Research Article

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# Biological activity of natural phytoecdysteroids from *Ajuga iva* against the sweetpotato whitefly *Bemisia tabaci* and the persea mite *Oligonychus perseae*

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## Abstract

**BACKGROUND: Ecdysteroids are steroid hormones that control moulting and govern several changes during metamorphoses in arthropods. The discovery of the same molecules (phytoecdysteroids) in several plant species displayed a wide array of rather beneficial agricultural impact. Many representatives of the genus *Ajuga* plants contain phytoecdysteroids with a 5$\beta$-7-ene-6-one system exhibiting physiological activities in insects.**

**RESULTS: By means of chromatographic (silica gel column, TLC) and LC-MS, two major ecdysteroids (20-hydroxyecdysone and cyasterone) have been isolated and identified from Israeli carpet bugle *Ajuga iva* (L.) Schreber (Lamiales: Lamiaceae) plants. *Ajuga iva* extract fractionated on the silica gel column yielded two fractions that showed high activity against the sweetpotato whitefly *Bemisis tabaci* and the persea mite *Oligonychus perseae*. A dose of 5 mg AI $L^{-1}$ of the purely identified *A. iva* ecdysterone significantly reduced fecundity, fertility and survival of these pests, while commercial 20-hydroxyecdysone at the same dose had lesser effects.**

**CONCLUSION: The results demonstrate considerable efficacy of natural phytoecdysteroids against major agricultural pests, and suggests that these materials should be considered for potential development of friendly control agents.**


**Keywords:** *Bemisia tabaci*; *Oligonychus perseae*; *Ajuga iva*; phytoecdysteroids

## 1 INTRODUCTION

Insect pests in many agricultural crops are a limiting factor that prohibits maximum productivity. The majority of insect pests are controlled using chemical insecticides that harm the environment, humans and many beneficial organisms. Thus, it is pivotal and well accepted that other control methods such as physical prevention, cultural actions and organic and environmentally friendly compounds are essential for integrated pest management (IPM) programmes. Many organic insecticides were derived from plant sources, and some exhibited very high toxicity against a diversity of agricultural pests. Examples include azadirachtin, alkaloids, non-proteic amino acids, steroids, phenols, flavonoids, glycosids, glucosinolates, quinones, tanins and terpenoids. An interesting discovery was the presence of compounds resembling steroids that interfere with the normal development of the insects. Among these are phytoecdysteroids, which are analogues of steroid hormones that control moulting, responsible for many developmental processes, mainly metamorphoses, in many arthropods.[1] Phytoecdysteroids are found in a wide range of species in the genus *Ajuga*, affect a wide range of insects at very low concentrations and are not harmful to humans or animal cells. Ecdysteroids are present in 5–6% of plant species,[2] generally at higher concentrations than those typically found in arthropods. Intensive research on the genus *Ajuga* revealed the presence of ecdysteroids in more than 100 species showing worldwide distribution, especially in China, Japan, Europe and Korea,[3] and appearing both as annuals and perennials. The discovery of phytoecdysteroids in several *Ajuga* species made them easily available in large amounts, and they displayed a wide beneficial use in folk medicine.[4] They have some pharmacological

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effects, e.g. against diabetes or asthenia,[5] and are considered non-toxic to mammals. They can be detected in mammalian tissues and fluids as a consequence of dietary intake of ecdysteroid-containing plants, insects or infection by invertebrate parasites.[5] On the other hand, most crop plant species do not contain phytoecdysteroids, although both spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*) do contain significant levels, especially in the seeds and in younger leaves.[2]

Previous studies have investigated the effects of phytoecdysteroids on insect pests. Application of these compounds to a number of species resulted in significant growth and developmental disruption such as in *Spodoptera frugiperda*,[6] *Bombyx mori*,[7,8] *Lobesia botrana*,[9] *Inachis io*, *Aglais urticae*[10] and *Bradysia impatiens*.[11] The effects included inhibition of growth and induced death before or after moulting. Certain insect species were not affected after phytoecdysteroid application such as in *Heliothis virescens*,[12] *Heliothis armigera*,[13] *Spodoptera littoralis*[14] and *Lacanobia*.[15] In these species, detoxification mechanisms were able to overcome the applied exogenous phytoecdysteroids.

