EFFECT OF *ARTEMISIA ANNUA* L. ON DETERRENCE AND NUTRITIONAL EFFICIENCY OF LESSER MULBERRY PYRALID (*GLYPHODES PYLOLAIS* WALKER) (LEPIDOPTERA: PYRALIDAE)

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Abstract: Methanolic extract of *Artemisia annua* L. were evaluated on growth and nutritional efficiency of lesser mulberry pyralid *Glyphodes pyloalis* (Lepidoptera: Pyralidae) in laboratory conditions (24±1°C, 75±5% RH and 16 L : 8 D). Feeding deterrence index was evaluated by a choice test method on < 24 h fifth instar larvae with 5, 2.5, 1.25 and 0.625 percent of the extract. The result indicated that increasing the concentration resulted in higher deterrence. The extract retarded larval growth significantly (EC_{50} = 3.63%) and also showed significant effects on the relative growth rate (RGR), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), approximate digestibility (AD) and consumption index (CI). Lipid, protein and carbohydrate in treated insects were significantly reduced compared to the controls. Hence, methanol extract of *A. annua* could be considered as a strong deterrent which also affects the biochemical metabolism of the target pest. Methanol extract of *A. annua* has the potential for development as a botanical insecticide.

Key words: *Artemisia annua* L., methanolic extract, deterrence, nutritional efficiency

INTRODUCTION

Lesser mulberry pyralid is a monophagous insect feeding solely on mulberry. It was reported for the first time from Punjab, Pakistan in 1928 (Mathur 1980) and in 2002 from mulberry orchards in northern Iran (Jafari Khaljiri *et al*. 2006). This pest is considered a nuisance in mulberry orchards in India, China, Japan, Malaysia, Pakistan and Uzbekistan (Madyarov *et al*. 2006). The larvae fold the leaves of mulberry by their secretions and feed on parenchyma, causing considerable damage. The larvae of *G. pyloalis* also act as alternate hosts to densovirus and picornavirus and are therefore considered as vectors to viruses in silkworm (Watanabe *et al*. 1988). Since mulberry is considered as the most important food for silkworm rearing, the use of insecticides should be restricted and alternative methods for pest control that are less hazardous to the environment are highly appreciated.

The vast use of chemical insecticides has led to many unpredictable problems: acute and chronic toxicity to user, farmers and even consumer, birds, fishes and other wild animals, toxicity to natural enemies and pollinators, pollution of underground waters, danger to human health and environment and gradual resistance of insect pests to insecticides (Isman 2006). Such difficulties have caused natural products to gain attention. Among the natural products, plant derived pesticides are more acceptable. This acceptance is due to their abundance, their being nature-friendly, being least toxic to natural enemies, their effect on limited species, fast degradation, low phytotoxicity and low toxicity to vertebrates. These factors are why natural products are considered suitable for developing new groups of healthy and safe insecticides for insect control (Kim *et al*. 2003).

A considerable number of plant families are found to be active insecticides against a number of insect pests. Among them are: Rutaceae, Asteraceae, Labiateae, Meliaceae and Annonaceae (Akhtar and Isman 2004).

The species *A. annua* known as sweet worm wood grows wild in Europe and America and is planted widely in China, Turkey, Vietnam, Afghanistan and Australia (Bhakuni *et al*. 2001). The plant also grows wild in the northern parts of Iran around paddy fields. Several isolated compounds from this species have shown antimalarial, antibacterial, anti-inflammatory, plant growth regulatory and cytotoxicity (antitumor) activities (Bhakuni *et al*. 2001). Studies show that products of this plant have insecticidal, antifeedant and repellency effect on insects (Shekari *et al*. 2008; Haghighian *et al*. 2008).

An important part of feeding ecology is the evaluation of feeding indices or efficiency indices illustrated by Waldbauer (1968). These indices demonstrate the digestion efficiency or utilization of diet or diet ingredients and in fact illustrate the conversion of food to the biomass of insects. These indices can provide valuable informa-

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tion about the positive or negative impact of ingredients or total food (Cohen 2005). The studies on feeding indices after feeding on secondary metabolites can help to determine whether a chemical compound is an antifeedant or toxic after feeding (Liu *et al*. 1990). The general feeding indices used are: approximate digestibility (AD), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), relative growth rate (RGR) and consumption index (CI). In the present investigation the effect of methanolic extract of *A. annua* has been investigated on growth and feeding physiology of lesser mulberry pyralid *G. pyloalis* Walker, in the hope of finding a safer and cheaper method for its control.

