Journal of Chemical Ecology, Vol. 31, No. 7, July 2005 (©2005)
DOI: 10.1007/s10886-005-5795-y

LABORATORY EVALUATION OF *Artemisia annua* L. EXTRACT AND ARTEMISININ ACTIVITY AGAINST *Epilachna paenulata* AND *Spodoptera eridania*

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(Received November 4, 2004; revised March 1, 2005; accepted March 23, 2005)

Abstract—Ethanolic extract of aerial parts of *Artemisia annua* L. and artemisinin were evaluated as anti-insect products. In a feeding deterrence assay on *Epilachna paenulata* Germ (Coleoptera: Coccinellidae) larvae, complete feeding rejection was observed at an extract concentration of 1.5 mg/cm^2 on pumpkin leaf tissue. The same concentration produced a feeding inhibition of 87% in *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae). In a no-choice assay, both species ate less and gained less weight when fed on leaves treated with the extract. Complete mortality in *E. paenulata* and 50% mortality in *S. eridania* were observed with extract at 1.5 mg/cm^2. Artemisinin exhibited a moderate antifeedant effect on *E. paenulata* and *S. eridania* at 0.03–0.375 mg/cm^2. However, a strong effect on survival and body weight was observed when *E. paenulata* larvae were forced to feed on leaves treated at 0.03 and 0.075 mg/cm^2. *Artemisia annua* ethanolic extract of aerial parts at 1.5 mg/cm^2 showed no phytotoxic effect on pumpkin seedlings.

Key Words—*Artemisia annua*, *Epilachna paenulata*, *Spodoptera eridania*, insecticide, artemisinin.

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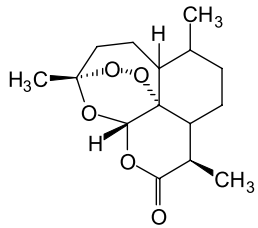
INTRODUCTION

Many wild plants are capable of synthesizing secondary metabolites with biological properties that are important in the fight against insect pests (Matthews, 1993; Enriz et al., 2000; Calderón et al., 2001; Céspedes et al., 2001; Gonzalez-Coloma et al., 2002). For these to be practical and economical, the selected species must meet some requirements, such as being cultivatable, and having a potent active principle, high stability, and a good yield. In our search for naturally occurring insecticide products, we previously studied the anti-insect activity of extracts of *Melia azadarach* L. (Meliaceae) (Valladares et al., 1997, 1999; Carpinella et al., 2002, 2003), and now we have focused on *Artemisia annua*.

The genus *Artemisia* is a rich source of biologically active natural products. Approximately 200 *Artemisia* sp. grow in China, where more than 50 of them have been used in traditional Chinese medicine (Tan et al., 1998). *Artemisia annua* L., known as "sweet Annie" or "annual wormwood," is an annual herb native to Asia (China) where it is known as "quinghao." The plant has become naturalized in many countries including Argentina, Bulgaria, France, Hungary, Italy, Spain, and the United States (Klayman, 1989, 1993), and it is well known for its antimalarial activity, attributed to the presence of artemisinin (**1**) (Klayman et al., 1984). This compound is also phytotoxic (Duke et al., 1987) in laboratory assays (Chen et al., 1991), but has performed poorly in the field (Duke et al., 2000). The essential oil from aerial parts of the plant has been used in cosmetics and pharmaceuticals, and it has been recently reported as protecting stored products and plants against insect attack (Rao et al., 1999; Tripathi et al., 2000, 2001).



Artemisinin **(1)**

As far as we know, neither organic extracts from aerial parts of this plant nor artemisinin has been assayed on insects of agronomical interest,

although some authors have apparently noted that artemisinin has anti-insect activity (Jia, 1997; Wang and Wang, 2002), but have not published in a peer-reviewed journal. In the present paper, we study the effects of an ethanolic extract of *A. annua* aerial parts on the feeding behavior, development, and survival of the leaf-feeding coccinelid, *Epilachna paenulata* Germ. (Coleoptera: Coccinellidae), and also on the polyphagous pest, southern armyworm, *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae). The anti-insect activity exhibited by **1** is also reported here.

