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Biological activity of *Ajuga iva* extracts against the African cotton leafworm *Spodoptera littoralis*

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Key Message

Insects cause severe damage to numerous crops and their control relies on pesticides. Green control is becoming increasingly popular due to concerns about the negative impacts of pesticides on the environment. Phytoecdysteroids are found in Ajuga plants and affect a wide range of insects at very low concentrations. Here we demonstrate that crude extract from *Ajuga iva* alters the development of *Spodoptera littoralis*. Phytoecdysteroids may therefore be beneficial in IPM programs.

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30 Abstract

31 The African cotton leafworm *Spodoptera littoralis*, a major crop pest worldwide, is
32 controlled by chemical insecticides, leading to serious resistance problems. *Ajuga* plants
33 contain phytoecdysteroids (analogs of arthropod steroid hormones that regulate
34 metamorphoses) and clerodanes (diterpenoids exhibiting antifeedant activity). We
35 analyzed phytoecdysteroids and clerodanes in leaf extracts of the Israeli *Ajuga iva* by LC-
36 TOF-MS and TLC, and their efficiency at reducing *S. littoralis* fitness. Castor bean leaves
37 were smeared with an aqueous suspension of dried methanolic crude extract of
38 phytoecdysteroid and clerodanes from *A. iva* leaves (50, 100 and 250 µg/µl). First and
39 third instars of *S. littoralis* larvae were fed with 1 treated leaf for 3 and 4 days, respectively.
40 Mortality, larval weight gain, relative growth rate and survival were compared to feeding
41 on control leaves. To evaluate and localize *A. iva* crude leaf extract activity in the insect
42 gut, we used DAPI and phalloidin staining. Crude extract of *A. iva* leaves (50, 100 and 250
43 µg/µl) significantly increased mortality of first instar *S. littoralis* larvae (36%, 70% and 87%,
44 respectively) compared to controls (6%). Third instar larval weight gain decreased
45 significantly (by 52%, 44% and 30%, respectively), as did relative growth rate (–0.05 g/g
46 day, compared to the relevant controls). *S. littoralis* larvae were further affected at later
47 stages, with few survivors. Insect-gut staining showed that 250 µg/µl crude leaf extract
48 reduces gut size, with relocation of nuclei and abnormal actin-filament organization. Our
49 results demonstrate the potential of *A. iva* extract for alternative, environmentally safe
50 insect-pest control.

51

52 Keywords *Ajuga*; Clerodane; Pest control; Phytoecdysteroid; *Spodoptera littoralis*

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55 ## Introduction

56

57 The African cotton leafworm *Spodoptera littoralis* is considered one of the most serious
58 pests of cotton, maize, rice, alfalfa, potato, tomato, ornamentals and orchard trees
59 (Martinez and van Emden 2001). It feeds year-round on the leaves of numerous old- and
60 new-world plant species (Adel El-Sayed et al. 2011). Today, insect pests are mainly
61 controlled by insecticides, which constitute a risk to human health and the environment
62 (Horowitz et al. 2005). Many organic insecticides have been derived from plant sources,
63 and some, such as alkaloids, terpenoids, phenols and steroids, exhibit very high toxicity
64 against a variety of agricultural pests. In this study, we examined the potential use of
65 phytoecdysteroids and clerodanes extracted from *Ajuga* (Lamiaceae) plants to control the
66 African cotton leafworm.

67 Phytoecdysteroids are plant-produced steroids that are analogs of the steroid
68 hormones that control molting and metamorphosis in arthropods (Dinan 2001).
69 Phytoecdysteroids are present in 5–6% of plant species (Sandlund et al. 2018), generally
70 at higher concentrations than those typically found in arthropods (Dinan 1995a). Most of
71 them possess a cholest-7-en-6-one carbon skeleton (C27), and are synthesized from
72 phytosterols in the cytosol through the mevalonic acid pathway (Dinan 2001). They can
73 mimic insect 20-hydroxyecdysteroid, bind insect ecdysone receptors and elicit the same
74 responses (Sadek 2003). Phytoecdysteroids may cause abnormal larval development,
75 feeding deterrence and ultimately, death (Sadek 2003). Ecdysteroids are not toxic to
76 mammals because their structure is quite different from mammalian steroids, and they
77 are not expected to bind to vertebrate steroid receptors (Lafont and Dinan 2003).