In this study, a Mediterranean *Ajuga iva* species was selected, and two phytoecdysteroids, 20-hydroxyecdysone and cyasterone, were isolated. Tests were then conducted on the insecticidal efficacy of these ecdysteroids against the sweetpotato whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) and the persea mite *Oligonychus perseae* (Fig. 1), as a first step in testing the potential activity of these natural insect growth regulators (IGRs) from plants. *Oligonychus perseae* is a piercing/sucking pest that damages avocado foliage and causes circular necrotic spots, and in high densities it can cause substantial economic losses.[16] Presently, chemical acaricides are the only way used to control this mite, but with very limited success. *Bemisia tabaci* is a cosmopolitan, highly polyphagous pest that inflicts damage in many crops by direct feeding, honeydew secretion and vectoring several hundreds of plant viruses, some of which are devastating.[17–19] *Bemisia tabaci* is a complex of more than ten biotypes, well defined by microsatellite polymerase chain reaction (PCR)-based methods and varying greatly with respect to host range, fecundity, insecticide resistance, ability to transmit plant viruses and induction of plant disorders.[17,19,20–22] It was found that the natural phytoecdysteroid 20-hydroxyecdysone significantly reduced fecundity, fertility and survival of these pests, while natural cyasterone and commercial 20-hydroxyecdysone at the same dose had no to moderate effect on the two pests tested. The work presented here demonstrates for the first time the considerable insecticidal efficacy of natural ecdysteroids against *B. tabaci* and *O. perseae*, and significant potential for the development of friendly control agents.

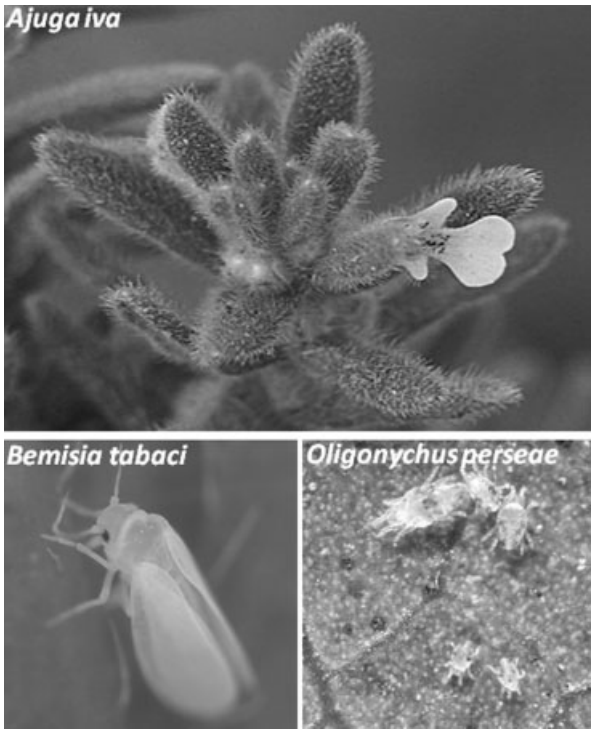


**Figure 1.** *Ajuga iva* plant and the insects used in this study: the whitefly *Bemisia tabaci* and the persea mite *Oligonychus perseae*.

## 2 EXPERIMENTAL METHODS

### 2.1 Plants and insects

*Ajuga iva* plants (Fig. 1) were collected during April 2010 from the Negev, southern Israel. Leaves and stems of fresh plants were air dried for 1 week and homogenised to a fine powder prior to extraction.

*Oligonychus perseae* mites (Fig. 1) were collected from young leaves of infected Avocado trees (Bet Haemek, Israel), placed in a chamber under controlled conditions (24 ± 2 °C, 43 ± 2% RH, 16 : 8 L : D) for 2 days for adaptation to lab conditions and then subjected to experiments with *A. iva* plant extract.

*Bemisia tabaci* [susceptible strain (biotype B)] used in the present bioassays was collected from cotton fields and thereafter reared in isolation on cotton seedlings (*Gossypium hirsutum* L. cv. Acala) under standard laboratory conditions of 26 ± 2 °C and a 14 : 10 h light : dark photoperiod.

