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Acetylcholinesterase inhibition by nootkatone and carvacrol in arthropods

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Abstract
The essential oils from many botanicals have been screened for insecticidal activity. Two constituents of the Alaskan yellow cedar tree, the monoterpenoid carvacrol and the sesquiterpenoid nootkatone, both are toxic against several arthropods. The mode of action through which nootkatone and carvacrol exert their insecticidal activity remains uncertain. It has been hypothesized that they may inhibit acetylcholinesterase enzyme activity. The degree of acetylcholinesterase inhibition of carvacrol and nootkatone was compared to that of carbaryl, a known acetylcholinesterase inhibitor, in the house fly (*Musca domestica*), yellow fever mosquito (*Aedes aegypti*), American dog tick (*Dermacentor variabilis*) and American cockroach (*Periplaneta americana*). The concentration of carbaryl, at which 50% of the acetylcholinesterase activity was inhibited (IC_{50}), was less than 2 μM in all four arthropod models. Carvacrol was observed to cause slight inhibition of the acetylcholinesterase enzyme in house flies, ticks and cockroaches, but it did not inhibit the mosquito acetylcholinesterase enzyme. Nootkatone did not inhibit the acetylcholinesterase enzyme in any of the four arthropod models tested. From this study, we conclude that the acetylcholinesterase inhibition is not likely the primary mode of action for insecticidal activity by nootkatone or carvacrol.

Keywords
Acetylcholinesterase, Nootkatone, Terpenoids, Carvacrol, Natural products

Disciplines
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Acetylcholinesterase inhibition by nootkatone and carvacrol in arthropods

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ABSTRACT

The essential oils from many botanicals have been screened for insecticidal activity. Two constituents of the Alaskan yellow cedar tree, the monoterpenoid carvacrol and the sesquiterpenoid nootkatone, both are toxic against several arthropods. The mode of action through which nootkatone and carvacrol exert their insecticidal activity remains uncertain. It has been hypothesized that they may inhibit acetylcholinesterase enzyme activity. The degree of acetylcholinesterase inhibition of carvacrol and nootkatone was compared to that of carbaryl, a known acetylcholinesterase inhibitor, in the house fly (*Musca domestica*), yellow fever mosquito (*Aedes aegypti*), American dog tick (*Dermacentor variabilis*) and American cockroach (*Periplaneta americana*). The concentration of carbaryl, at which 50% of the acetylcholinesterase activity was inhibited (IC_{50}), was less than 2 µM in all four arthropod models. Carvacrol was observed to cause slight inhibition of the acetylcholinesterase enzyme in house flies, ticks and cockroaches, but it did not inhibit the mosquito acetylcholinesterase enzyme. Nootkatone did not inhibit the acetylcholinesterase enzyme in any of the four arthropod models tested. From this study, we conclude that the acetylcholinesterase inhibition is not likely the primary mode of action for insecticidal activity by nootkatone or carvacrol.

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1. Introduction

In recent years, there has been an increased demand for safe, effective, environmentally friendly alternatives to synthetic insecticides and repellents [1]. To date, a variety of natural plant extracts and essential oils have been studied to determine their efficacy as insecticides and repellents, and many have been shown to be effective against a wide variety of insect pests. For example, vetiver oil extracted from the roots of vetiver grass has been shown to kill or repel cockroaches, ants, ticks [2] and repel termites [3]. Menthol and tea tree oil have both been shown to be an effective acaricide [4, 5, respectively]. Oil extracted from turmeric, citronella grass and hairy basil were shown to repel multiple species of mosquito under laboratory conditions [6]. Likewise, essential oils from rosemary, eucalyptus, clove, thyme, and citrus have also been widely studied as insecticides and repellents [7, 8].

Recently, carvacrol and nootkatone, two terpenoid compounds extracted from the heartwood of the Alaskan yellow cedar (*Chamaecyparis nootkatensis)*, were shown to be effective in repelling the blacklegged tick (*Ixodes scapularis*) [9] and the lone star tick (*Amblyomma americanum*) [10]. In addition to ticks, nootkatone (extracted from various botanicals) has been shown to kill the Formosan subterranean termite [3], fleas [11], rice and maize weevil [12], as well as kill and repel ants and cockroaches [2]. Similarly, carvacrol has also been shown to kill fleas and mosquitoes [11]. While both nootkatone and carvacrol are effective insecticides/ acaricides against several insect and tick pests, their primary modes of action remain unclear, and further research is needed to understand the mechanism of toxicity.