78 Ecdysone, a natural molting hormone of insects derived from enzymatic
79 modification of cholesterol by p450 enzymes (Dinan 1989), controls developmental
80 events by changing the levels of other ecdysteroids (Lafont 1997). The ecdysone receptor
81 is a nuclear receptor (a ligand-activated transcription factor) that binds to and is activated
82 by ecdysteroids. In *Manduca sexta* larvae, 20-hydroxyecdysone is primarily produced in
83 the prothoracic gland, gut and fat bodies (Grieneisen et al. 1991) from dietary cholesterol,

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84 and acts through the ecdysone receptor (Thummel and Chory 2002). In addition, the
85 ecdysone receptor controls development, and contributes to other processes (such as
86 reproduction) (Riddiford et al. 2000), and to interactions between the cytoskeleton (the
87 effector of cell movement and changes in cell shape) and changes in the distribution of
88 actin staining and microfilaments (Otey et al. 1990).

89 Discovery of the same molecules (phytoecdysteroids) in several plant species
90 suggests that they may be effective against insect herbivores by acting as antifeedants
91 and/or disrupting the insects' endogenous endocrine levels (Blackford et al. 1996;
92 Dinan 2001; Belles and Piulachs 2014). Low concentrations (2–25 ppm) of
93 phytoecdysteroids deter some insects, while others are resistant to even very high
94 concentrations (400–1000 ppm) (Blackford et al. 1996). Kubo (1997) reported that an
95 extract of *Ajuga remota* containing 20-hydroxyecdysone and cyasterone, added to the
96 diet of *Bombyx mori*, inhibited ecdysis, resulting in larval retention of the exuvial head
97 capsule and the insect's death. Similarly, larvae of the greenhouse whitefly exhibited 100%
98 mortality when fed on *Ajuga reptans* plants. High levels of the three major
99 phytoecdysteroids, 20-hydroxyecdysone (ecdysterone), makisterone A and cyasterone,
100 have been found in several plants, including *Ajuga* (Tomás et al. 1992; Wessner et al. 1992;
101 Dinan 2001; Coll and Tandrón 2005; Castro et al. 2008, 2011; Grace et al. 2008; Sun et al.
102 2012; Lva et al. 2014; Guibout et al. 2015; Taha-Salaime et al. 2019), quinoa and spinach
103 (Dinan 1995b, 2001). An extract of 20-hydroxyecdysone and cyasterone from *A. iva*
104 showed high activity against *Oligonychus perseae* (Kubo and Klocke 1983; Aly et al. 2011);
105 a dose of 5 μg/ml of pure extracted *A. iva* ecdysterone significantly reduced fecundity,
106 fertility and survival of this pest, while commercial 20-hydroxyecdysone at the same dose
107 had lesser effects (Aly et al. 2011).

108 In addition to phytoecdysteroids, species of the genus *Ajuga* also contain the
109 bioactive compounds clerodane diterpenes (include clerodanes) and iridoid glycosides
110 (Camps and Coll 1993). Clerodanes (diterpenoids) are a large group of C20 terpene
111 compounds derived from geranylgeranyl diphosphate and biosynthesized through the
112 deoxyxylulose phosphate pathway in the cytoplasm, mostly in the leaves and stems of
113 the Lamiaceae and Asteraceae families (Hussain et al. 2012). Clerodin was originally

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114 isolated from *Clerodendrum infortunatum* L. (Lamiaceae), and has potential as a natural
115 pesticide due to its insect antifeedant and repellent activities (Pereira and Gurudutt 1990;
116 Kubo et al. 1991; Coll and Tandrón 2005; Koul 2016; Li et al. 2016). Koul (2016) showed that
117 the most active compounds, dihydroclerodin and clerodin hemiacetal, from *Caryopteris*
118 *divaricata* exhibit 100% antifeedant activity at 50 ppm. These clerodanes were deadly to
119 *Spodoptera litura* larvae.

