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ORIGINAL ARTICLE

Artemisia annua extracts, artemisinin and 1,8-cineole, prevent fruit infestation by a major, cosmopolitan pest of apples

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Abstract

Context: Extracts of *Artemisia annua* (L.) (Asteraceae) and artemisinins are used for treatment of malaria, parasitic infections and have potent anticancer properties in cell lines. Eucalyptus oil and 1,8-cineole have antimicrobial, immune-stimulatory, anti-inflammatory, antioxidant, analgesic, and spasmolytic effects. Codling moth, *Cydia pomonella*, (L.) (Tortricidae), is a major cosmopolitan pest of the apple, potentially causing damage translating to 40 billion US dollars per year, globally. Currently used control measures are either hazardous to agricultural workers and harmful to environment, or ineffective. The potential of plant-derived semiochemicals for codling moth control is heavily understudied.

Objective: This study evaluated the potential of *A. annua* extracts, and two chemicals that this plant contains, artemisinin and 1,8-cineole, for preventing apple feeding and infestation by neonate *Cydia pomonella* larvae.

Methods: We studied effects of *A. annua* extracts, artemisinin and 1,8-cineole on apple infestation by neonate codling moth larvae using fruit choice assay in laboratory experiments. Preference of fruit treated with test solutions versus fruit treated with solvent was recorded and analyzed.

Results: Crude *A. annua* extracts prevented fruit feeding at 1, 3, and 10 mg/ml. Artemisinin had feeding deterrent effects at 10 and 30 mg/ml, and 1,8-cineole at 100 and 300 mg/ml.

Discussion and Conclusions: A. annua contains chemicals that prevent apple infestation by codling moth neonates. Artemisinin and 1,8-cineole are among them, but there are other, polar constituents of *A. annua*, which have similar effects. There is a potential of using our findings in codling moth control and production of codling moth-resistant apples.

Keywords: Codling moth, *Cydia pomonella*, sweet annie, apple, fruit feeding, eucalyptol

Introduction

Extracts of sweet wormwood, *Artemisia annua* (L.) (Asteraceae), and artemisinins derived from this plant are well established as safe and cheap drug class for combinatory treatment of malaria, including highly drug-resistant strains. Their efficacy also extends to parasitic infections such as schistosomiasis. They have also shown potent and broad anticancer properties in cell lines and animal models (Krishna et al., 2008). Eucalyptus oil and its major component, 1,8-cineole, have antimicrobial effects against many bacteria, including antibiotic-resistant strains, viruses, and fungi. Immune-stimulatory, anti-inflammatory, antioxidant, analgesic, and spasmolytic effects were also reported (Sadlon & Lamson, 2010).

A. annua potential effects on development and behavior in insects (and particularly in pest insects) are understudied, which is strange, since the fact that this plant and derived chemicals are used in medicine should facilitate the process of their registration for pest management due to minimum side effects on humans.

The codling moth, *Cydia pomonella* (L.) (Tortricidae), is the major cosmopolitan pest of apples, which if not controlled may cause annual loses in excess of 40 billion US dollars globally. The grower has limited options

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for control of this insect at the neonate stage, which shortly after hatching from the egg burrows into the fruit and stays there until its development is completed. This limitation is especially true in apple growing areas that have partial third generations of codling moth, where neonates are attempting to penetrate apples within days of harvest (the time when using insecticides is not allowed). Sprays with broad-spectrum organophosphate neurotoxin, azinphos-methyl, are still popular control measure, even though this insecticide has to be applied in excessive amounts of 1.7 kg per hectare due to codling moth resistance against azinphos-methyl that has accumulated over years. This pesticide has been linked with health problems of agricultural workers and raises serious concerns from U.S. Environmental Protection Agency. In fact, the outer layer of waxes covering the apple is washed off to minimize azinphos-methyl residues before the fruit reaches the consumer. This insecticide has been already banned in European Union and The New Zealand Environmental Risk Management Authority made a decision to phase out azinphos-methyl by 2014. Insecticides based on natural pathogens of codling moth such as *Bacillus thuringiensis* or *Carpocapsa pomonella* are expensive and to become effective must be ingested in large quantities. In such situations, the fruit damage is often done before the larvae die. Some newer chemical agents such as spinetoram or neonicotinoids, are in the implementation stage. Pheromone-based insect control measures such as mating disruption or attract-and-kill do not resolve problems caused by migration of moths from adjacent areas (Wolfgang, 1989) or insecticide resistance (Poullot et al., 2001). Research on alternative strategies of combating codling moth is needed. Discouraging the neonates from burrowing into the fruit with feeding deterrents of plant origin may become a new strategy.