### 2.2 Extraction and purification of phytoecdysteroids

Leaves and stem powder (124 g) were soaked in 1 L of methanol and homogenised. The filtered extract was concentrated in vacuum and treated with $H_2O$ to give 30% aqueous methanol. This solution was extracted with *n*-hexane 4 times, and the hexane was discarded. The aqueous methanol was concentrated to about half volume and extracted twice the volume 4 times with ethyl acetate. The yellow-green methanol extract was evaporated by dryness in a TurboVap II concentration workstation (Zymark Corporation, USA) and then 5 mL of methanol was added, and 3 mL was subjected to chromatography on a silica gel column (2 × 40 cm). Ten fractions of 100 mL eluent ($CHCl_3$:MeOH 9 : 1) were collected and evaporated by dryness in the TurboVap II concentration workstation. The final sample was kept in 1 mL of methanol.

### 2.3 Thin-layer chromatography

The crude extract, the pure isolated compound and a commercial sample for ecdysterone were applied to silica gel GF-254 plates (0.25 mm; 20 × 20 cm) and developed with $CHCl_3$:EtOH : acetone (6 : 2:1). Observation under UV (365 nm) revealed ecdysterone as dark spots on a yellow-green fluorescent background. The $R_f$ value was 0.54.

### 2.4 UPLC-QTOF-MS

UPLC-QTOF-MS (Waters Premier QTOF, Milford, MA) was used for metabolite analyses. First, separation of metabolites was performed on a UPLC BEH C18 column, and accurate masses of the eluted compounds were detected by a QTOF Premier

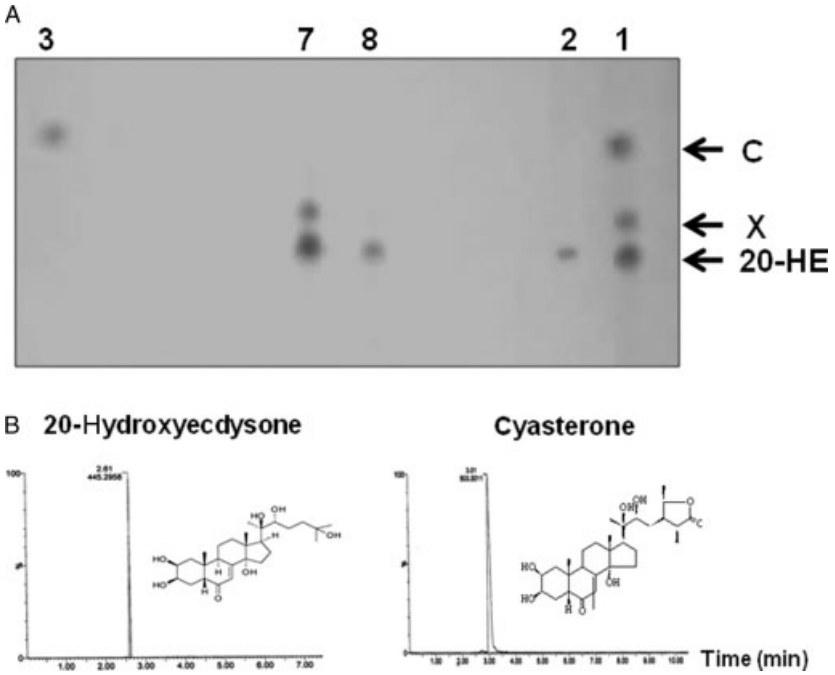


**Figure 2.** Identification of *Ajuga iva* phytoecdysteroids by TLC and UPLC-QTOF-MS analyses (see the text for details). (A) TLC plate showing the separation of crude extract (1), commercial ecdysterone standard (2), fraction 8, which was used in the present experiments, showing the presence of only pure ecdysterone (8) and fraction 7 showing the presence of ecdysterone and another unidentified spot (7). The last lane on the plate (3) shows a spot representing cyasterone after ethyl acetate extraction from crude extract, which was used in the present bioassays. C: cyasterone; 20-HE: 20-hydroxyecdysone; X: unidentified spot. (B) UPLC-QTOF-MS specific peaks for ecdysterone and cyasterone and the retention time for each material.

MS equipped with an ESI source. Acquisition was performed separately in positive and negative ESI modes. MassLynx software v.4.1 (Waters Inc.) was used to control the instrument and calculate accurate masses. Markerlynx software v.4.1 (Waters Inc.) was used for peak picking, alignment and integration.