120 We previously identified and quantified high contents of three phytoecdysteroids
121 and two clerodanes in *A. iva* growing in Israel (Taha-Salaime et al. 2019). We hypothesized
122 that crude extract of *A. iva* leaves that includes the three phytoecdysteroids (20-
123 hydroxyecdysone, makisterone A and cyasterone), which specifically interfere by
124 controlling molting, and are responsible for the metamorphosis and antifeedant activities
125 in insects, might be a promising pest-control agent. We evaluated the efficiency of *A. iva*
126 extracts (containing phytoecdysteroids and clerodanes) at reducing the damage caused
127 by *S. littoralis* larvae by addressing the following questions: Does *A. iva* crude leaf extract
128 affect *S. littoralis* larvae? Do phytoecdysteroids isolated from the crude leaf extract and
129 commercial standards have different effects on the larvae? Do phytoecdysteroids have a
130 direct effect on the larvae's gut?

131

132 Materials and Methods

133

134 Plants and insects

135

136 *A. iva* plants were collected in April 2014 from a wild population in the Negev, southern
137 Israel, and then cultivated and acclimated in an open field at Newe Ya'ar Research Center.
138 Young and mature leaves and stems of fresh plants were collected after blooming (July–
139 November) and oven-dried at 55°C for 3–4 days, then homogenized to a fine powder
140 prior to extraction. The first and third instars of *S. littoralis* larvae used for the bioassays
141 were from Murad Ghanim's laboratory, Department of Entomology, Agricultural Research
142 Organization (African cotton leafworm colony) reared on castor bean leaves.

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143

144 Extraction and purification of phytoecdysteroids

145

146 *A. iva* crude extracts were prepared according to our recently published procedure (Taha-
147 Salaime et al. 2019). Leaf and stem powder were pooled (24 g) and soaked in 240 ml of
148 100% MeOH, sealed and homogenized with shaking (2500 rpm) for 1 h. The extract was
149 then centrifuged (112 *g)* for 10 min, filtered and concentrated under vacuum. The final
150 filtered methanol solution was analyzed by liquid chromatography-time of flight-mass
151 spectrometry (LC-TOF-MS) and dried in a chemical vaporizer for 5 days. For purification
152 of phytoecdysteroids from the crude extract, leaf and stem powder (100 g) was soaked in
153 300 ml methanol and homogenized. The filtered extract was vacuum-concentrated and
154 treated with H_2O to give 30% aqueous methanol. This solution was extracted as previously
155 described (Aly et al. 2011).

156

157 Identification phytoecdysteroids and clerodanes

158

159 LC-TOF-MS analysis was used to identify and confirm the presence of phytoecdysteroids
160 and clerodanes in three concentrations of *A. iva* crude leaf extract (50, 100 and 250 μg/μl).
161 We analyzed the profile of phytoecdysteroids and clerodanes in the *A. iva* crude leaf
162 extract before each test for biological activity. Extracts of the plant material (1 μl) were
163 injected into an Agilent 1290 Infinity Series liquid chromatograph coupled with an Agilent
164 1290 Infinity DAD and Agilent 6224 Accurate Mass TOF mass spectrometer (Agilent
165 Technologies, Santa Clara, CA, USA) (Dinan 1989). Thin-layer chromatography (TLC) was
166 used to separate the components into well-defined spots. The crude leaf extract, the pure
167 isolated compounds (20-hydroxyecdysone [ecdysterone], makisterone A and cyasterone)
168 and a commercial ecdysterone sample were applied to silica gel GF-254 plates (0.25 mm;
169 20 × 20 cm) as described in Aly et al. (2011).