MATERIALS AND METHODS

Experimental animals

The larvae of *G. pyloalis* were collected from mulberry orchards in the vicinity of Rasht city, north of Iran, and were reared on fresh mulberry leaves (Kenmochi variety) in the laboratory at 24±1°C, 75±5 RH and 16 : 8 LD. They were kept in transparent plastic boxes 18x15x7 cm which were covered with muslin cloth for aeration. On adult emergence, they were separated and placed in transparent plastic boxes 18x7 cm. They were provided with 10% honey on cotton wool and mulberry leaves as oviposition site as feed. The leaves with eggs were transferred to transparent plastic boxes 18x15x7 cm provided with humid cotton wool. The hatched larvae were transferred to transparent plastic box with the help of a delicate and soft brush. The boxes and leaves were changed every two days for the first and second instar, and daily for older instars.

Preparation of *A. annua* extract

The *A. annua* were collected from the fields of the Guilan University campus and after separating the leaves, they were washed with distilled water and left to dry in the shade. Finally, they were transferred to an oven (45°C) for 24 h and made into a fine powder with a blender. Briefly, 30 g of dried powder were stirred with 300 cc of 85% methanol (Merck Company) for 1 h and incubated for 48 h at 4°C and then stirred for an additional 1 h and filtered twice through whatman No. 4 filter paper. The solvent was removed by vacuum in a rotary evaporator and the dark green residue was dissolved in 10 ml acetone and used as a starting stock solution.

Bioassays

Bioassays were performed on newly moulted fifth instar larvae of lesser mulberry pyralid *G. pyloalis* Walker. These larvae were starved for 4 h before the start of the experiments. In this experiment four concentrations of 5, 2.5, 1.25 and 0.625 percent methanol extract of *A. annua* were used. The experiments were performed in four replicates of 10 larvae each. The mulberry leaves were circularly cut (R = 8 cm) and were dipped for 10 seconds in the desired concentrations. The control leaves were impregnated in the same way with methanol alone. The leaves were left to dry at room temperature for 30 minutes and then placed over humid filter paper to avoid dryness inside the petri dishes (R = 8 cm). After four days from the onset of the experiments, the weight of treated and control leaves were recorded.

Deterrence index

Deterrence activity of methanolic extract of *A. annua* was evaluated by choice test. For this purpose the concentrations of 0.625, 0.312, 0.156 and 0.078 of methanol extract was prepared. The leaf disc of (R = 8 cm) were impregnated for 10 s in the desired concentrations and the control leaf disc was impregnated with methanol alone. One treated leaf disc and one control which was dried at room temperature were randomly placed at 5 cm distance in a transparent plastic box 21x15x5 cm over humid filter paper to avoid dryness. This experiment was replicated 4 times by 10 fifth instar larvae starved for 4 h before the start of the experiments. The dishes were similarly placed in a controlled room as above. After 24 h the larvae were removed and leaf area was measured by leaf area meter. Feeding deterrence index was calculated according to Isman *et al.* (1990):

$$FDI = (C–T)/(C+T) \times 100$$

where:
C – fed leaf area of control,
T – fed leaf area of treated.

Evaluation of the effect of methanol extract on feeding indices

For evaluation of the effect of plant extract on feeding indices of *G. pyloalis*, four concentrations of 5, 2.5, 1.25 and 0.625 percent were used. The fifth instar larvae of < 24 h were starved 4 h prior to the onset of the experiment to exude gut contents. Mulberry leaf discs (R = 8 cm) were impregnated with the above concentrations of plant extract for 10 s and were dried at room temperature. Control leaf discs received methanol alone. The experiments were replicated six times with five larvae of fifth instar in each replication. The initial weight of larvae was recorded and then were let to feed on treated and untreated leaves. After 24 h the remains of leaves were replaced by new treated leaves. The remaining leaves were weighed at the end of 24 h and then placed in an oven (45°C) for 48 h and reweighed in order to calculate the dry weight of consumed food. The dry weight of consumed food is estimated on the basis of dried weight of total food provided to the insect. The feces produced each day were collected, and then oven dried and weighed to estimate the dry weight of excreta. The weights of larvae were recorded at the end of the day. A few larvae which had similar biological and physiological conditions to the experimental insects were weighed, and dried in the oven (45°C) for 48 h then reweighed for determining the dry weight of each larva. The duration of the experiment was four days and the observation was recorded each day.