### 2.5 Biological activity of crude phytoecdysteroids against *Oligonychus perseae*

HPLC was used to identify and quantify the specific peaks in the active fractions. Quantification was performed by comparison with a linear line prepared from a commercial standard and run in the same HPLC analysis. Fractions that showed the presence of ecdysteroids in the TLC and subsequent analyses were used to assess the activity on *O. perseae*. Avocado leaf discs (3 cm diameter) were coated with 5 mg AI $L^{-1}$ of 20-hydroxyecdysone or cyasterone with DX spreader. Leaf discs were air dried for 1 h at 25 °C. In parallel, control discs were coated with $ddH_2O$ and DX spreader. After drying, leaf discs were laid floating bottom side up on polyacrylamide gel (5 g $L^{-1}$) to keep the leaf discs viable for a sufficient time.[16] Three female *O. perseae* were then placed on the treated and control leaf discs and kept in a chamber under controlled conditions (24 ± 2 °C, 43 ± 2% RH, 16 : 8 L : D) for a week. Each three females were considered one replicate, and this was replicated 10 times, with a total of 30 females in the treatment and 30 in the control. Female mortality and fecundity were monitored a week after the start of the experiment.

### 2.6 Biological activity of crude phytoecdysteroids against *B. tabaci*

Leaves of cotton seedlings were treated with a paint brush on their lower surfaces with 5 mg AI $L^{-1}$ of either 20-hydroxyecdysone or cyasterone with DX spreader. In parallel, control leafs were treated with $ddH_2O$ and DX spreader. After air drying, 15 whiteflies (females, sexed under a stereoscope) were confined on each leaf in a leaf clip cage, and the number of eggs per female was determined after 24 h. Adult mortality was recorded after 3 days, the percentage of hatched eggs (fertility) was determined after 8 days and suppression of pupations was determined after 18 days from egg lay.

### 2.7 Statistical analysis

Data in each experiment represent the results of 5–10 separate replicates. Data were analysed using JMP® software v.4.0.3 (SAS Institute Inc., Cary, NC). All results comparing differences in adult mortality, fertility and fecundity were statistically analysed using a paired *t*-test with $\alpha = 0.05$. Error bars in all graphs represent the standard error of the mean (SEM), and significance is indicated in each experiment.

## 3 RESULTS

### 3.1 Identification of natural ecdysteroids from *Ajuga iva*

Ecdysterone and cyasterone were isolated using flash chromatography on silica gel as previously described. The pure isolated compounds were identified by comparing the $R_f$ (TLC) and RT and MS (UPLC-QTOF-MS) with a commercial sample of ecdysterone.[23] Cyasterone was identified in the crude extracts after TLC analysis as a single spot and then in the UPLC-QTOF-MS analysis as a single pure pick, after further extraction by the ethyl acetate method.[24]

### 3.2 Biological activity of ecdysteroids from *A. iva* against insect pests

Two major phytoecdysteroids, ecdysterone and cyasterone, were identified from *A. iva* crude extracts by means of chromatography

  

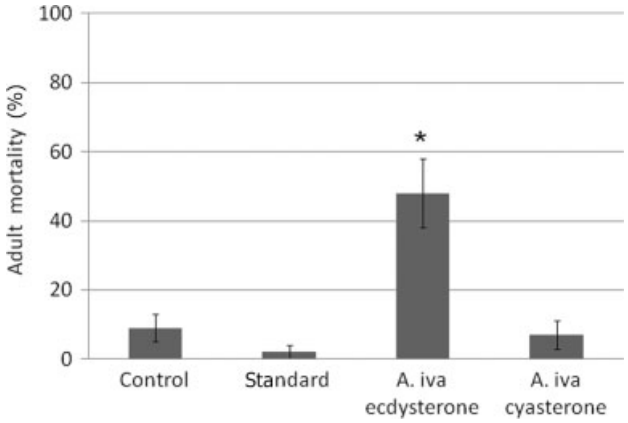


**Figure 3.** Adult *Bemisia tabaci* mortality rates obtained after ecdysterone and cyasterone treatments, compared with a commercial ecdysterone standard and a water control.