170

171 Biological activity of *A. iva* crude leaf extract against *S. littoralis*

172

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173 To assess effects on the larvae, mature castor bean leaves were smeared, using a paint
174 brush, with aqueous *A. iva* crude leaf extract (24 g of dried pooled leaves and stems
175 dissolved in 240 ml MeOH, 1:10) and Tween 20 (1.5 mg). The leaves were dried in a
176 chemical hood for 2 h. Then 10 first instar *S. littoralis* larvae were placed on 1 treated
177 castor bean leaf in a petri dish and allowed to feed for 3 days in a climate-controlled
178 room at 25°C. Control leaves were similarly smeared with double-distilled water (ddH_2O)
179 and Tween 20. The larvae were exposed to three concentrations of crude leaf extract (50,
180 100 and 250 µg/µl), one concentration per treatment. After preparing the *A. iva* crude leaf
181 extract, the methanolic extract was dried in a chemical hood; 1.2 g dried extract powder
182 was dissolved in 4.8 ml ddH_2O and Tween 20 (0.5 mg/ml) for the 250 µg/µl concentration;
183 0.83 ml of the high concentration (250 µg/µl) extract was dissolved in 3 ml ddH_2O to
184 obtain the 100 µg/µl concentration; and 0.67 ml of the 100 µg/µl solution was dissolved
185 in 3 ml ddH_2O to obtain the 50 µg/µl concentration. At the end of the experiment, larval
186 mortality was compared to that of controls. Data in this experiment represent the results
187 of 11 replicates (10 larvae/replicate). Differences are reported as percent mortality of first
188 instar larvae after feeding on the three concentrations of *A. iva* crude leaf extract using a
189 t-test and significance was determined by t-test.

190 For the third instars, 1 or 10 larvae were fed on 1 treated caster bean leaf for 4 or
191 8 days in a climate-controlled room at 25°C (more freshly treated leaves were provided
192 after 4 days of feeding to avoid feeding on decayed leaves). Four different treatments
193 were tested, where larvae were fed on castor bean leaves treated with: (1) 250 µg/µl *A.*
194 *iva* crude leaf extract for 4 days, with a freshly treated leaf for 4 more days; (2) the same
195 treatment as (1) with 250 µg/µl of a fractionated mixture of three phytoecdysteroids from
196 *A. iva* leaf extract; (3) *A. iva* crude leaf extract for 4 days, and then a control castor bean
197 leaf treated with ddH_2O for the next 4 days; (4) a control castor bean leaf for 4 days and
198 then *A. iva* crude leaf extract for the next 4 days. In parallel, control leaves were smeared
199 with ddH_2O and Tween 20 for 8 days. We recorded the different reactions of *S. littoralis*
200 larvae in all treatments after 4 days, and if some larvae can recover again if provided
201 control castor bean leaf larvae (first were fed on treated leaf), or were adversely affected
202 and dead when provided a treated castor bean leaf after 4 days (first were fed on control
203 leaves. In another experiment, third instar larvae were exposed to three concentrations of