Many plants belonging to *Artemisia* genus, which contains around 300 species, are used as spices or traditional medicines and some exhibit insect repellent properties (Klayman, 1993). For instance, terpinen-4-ol isolated from *Artemisia vulgaris* (L.) (Asteraceae) repelled the yellow fever mosquito *Aedes aegypti* (L.) (Culicidae) (Hwang et al., 1985). Coumarin and thujyl alcohol found in *Artemisia abrotanum* (L.) (Asteraceae) were shown to repel the tick *Ixodes ricinus* (L.), (Ixodidae) (Tunón et al., 2006). Essential oils from *A. vulgaris* are repellent to *Tribolium castaneum* (Herbst) (Tenebrionidae) (Wang et al., 2006), and those from *A. annua* have been shown to be both repellent and toxic to all life stages of the pulse beetle, *Callosobruchus maculatus* (F.) (Chrysomelidae) and the red flower beetle, *T. castaneum* (Tripathi et al., 2000).

There is also growing body of evidence that chemicals from *Artemisia* plants affect feeding behavior in insects. Compounds from growing buds of *Artemisia capillaris* (Thunb.) (Asteraceae) (among them phenylacetylenes) act as insect antifeedants (Yano, 1983, 1987). 1, 8-cineole (Figure 1) from *A. annua* has toxic and feeding deterrent activities against *T. castaneum* (Tripathi et al., 2001). Ethanol extracts from *A. annua* as well as

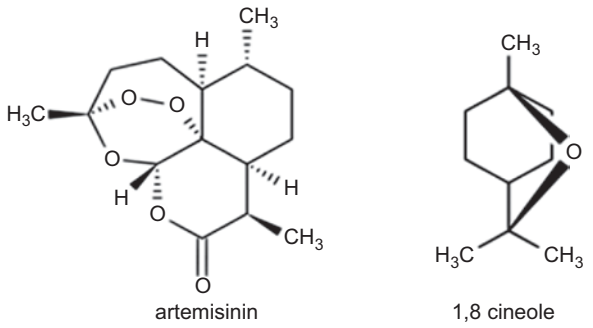

Figure 1. Chemical structures of two secondary metabolites of *Artemisia annua*, artemisinin and 1,8-cineole, tested for codling moth deterrence in this study. Both these compounds exhibit weak deterrent effects, which do not explain deterrent activity of *A. annua* extracts toward codling moth neonates, suggesting presence of other codling moth deterrent compounds in this plant.

artemisinin (Figure 1), a sesquiterpene lactone isolated from this plant, were found to inhibit feeding of the South American ladybird beetle, *Epilachna paenulata* (Germar) (Coccinellidae) and caterpillars of southern armyworm *Spodoptera eridania* (Stoll) (Noctuidae) on pumpkin, *Cucurbita maxima* (Duchesne) (Cucurbitaceae) (Maggi et al., 2005).

Suomi et al. (1986) found that extracts from *Artemisia absinthium* (L.) (Asteraceae) inhibit apple infestation by codling moth neonates, and Durden et al. (2008, 2009) showed that *Artemisia arborescens x absinthium* and *Artemisia ludoviciana* (Nutt.) (Asteraceae) have the same effect. Influence of *A. annua*, and two of its metabolites, artemisinin or 1,8-cineole on feeding by codling moth neonates were not previously studied. Here, we show that *A. annua* extracts, artemisinin, and 1,8-cineole prevent fruit infestation by codling moth neonates. However, artemisinin and 1,8-cineole are not the only components of crude *A. annua* extracts responsible for this effect.

