Aedes aegypti* have become an important worldwide public health problem (Warikoo *et al.*, 2011). Personal protection and reduction in mosquito populations through chemical control are the effective ways to eliminate mosquito borne diseases (Faradin and Day, 2002). The common approach for the control of mosquito vectors and reducing arthropod transmitted diseases is based on the use of chemical insecticides from different chemical classes. However, frequent use of insecticides has failed to achieve these objectives due to the development of insecticide resistance among mosquito populations. Pesticides also pose concerns on their toxic effects on human and animals and deterioration of nontarget species in the ecosystem.

Hydrazone derivatives have been reported to exhibit a wide spectrum of biological effects including antifungal and insecticidal activity in the literatures. Legocki *et al.* (2003) reported that 2,4-dihydroxythiobenzoyl derivatives substituted with amide, hydrazine, hydrazide, hydrazone, and semicarbazide groups showed different levels of antifungal activity depending on functional groups against *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium culmorum*, *Phytophthora cactorum*, and *Erysiphe graminis*. More recently, the research on nalidixic acid-based hydrazones revealed that hydrazones with a monosubstituted phenyl ring exhibited better activity than compounds with a disubstituted phenyl ring against *Rhizoctonia bataticola*, *Sclerotium rolfsii*, *R. solani*, *F. oxysporum*, and *Alternaria porii* (Agarwal *et al.*, 2010). Pyrazole and hydrazone derivatives possess good insecticidal activities, and they are widely used in pesticide design. For example hydramethylnon containing a hydrazone moiety and commercially known as Amdro[®], Combat[®], and Maxforce[®] is used for fire ants, cockroaches, and termites control (Hollingshaus, 1987). Another hydrazone type derivative, metaflumizone, was discovered by Nihon Nohyaku in the 1993 and

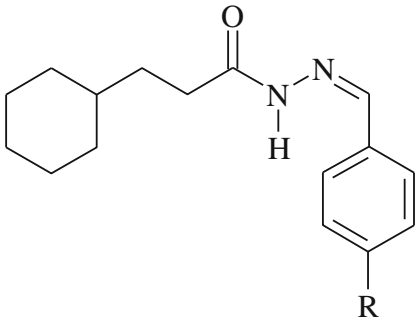
developed by BASF. The commercial product of metaflumizone, ProMeris[®] and ProMeris Duo[®], is used to control fleas, ticks, lice, and *Demodex* mites (Takagi *et al.*, 2007). Boger *et al.* (2001) reported that several benzophenone hydrazone derivatives possessed good activity against selected chewing insect pest. Liu *et al.* (2010) described phthalamides containing hydrazone substructures showing good larvicidal activity against *Plutella xylostella*. Wu *et al.* (2012) reported that pyrazole amide derivatives containing hydrazone substructures demonstrated notable control of *P. xylostella*, *Helicoverpa armigera*, *Culex pipiens pallens*, *Laphygma exigua*, *Spodoptera litura*, *Nilaparvata lugens*, and *Rhopalosiphum maidis* larvae.

The present study was undertaken to evaluate *N*-(benzylidene)-3-cyclohexylpropionic acid hydrazide derivatives for their antifungal activity against the plant pathogens, *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *B. cinerea*, *Phomopsis obscurans*, *Phomopsis viticola*, and *F. oxysporum* and insecticidal activity against *Ae. aegypti*.

Results and discussion

Ten hydrazone derivatives (**1–10**) synthesized via the nucleophilic addition–elimination reaction of 3-cyclohexylpropionic acid hydrazide with 4-substituted benzaldehydes (Table 1) were tested for their antifungal and insecticidal activity.

The 96-well micro-dilution broth assay was used in a dose–response format to evaluate each compound in more detail against *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis* species. Hydrazone derivatives (**1–10**) exhibited selective activity against *Phomopsis* species and no activity was observed against the other tested fungi (Fig. 1). *Phomopsis obscurans* is a common fungus that causes leaf blight in strawberry and other plants, while *P. viticola* causes *Phomopsis* cane and leaf spot of grapes worldwide. Compounds **3**, **5**, **7**, **9** at the highest concentration (30 μ M) caused 86.7, 86.9, 74.1, 99.5 % growth inhibition of *P. obscurans*, respectively. Compounds **5** and **9** caused 83.6 and 94.0 % growth inhibition of *P. viticola*, respectively, whereas captan standards showed 99.2 % activity against the same pathogen at the highest concentration (Fig. 1). The antifungal activity of the most effective derivative (**9**) against two pathogens can be attributed to increased lipophilicity associated with isopropyl group. Captan showed 100 % fungal growth inhibition of *P. obscurans* and *P. viticola* at 30 μ M. This level of activity is consistent with captan in our bioassay of these pathogens. However, we believe that new product-based fungicides must be active at 3.0 μ M or even 0.3 μ M to be commercially viable.