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204 *A. iva* crude leaf extract (50, 100 and 250 µg/µl) for 4 days. At the end of the experiment,
205 we recorded larval survival and relative growth rate (RGR) as $(\ln W_2 - \ln W_1)/ (t_2 - t_1)$, where
206 W_1 and W_2 are weights at times t_1 and t_2 (n = 20 replicates). In each treatment, pupation
207 rate was evaluated for an additional 15 days. RGRs were compared using a mixed-model
208 ANOVA (repeated measures ANOVA until day 4 and two-way ANOVA from day 4 to the
209 end of the experiment). Survival rates were analyzed using a Friedman test, since the data
210 for larval survival did not follow a normal distribution. Data for larval weight gain (LWG)
211 are the result of 10 replicates (10 larvae in each replicate). Comparisons of LWG were
212 assessed using a repeated measures ANOVA. Statistical significance was reported at $p <$
213 0.05. Error bars in all graphs represent the standard error of the mean (SEM), and
214 significance is indicated in each experiment. All statistical analyses were performed with
215 IBM SPSS software v.20 for Windows (IBM, Armonk, NY, USA).
216
217 The effect of *A. iva* crude leaf extract and purified phytoecdysteroid mixture on larval gut
218 of *S. littoralis*
219
220 To examine the gut morphology of *S. littoralis* larvae, we used DAPI and phalloidin
221 staining. Larvae fed on castor bean leaves treated with *A. iva* crude leaf extract and
222 controls (leaves treated with water) were tested after 7 days of treatment. Guts were
223 dissected in phosphate buffered saline (1X PBS), then fixed in 4% paraformaldehyde in 1X
224 PBS for 30 min, washed in 0.1% Triton X-100 for 30 min, washed three times in PBS Tween-
225 20 (PBST) (https://www.usbio.net/protocols/phosphate-buffered-saline-tween-20),
226 incubated in 0.1% phalloidin in PBST for 30 min, washed three times with PBST and
227 mounted whole in 0.1% DAPI in hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M NaCl,
228 0.01% w/v sodium dodecyl sulfate, 30% v/v formamide). Changes in actin fibers and nuclei
229 were visualized under a Leica confocal microscope (Leica SP8 and Olympus IX 81 confocal
230 laser scanning microscope).
231
232 Results
233
234 Identification of natural phytoecdysteroids from *A. iva* using TLC analysis

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235

236 *A. iva* crude leaf extract was subjected to flash chromatography on a silica gel (TLC),
237 yielding three individual isolated compounds (20-hydroxyecdysone, makisterone A and
238 cyasterone). The retention factor (Rf) values, i.e., the distance migrated over the total
239 distance covered by the solvent, of the phytoecdysteroid spots were similar to those of
240 the respective commercial ecdysteroids (Fig. 1).

241

242 The effect of *A. iva* crude leaf extract on first instar larval survival

243

244 First instar *S. littoralis* larvae showed a significant increase in mortality (25, 65, 85%) after
245 feeding on the three concentrations of *A. iva* crude leaf extract (50, 100 and 250 µg/µl,
246 respectively), compared to the control (treated with water, 5%) (Fig. 2a).

247

248 The effect of *A. iva* crude leaf extract on third instar larval survival and development

249

250 Third instar *S. littoralis* larvae fed on crude leaf extract (50, 100 and 250 µg/µl) showed
251 reduced LWG ($F_{3.104}$ = 20.334, 17.246 and 13.007, respectively, $p < 0.001$; Fig. 2b) compared
252 to the control.

253 All concentrations of crude leaf extract significantly decreased ($p < 0.001$) larval
254 RGR compared to the normally developing larvae on the control diet (Fig. 3a, arrow).
255 Reduced RGR was recorded as early as 2 days into the experiment. With the highest
256 concentration of crude leaf extract, RGR decreased by 0.05 and 0.20 g/g day on days 6
257 and 8, respectively, compared to the control ($F_{3.16,\ 18}$ =12.641, $p < 0.001$; Fig. 3a). All
258 concentrations of *A. iva* crude leaf extract significantly reduced third instar larval survival
259 after 11 days (X^2_3 = 6.221, p = 0.038; Fig. 3b). Whereas all larvae survived on the control
260 leaves, the effect of the crude extract was apparent after 3 days. In fact, none of the
261 treated larvae survived more than 8 days for the highest concentration of crude leaf
262 extract and 10 days for the other concentrations (Fig. 3b).