For determination of feeding indices, the formulae of Waldbauer (1968) were used as follows:

Approximate digestibility (AD) = 100 (E–F)/F,
Efficiency of conversion of ingested food (ECI) = 100P/E,
Efficiency of digested food (ECD) = 100 P/(E–F),
Consumption index (CI)= E/TA
and
Relative growth rate (RGR) = P/TA

where:
A = average of dry weight of larvae during the experiment,
E = dry weight of consumed food,
F = dry weight of produced feces,
P = dry weight of the biomass of larvae,
T = duration of the experiment (4 days).

Total protein, lipid and carbohydrate quantification

For determination of total protein, the method of Bradford (1976) was adopted. Each larva that consumed treated leaves with methanol extract for 48 h was homogenized with 350 µl of distilled water and then were centrifuged for 5 m in 10 000 rpm under 4°C. 10 µl of the supernatant was mixed with 90 µl of distilled water and 2 500 µl dye. The absorbance was read at 630 nm in an Elisa reader (Awareness Technology INC, USA). Protein was quantified using BSA as the standard.

For determination of lipids and carbohydrates the method presented by van Handel and Day (1988) and Yuval *et al.* (1994) were adopted. Each larva was individually homogenized in 100 µl sodium solphate (2%) and 750 µl chloroform: methanol (2 : 1). Then, they were centrifuged for 10 m in 8 000 rpm under 4°C. The supernatant (250 µl) was dried at 40°C. Then 500 µl H_2SO_4 was added and were placed in a hot bath for 10 m at 90°C. Briefly, 30 µl of sample was mixed with 270 µl of vanillin solution (600 mg of vanillin in 100 µl distilled water and 400 ml 85% H_3PO_4) and was placed in an Elisa plate. After 30 minutes the absorbance was read at 545 nm. Total lipid was calculated based on the standard curve. In the case of carbohydrate to 150 µl of supernatant, 100 µl distilled water was added along with 500 µl of anthrone (500 mg of anthrone dissolved in 500 ml H_2SO_4). The samples were placed at 90°C for reaction to take place, and after that 250 µl of sample were placed in the Elisa plate. The absorbance was read at 630 nm and carbohydrate level was calculated by a standard curve.

Statistical analysis

The required concentration for growth and feeding inhibition was calculated by Polo-PC software (LeOra 1987). Deterrence and feeding indices in different treatments were analyzed using ANOVA. The least significant among treatments were compared using Tukey multiple range test at 5 percent using SAS software (SAS 1997).

RESULTS

Effective concentration

The EC_{50} values, confidence limit (95%) and regression slope after 4 days of feeding of plant extracts on *G. pyloalis* are depicted in table 1. The EC_{50} value calculated was 3.63%.

Table 1. The EC_{50} value, confidence limit (95%) and regression slope after a 96 h exposure to plant extract in larvae of lesser mulberry pyralid *G. pyloalis*

Stage	N	Slope ±SE	EC_{50}(95% CL)	X2(df)
Vth Instar Larva	30	2.12±0.23	3.63 (2.34–10.23)	3.29(2)

df – stands for degree of freedom

Feeding deterrence index

The deterrence index for *A. annua* extract was calculated in 24 h. As it is depicted in figure 1, the increasing concentration increases percent deterrence and in the highest concentration it is about 90%.