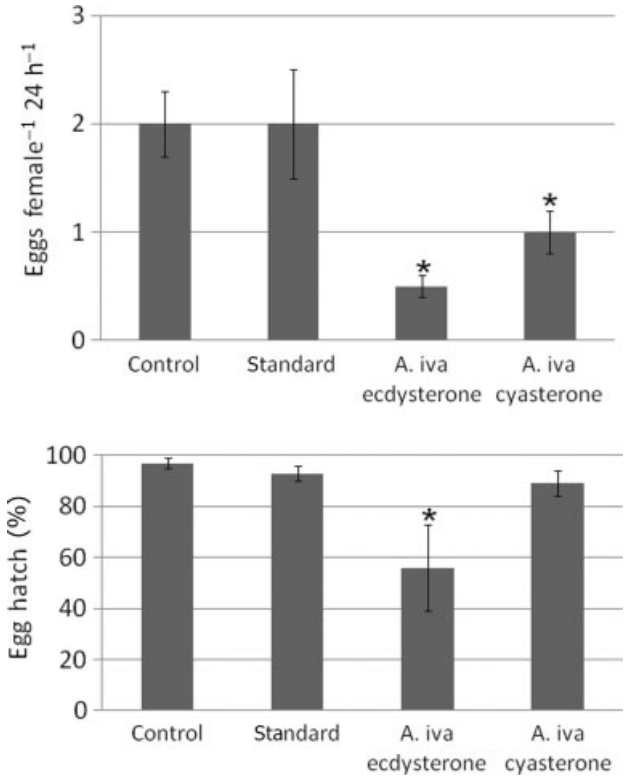


**Figure 4.** Effect of ecdysterone and cyasterone from *Ajuga iva* on *Bemisia tabaci* fecundity (A) and fertility (B). The treatments were compared with a commercial ecdysterone standard and a water control. Asterisks above columns indicate a statistically significant effect of the tested materials compared with the control.

and MS (UPLC-QTOF-MS). Ecdysterone was identified in fraction 8 after methanolic extraction (Fig. 2), and cyasterone after ethyl acetate extraction from the crude extract, and using subsequent extraction methods they were separated and identified with high purity. Given the fact that steroid hormones and their derivates were shown to have some activity against arthropod pests such as *Spodoptera frugiperda, Bombyx mori, Lobesia botrana, Inachis io, Aglais urticae* and *Bradysia impatiens*,[6–11] tests were conducted to establish whether the two steroids identified had some activity against two important insect pests: *B. tabaci* and *O. perseae*. First, mortality tests on *B. tabaci* adults showed that, while the control (water), 10 mg AI $L^{-1}$ of commercial 20-hydroxyecdysone standard and extracts from *A. iva* that contained 5 mg AI $L^{-1}$ of cyasterone had no effect on adult *B. tabaci* mortality, the extract that contained only 5 mg AI $L^{-1}$ of ecdysterone had a significant effect that reached up to ∼50% (Fig. 3), and this effect was highly statistically significant ($P < 0.001$), suggesting a strong effect of the ecdysterone from *A. iva* on adult *B. tabaci* mortality. The same treatments were used to test the effect on fecundity (egg lay) and fertility (egg hatch) of *B. tabaci*. Fig. 4A shows that both the ecdysterone and cyasterone from *A. iva* had a significant effect on fecundity compared with the other treatments (water and the commercial 20-hydroxyecdysone standard); however, the effect of ecdysterone was significantly stronger and caused 75% reduction in fertlity ($P < 0.002$), while cyasterone caused 50% reduction ($P < 0.032$). A significant effect on fertility (egg hatch) was also observed with *A. iva* ecdysterone treatment, which resulted in only 56% egg hatch compared with more than 90% egg hatch with the water control, standard or cyasterone (Fig. 4B). This effect was statistically significantly ($P < 0.012$), suggesting a strong suppression of egg hatch caused by the application of the ecdysterone from *A. iva*. A similar result was obtained when the effect of *A. iva* ecdysterone and cyasterone was tested on the suppression of pupation, compared with water and commercial standard controls. While ∼90% pupation was obtained with the control, commercial ecdysterone and cyasterone, only 50% pupation was obtained after exposure to ecdysterone, resulting in ∼40% suppression of pupation, a result that was highly significant compared with the water control ($P < 0.001$) (Fig. 5).