METHODS AND MATERIALS

Plant Material. Aerial parts of the *A. annua* plant were collected in the hill area of Córdoba, Argentina, after the plants bloomed in March 2000, and were identified by Prof. Luis Ariza Espinar from the Botanic Museum of Córdoba National University, Argentina. The vegetable material was air-dried at 20°C, crushed and Soxhlet extracted with ethanol (133.68 g). After exhaustive solvent removal, a viscous residue was obtained (22.21 g, 16.6% yield), and then a weighed amount of extract was dissolved in enough 95% ethanol to make concentrations of 1% and 10%. The presence of artemisinin in the extract was confirmed and quantified by high-performance liquid chromatography (HPLC) (Na-Bangchang et al., 1998), yielding 0.53 g (2.41%). Pure artemisinin was dissolved in 95% ethanol at concentrations of 2, 5, and 25 mg/ml for testing for anti-insect activity.

Insects. Larvae for the experiments were obtained from continuous colonies, reared on a natural diet of pumpkin, *Cucurbita maxima*, maintained in a growth chamber at 24 ± 1°C, 70–75% relative humidity, with a photoperiod of 16:8 light–dark cycle, and periodically renewed with field specimens (Aranda et al., 1996).

Chemicals. Artemisinin was purchased from Sigma (St. Louis, MO, USA). All organic solvents were of HPLC-grade and purchased from Merck (Darmstadt, Germany). Acetonitrile and acetic acid were obtained from Fisher Scientific (New Jersey, NJ, USA).

Equipment. HPLC was performed on a Shimadzu liquid chromatography system equipped with an LC-10AS pump and an L-ECD-6A electrochemical detector. A Phenomenex Luna C18 5 μm particle size (250 × 4.6 mm i.d.) reversed-phase column was used. The compound was eluted with 45% (v/v) MeCN in water containing 0.1 M acetic acid (pH 4.8), as previously reported (Na-Bangchang et al., 1998). The electrochemical detector was operated in the reductive mode at an applied potential of 1.0 V.

Phytotoxicity Assay. Pumpkin, *Cucurbita maxima*, seedlings were used for a phytotoxicity assay. Pumpkin seeds were germinated, and seedlings grown in a growth chamber for 5 d, in pots (250 ml) containing a sterile soil mix (2:2:1 sand/loam/peat, by volume). For every 2 d, the 5 d-old seedlings (N = 15) were sprayed with a 10% solution of *A. annua* ethanolic extract (approximately 2.4 mg/ml of **1**) dissolved in water (~500 µl per plant), during 20 d. This dosage was equivalent to 1.5 mg/cm^2 of extract and 0.03 mg/cm^2 of **1**. A control, sprayed with water, was run in parallel. The number of new leaves and symptoms of chlorosis were determined. Data were analyzed with analysis of variance (ANOVA) and Tukey's honestly significant difference test.

Feeding Deterrence Assay. To survey the antifeedant properties of the extract and of artemisinin, a modified leaf-disk choice test (Carpinella et al., 2003) was used. Two cotyledon leaves from a *C. maxima* seedling (both the same size, age, and from the same plant) were placed in a 9-cm-diam. Petri dish, and a glass disk with two 1.3-cm-diam. holes was placed on top. A third-instar *E. paenulata* or *S. eridania* larva was placed equidistant from a leaf disk treated with 20 µl of the test solution, and a control leaf disk treated with 20 µl ethanol. The larvae were allowed to feed for 24 hr. Ten replicates were used for each treatment. The relative amounts of the treated and untreated leaf areas eaten (recorded in percentages from 0 to 100) were visually estimated by dividing the leaf area into imaginary quarters. Measurements were always made by the same operator. Data were then compared by using the Wilcoxon paired-sample test (α = 0.05). The Antifeedant Index (AI%) was calculated as $(1 - T/C) \times 100$, where T and C represent the consumption on treated and untreated disks, respectively (Gonzalez-Coloma et al., 1998). After calculation of the AI% for compound **1**, the relative potency (ED_{50} values, the effective dosage for 50% feeding reduction) for **1** was determined by linear regression of AI% on log dose. These, and all the following experiments, were carried out at 24 ± 1°C and with a 16:8 (L/D) photoperiod.

