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RESEARCH ARTICLE

INSECTICIDAL AND REPELLENT ACTIVITIES OF CINNAMATES AGAINST MOSQUITOES AND TICKS

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ABSTRACT

Three natural flavor and fragrance compounds**,** *n*-butyl cinnamate, benzyl cinnamate, and benzyl cinnamate, were evaluated for toxicity and repellency against mosquitoes and ticks in this study. *n*-Butyl cinnamate showed the highest level of toxicity among the tested cinnamates with LC_{50}value of 7 ppm followed by benzyl cinnamate (LC_{50}= 8.4 ppm) and phenethyl cinnamate (LC_{50}= 10.3 ppm) against 1-d old *Aedes aegypti* larvae. In Klun & Debboun (K&D) biting deterrence bioassay, these three compounds showed biting deterrent activity above the solvent control. *n*-Butyl cinnamate and benzyl cinnamate with proportion not biting (PNB) values of 0.8 and 0.74, respectively, were similar to DEET while the activity of phenethyl cinnamate was lower than DEET and the other compounds at a rate of 25 nmol/cm^2. In Ali and Khan (A&K) bioassay, *n*-butyl cinnamate was active at the lowest dose of 5.9 µg/cm^2 followed by DEET and benzyl cinnamate that were active at 11.7 µg/cm^2 whereas phenethyl cinnamate did not show repellent activity at the highest dose of 93.7 µg/cm^2. Based on repellency data, *n*-butyl cinnamate was tested for residual repellency. Both DEET and *n*-butyl cinnamate were within the limits of the minimum effective dose (MED) up to 120 min at a dose of 23 .4 µg/cm^2. At 11.7 µg/cm^2, DEET crossed MED threshold after 30 min whereas *n*-butyl cinnamate was active up to 120 min. In tick bioassays, the repellent activity of *n*-butyl cinnamate at the concentration of 2.5% was similar to DEET at 1.25%. *n*-butyl cinnamate demonstrated reasonably good concentration-repellency response. In contrast, benzyl cinnamate did not demonstrate significant repellency when compared to DEET at the highest dose of 5%. High residual repellency of *n*-butyl cinnamate indicated its potential to be used as mosquito repellent.

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INTRODUCTION

Insect vectors of human diseases are important in global public health because they transmit many disease pathogens. Mosquitoes, *Aedes aegypti* (L) and *Ae. albopictus* (Skuse) are considered primary and secondary vectors of Zika virus (ZIKV), respectively, as well as other viruses (Ali *et al.,* 2017). *Anopheles* spp. transmits malaria (Sanders *et al.,* 1996; Meslin, 1997) and *Culex quinquefasciatus* Say West Nile virus (Godsey *et al.,* 2005). Similarly, ticks are vectors of important human diseases and thousands of Americans are infected each year with tick-borne diseases. Lone star ticks, *Amblyomma americanum* (L.), have recently become important human health risk (Childs *et al.,* 2003; Goddard *et al.,* 2009). The use of synthetic insecticides in mosquito control has proved to be one of the major components for the prevention and reduction of mosquito-borne disease incidence (Bhatt *et al.,* 2015). Insect repellents play an important role in the reduction of disease incidence by preventing infected mosquitoes from biting humans (Leal, 2006). Similarly, **s**ynthetic chemical repellents are accepted means of human protection against tick bites (Vazquez *et al.,* 2008).

Moreover, repellents have always been used against host seeking vectors as they provide immediate and localized personal protection. *N*,*N*-Diethyl-3-methylbenzamide (DEET) has been in use for more than 60 years and is the standard to which all repellents are measured in the market place (Frances, 2007). Some cinnamates have been reported as part of repellent combinations (Thireou *et al.,* 2018). This is the first detailed study of the repellent activity of these cinnamates. This paper reports insecticidal and repellent activity of *n*-butyl cinnamate, benzyl cinnamate and phenethyl cinnamate (Figure 1) against the yellow fever mosquito, *Ae. aegypti* L. and the lone star tick, *A. americanum* (L.).

MATERIALS AND METHODS

Arthropods and chemicals: *Aedes aegypti* used in these studies were from a laboratory colony maintained at the Mosquito and Fly Research Unit at the Center for Medical,

Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida (Pridgeon *et al.,* 2007). For biting deterrence bioassays, pupae were maintained in the laboratory at 27 ± 2°C and 60 ± 10% RH, and 6-15-d-old females were used. For larval bioassays, the eggs were hatched and the larvae were maintained under the above conditions. *n*-Butyl cinnamate (Cas # 538-65-8), benzyl cinnamate (Cas # 103-41-3)and phenethyl cinnamate (Cas # 103-53-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nymphs of the lone star tick, *A. americanum*, were obtained from a colony maintained in a tick rearing facility at Oklahoma State University, Stillwater, OK, USA. Nymphs were held at 22–23°C, 95 ± 2% RH, and 16:8 h (L:D) photoperiod for at least 7 days prior to use in bioassays. Nymphs were 2–3 months old (age from the last molt) at the time of bioassays.