263 In addition, when *S. littoralis* larvae were first fed on control leaves for 4 days and
264 then on leaves treated with *A. iva* crude leaf extract for an additional 4 days, their RGR
265 was affected by feeding on the crude leaf extract after day 5 of the experiment ($F_{3.16,\ 18}$

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266 =7.310, $p < 0.001$; Fig. 4a), and continued to decrease until the end of the experiment,
267 with no surviving larvae ($X^2_3 = 9.282$, $p = 0.021$; Fig. 4b).
268 The same result was obtained when the order of the treatments was reversed (Fig.
269 4c, d). When the larvae were first fed on leaves treated with *A. iva* crude extract for 4 days
270 and then fed on control leaves (for an additional 4 days), a significant decrease in RGR
271 was obtained on days 2–4 ($F_{3.16, 18}$ = 4.595, 3.608 and 8.113, p = 0.034, 0.02 and 0.001 for
272 50, 100 and 250 µg/µl crude leaf extract, respectively; Fig. 4c). Moreover, castor bean
273 leaves treated with 250 µg/µl of the mixture of the three fractionated and purified
274 phytoecdysteroids from the *A. iva* crude leaf extract significantly reduced RGR ($F_{2.20, 18}$ =
275 6.172, $p = 0.001$) compared to the control (Fig. 5a). Few larvae survived on the leaves
276 treated with purified phytoecdysteroid fraction ($X^2_3 = 11.305$, $p = 0.04$) (Fig. 5b).
277 Overall, larvae fed on castor bean leaves treated with 250 µg/µl *A. iva* crude leaf
278 extract or 250 µg/µl of the phytoecdysteroid mixture lost weight, stopped growing and
279 ultimately died (Fig. 5a, larvae depicted above columns).
280
281 The effect of *A. iva* crude leaf extract and purified phytoecdysteroid mixture on larval gut
282 of *S. littoralis*
283
284 Larval guts were stained with phalloidin, an actin-specific marker that binds to the
285 interface between adjacent actin monomers in the F-actin polymer, and with DAPI, which
286 stains the nuclei. Larvae feeding on 250 µg/µl *A. iva* crude leaf extract for 8 days had
287 smaller nuclei with an abnormal shape—the nuclei moved to the edges of the cell and
288 were thinner than normal (Fig. 6d–f). Phalloidin staining showed normal actin-filament
289 organization in the control treatment (Fig. 6a–c). In contrast, in guts dissected from larvae
290 treated with 250 µg/µl crude leaf extract or 250 µg/µl of the three phytoecdysteroids (20-
291 hydroxyecdysone, makisterone A and cyasterone) isolated from the leaf extract, the actin
292 filaments were smaller and their amount reduced (Fig. 6d–i). The damage observed in
293 these experiments continued until the insects died. Overall, larvae exposed to crude leaf
294 extract or its phytoecdysteroid fraction had less actin fibers and smaller, abnormally
295 shaped nuclei.
296

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297 Pupation of *S. littoralis* larvae following biological activity treatments

298

299 Larvae fed 250 μg/μl of crude leaf extract or 250 μg/μl of its phytoecdysteroid fraction
300 for 8 days were unable to complete their development and pupate after 15 days (Fig. 7).
301 Figure 7b and c shows the incomplete pupae obtained; the dying larvae had short limbs,
302 small heads, decreased weight and only stomach and chest pupated, considered non-
303 pupation. None of them completed their development to adult moths.

304

305 Discussion and Conclusions

306

307 As predicted by Taha-Salaim et al. (2019), we found that Israeli *A. iva* crude leaf extract
308 and its fractionated phytoecdysteroids (20-hydroxyecdysone, makisterone A and
309 cyasterone) significantly reduce the development and survival of *S. littoralis* larvae. These
310 effects were pronounced throughout all larval developmental stages, including pupation.
311 It has been shown that phytoecdysteroids negatively affect lepidopteran pests (Schmelz
312 et al. 2002), whereas other insect species tolerate them (Schmelz et al. 2002; Taha-Salaime
313 et al. 2019). We found that *S. littoralis* first and third instar larvae fed on *A. iva* crude leaf
314 extract (50, 100 and 250 μg/μl) for 3 and 11 days, respectively, had increased mortality,
315 reduced LWG and decreased RGR compared to the control treatment. Similarly,
316 phytoecdysteroids from *A. iva* have been found to reduce the fertility and fecundity of
317 *Bemisia tabaci* and *Oligonychus perseae* (Aly et al. 2011).