The effect of ecdysterone and cyasterone from *A. iva* on the mite *O. perseae* was tested and compared with a water control and a commercial 20-hydroxyecdysone standard. The results given in Fig. 6 show that this ecdysterone had a strong effect on both mortality and fecundity. Mortality rates reached 63.3% after treatment with ecdysterone ($P < 0.002$ compared with the water control), 50% after treatment with commercial 20-hydroxyecdysone standard ($P < 0.01$ compared with the water control) and 23.3% in the control. Ecdysterone from *A. iva* also showed a strong effect on *O. perseae* fecundity, and treatment with this compound caused ∼80% reduction in fecundity compared with the control ($P < 0.0034$), while the commercial standard caused ∼50% suppression in egg lay ($P < 0.021$). These effects further demonstrate the potency of ecdysterone on *O. persea*, as was also shown on *B. tabaci*, and suggest a specific mechanism by which phytoecdysterones affect mortality, fecundity and fertility in these insect pests.

## 4 DISCUSSION AND CONCLUSIONS

Previous studies have demonstrated the potency of ecdysteroids from plants on a number of lepidopteran pests. These pests include some very economically important ones that are known for their ability to detoxify chemical insecticides, including *S. frugiperda, Pectinophora gossypiella*,[6] *Acrolepiopsis assectella*,[25] *A. urticae* and *I. io*,[11] while other species showed complete tolerance to these phytoecdysteroids, including *H. virescens*,[12] *H. armigera*[13] and *S. littoralis*.[14] The data presented in the present study demonstrate that, in the concentrations studied, the mortality, fecundity and fertility of both *B. tabaci* and *O. persea*, two economically important agricultural pests, are affected by ecdysterone and sometimes cyasterone. The potency of ecdysterone was higher than cyasterone in all the parameters tested. Morphological effects

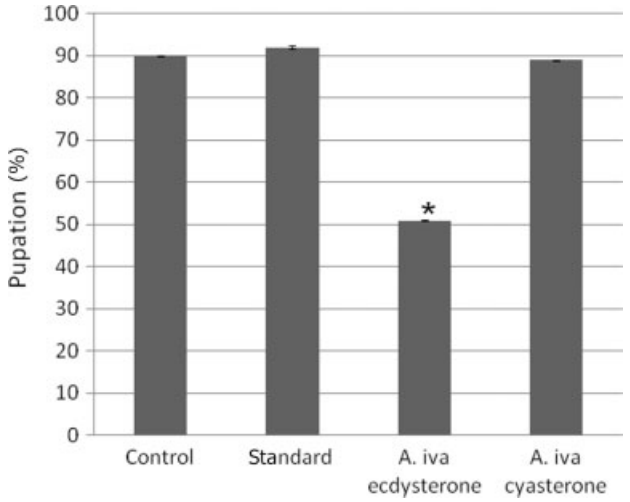


**Figure 5.** Effect of ecdysterone and cyasterone from *Ajuga iva* on *Bemisia tabaci* pupation. The treatments were compared with a commercial ecdysterone standard and a water control. Asterisks above columns indicate a statistically significant effect of the tested materials compared with the control.

of phytoecdysteroid application on the studied pests were not studied, but effects were observed on three parameters: adult mortality, fecundity (number of eggs laid) and fertility (number of hatched eggs). It was previously shown that the mortality of some pests in the larval stage could result from the effect of applied ecdysteroids exogenously. Ingestion of these compounds results in toxicity of midgut epithelial cells, and severe toxicity could be observed in midgut epithelial cells of *P. interpunctella* and *B. mori*.[26,27]

One of the main effects of ecdysteroids on insects is their interference with development and the important role they play in the transition from one developmental stage to another, especially during metamorphosis. In the present study, suppression in pupation was observed in *B. tabaci*, and reduction in fertility and fecundity in both *B. tabaci* and *O. persea*. The effect on pupation could be a consequence of hormonal balance disruption with internal levels of ecdysone. External ecdysteroid detoxification is one of the main ways by which insects overcome the toxic effects of these compounds. A previous hypothesis suggested that sensitivity to phytoecdysteroids correlates with the ability of the insect to feed on host plants when monophagous insects are most sensitive and polyphagous are most tolerant to phytoecdysteroids.[16] The present results and those of other studies with *P. interpunctella*[27] do not fully agree with this hypothesis. *Bemisia tabaci* is an extremely polyphagous insect, feeds on more than 600 plant species and has developed resistance to major insecticide groups, including organophosphates, pyrethroids, neonicotinoids and several IGRs.[28] In spite of this, the present results show that *B. tabaci* is highly sensitive to phytoecdysteroid from *A. iva*. Along with this hypothesis, several polyphagous insect pests, such as *S. littoralis* and *L. oleracea*, feed on plants containing high levels of ecdysteroids; however, they are normally not affected owing to an efficient detoxification mechanism that enables them to survive when exposed to high doses of phytoecdysteroids.[16]