The mode of action of many synthetic chemical pesticides, including organophosphates (OPs) and carbamates, is inhibition of acetylcholinesterase (AChE) enzymes. Both OPs and

carbamates are known to bind to and inhibit AChE enzymes, causing overstimulation of the neurons, which leads to rapid twitching of the muscles, convulsions and insect death [13]. In addition to OPs and carbamate insecticides, a variety of terpenoid compounds have also been shown to inhibit AChE (see [14] for a review). The ability of nootkatone or carvacrol to inhibit acetylcholinesterase activity has not been assessed to date. The goal of this study is to characterize the degree of AChE inhibition of nootkatone and carvacrol, using four arthropod model organisms: house fly (*Musca domestica*), American dog tick (*Dermacentor variabilis*), yellow fever mosquito (*Aedes aegypti*), and American cockroach (*Periplaneta americana*). By understanding the mechanisms of action, nootkatone and carvacrol can be better used to control public health pests.

2. Methods

Acetylcholinesterase activity was measured using a spectrophotometric assay, modified from Ellman et al., [15] and Beauvais et al., [16]. The concentrations of acetylthiocholine (AThCh) substrate and 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) reagent were optimized for each arthropod species tested. Acetylcholinesterase enzyme homogenates were prepared from each arthropod model, as follows: approximately 250 adult, mixed sex house flies (from a colony maintained at Iowa State University) were decapitated on dry ice and the house fly heads were homogenized in Tris buffer (pH 7.4). Twenty adult, mixed sex American dog ticks (purchased from El Laboratories, Soquel, CA) were cut into pieces using scissors. Tick pieces were homogenized for one minute in 5 ml of tick extraction buffer (10 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 0.5% Triton X-100) and extracted for one hour at 4^{o}C on a plate shaker, following the method of Pruet and Pound, 2005 [17]. Eleven adult, mixed sex American

cockroaches (from a colony maintained at Iowa State University) were decapitated, and the heads were stored in insect Ringer's solution (214 mM sodium chloride, 1.2 mM magnesium sulfate, 9 mM calcium chloride, 3.1 mM potassium chloride, 0.4 mM potassium phosphate monobasic, 25 mM sodium bicarbonate, 10 mM D-glucose) at -20^{o}C. Upon thawing, the cockroach heads were drained, rinsed, and homogenized in 22 ml of 50 mM Trimza 7.4 buffer. Fifty mixed sex 4th instar yellow fever mosquito larvae (from a colony maintained in the Medical Entomology Lab at Iowa State University) were homogenized in 6 ml of mosquito extraction buffer [18]; 0.1 M sodium phosphate buffer (pH 7.8), 1% Triton X-100). All enzyme homogenates were clarified by centrifugation, and stored at -20^{o}C prior to use in the assay.

Solutions of nootkatone (Alfa Aesar, >98% purity), carvacrol (Sigma, 98% purity) and carbaryl (used as a positive control) were prepared in acetone. Multiple concentrations of test inhibitors nootkatone and carvacrol (ranging from 0 µM to 30,000 µM) and the carbaryl (0 µM to 30 µM) were tested to determine the concentration at which 50% of the acetylcholinesterase enzyme activity was inhibited (IC_{50}). For the house fly assay, 10 mM AThCh was added to microplate wells containing 167 µl of Tris buffer (pH 8.0), 0.32 mM DTNB, 30 µl of house fly enzyme homogenate, and 3 µl of the test inhibitor solution. For the tick assay, 20 mM AThCh was added to microplate wells containing 77 µl of sodium phosphate buffer (50 mM, pH 7.5), 0.32 mM DTNB, 90 µl of tick enzyme homogenate, and 3 µl of the test inhibitor solution. For the cockroach assay, 10 mM AThCh was added to microplate wells containing 167 µl of Tris buffer (50 mM, pH 7.4), 0.32 mM DTNB, 30 µl of cockroach enzyme homogenate, and 3 µl of the test inhibitor solution. For the mosquito assay, 10 mM AThCh was added to microplate wells containing 157 µl of mosquito extraction buffer, 0.32 mM DTNB, 40

106 µl of mosquito enzyme homogenate, and 3 µl of the test inhibitor solution. Blank controls
107 (enzyme homogenate was replaced with buffer) were included for each arthropod assay, to
108 control for non-specific hydrolysis of the AThCh. Negative controls (containing acetone in the
109 place of the test inhibitor) were also used to determine enzyme activity in the uninhibited
110 sample.