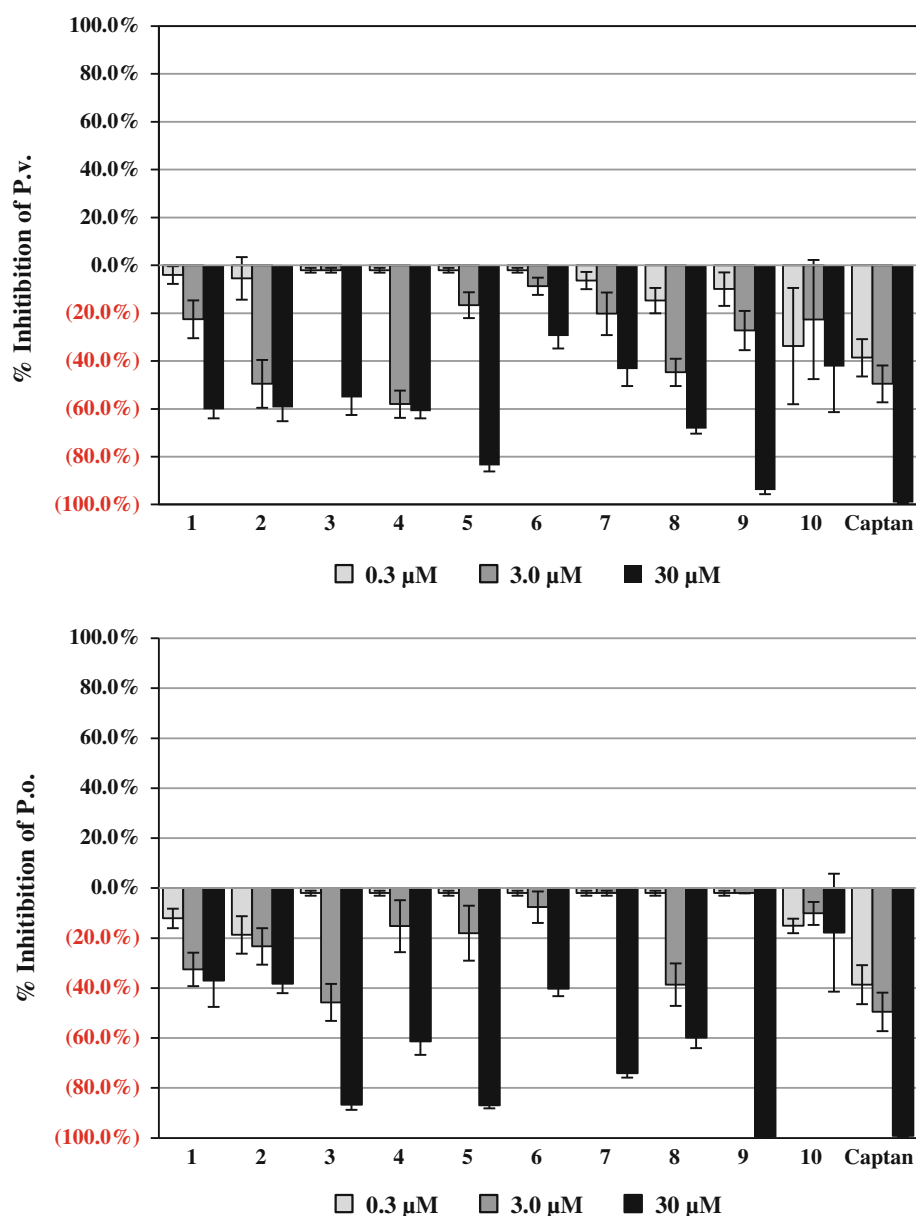
Table 1 Properties of hydrazone derivatives (1–10)