Materials and methods

Insects

Codling moth pupae from USDA-ARS Yakima Agricultural Research Laboratory in Wapato, WA, USA were stored at 25°C, 70–80% relative humidity and 16:8 (L:D). Moths were allowed to oviposit on polypropylene sheets cut out from Ziploc bags (S.C. Johnson, Racine, WI, USA). Neonates were collected 0.5–1.0 h posthatch and subjected to bioassays immediately.

Chemicals

Dehydration alcohol (a V/V mixture of 80% ethanol, 10% isopropanol, and 10% of methanol) was purchased from EMD, Gibbstown, NJ, USA. Artemisinin, 1,8-cineole, and ACS grade solvents used in chromatography were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Crude plant extract

Powdered *A. annua* (lot number 121101) was purchased from Solaray (Park City, UT, USA). This product is readily

available over the counter in US drugstores and internationally via the Internet. The powder (150 mg) was placed in a 2 ml Eppendorf tube with 1 ml of dehydration alcohol, vortexed briefly, allowed to rest for 10 min at room temperature and centrifuged at 2000*g* for 10 min. The liquid fraction was pipetted off, transferred to a preweighed Eppendorf tube, and evaporated in a SpeedVac rotary evaporator. After desiccation, Eppendorf tubes were reweighed to determine the final mass of the residue, which was subsequently resuspended with dehydration alcohol to the desired concentration.

The presence of artemisinin in crude extract was confirmed by high performance thin layer chromatography (HPTLC) using procedure of Widmer et al. (2007). Briefly, *A. annua* crude extracts and artemisinin standards were applied onto Merck 10 × 10 cm silica gel 60 F_{254} glass plates with Camag Nanomat 4 HPTLC plate spotter. The plates were developed for 6 min in Camag Twin Trough 10 × 10 cm Horizontal Development Chamber (CAMAG Scientific Inc., Wilminton, NC, USA) with 5 ml of mobile phase (cyclohexane, ethyl acetate, acetic acid in a V/V ratio of 20:10:1) and allowed to air dry. Next, the plates were immersed for 1 sec in modified vanillin reagent (20 ml acetic acid, 4 ml sulfuric acid, and 2 ml of vanillin added to a 100 ml of ethanol and mixed with 80 ml of double distilled water), air dried for 5 min, heated at 100°C for 12 min using Camag Plate Heater III, and visually inspected in daylight.

To confirm presence of 1,8-cineole in crude extract, we used the same equipment as for artemisinin; however, the methods were adapted from Wagner and Bladt (2001). Mobile phase contained toluene and ethyl acetate in a V/V ratio of 93 to 7 and vanillin reagent was prepared from 95 ml of ethanol in which 0.5 g vanillin and 5 ml of sulfuric acid were dissolved. Developed plates were immersed in vanillin reagent for 1 sec, air dried for 5 min, heated at 100°C for 10 min. and visually inspected in daylight.

Maximal concentration of artemisinin and 1,8-cineole in *A. annua* crude extract were determined using aforementioned HPTLC procedures and serial dilution method (Kirchner et al., 1954).

Partitioning of crude *A. annua* extract

In our previous study (Durden et al., 2009), we succeeded in partitioning codling moth deterrent constituents from *A. absinthium* crude extract to hexane for further isolation. We used the same method for partitioning crude *A. annua* extract in this study. Briefly, 400 µl of crude extract was placed in an Eppendorf tube and 100 µl of double-distilled water was added. Next, 1 ml of hexane was added to the tube, the mixture was vortexed for 1 min, allowed to rest at room temperature for 10 min, centrifuged at 2000*g* for 10 min and hexane fraction was collected to a separate tube. The procedure of partitioning was applied thrice to each sample of the crude extract, the alcohol fraction collected to a separate tube, and the hexane fractions combined for each sample. Alcohol and hexane fractions were evaporated in Speedvac rotor evaporator and dissolved to desired concentration in dehydration alcohol. To ascertain whether artemisinin or 1,8-cineole partitioned from alcohol fraction to hexane, the partitioned fractions were subjected to HPTLC at presence of artemisinin and 1,8-cineole standards as described above.