In vitro K&D bioassay: Bioassays were conducted using a six-celled *in vitro* Klun and Debboun (K&D) bioassay system (Klun *et al.* 2005). Briefly the bioassay system consists of six 3 × 4 cm wells each of which contain approximately 6 mL of the feeding solution. As described by Ali *et al*. (2012), a feeding solution consisting of CPDA-1 and ATP was used instead of blood. All the compounds were tested in this study and DEET, 97%, *N*,*N*-Diethyl-3-methylbenzamide (Cas # 134-62-3, Sigma-Aldrich, St. Louis, MO, USA) at 25 nmol/cm^2 was used as a positive control. All the treatments were freshly prepared in molecular biology grade 100% ethanol. The temperature of the feeding solution in the reservoirs was maintained at 37°C by using a circulatory bath. The reservoirs were covered with a layer of collagen membrane (Devro, Sandy Run, SC). The test samples were randomly applied to six 4 × 5 cm marked areas of organdy and positioned over the membrane-covered CPDA-1+ATP solution with a Teflon separator placed between the treated organdy and the module to prevent the contamination. The K&D module containing five female mosquitoes per cell was positioned over treated organdy and trap doors were opened to expose the treatments to the females. The number of mosquitoes biting through treated organdy in each cell was recorded after a 3 min exposure and mosquitoes were prodded back into the cells to check the actual feeding. These mosquitoes were then squashed to determine the numbers that had imbibed the solution. A replicate consisted of six treatments: four test samples, DEET and ethanol treated organdy as solvent control. Two sets of 5 replications each with 5 females per treatment were conducted on 2 different days using a newly treated organdy and a new batch of females in each replication. Treatments were replicated 10 times.

In vitro A&K repellent bioassay: Bioassays were conducted using Ali & Khan (A&K) bioassay system developed by Ali *et al*. (2017) for quantitative evaluation of repellency against mosquitoes. Briefly the bioassay system consists of a 30 × 30 × 30 cm collapsible aluminum cage having one penal of clear transparent acrylic sheet with 12× 3.5 cm slit through which the blood box containing a removable feeding device was attached. The top of the blood box had a sliding door used to expose the females to the treatment during the bioassay. Rectangular areas of either 3 × 4-cm were marked on the collagen sheet that matched the measurement of the rectangular liquid reservoirs. Treated collagen was secured on the feeding reservoir containing the feeding solution using a thin layer of grease. The feeding device was then pushed inside the blood box and the sliding door was opened to expose the females to the treatment. The number of females biting through the treated collagen during 1 min exposure was recorded. Means and standard errors were calculated using Microsoft Excel version 10 or SAS.

Larvicidal bioassays: Bioassays were conducted using the bioassay system described by Pridgeon et al. (2009). Further methods and statistical analyses were described in (Ali *et al.,* 2013). DMSO was used as a solvent to prepare the treatments and was also used as a negative control. Permethrin (95.7%) (Chem Service, Inc. West Chester, PA) was used as a positive control.

Statistical analyses: Proportion not biting (PNB) values in K&D data were calculated using the following formula:

$$\text{PNB} = 1 - \left(\frac{\text{Total number of females biting}}{\text{Total number of females}}\right)$$

Data on the PNB were analyzed using SAS Proc ANOVA (SAS Institute, Inc., Carry, NC 2007) and means were separated using Ryan-Einot-Gabriel-Welsch multiple range test. Means and standard errors of MED values were calculated using SAS Proc Means or Microsoft Excel 2010. LC_{50} values for larvicidal data were calculated by using SAS, Proc Probit.

Tick repellency bioassay: Each of the test compounds (*n*-butyl cinnamate, phenethyl cinnamate, and benzyl cinnamate) was diluted in ethanol to generate 7 test concentrations. Ethanol alone was tested as a negative control, and 1.25% DEET was used as a positive control. Repellency tests of these compounds against nymphs of the lone star tick were carried out using the vertical paper assay described by Carroll *et al*. (2011). Briefly, a 4 × 7 cm rectangle of Whatman No. 4 filter paper was prepared by treating the central 4 × 5 cm zone with a volume of 165 µL of the test solution. After drying, the paper strip was suspended from a bulldog clip hung from a holder. Ten nymphs were released from a glass vial on the lower untreated end of the paper strip. Nymphs crawl upward and their locations on the filter paper were recorded at 1, 3, 5, 10, and 15 min of release. Ticks were considered repelled if they stayed on the lower untreated zone or fell off the filter paper without having crossed into the upper untreated zone (Meng *et al.,* 2016; Machtinger *et al.,* 2017). Each treatment included three replicates. Percentage repellency was converted into corrected repellency using Abbott's formula (Abbott, 1925) for each test. Data is presented as corrected percent repellency.