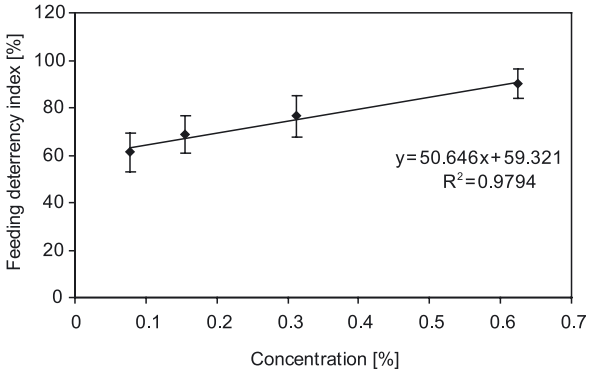

Fig. 1. Mean percent of feeding inhibition of fifth instar larvae of *G. pyloalis* after treatment with methanol extract of *A. annua*

Effect of *A. annua* extract on nutritional indices

The feeding efficiency of fifth instar larvae of *G. pyloalis* feeding on *A. annua* extract treated leaves is intensely affected. It was shown that *A. annua* extract had a strong effect on feeding behaviour and growth of this pest. The results of the effect of *A. annua* extract on feeding efficiency are presented in table 2. By using the plant extract RGR, ECI and ECD were reduced. The RGR index is significantly reduced compared to the control even in the lowest concentration used. The amount of the AD was also significantly different in all concentrations compared to the control; the maximum was 5 percent (93.83) and also the lowest concentration being significantly higher than the control. The CI was lowest at 5 and 2.5 percent (1.035 and 1.093 mg/day, respectively) being significantly different than the control. The other treatments, however, did not show differences compared to the control.

Effect on total protein, lipid and carbohydrates

The results of the total protein, lipid and carbohydrates in fifth instar larvae of lesser mulberry pyralid *G. pyloalis* after treatment with *A. annua* L. extract are depicted in figures 2–4. The mean total protein in all treatments shows significant differences compared with that of the control (Fig. 2). Similarly carbohydrate and lipid contents were significantly reduced (Figs. 3–4).

Table 2. Nutritional indices of fifth instar larvae of *G. pyloalis* after treatment with *A. annua* extract

Treatment	AD [%]	ECI [%]	ECD [%]	RGR [mg/mg/day]	CI [mg/mg/day]
Control	79.84±1.04d	4.041±0.21a	5.086±0.32a	0.052±0.002a	1.301±0.04a
0.625	84.34±1.14c	3.182±0.20ab	3.785±0.26b	0.035±0.002b	1.137±0.05ab
1.25	87.12±0.53bc	2.525±0.22bc	2.906±0.26bc	0.028±0.002bc	1.136±0.03ab
2.5	88.69±1.09b	2.246±0.23c	2.541±0.26c	0.024±0.002c	1.093±0.03b
5	93.83±0.81a	0.909±0.22d	0.978±0.24d	0.0097±0.002d	1.035±0.05b

Within columns, means followed by the same letter do not differ significantly ($p \leq 0.05$); AD – approximate digestibility; CCI – efficiency of conversion of ingested food; CCD – efficiency of conversion of digested food; RGR – relative growth rate; CI – consumption index

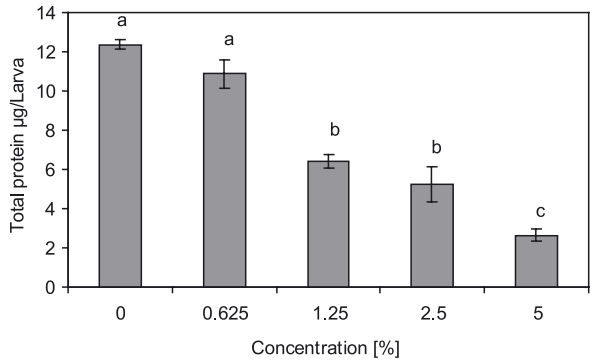

Fig. 2. Amount of protein in fifth instar larvae of *G. pyloalis* after treatment with methanolic extract of *A. annua* (a, b and c means significant at $p \leq 0.05$)

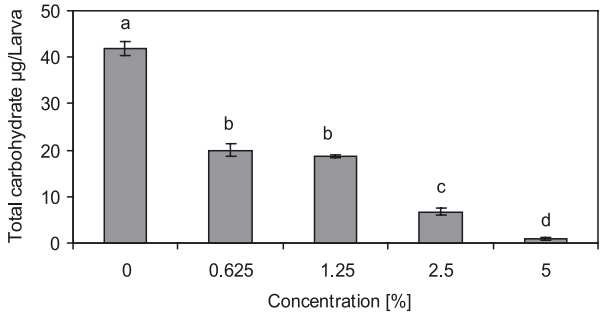

Fig. 3. Amount of carbohydrate in fifth instar larvae of *G. pyloalis* after treatment with methanolic extract of *A. annua* (a, b and c means significant at $p \leq 0.05$)