No-Choice Assay. This test was carried out to analyze the effects of the extract and of artemisinin on the development and survival of larvae of both insect species. A first-instar *E. paenulata* and a third-instar *S. eridania* were each placed into a Petri dish and fed with fresh pumpkin leaves (renewed every 48 hr), treated either with 20 µl of the ethanolic extract solution, artemisinin solution, or with ethanol (control). Ten replicates were used for each treatment. A similar set of larvae of both insects were not fed at all, and acted as starved controls. Leaf consumption, development, and survival were recorded regularly. Data were analyzed with analysis of variance (ANOVA), Tukey's honestly significant difference test (Zar, 1996) and the Kaplan–Meier method (Hollander and Wolfe, 1999).

RESULTS AND DISCUSSION

In order to assess the insect control effects of *A. annua* extract, and the possibility of developing it as a natural insecticide for use in pest management programs, two phytophagous species belonging to two different orders, *E. paenulata* and *S. eridania*, were used.

Before starting with the insecticide assays, an evaluation of phytotoxicity was made on 5-d-old pumpkin seedlings, and no differences were observed in the development of new leaves or in phytotoxicity symptoms, such as chlorosis and death, between treatment and control (data not shown) during 20 d. These results suggest that phytotoxicity did not occur at the highest concentration (1.5 mg/cm^2 of extract, *vide infra*) evaluated in our feeding deterrence and no-choice assays.

Both species, *E. paenulata* and *S. eridania*, were deterred from feeding on pumpkin leaves treated with 1.5 mg/cm^2 *A. annua* extract (approximately equivalent to 1% w/w), consuming significantly less food (Wilcoxon, $P < 0.05$) than the control (Table 1). Feeding inhibition of *E. paenulata* larvae was almost complete at this concentration, and a significant difference was still observed at 0.15 mg/cm^2 (approximately equivalent to 0.1% w/w), where an AI of 78.8% was observed. These values indicate the potent antifeedant activity of *A. annua* extract, at the same level of activity as that previously shown by *M. azedarach* extract against *E. paenulata* (Carpinella et al., 2003).

After 3 d, larvae of *E. paenulata* confronted with leaves treated with 0.15 mg/cm^2 of ethanolic extract had consumed approximately half the leaf area of those receiving untreated leaves, and this trend was maintained throughout the experiment (Table 2). When larvae were fed on leaves treated with 1.5

TABLE 1. FEEDING DETERRENCE EFFECTS OF *Artemisia annua* AERIAL PARTS ETHANOLIC EXTRACT ON *Epilachna paenulata* AND *Spodoptera eridania*

	Leaf area eaten (%) ± SE[a]		
Extract dosage (mg/cm^2)	Treated	Control	AI%[b]
Epilachna paenulata			
0.15	7 ± 6.8**	33 ± 10.3	78.8
1.5	0.5 ± 1.6**	31.3 ± 15.7	98.5
Spodoptera eridania			
0.15	15.3 ± 8.3	16.3 ± 12	6.1
1.5	2.5 ± 4.3*	19.5 ± 22.7	87.1

[a] Consumption significantly lower on extract-treated food, *$P < 0.05$; **$P < 0.01$, Wilcoxon signed paired rank test.

[b] Results observed at 24 hr. Antifeedant Index (AI) = $(1 - T/C) \times 100$. Means of ten replications are presented.

TABLE 2. MEAN LEAF AREA CONSUMED BY *Epilachna paenulata* AND *Spodoptera eridania* LARVAE ON LEAVES TREATED WITH *Artemisia annua* AERIAL PARTS ETHANOLIC EXTRACT IN NO-CHOICE ASSAY (% PER INDIVIDUAL/DAY)

Days	Area consumed (%)[a]		
	Control	0.15 mg/cm^2	1.5 mg/cm^2
Epilachna paenulata			
3	21.2a	11.3b	2.4c
5	22.5a	24.6a	5.3b
7	40.1a	23.3b	5.0c
13	33.3a	17.6b	0.0
Spodoptera eridania			
2	16.1a	14.5a	18.6a
6	20.2a	13.7b	13.2b
9	48.6a	38.4b	22.1c
13	72.5a	53.3b	9.6c

[a] Means within rows followed by the same letter are not significantly different in ANOVA test with repeated measures (Tukey's test, $P < 0.05$).

mg/cm^2, an abrupt decrease in the area consumed was observed during the experiment, amounting to at least four times less than that consumed by the control larvae ($F = 52.9$; $df = 27$; $P < 0.001$).