The observed effects on fecundity and fertility exhibit another possible mode of action of phytoecdysteroids. The target site is ovarian development, which, if affected, can lead to egg abortion during oogenesis, leading to a reduction in the number of eggs laid (less fecundity), mortality of adults as an indirect effect or reduction in egg hatch if eggs are affected in later stages during oogenesis (less fertility), where effects are seen after egg lay. Indeed, it was shown in *B. mori, Drosophila* and other insects that ovarian development in adults is induced by 20-hydroxyecdysone through the activation of several molecular cascades. Furthermore, the transition from one stage in ovarian development to another, such as from previtellogenesis to vitellogenesis to chorionogenesis, is governed by the action of several pathways that respond to different titres of 20-hydroxyecdysone. The follicle developmental arrest after treatment with the ecdysone agonist tebufenozide indicates the requirement for a decline in the titres of 20-hydroxyecdysone. The steroid hormone, therefore, seems to be a crucial factor in regulating the development of ovarioles in insects.[29] The above facts suggest that the effects observed in *B. tabaci* and *O. persea* could be a consequence of disruption in ecdysteroid titres, affecting both the number of eggs laid and their ability to hatch.

To summarise, the results of the present work suggest a strong and potent effect of phytoecdysteroids from *A. iva* on both *B. tabaci* and *O. persea*. Both insect pests are polyphagous and are able to cope with many plant hosts, suggesting their ability to detoxify many plant secondary metabolites. However, the phytoecdysteroids from *A. iva* that were tested, ecdysterone and cyasterone, caused significant effects on both insects, with the first being more potent in both insects, confirming that these materials may be an important plant defence mechanism against insect pests. As the source of plant material was very limited, it was not possible to test a series of concentrations and show a dose–response effect; thus, only the 5 mg AI $L^{-1}$ concentration

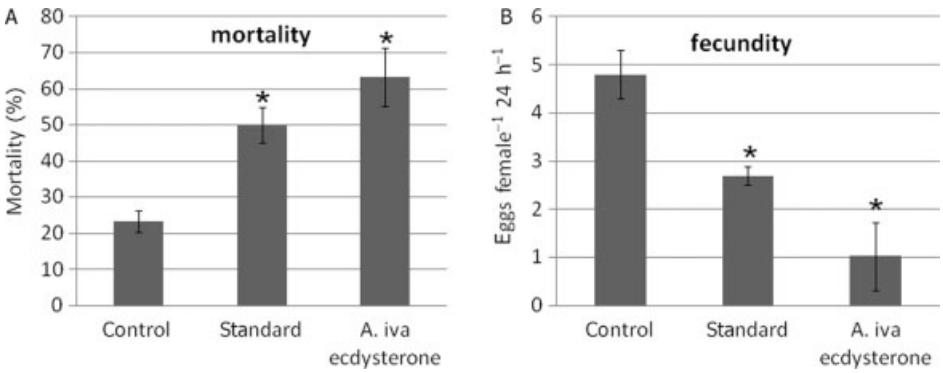


**Figure 6.** Effect of ecdysterone from *Ajuga iva* on adult mortality (A) and fecundity (B) of *Oligonychus perseae*. This effect was compared with a commercial ecdysterone standard and a water control. Asterisks above columns indicate a significant effect of the treatment compared with the controls.

  

was used. It could be interesting to test other concentrations for both phytoecdysteroids. Phytoecdysteroids seem to play an important role in plant defence, and using plants as a source for the production of these materials for subsequent use in organic agriculture will be realistic once cultivation and growing methods have been adapted for *A. iva* plants. Another promising alternative is to breed plants that produce these materials for self-defence through classical breeding or by developing transgenic plants. The diversity of plants that are able to produce natural ecdysteroids suggests that the genetic make-up underlying phytoecdysteroid production in the plant kingdom is highly diverse, and full or partial make-up of the pathways responsible for the production of these materials is found in many plant species.[1] The use of these natural materials in integrated pest management programmes may complement other methods used.

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