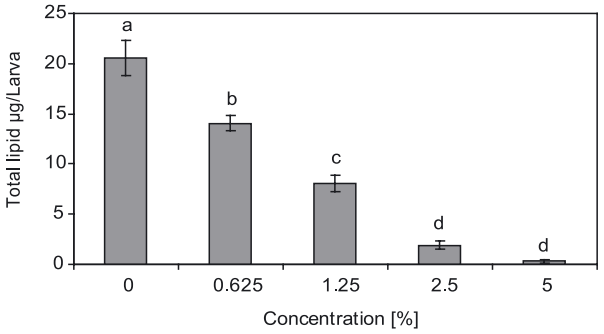

Fig. 4. Amount of lipid in fifth instar larvae of *G. pyloalis* after treatment with methanolic extract of *A. annua* (a, b and c means significant at $p \leq 0.05$)

DISCUSSION

The results of the present study show a high deterrence effect of *A. annua* extract. This study clearly shows that *A. annua* extract severly affects larval feeding behavior and mainly acts as an antifeedant. Certain compounds of plant origin on contact, like monoterpinoids, may affect the nervous system (Isman 2000). By increasing concentration, deterrence index increases and a good dose-response is achieved. Deterrence index in the highest concentration reaches 90.2% and in the lowest concentration it is 61.13%. In higher concentrations, the larvae leaving treated food may be due to a rapid deterrence evoked by chemical sensila on mouth parts or retracted pulses coming from stomodael nervous system after ingestion (Sadek 2003) or toxic effects after ingestion. Liao and Chiu (1986) reported that toosendanin is not toxic for *Mythimna separata* but acts as a strong feeding deterrent. This activity may be due to the effect of the plant compound on the chemical receptors of the mouth parts. It may also be due to the involvement in the energy transmission process in the central nervous system. A number of researchers have reported on the deterrence effect of plant extracts on insects (Liu *et al*., 2006; Senthil Nathan 2006; Kostic *et al*., 2008). Shekari *et al*. (2008) also reported on *A. annua* extract deterrency against the elm leaf beetle *Xanthogaleruca luteola*. Some researchers believe that the deterrence may be due to the presence of a number of chemical compounds like, flavonoids, terpenes, tanins and sterols (Salama and Sharaby 1988). These researchers believe the reasons for some of the plant's defense against insects is the presence of some special chemicals in the plant's defense system (Zapata *et al*. 2009). In field conditions, deterrence retards the growth stages of the insect, and prolonging the insect's search for food increases the probability of insect mortality. The theory of using non-toxic antifeedant chemicals is important in plant protection but in fact, there are a number of problems associated with their use (Akhtar and Isman 2004). Evaluation of feeding indices under methanol extract of *A. annua* show that approximate digestibility in larval feeding on treated leaves is increased compared to the controls. Probably the insect tries to compensate for the low consumption. Senthil Nathan (2006) reported similar results with *Melia azedarach* on *Cnaphalocrocis medinalis* in longer larval duration, low consumption and maintaining food for a longer duration in the gut, and as a result, leading

to higher approximate digestibility. Stoyenoff (1994) reported that when gypsy moth larvae feed less, the food will pass slowly through the gut hence, it enhances the digestibility. Relative growth rate (RGR) which follows weight gain in control larvae is significantly higher than treated larvae. The lower RGR could be due to irreparable damages made to midgut lumen cellular surfaces (Jansen and Groot 2004). Gusmao *et al*. (2002) reported the discrepancy in peritrophic membrane and damages of gut cells in *Aedes aegypti* treated with methanol extract of *Derris uruca*. Low RGR with increasing concentration shows low quality of food and probably it acts as an inhibitor. Lack of specific components particularly nitrogen or water leads to a lower growth rate and lower metabolic efficiency (Schoonhoven *et al*. 2005).