111 For all assays, the initial reading (time 0) was taken immediately following addition of
112 the AThCh substrate, at an absorbance of 405 nm (THERMOmax microplate; Softmax software).
113 For the house fly assay, the final reading was taken after incubating for 6 minutes at room
114 temperature (with microplate mixing every 9 seconds). Whereas, for the cockroach assay, the
115 final reading was taken after incubating for 20 minutes at room temperature (with microplate
116 mixing every 30 seconds), and for both the tick and mosquito assays, the final reading was
117 taken after incubating for 30 minutes at room temperature (with microplate mixing every 45
118 seconds).

119 **2.1. Data Analysis**

120 All analyses were run in triplicate with one exception (one assay using carbaryl as
121 an inhibitor was only conducted in duplicate due to limited house fly homogenate).
122 The coefficient of variation between triplicate data points was analyzed. If the CV
123 between the triplicate analysis at both the time 0 and the final reading was greater
124 than 10%, data points that had a relative percentage difference greater than 15% were
125 excluded. The change in absorbance (Δ Abs) was determined by subtracting the final
126 reading from the initial reading, and dividing by the incubation time. The average Δ Abs
127 in the blank samples was subtracted from the Δ Abs of each sample, and the blank

subtracted Δ Abs was used to calculate percentage inhibition of the treatment samples, relative to the 0 µM control samples (where percentage inhibition = [average Δ Abs of 0 µM control – average Δ Abs of treatment/ average Δ Abs of 0 µM control]*100). The IC_{50} of nootkatone, carvacrol and carbaryl were determined in each arthropod model by plotting the concentration (log µM) against the probit-transformed percentage inhibition data.

3. Results

The IC_{50} for the positive control, carbaryl, was 1.2 µM in the house fly assay, 1.8 µM in the American dog tick assay, 0.4 µM in the American cockroach assay, and 1.8 µM in the yellow fever mosquito assay (Table 1). The calculated IC_{50} for carvacrol in the house fly assay was roughly three orders of magnitude higher than the IC_{50} that was observed with the positive control carbaryl. However, it should be noted that the maximum inhibition of the acetylcholinesterase enzyme was only 57% by carvacrol in the house fly assay, even at the highest exposure concentration tested (30,000 µM, Figure 1). The IC_{50} values for carvacrol in the American dog tick and the American cockroach assays were 224 µM and 51 µM, respectively. The maximum inhibition of the AChE enzyme by carvacrol in the tick assay was 85% at a concentration of 3,000 µM. The level of inhibition in the 30,000 µM carvacrol treatment could not be assessed because the carvacrol was observed to come out of solution at this high concentration. The maximum inhibition of the AChE enzyme in the cockroach assay was 81% at a concentration of 300 µM carvacrol, and the percentage inhibition at 3,000 and 30,000 µM was lower, indicating that a true concentration-response was not attained, probably due to exceeding of solubility limits. Due to the large standard error, the results at the high

concentration should be interpreted with caution. The IC_{50} for carvacrol in the yellow fever mosquito assay was > 3000 µM (the level of inhibition in the 30,000 µM carvacrol treatment could not be assessed because the carvacrol was observed to come out of solution at this concentration). For nootkatone, the IC_{50} was > 30,000 µM for all four arthropod models.

4. Discussion

To date, the colorimetric assay [15] has been used to screen acetylcholinesterase activity in a wide variety of insects. For each arthropod species used in this study, the AThCh substrate concentration, DTNB concentration and incubation time were optimized. Carbaryl was used as a positive control to monitor the performance of the assay, as carbamates are known to bind to the acetylcholinesterase enzyme and prevent hydrolysis of the acetylcholine neurotransmitter. The IC_{50} for carbaryl was less than 2 µM in all four arthropod models, indicating that when exposed to a known AChE inhibitor, the AChE enzymes used in this study were strongly inhibited.