From d 3 onwards, there were significant differences in the mean weight of *E. paenulata* larvae between treatments and control (data not shown). Whereas control larvae steadily increased their body weight, larvae treated with 1.5 mg/cm^2 lost weight. At d 5, significant differences were observed between both dosages ($F = 8.14$; $df = 27$; $P < 0.001$).

The distribution of survival for *E. paenulata* was significantly different between the treatments at 5, 7, and 13 d (data not shown). On the fifth day, 30% survival was observed at the 1.5 mg/cm^2 dosage, while 90% of the starved larvae had survived. These data suggest the presence of one or more toxic compounds, whose toxicity was evident at the highest concentration. The small amount of leaf ingested was enough to incorporate these compounds in toxic doses. At d 7, complete mortality was observed for these two treatments showing significant differences with both control and 0.15 mg/cm^2 dosages. At this time, the mortality could be attributed either to toxic metabolites present in the extract, or to the highest concentration provoking a strong antifeedant effect on larvae, causing 100% mortality through starvation.

In the no-choice assay, *S. eridania* larvae consumed less of leaves treated with 0.15 mg/cm^2 than controls (Table 2). When larvae were fed on 1.5 mg/cm^2 treated leaves, there were significant differences in the consumed area compared with controls from d 6 to the end of the study ($F = 3.86$, $df = 54$, $P = 0.04$).

These findings show that the inhibitory effect of the *A. annua* extract was more effective on *E. paenulata* larvae than on *S. eridania*, and probably acted in the latter as a secondary antifeedant, where reduction of food intake follows some initial consumption (Ascher, 1993).

There was a great difference in mean body weight of *S. eridania*, which began to be significant on d 2 and continued until the end of the assay for both treatments (data not shown). Moreover, from d 9 the body weight of larvae eating 1.5 mg/cm^2 was half or less than that of those fed with the lower dose ($F = 5.18$, $df = 54$, $P = 0.021$). The distribution of survival for *S. eridania* was significantly different between treatments at d 9 and 13 (data not shown). The survival rate by d 2 of larvae fed on 1.5 mg/cm^2 was 70%. However, survival declined to 50% at the end of the study, while, with starved larvae, 0% survived at d 9.

The dose of 1.5 mg/cm^2 artemisinin contained approximately 0.03 mg/cm^2 of **1**. We, therefore, checked the antifeedant effect of **1** at concentrations around this value. Artemesinin exhibited a moderate antifeedant effect on *E. paenulata* and *S. eridania* at 0.375 mg/cm^2 with an AI% of 87.9 and 81.0, respectively (Table 3). Lower doses (0.075 and 0.03 mg/cm^2) showed an AI% of approximately 60% to 75%. This did not account for the strong feeding inhibition of the extract, suggesting that the activity is only partially attributable to the presence of **1**, and that an additive or synergistic effect between **1** and other active principles could be present. From the data from both species (Table 3), the effective dosage for 50% feeding reduction (ED_{50}) calculated for compound **1** was 0.0136 mg/cm^2. Comparing the antifeedant activity of

TABLE 3. ANTIFEEDANT INDEX OF ARTEMISININ ON *Epilachna paenulata* AND *Spodoptera eridania* LARVAE UNDER FEEDING DETERRENT ASSAY

Dosage (mg/cm^2) artemisinin	Leaf area eaten (%) ±SE[a]		AI%[b]
	Treated	Control	
Epilachna paenulata			
0.01	29.5 ± 37.9	54.5 ± 42.3	45.9
0.03	17.5 ± 28.5	46 ± 34.4	61.9
0.075	33.3 ± 14.4*	96.5 ± 4.1	65.4
0.375	11.5 ± 4.6*	95.3 ± 3.5	87.9
Spodoptera eridania			
0.01	16 ± 17.1	19 ± 10.4	15.8
0.03	7.5 ± 14.8	18.5 ± 26.8	59.4
0.075	12.3 ± 15.2*	51 ± 33.5	75.8
0.375	7.5 ± 9.5*	49 ± 23.31	81.0

[a] Consumption significantly lower on extract-treated food, *$P < 0.01$, Wilcoxon's signed paired rank test.