Compound	R	¹ H data in DMSO-d ₆ (δ ppm)
1	H	0.87–1.75 (11H, m, cyclohexane), 2.19–2.22 (2H, cyclohexane–CH ₂), 2.60–2.65 (2H, t, CH ₂ –CO), 7.40–8.18 (6H, m, phenyl and CH=N protons), 11.19–11.35 (1H, two s, N–H)
2	NO ₂	0.88–1.75 (11H, m, cyclohexane), 2.24–2.27 (2H, m, cyclohexane–CH ₂), 2.65–2.68 (2H, t, CH ₂ –CO), 7.91–8.30 (5H, m, phenyl and CH=N protons), 11.53–11.66 (1H, two s, N–H)
3	CH ₃	0.85–1.75 (11H, m, cyclohexane), 2.18–2.21 (2H, m, cyclohexane–CH ₂), 2.33–2.34 (3H, CH ₃), 2.60–2.63 (2H, t, CH ₂ –CO), 7.24–8.12 (5H, m, phenyl and CH=N protons), 11.13–11.27 (1H, two s, N–H)
4	Br	0.87–1.74 (11H, m, cyclohexane), 2.19–2.23 (2H, m, cyclohexane–CH ₂), 2.60–2.65 (2H, t, CH ₂ –CO), 7.59–8.14 (5H, m, phenyl and CH=N protons), 11.28–11.41 (1H, two s, N–H)
5	F	0.87–1.74 (11H, m, cyclohexane), 2.19–2.22 (2H, m, cyclohexane–CH ₂), 2.60–2.63 (2H, t, CH ₂ –CO), 7.26–8.17 (5H, m, phenyl and CH=N protons), 11.21–11.35 (1H, two s, N–H)
6	OH	0.85–1.74 (11H, m, cyclohexane), 2.15–2.19 (2H, m, cyclohexane–CH ₂), 2.57–2.60 (2H, t, CH ₂ –CO), 6.80–8.04 (5H, m, phenyl and CH=N protons), 9.86–9.89 (1H, O–H), 10.98–11.12 (1H, two s, N–H)
7	OCH ₃	0.87–1.74 (11H, m, cyclohexane), 2.17–2.20 (2H, m, cyclohexane–CH ₂), 2.59–2.62 (2H, t, CH ₂ –CO), 3.79–3.80 (3H, OCH ₃), 6.99–8.10 (5H, m, phenyl and CH=N protons), 11.07–11.20 (1H, two s, N–H)
8	Cl	0.85–1.74 (11H, m, cyclohexane), 2.19–2.23 (2H, m, cyclohexane–CH ₂), 2.61–2.65 (2H, t, CH ₂ –CO), 7.49–8.15 (5H, m, phenyl and CH=N protons), 11.27–11.40 (1H, two s, N–H)
9	CH(CH ₃) ₂	0.85–1.74 (17H, m, cyclohexane, isopropyl (CH ₃) ₂), 2.18–2.21 (2H, m, cyclohexane–CH ₂), 2.60–2.65 (2H, t, CH ₂ –CO), 2.89–2.95 (1H, m, isopropyl CH), 7.30–8.12 (5H, m, phenyl and CH=N protons), 11.15–11.27 (1H, two s, N–H)
10	N(CH ₃) ₂	0.84–1.75 (11H, m, cyclohexane), 2.15–2.18 (2H, m, cyclohexane–CH ₂), 2.57–2.59 (2H, t, CH ₂ –CO), 2.92–3.05 (6H, N(CH ₃) ₂), 6.73–8.00 (5H, m, phenyl and CH=N protons), 10.90–11.02 (1H, two s, N–H)

The biting deterrent activity of the compounds (1–10) was evaluated using in vitro Klun & Deboun (K & D) module system against *Ae. aegypti*. Mean biting deterrence index (BDI) values for hydrazone derivatives are presented in Fig. 2. The experimental design was set up with *N,N*-diethyl-*meta*-toluamide (DEET) at 25 nmol/cm² as the positive control and ethanol as solvent control. BDI values indicated that all the compounds tested have an activity, which is higher than solvent control. Compound 5 carrying 4-fluoro substituent on phenyl ring showed the highest biting deterrent effect. However, this activity was significantly lower than DEET at 25 nmol/cm². Compounds 1, 4, and 8 showed more than 50 % activity when compared with DEET. This outcome confirms that phenyl and halogen-substituted phenyl groups may have a role in this biting deterrent activity. Biting deterrent effects in the other compounds ranged between 22 and 39 %.