Bioassay

We used a laboratory bioassay described in detail by Durden et al. (2008). Briefly, apple plugs were formed by forcing a plastic soda straw through a 20-mm thick section of apple containing both epidermis and flesh of the apple. The straw with a plug in it was cut to a length of approximately 15 mm, the plug positioned with the epidermis of the apple protruding 2 mm from the straw and entire assembly was dipped in liquid paraffin wax. Excess wax was removed from the epidermis of the plug using a warm spatula. Five microliters of either control (dehydration alcohol only) or experimental solution was applied to the epidermis of each apple plug and allowed to dry. Using a small amount of modeling clay, four plugs (two controls, two experimental) were arranged with one control and one experimental plug facing each other at either side of a 60 × 15 mm polyurethane petri dish (Figure 2). A short piece of glass rod (1.3 mm diameter) was placed between the two pairs of plugs to form a bridge allowing tested codling moth neonate to choose between either control or experimental plug regardless of which direction the larva traveled along the glass rod. Single codling moth neonate was placed with a fine camel brush, on the glass rod, equidistant from the apple plug pairs, the petri dish was covered, placed upon a light table and covered with a semitranslucent dome to avoid a nondirectional light source, which could bias the results as codling moth neonates are known to exhibit mild phototropism

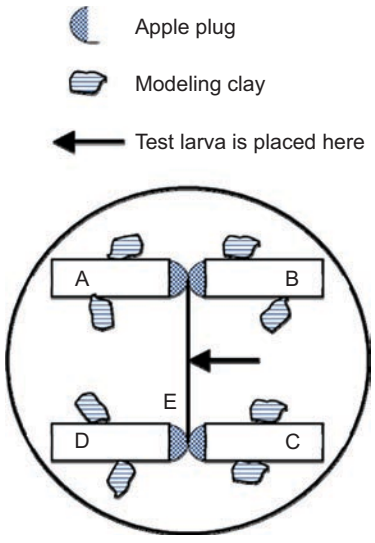

Figure 2. Test arena in our bioassay. (A, C) Plugs treated with experimental solutions. (B, D) Plugs treated with control solutions. (E) Glass rod.

(Jackson, 1982). Assays were evaluated after 20 h to determine which plug was fed upon. Feeding deterrence index was calculated according to Jones (1952) by dividing the number of the neonates feeding on apple plugs treated with *Artemisia* extracts by the number of the neonates feeding on the plugs treated with dehydration alcohol only, subtracting this figure from 1, and multiplying the result by 100.

A. annua crude extract was tested for deterrent effects at concentrations of 10, 3, 1, and 0.1 mg/ml. Artemisisin was tested at 30, 10, 3, 1, and 0.1 mg/ml and 1,8-cineole at 300, 100, 30, 10, 3, 1, and 0.1 mg/ml. Hexane and alcohol fractions of partitioned *A. annua* crude extract were tested at 10 μg/ml. Each solution was prepared immediately before testing. The number of tests per concentration (each test performed with a different neonate) varied between 42 and 83.

Statistical analysis

The null hypothesis that 50% of neonates would choose control plugs and 50% would choose experimental plugs was tested using Fisher's Exact test (α=0.05).

Results

High-performance thin layer chromatography

Visual comparison of *A. annua* chromatograms with artemisinin and 1,8-cineole standards indicated that both these compounds were present in crude *A. annua* extracts. We estimated that artemisinin concentration in crude *A. annua* extracts was less than 0.02% and that of 1,8-cineole was less than 3%.

Feeding deterrent effects of A. annua crude extract

Crude extracts from *A. annua* prevented fruit feeding at concentration of 1 mg/ml (Table 1, N = 43, P < 0.05, Fisher's Exact test). This deterrent effect was even more pronounced at concentrations of 3 mg/ml (Table 1, N = 45, P < 0.01, Fisher's Exact test) and 10 mg/ml (Table 1, N = 45, P < 0.001, Fisher's Exact test).

Feeding deterrent effects of artemisinin and 1,8-cineole

Artemisinin had feeding deterrent effects against codling moth neonates at 30 and 10 mg/ml (Table 2, N = 43–61, P < 0.05). Lower concentrations of artemisinin had no effect (Table 2). Deterrence index of 10 mg/ml artemisinin was only slightly higher than that of 1 mg/ml crude *A. annua* extract (Tables 1 and 2). 1,8-Cineole also prevented feeding; however, deterrent effects of this compound were found at much higher concentrations. Only 100 and 300 mg/ml were effective (Table 3, N = 48–66, P < 0.05, Fisher's Exact test).