[b] Results observed at 24 h. Antifeedant Index (AI) = $(1 - T/C) \times 100$, 10 replications.

TABLE 4. MEAN LEAF AREA CONSUMED BY *Epilachna paenulata* LARVAE ON LEAVES TREATED WITH ARTEMISININ IN NO-CHOICE ASSAY (% PER INDIVIDUAL/DAY)

Days	Area consumed (%)[a]		
	Control	0.03 mg/cm^2	0.075 mg/cm^2
3	15.5a	3.6b	6.8b
5	46.7a	24.5b	26.2b
7	39.5a	1.4b	–
9	47.8	–	–
11	32.5	–	–

[a] Means within rows followed by the same letter are not significantly different in ANOVA test with repeated measures (Tukey's test $P < 0.05$).

1 with that reported (Carpinella et al., 2002) for meliartenin (ED_{50} = 0.0008 mg/cm^2), azadirachtin (ED_{50} = 0.00072 mg/cm^2), and toosendanin (ED_{50} = 0.0037 mg/cm^2), **1** is approximately 17, 19, and 4 times less active than meliartenin, azadirachtin, and toosendanin, respectively.

Larvae of *E. paenulata*, forced to feed on pumpkin leaves treated with 0.03 and 0.075 mg/cm^2 of **1**, consumed less than the control ($F = 16.15$; $df = 2$; $P < 0.001$) (Table 4). At d 7, the 0.03 mg/cm^2-leaf area consumed by larvae was 28 times lower than that ingested of the control. At the same time, larvae exposed to 1.5 mg/cm^2 of extract ate eight times less than the larvae fed with untreated leaves (Table 2). Although **1** showed a moderate antifeedant effect, it provoked a strong inhibition of feeding once a significant quantity of treated food was ingested in no-choice assays.

Body weight of control larvae increased throughout the experiment (data not shown), while the two treatments showed almost no change in body weight ($F = 53.47$, $df = 2$, $P < 0.001$), and at d 5 weighed at least four times less than the controls.

Larvae were observed to tremor, move without coordination, and collapse when they were forced to feed on **1**-treated leaves. This behavior was similar to symptoms of insects under the effects of neurotoxins (Wang et al., 2000).

The distribution of survival for *E. paenulata* (data not shown) was significantly different $P < 0.001$ between the treatments at d 7, 9, and 11. Complete mortality was reached at d 7 and 9 for 0.075 and 0.03 mg/cm^2, respectively, while all starved larvae died at d 7. These data would suggest that death was attributable to the inhibition of feeding by **1**, although the possible neurotoxic effect observed suggests that other mechanisms may be involved.

In summary, *A. annua* ethanolic extract inhibited feeding and produced negative effects on the survival and development of the leaf-feeding coccinelid, *E. paenulata*, and the polyphagous pest, southern armyworm, *S. eridania*.

High mortality was observed in both *E. paenulata* and *S. eridania* with the extract at a dose of 0.15 mg/cm^2. Its activity at concentrations of 0.15 and 1.5 mg/cm^2 opens up the possibility of using *A. annua* ethanolic extract for the control of these pests. Artemisinin affected the development and survival of *E. paenulata* at a dose of 0.03 mg/cm^2, equivalent to 1.5 mg/cm^2 of *A. annua* extract, indicating that **1** is its main active principle. The strange behavior of larvae of this species fed on **1**, as if under a neurotoxic effect, warrants further investigation.

Acknowledgments—Financial support for this work was provided by Agencia Córdoba Ciencia-Unidad CEPROCOR (Centro de Excelencia en Productos y Procesos de la Provincia de Córdoba). MCC gratefully acknowledges receipt of a fellowship from CONICET.

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