All synthetic hydrazone derivatives were also screened against *Ae. Aegypti* for their larvicidal activity. The compounds were first screened in larval bioassays at concentrations of 100, 50, and 25 ppm in a dose-dependent manner and percent mortality was observed. Only compound 9 was active with 80, 70, and 30 % mortality at 100, 50, and 25 ppm concentrations, respectively, at 24-h post treatments. Compound 9 was further evaluated to develop dose response bioassays. Mean percentage mortality of compound 9 at each dose at 24- and 48-h post treatment is presented in Fig. 3. At concentration of 0.8 and 1.6 ppm, there was no mortality at 24-h post treatment, whereas mortality at these dosages was 4 and 14.7 % at 48-h post treatment. At all the other dosages, mortality was significantly higher at 48-h post treatment when compared with 24-h. LD₅₀ values were 57.4 and 4.35 and LD₉₀ values were 297.8 and 19.1 ppm, respectively, at 24- and 48-h

Fig. 1 Growth inhibition of *P. viticola* (Pv) and *P. obscurans* (Po) after 144 h using 96-well microdilution broth assay in a dose response to compounds **1–10** and the commercial fungicide standard captan



post treatment. Although efficacy of this compound was low at 24-h post treatment, efficacy at 48-h post treatment was significantly high. This significantly higher mortality indicates that the compound acts slowly and this phenomenon should be further explored to find the utility of this compound or its analog in integrated pest management systems.

Conclusions

In this study, we evaluated ten hydrazone derivatives for their antifungal and insecticidal effects, which have not

been reported previously. The antifungal activity against various fungal species and larvicidal activity against *Ae. aegypti* indicate that compound **9** is the most active derivative among the tested compounds. This outcome confirms that the isopropyl substituent on benzene ring may have a considerable influence on both antifungal and larvicidal activity. Compound **5**, which has fluorine substituent as an electron withdrawing substituent on benzene ring, also showed good activity in the in vitro biting deterrent activity against *Ae. aegypti*. However, further research can be carried out on the development of new effective antifungal and insecticidal agents bearing hydrazone moiety by the modification of compounds **9** and **5**.

Fig. 2 Mean biting deterrence index (BDI $\pm$ SE) of hydrazone derivatives (1–10) tested against *Ae. aegypti* females. All compounds were tested at the concentration of 25 nmol/cm². Ethanol was the solvent control and DEET at 25 nmol/cm² was used as positive control. Proportion not biting in DEET ranged between 0.86 and 0.94 while these values were 0.36–0.38 in control