Table 1. Effects of crude extract from *Artemisia annua* on apple feeding by codling moth neonates.

Concentration (mg/ml)	Number of neonates feeding		Deterrence index
	Treated	Control	
10	2***	43	95.3
3	9**	36	75.0
1	12*	31	61.3
0.1	26	32	18.8

*P < 0.05, **P < 0.01, ***P < 0.001 in Fisher's Exact test.

Experiments with hexane and alcohol fractions of crude *A. annua* extract

Both alcohol and hexane fractions, at 10 mg/ml, exhibited similar feeding deterrence indexes in bioassays with codling moth neonates; 77.8 and 76.1, (Table 4, N = 66–83, P < 0.001, Fisher's Exact test). HTPLC showed presence of both 1,8-cineole and artemisinin in hexane fractions only.

Discussion

There is an increasing body of evidence that some constituents extractable from the plants of *Artemisia* family influence insect behavior and longevity. However, although anticancer and antimalarial activities of *A. annua* attracted attention of researchers in medical science area (Eastman & Fidock, 2009; Firestone & Sundar, 2009), insect deterrent potentials of this plant and its metabolites are understudied. Maggi et al. (2005) showed that both *A. annua* extracts and synthetic artemisinin reduce pumpkin foliage consumption by larvae of *E. paenulata*

Table 2. Effects of alcoholic solutions of artemisinin on apple feeding by codling moth neonates.

Concentration (mg/ml)	Number of neonates feeding		Deterrence index
	Treated	Control	
30	9**	39	79.5
10	11*	32	65.6
3	21	40	47.5
1	18	25	28.0
0.1	22	27	18.5

*P < 0.05, **P < 0.01 in Fisher's Exact test.

Table 3. Effects of alcoholic solutions of 1,8-cineole on apple feeding by codling moth neonates.

Concentration (mg/ml)	Number of neonates feeding		Deterrence index
	Treated	Control	
300	9**	39	76.9
100	20*	46	56.5
30	26	46	45.6
10	43	37	Not deterrent
3	22	23	4.4
1	31	34	8.8
0.1	27	21	Not deterrent

*P < 0.05, **P < 0.01 in Fisher's Exact test.

Table 4. Effects of partitioning on codling moth deterrent effects of *Artemisia annua* extract.

Fraction (10μg/ml)	Number of neonates feeding		Deterrence index
	Treated	Control	
Alcohol	12***	54	77.8
Hexane	16***	67	76.1

***P < 0.001 in Fisher's Exact test.

and *S. eridania*. Tripathi et al. (2001) reported that 1,8-cineole from *A. annua* has toxic and feeding deterrent activities against a stored product pest, *T. castaneum*. To the best of our knowledge, our current study is first demonstration that *A. annua* and artemisinin prevent fruit infestation by internal fruit feeding larvae.

In our experiments, crude extract from *A. annua* prevented apple infestation by codling moth neonates in a dose-dependent manner (Table 1). At 10 mg/ml, more than 90% of neonates avoided the fruit treated with the extract and insect deterrent activity of the extract was still observed at 3 and 1 mg/ml. In our earlier work, crude extract from *A. absinthium* exhibited deterrent properties against codling moth neonates only at 30 and 10 mg/ml; concentrations of 3 and 1 mg/ml were inactive (Durden et al., 2009). It seems that *A. annua* has more pronounced deterrent properties against codling moth neonates than *A. absinthium*.