Terpenoids are found in a wide variety of plants and consist of repeating isoprene units [19]. The aromatic monoterpenoid, carvacrol, contains ten carbon atoms, whereas the sesquiterpenoid, nootkatone, contains 15 carbon atoms, originally biosynthesized from the combination of three isoprene units. Previously, several different terpenoid compounds have been shown to have anti-acetylcholinesterase activity. For instance, 1,8-cineole and α-pinene, two monoterpenoid constituents of sage (*Salvia officinalis* and *S. lavandulaefolia*), were shown to inhibit erythrocyte acetylcholinesterase activity, with 50% inhibition of the enzyme occurring at concentrations of 0.67 and 0.63 mM, respectively [20]. More recently, 1,8-cineole has been shown to inhibit AChE enzyme activity from electric eel and head louse, with 50% inhibition of

the enzyme occurring at concentrations of 6 mM and 77 mM in eel and head louse, respectively [21]. The monoterpenoids (+)2-carene, (+)3-carene, and (+)-pulegone have also been reported to inhibit acetylcholinesterase activity; however, the lowest IC_{50}s ranged from 200 µM for (+)-3-carene to 890 µM for (+)-pulegone, indicating that the level of inhibition was relatively weak [14], compared to carbamates and organophosphates, but similar to other terpenoid studies. Likewise, eugenol, extracted from *Inula graveolens* L. has been shown to inhibit AChE in eel at a concentration 2.9 mM [22], and pulegone -1,2-epoxide, extracted from the poleo plant is also reported to inhibit eel AChE [23]. Several terpenoids extracted from water mint (*Mentha aquatic),* including viridiflorol, elemol, 1,8-cineole, and pulegone, were also screened for acetylcholinesterase inhibition using the Ellman colorimetric assay, and inhibited 50% of the enzyme concentrations of 0.11, 0.15, 0.27, 0.89 mM, respectively [24].

In the present study, we observed that nootkatone did not inhibit the AChE enzyme in any of the arthropod models. Carvacrol did display limited inhibitory activity in house fly, tick and cockroach, however the level of inhibition by carvacrol was several orders of magnitude lower that the inhibitory potential of the positive control carbaryl. The inhibitory potential of carvacrol is comparable to other monoterpenoid compounds extracted from water mint, which ranged from 0.11 to 0.89 mM. This indicates that while there appears to be some inhibitory effect of carvacrol on acetylcholinesterase enzyme activity, it is not likely the primary mode of action of this compound. A recent study confirmed that both nootkatone and carvacrol did not share a similar mode of action as OPs and carbamates using *Anopheles gambiae* with ACE-1 gene mutations [25]. This study also reported that nootkatone and carvacrol did not share a similar mode of action as permethrin or dieldrin, using *Anopheles gambiae* containing

194 mutations of the sodium channel para-locus and of the y-aminobutyric acid (GABA) receptor,
195 respectively [25]. Recently, Tong and Coats [26] also characterized the binding of carvacrol and
196 other monoterpenoids to the GABA receptor. They reported that while carvacrol, pulegone and
197 thymol were able to bind to the GABA receptor, the observed binding was different than typical
198 of cyclodiene and organochlorine pesticides. Results from a separate study suggest that
199 nootkatone may in fact enhance the activity of the acetylcholinesterase enzyme [27].
200 Because we investigated both carvacrol and nootkatone independently, we did not test
201 for possible synergistic effects between these two compounds or other constituents of Alaska
202 yellow cedar extract. In many cases essential oils have been shown to support higher
203 acetylcholinesterase inhibition than the individual constituents of the oil [24], and synergism
204 was previously reported among terpenoids in *Salvia lavandulaefoilia* essential oil [20]. The
205 potential synergistic effect of these two test compounds remains to be determined in later
206 studies. In this study, we found that nootkatone did not cause inhibition of
207 acetylcholinesterase activity, whereas carvacrol did show limited inhibition in house fly, tick and
208 cockroach. Because of the low level of inhibitiory activity observed with carvacrol, relative to
209 the positive control carbaryl, acetylcholinesterase inhibition is not likely the primary mode of
210 action. Studies characterizing additional potential modes of action of nootkatone and carvacrol
211 in insects, including octopamine receptors, sodium channels, nicotinic acetylcholine receptors
212 and GABA receptors are currently underway, in an effort to better understand the primary
213 action site of these terpenoids.
214

215 **5. Acknowledgements**

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