As for results obtained, it can be said that *G. pyloalis* feeding on treated food faces lower feeding indices. Senthil Nathan (2006), Shekari *et al*. (2008) and Zapata *et al*. (2009), all reported low feeding indices after treatment of their respective insects by plant extract or essential oils. Hala and Reem (2007) reported that the extract of three plants namely Oshar (*Calotropis procera*), Harmal (*Rhazya stricta*) and Hargal (*Solenostemma* argel) severely reduces RGR, CI and ECI in treated larvae of *Spodoptera littoralis.* These authors believe that the mechanisms of an antifeedant agent may be due to disruption in the cellular surfaces of the midgut. Hence, reduction of digestion as a result of covalent bands with food proteins or digestive enzymes.

Unsuitable food ingredients and the food regiments lacking essential components for growth lead to a higher digestibility and consumption index but reduced RGR and ECI (Cohen 2001). Lower RGR, ECI and ECD probably lead to delay in larval growth and formation of smaller pupa which have a direct relation to fecundity and longevity of the adult insect and make them susceptible to diseases and natural enemies.

In physiological studies, determination of total protein is important. Many of the insecticides lead to antifeedant in insects and reduce feeding efficiency which in turn reduces some of the vital components like proteins in the body. Ultimately, insects die due to reduced energy metabolism (Etebari *et al*. 2006). In the present investigation it was shown that total protein of treated larvae with *A. annua* extract was considerably reduced after 48 h compared to the controls. It is likely that the insect degrades proteins to resultant amino acids in order to let them enter into the TCA cycle as a keto acid for compensation for the lower energy caused by stress (Nath *et al*. 1997). Shekari *et al*. (2008) also reported lower protein and glucose in third instar larva of *X. luteola* treated with *A. annua*. Etebari *et al*. (2006) showed considerable reduction in total protein of fifth instar silkworm treated by pyriproxyfen after five days. Similar results were obtained by Schmidt *et al*. (1998) by methanolic extract of *M. azedarach* on hemolymph protein of *Spodoptera littoralis* and *Agrotis ipsilon*.

Lipids are an important source of energy and are reserved in fat bodies. Their amount can vary with growth stage and feeding condition (Chapman 1998). The reserve of lipids during the feeding period increases but is reduced in the non-feeding stage. In this investigation lipid reduction is dose dependent. Etebari *et al*. (2006) reported reduction of some compounds like glucose and cholesterol on the first day after treatment and correlated that to disruption in the absorption system.

Reduced carbohydrate rate could be related to a strong deterrence effect of *A. annua*. Etebari and Matindoost (2004) showed that starvation may reduce biochemical components in hemolymph.

In conclusion, methanol extract of *A. annua* showed a significant effect on *G. pyloalis* larvae and caused reduced growth rate, lower food efficiency and reduced key metabolic components. This extract may thus serve as an alternative to conventional insecticide for the control of lesser mulberry pyralid.

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POLISH SUMMARY

DZIAŁANIE *ARTEMISIA ANNUA* L. NA ODSTRASZANIE I WYDAJNOŚĆ ŻYWIENIOWĄ *GLYPHODES PYLOLAIS* WALKER (LEPIDOPTERA: PYRALIDAE)

Oceniano działanie wyciągu metanolowego *Artemisia annua* L. na wzrost i wydajność żywieniową zadarliby morwowej *Glyphodes pylolais* (Lepidoptera: Pyralidae) w warunkach laboratoryjnych (24±1°C, 75±5% wilgotności względnej fotoperiod – 16 godz. Światła i 8 godz. ciemności). Wskaźnik odstraszania żywieniowego, określano wybraną metodą na piątym pokoleniu larw przy wykorzystaniu wyciągu o stężeniu 5.25, 1.25 i o.625%. Wyniki wykazały, że wzrost stężenia powodował większe odstraszanie (DC_{50} = 0.48%). Wyciąg istotnie opóźniał wzrost larw (EC_{50} = 3.63%) i także wykazywał istotne działanie na względne tempo wzrostu (RGR), wydajność konwersji pobranego pokarmu (ECI), przybliżoną strawność (AD) i wskaźnik konsumpcji (IC). Tłuszcz, białko i węglowodany w traktowanych owadach były znacznie zredukowane w porównaniu do kontroli. Więc wyciąg metanolowy *A. annua* mógłby być uznany za silny związek odstraszający, wpływający także na metabolizm biochemiczny docelowego szkodnika. Wyciąg metanolowy *A. annua* ma potencjał stania się insektycydem botanicznym.