RESULTS AND DISCUSSION

The three cinnamates tested in this study showed larvicidal activity. *n*-Butyl cinnamate with LC_{50} value of 7 ppm showed the highest level of toxicity followed by benzyl cinnamate (LC_{50}= 8.4 ppm) and phenethyl cinnamate (LC_{50}= 10.3 ppm) against 1-d old *Ae. aegypti* larvae (Table 1). Similarly based on $LC_{90,}$ *n*-butyl cinnamate with a value of 13.7 ppm were significantly more toxic than benzyl-or phenethyl cinnamate against *Ae. aegypti* larvae. In K&D biting deterrence bioassay all compounds showed biting deterrent activity above the solvent control (Figure 2). *n*-Butyl cinnamate and benzyl cinnamates with PNB values of 0.8 and 0.74, respectively, were statistically similar to DEET while the activity of phenethyl cinnamate was lower than DEET at a rate of 25 nmol/cm^2. In A&K repellent bioassay, *n*-butyl cinnamate was the most active at the lowest dose of 5.9 µg/cm^2 followed by DEET and benzyl cinnamate that were active at 11.7 µg/cm^2

Table 1. Toxicity of cinnamates against first-instar larvae of *Aedesaegypti*

Compound	LC_{50} (95% CI)[a]	LC_{90} (95% CI)	χ^2	df
n-Butyl cinnamate	7.0 (6.2 - 8.0)	13.7 (11.6 - 17.3)	81.7	48
Benzyl cinnamate	8.4 (7.2 - 9.8)	21.7 (17.6 - 28.9)	87.8	48
Phenethylcinnamate	10.3 (8.7 - 12.2)	30.3 (24.1 - 41.9)	89.3	48

[a]LC_{50} and LC_{90}values are given in ppm (95% confidence interval).

Table 2. Repellent activity of DEET and cinnamates against *Aedesaegypti*in *in vitro* A & K bioassay

Compound	N	%age females feeding out of 200					
		Dose (µg/cm^2)					
		93.7	46.9	23.4	11.7	5.9	2.92
DEET	15	0	0	0	0.30 ± 0.5	>1	>1
n-Butyl cinnamate	15	0	0	0	0	0.57 ± 0.13	>1
Benzyl cinnamate	15	0	0	0.25 ± 0.08	0.7 ± 0.08	>1	>1
Phenethylcinnamate	15	>1	>1	>1	>1	>1	>1

[1] N is the number of replications. [2]Data are %age (mean ± SEM) females biting. The minimum effective dose is ≤ 1% biting which are 2 females out of 200 in the cage. Data is from A & K bioassay using 12 cm^2 treated surface area. Ethanol was regularly tested at the beginning and after every 5 replications as solvent control. The bioassays were continued only if the ethanol treatment failed (feeding ≥ 1%).

Table 3. Residual repellent activity of DEET and n-butyl cinnamate against *Aedesaegypti* females at different dosages in an *in vitro*, A & K bioassay

Compound	Dose (µg/cm^2)	%age females feeding out of 200				
		Time after treatment (Min)				
		0	30	60	90	120
DEET	23.4	0	0	0	0.03 ± 0.03	0.43 ± 0.07
n-Butyl cinnamate	23.4	0	0	0	0	0.17 ± 0.06
DEET	11.7	0.26 ± 0.07	0.53 ± 0.08	>1	>1	>1
n-Butyl cinnamate	11.7	0.07 ± 0.04	0.1 ± 0.05	0.1 ± 0.05	0.23 ± 0.07	0.09 ± 0.1

[1] N is the number of replications. [2]Data are %age (mean ± SEM) females biting. The minimum effective dose is ≤ 1% biting which are 2 females out of 200 in the cage. Data is from A & K bioassay using 12 cm^2 treated surface area.

Table 4. Repellency of cinnamates against nymphs of thelone star tick

Compound	Conc. (%)	% Corrected repellency			
		R1	R2	R3	Mean
Experiment 1					
DEET	1.25	75.0	71.4	100.00	82.14*
n-Butyl cinnamate	0.16	12.5			12.5
n-Butyl cinnamate	0.31	12.5	0.0	-33.33	-6.9
n-Butyl cinnamate	0.63	0.0	42.9	50.00	31.0
n-Butyl cinnamate	1.25	25.0	28.6	-16.67	12.3
n-Butyl cinnamate	2.50	87.5	85.7	83.33	85.5*
n-Butyl cinnamate	5.00		100.0	100.00	100*
Experiment 2					
DEET	1.25	60	87.5	42.86	63.5*
Benzyl cinnamate	0.16	-20	25	28.57	11.19
Benzyl cinnamate	0.31	-60	0	-42.86	-34.29
Benzyl cinnamate	0.63	-100	-12.5	14.29	-32.74
Benzyl cinnamate	1.25	-40	0	-14.29	-18.10
Benzyl cinnamate	2.50	40	0	28.57	22.86
Benzyl cinnamate	5.00	40	-25	-14.29	0.24

Means within an experiment marked with * are statistically similar.