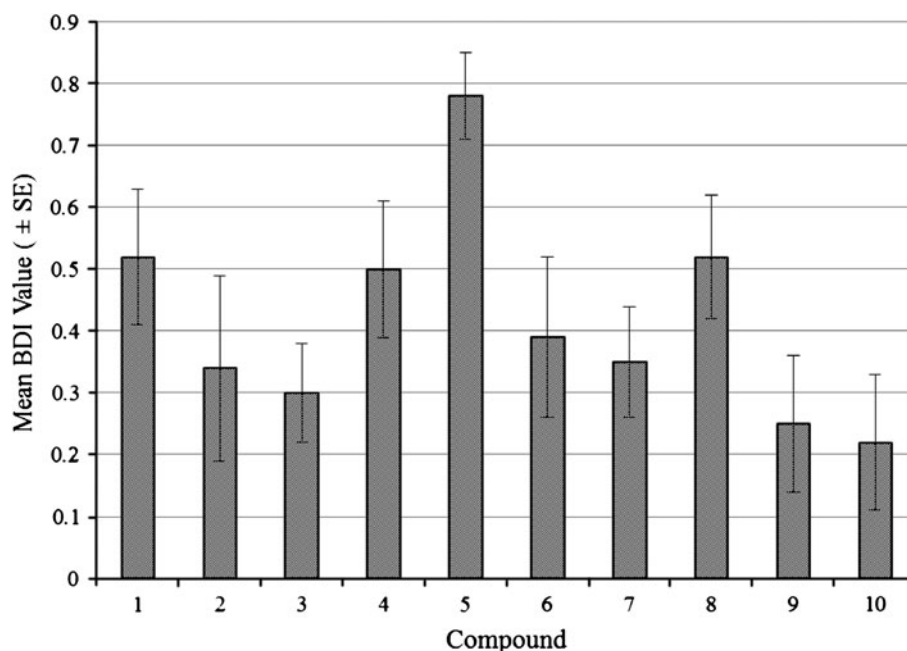
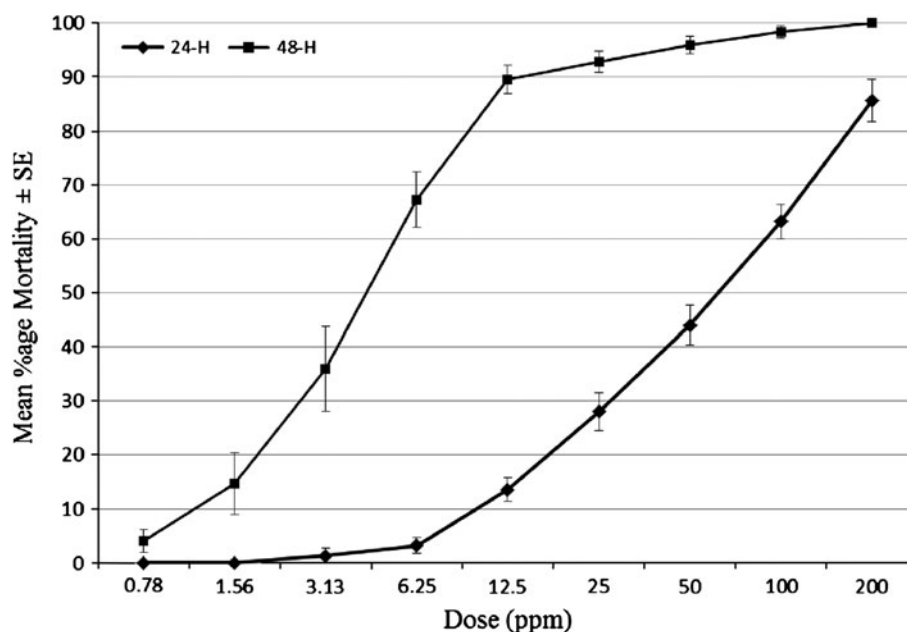


Fig. 3 Mean % age ($\pm$ SE) mortality of compound **9** against 1-d *Ae. aegypti* at 24- and 48-h post treatment



Experimental

Synthesis of test compounds

N-(Benzylidene)-3-cyclohexylpropionic acid hydrazide (**1**), *N*-(4-nitrobenzylidene)-3-cyclohexylpropionic acid hydrazide (**2**), *N*-(4-methylbenzylidene)-3-cyclohexylpropionic acid hydrazide (**3**), *N*-(4-bromobenzylidene)-3-cyclohexylpropionic acid hydrazide (**4**), *N*-(4-florobenzylidene)-3-cyclohexylpropionic acid hydrazide (**5**), *N*-(4-hydroxy-

benzylidene)-3-cyclohexylpropionic acid hydrazide (**6**), *N*-(4-methoxybenzylidene)-3-cyclohexylpropionic acid hydrazide (**7**), *N*-(4-chlorobenzylidene)-3-cyclohexylpropionic acid hydrazide (**8**), *N*-(4-isopropylbenzylidene)-3-cyclohexylpropionic acid hydrazide (**9**), *N*-(4-dimethylaminobenzylidene)-3-cyclohexylpropionic acid hydrazide (**10**) were obtained by the reaction of 3-cyclohexylpropionic acid hydrazide with 4-substituted benzaldehydes as reported previously (Kaplanickli *et al.*, 2012). The list of the compounds evaluated in this study is given in Table 1.

Biological assays

Pathogen production

Isolates of *C. acutatum* Simmonds, *Colletotrichum fragariae* Brooks, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from Barbara J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville, MS. Cultures of *P. viticola* and *P. obscurans* were obtained from Mike Ellis (Ohio State University, OH), and *B. cinerea* Pers. and *F. oxysporum* Schlechtend were isolated in our laboratory. *Fusarium oxysporum* identification was confirmed by Wade H. Elmer (Connecticut Agricultural Experiment Station, New Haven, CT), and the identity of *B. cinerea* was confirmed by Kenneth Curry (University of Southern Mississippi, Hattiesburg, MS). The three *Colletotrichum* species and *P. obscurans* were isolated from strawberry (*Fragaria ananassa* Duchesne), while *P. viticola* and *B. cinerea* were isolated from commercial grape (*Vitis vinifera* L.) and *F. oxysporum* from orchid (*Cynoches* sp.). Fungi were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in 9 cm Petri dishes and incubated in a growth chamber at $24 \pm 2^\circ\text{C}$ under cool-white fluorescent lights ($55 \pm 5 \mu\text{mol}/(\text{m}^2 \text{ s})$) with a 12 h photoperiod.