Comparison of our data with those of Maggi et al. (2005) indicates that feeding deterrent properties of artemisinin against codling moth neonates are comparable with those against *E. paenulata* and *S. eridania* larvae. Maggi et al. (2005) needed 0.375 mg of artemisinin per square centimeter (100 mm^2) of foliage to obtain deterrence index (calculated as in our study) in the range of 81–87. In our experiments, comparative results were obtained when the surface area of one apple plug was treated with 5 µl of 30 mg/ml concentration of artemisinin. This dose equals to 150 µg per plug, which has surface area of approximately 30 mm^2. Our experiments showed codling moth neonates were deterred by artemisinin dosage of about 500 µg (0.5 mg) per square centimeter, about 1.3 times the dose used by Maggi et al. (2005). Codling moth neonates are only slightly less sensitive to artemisinin than the larvae of *E. paenulata* and *S. eridania*.

Interestingly, Maggi et al. (2005) have found that synthetic artemisinin exerts stronger feeding inhibitory effects against *E. paenulata* and *S. eridania* larvae than *A. annua* extract. In their study, artemisinin deterred insects at concentrations 4–50 times lower than crude plant extract. In our experiments, however, artemisinin had generally lower insect deterrent properties than crude *A. annua* extract; only 30 and 10 mg/ml significantly reduced fruit infestation (Table 2). Because we estimated the concentration of artemisinin in our crude extracts as lower than 0.02% [which is in accordance with literature data, see Bhakuni et al. (2001)], it is unlikely that deterrent properties of crude *A. annua* extract against codling moth neonates could be attributed solely to deterrent activity of artemisinin. Experiment with 1,8-cineole produced similar results; this compound reduced feeding on apple at 100 and 300 mg/ml (Table 3). Interestingly, feeding deterrence indexes at these concentrations (about 56 and 77) that we observed in codling moth neonates are close to those reported by Tripathi et al. (2001) for *T. castaneum* larvae. In their experiments (Tripathi et al., 2001), feeding deterrence indexes for the larvae fed flour wafers treated with 100–120 mg/ml 1,8-cineole varied between 50 and 70. Our results from the experiment with 1,8-cineole also corroborate with previously published results of Landolt et al. (1999), who showed arresting and repellent effects of essential oil from eucalyptus, *Eucalyptus globulus* (Labille) (Myrtaceae), against codling moth neonates. However, considering that 1,8-cineole exhibits low deterrence against codling moth neonates and is present at 3% or lower concentration in our crude *A. annua* extracts we think that this compound may only slightly contribute to overall deterring properties of this plant.

Apparently, there are other insect deterrent compounds in crude *A. annua* extract, which alone or collectively prevent fruit infestation by neonate larvae of codling moth. At this stage of our study, we can only suggest what these components could be. Our experiments with partitioning *A. annua* crude extract suggest that in addition to hexane-extractable artemisinin, 1,8-cineole and other nonpolar compounds, some substances that deter codling moth neonates are present among polar compounds from *A. annua*, collected in the alcohol fraction. Highly polar compounds of *Artemisia* plants, such as phenylacetylenes, were reported as insect feeding deterrents previously (Yano, 1983).

In conclusion, our research showed that *A. annua* contains substances that prevent fruit infestation by internal fruit feeding larvae, codling moth neonates, and identified artemisinin and 1,8-cineole as codling moth feeding deterrents from *A. annua*. Because both artemisinin and 1,8-cineole exert their codling moth deterrent properties at relatively high concentrations, their use in codling moth control as direct sprays would be probably impractical, with an exception of environmentally conscious amateur growers. However, on the one hand, cloning and expression of the artemisinin biosynthetic genes in microbes as *Saccharomyces cerevisiae* and *Escherichia coli* have already led to large-scale microbial production of some artemisinin precursors such as amorpha-4-,11-diene and artemisinic acid (Zeng et al., 2008). Some progress has been made toward deciphering the synthetic pathways for 1,8-cineole (Roeder et al., 2007). On the other hand, apple genome has been recently sequenced (Velasco et al., 2010) and reconstruction of the complete artemisinin or 1,8-cineole biosynthetic pathway in transgenic apple is only a matter of time. Perhaps, artemisinin or 1,8-cineole could be expressed in apple waxes (that would be removed before the fruit reaches the consumer as it is done presently), making the fruit unpalatable to codling moth larvae, but still acceptable for consumers.

Also, there are other constituents of *A. annua* crude extract, which deter codling moth neonates from feeding and discourage from entering the fruit, and further attempts of isolation of these constituents are warranted.

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Declaration of interest

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