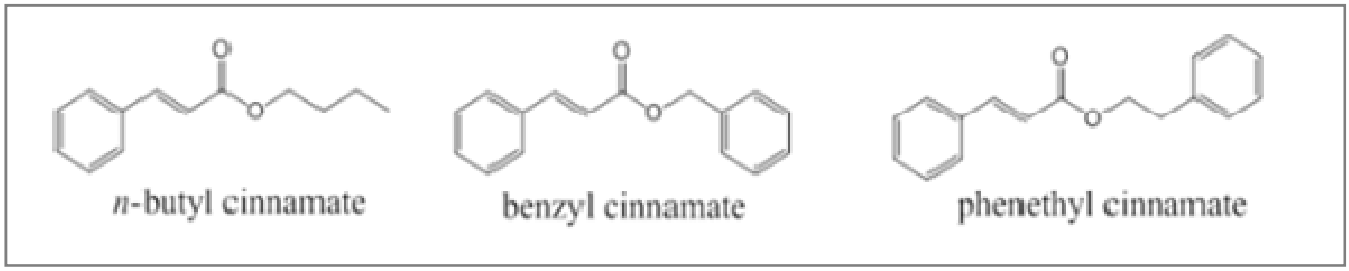

Figure 1. Structures of cinnamates tested in this study

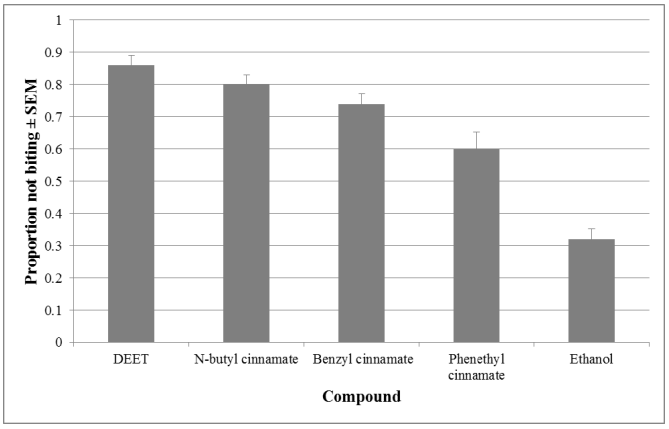

Figure 2. Proportion not biting values of n-butyl cinnamate, benzyl cinnamate, phenethyl cinnamate, and DEET against *Ae. aegypti* females. All compounds and DEET as a positive control were tested at 25 nmol/cm^2 while ethanol was the negative control

whereas phenethyl cinnamate did not show any repellent activity at the highest dose of 93.7µg/cm^2 (Table 2). A&K bioassay compares the data based on the minimum effective dose (MED) values. Based on repellency data, *n*-butyl cinnamate was further tested for residual repellency.

Both DEET and *n*-butyl cinnamate were within the limits of the MED up to 120 min at 23 .4 µg/cm^2 (Table 3). At 11.7 µg/cm^2, DEET crossed the MED threshold after 30 min whereas *n*-butyl cinnamate was active up to 120 min. These data indicated a strong potential of *n*-butyl cinnamate to be developed as a commercial repellent. In repellent studies some cinnamates have been used as parts of the formulations of repellents against mosquitoes (Thireou *et al.,* 2018). However, this is the first detailed study on the repellent activity of these compounds.

In tick bioassays, solvent (ethanol) alone resulted in an average of ~30% repellency, which was much higher than desired. n-Butyl cinnamate demonstrated reasonably good concentration-repellency response (Table 4). Repellencies of lower concentrations of *n*-butyl cinnamate were not statistically different from that of the solvent alone. *n*-Butyl cinnamate at the concentration of 2.5% reached the same level of repellency as 1.25% DEET, and reached a 100% repellency at 5.0%. In contrast, benzyl cinnamate did not demonstrate significantly higher repellency than that of solvent alone at any of the concentrations tested, suggesting that it is not an effective repellent against the lone star tick. In conclusion, cinnamates especially *n*-butyl cinnamate showed great potential to be used as a repellent against mosquitoes and lone star tick. Residual activity of *n*-butyl cinnamate is longer than DEET which is a strong positive attribute. Further studies will be continued to explore the repellent activity of *n*-butyl cinnamate by testing in different formulations under large cage laboratory bioassays and field tests against mosquitoes and ticks.

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