Conidia preparation

Conidia were harvested from 7 to 10 days old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped plastic rod. Aqueous conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) to remove mycelia. Conidia concentrations were determined photometrically (Wedge and Kuhajek, 1998) from a standard curve based on absorbance at 625 nm, and suspensions were adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/mL. Standard conidial concentrations were determined from a standard curve for each fungal species. Standard turbidity curves were periodically validated using both a Bechman/Coulter Z1 particle counter and hemocytometer counts.

Microdilution broth assay

A standardized 96-well micro-dilution broth assay developed by Wedge and Kuhajek (1998) was used to evaluate antifungal activity against *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *P. viticola*, *P. obscurans* and *F. oxysporum*. Captan was used as an internal fungicide standard in all assays. Each fungus was challenged in a dose–response format using test compounds where the final treatment concentrations were 0.3, 3.0 and 30.0 μM .

Microtiter plates (Nunc MicroWell, untreated; Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber as described previously (Crockett *et al.*, 2011; Sobolev *et al.*, 2011). Fungal growth was then evaluated by measuring absorbance of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL).

Microtiter assay experimental design

Chemical sensitivity of each fungus was evaluated using 96-well plate microbioassay format. Each chemical was evaluated in duplicate at doses 0.3, 3.0, and 30.0 μM . Sixteen wells containing broth and inoculum served as positive controls, eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. The experiments were repeated at three times over time. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 and 72 h except for *P. obscurans* and *P. viticola* where the data were recorded at 144 h. Means for percent inhibition of each fungus at each dose of test compound relative to the untreated positive growth controls were used to evaluate fungal growth. The SAS, Proc ANOVA (Statistical Analysis System, Cary, North Carolina) was used to identify significant factors, and Fisher's protected LSD was used to separate means (Steel and Torrie, 1980).

Mosquito activity

Insects

Aedes aegypti (L.) used in these studies was from a laboratory colony maintained since 1952 at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida. This colony has been maintained since 1952 using standard procedures (Pridgeon *et al.*, 2007). For repellent bioassays, pupae of *Ae. aegypti* were maintained in the laboratory at $28 \pm 1^\circ\text{C}$ and 30–60 % RH, and the emerging adults females aged 5–9 days were used. For larval bioassays, the eggs were hatched as per need and the larvae were maintained at a temperature of $27 \pm 2^\circ\text{C}$ and 70 ± 5 % RH in a photoperiod regimen of 12:12 (L:D) h.

Mosquito biting bioassays

Experiments were conducted using a six-celled in vitro K & D module bioassay system developed by Klun *et al.* (2005) for quantitative evaluation of bite deterrent properties of candidate compounds for human use. This bioassay method determines specifically measured biting (feeding) deterrent properties of the chemicals. In brief, the

assay system consists of a six well blood reservoir with each of the 3×4 cm wells containing 6 mL of blood. As reported earlier (Klun *et al.*, 2005; Klun *et al.*, 2008), female mosquitoes feed as well on the CPDA-1 (citrate-phosphate-dextrose-adenine) + ATP as they do on blood. Therefore, we used the CPDA-1 + ATP instead of human blood. CPDA-1 was prepared by dissolving 3.33 g sodium citrate, 0.376 g citric acid, 4.02 g dextrose, 0.28 g mono-basic sodium phosphate (Fisher Scientific Chemical Co. Fairlawn, NJ) and 0.346 g of adenine (Sigma–Aldrich, St. Louis, MO) in 1026 mL of deionized water. ATP was added to CPDA-1 to yield 10^{-3} M ATP (AABB 2005). CPDA-1 and ATP preparations were freshly made on the day of the test. DEET (99.1 % purity; *N,N*-diethyl-3-methylbenzamide) was obtained from Sigma–Aldrich (St. Louis, MO) and used as a positive control. Molecular biology grade acetone was obtained from Fisher Scientific Chemical Co. (Fairlawn, NJ). Compounds **1–10** were tested at the concentration of 25 nmol/cm² and DEET at 25 nmol/cm² was used as positive control. All the treatments were prepared in ethanol. The stock solutions were kept in a refrigerator at 3–4 °C. Treatments were prepared fresh at the time of bioassay. The temperature of the solution in the reservoirs was maintained at 37 °C by continuously passing the warm water through the reservoir using a circulatory bath. The reservoirs were covered with a layer of collagen membrane. This CPDA-1 + ATP solution membrane unit simulated a human host for mosquito feeding. The test compounds were randomly applied to six 4×5 cm areas of organdy cloth and positioned over the membrane-covered CPDA-1 + ATP solution with a separator placed between the treated cloth and the six-celled module. A six-celled K & D module containing five females per cell was positioned over cloth treatments covering the six CPDA-1 + ATP solution membrane wells, and trap doors were opened to expose the treatments to these females. The number of mosquitoes biting through cloth treatments in each cell was recorded after a 3 min exposure and mosquitoes were prodded back into the cells. These mosquitoes were then squashed to determine the number which has actually engorged the solution. A replicate consisted of six treatments: four test compounds, DEET (a standard bite deterrent compound) and ethanol treated cloth as solvent control. The 25 nmol DEET/cm² cloth dose was used as a standard, because it suppresses mosquito biting by 80 % as compared to controls (Klun *et al.*, 2005). A set of replications was conducted on different days using new lots of the insects.

Larval bioassays

Bioassays were conducted using the bioassay system described by Pridgeon *et al.* (2009) to determine the

larvicidal activity of the compounds (**1–10**) against *Ae. aegypti*. In brief, the eggs were hatched under vacuum (1 ~ h) by placing a piece of a paper towel with eggs in a cup filled with 100 mL of deionized water containing small quantity of larval diet. Larvae were removed from vacuum and held overnight in the cup in a temperature-controlled chamber maintained at a temperature of 27 ± 2 °C and 70 ± 5 % RH at a photoperiod regimen of 12:12 (L:D) h. Five 1-day-old first instar *Ae. aegypti* were added to each well of 24-well plates placed on illuminated light box using a disposable 22.5 cm Pasteur pipette with a droplet of water. 50 µL of larval diet (2 % slurry of 2:1 alfalfa pellets and hog chow) were added to each well using a Finnpiptette stepper (Thermo Fisher, Vantaa, Finland). All chemicals to be tested were diluted in dimethyl sulfoxide (DMSO). 11 µL of the test chemical was added to the labeled wells, and in control treatments 11 µL of DMSO alone was added. Each well had a total volume of 1.1 mL. After the treatment, the plates were swirled in clockwise and counter clockwise motions and front and back and side to side five times to insure even mixing of the chemicals. Larval mortality was recorded 24- and 48-h after treatment. Larvae that showed no movement in the well after manual disturbance of water by a pipette tip were recorded as dead. A series of dosages (4–5 concentrations) were used in each treatment to get a range of mortality. Treatments were replicated 15 times in each compound.

Statistical analyses

Since the K & D module bioassay system can handle only four treatments along with negative and a positive control, to make direct comparisons among more than four test compounds and to compensate for variation in overall response among replicates, repellency was quantified as BDI. BDI's were calculated using the following formula:

$$[BDI_{i,j,k}] = \left[\frac{PNB_{i,j,k} - PNB_{c,j,k}}{PNB_{d,j,k} - PNB_{c,j,k}} \right]$$

where $PNB_{i,j,k}$ denotes the mean proportion of females not biting test compound *i* for replication *j* and day *k* (*i* = 1–4, *j* = 1–5, *k* = 1–2), $PNB_{c,j,k}$ denotes the mean proportion of females not biting the solvent control for replication *j* and day *k* (*j* = 1–5, *k* = 1–2) and $PNB_{d,j,k}$ denotes the mean proportion of females not biting in response to DEET (positive control) for replication *j* and day *k* (*j* = 1–5, *k* = 1–2). This formula adjusts for variation in response among replication days and incorporates information from the solvent control as well as the positive control.

A BDI value of zero indicates an effect similar to acetone. A BDI value significantly greater than zero indicates an anti-biting effect relative to acetone. BDI values not significantly different from one are statistically similar to

DEET. BDI values were analyzed using SAS Proc ANOVA (SAS Institute, 2007) and means were separated using the Ryan-Einot-Gabriel-Welsch Multiple Range.

LD₅₀ values for larvicidal data were calculated using SAS, Proc Probit. Control mortality was corrected using Abbott's formula.

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