318 Our results are in agreement with previous studies suggesting that insect
319 herbivores cannot develop and survive when fed on phytoecdysteroid-treated leaves.
320 Ecdysteroids inhibited feeding of *Pieris brassicae* and *Mamestra brassicae* larvae when
321 given at 200 mg/kg fresh weight in sucrose solution (Ma 1972), and inhibited drinking in
322 *Dysdercus koenigii*, *Dysdercus fulvoniger* and *Spilostethus pandurus* adults at a
323 concentration of 100 mg/kg (Schoonhoven and Derksen-Koppers 1973). Jones and Firn
324 (1978) reported that ecdysone and 20-hydroxyecdysone deter feeding in *Pieris brassica*
325 when incorporated above 5 mg/kg diet. Exogenous application of ecdysteroids was
326 shown to be lethal to *Plodia interpunctella* and *Bombyx mori* larvae; ingestion of these
327 compounds was toxic to the midgut epithelial cells (Tanaka and Yukuhiro 1999; Rharrabe

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328 et al. 2009; Wadsworth et al. 2014). In our study, *A. iva* crude leaf extract was most
329 effective at the highest concentration applied, indicating a dose-dependent effect, in
330 agreement with other studies (Tanaka and Takeda 1993). In contrast, *S. littoralis* was not
331 deterred from feeding by 20-hydroxyecdysone at 50–70 mg/kg (Jones and Firn 1978).
332 In our study, LWG and RGR of *S. littoralis* larvae were affected by feeding
333 on 250 µg/µl methanolic crude leaf extract dissolved in ddH_2O, regardless of larval age,
334 in agreement with recent research using a methanolic extract of *Ajuga*
335 *remota* leaves containing cyasterone and ecdysterone, which disrupted the
336 molting cycle in *Bombyx mori* and *Spodoptera frugiperda* (Kubo et al. 1981).
337 Moreover, Slama et al. (1993) found that cyasterone and turkesterone are the most
338 effective lepidopteran- and coleopteran-specific ecdysteroids, and ingesting the
339 phytoecdysteroid 20-hydroxyecdysone caused death before and during *Bombyx mori*
340 molting (Chou and Lu 1980).
341 In the current study, we did not fractionate clerodanes from *A. iva* crude leaf
342 extract due to the difficulty involved in calibrating the protocol for fractionation, and to
343 the high cost of commercial clerodane standards. Kubo (1993) conducted an artificial
344 diet-feeding assay with the wheat aphid *Schizaphis graminum*, and showed that
345 ajugasterone C (a clerodane) was 10-fold more potent as a feeding deterrent than
346 20-hydroxyecdysone, and 30-fold more potent than cyasterone (Kubo 1993).
347 In our study, we only used a few commercial standards because they are very
348 expensive and are not feasible as a control treatment. We could not use them at the same
349 concentrations as the applied treatment. Therefore, we conducted an experiment with a
350 mixture of three commercial standards (ecdysterone, makisterone A and cyasterone) at a
351 maximum concentration of 100 ppm each, which is very low compared to the
352 concentration in the (crude leaf extract and phytoecdysteroid fraction treatments
353 (250,000 ppm). *S. littoralis* larvae were fed on castor bean leaf treated with 100 ppm of
354 the mixture for 8 days. No significant effect of the mixture on *S. littoralis* larvae was seen.
355 Tanaka (1995) reported altered epidermal sensitivity to 20-hydroxyecdysone at 300
356 ppm ecdysone, higher than the standard concentration used in our study.
357 Based on the results, phytoecdysteroids may affect insects by interfering with their
358 developmental stages, especially during metamorphosis (Chou and Lu 1980). In the

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359 present study, we observed suppressed pupation of *S. littoralis* due to the reduction in
360 LWG; the larvae did not reach the threshold weight for pupation and they died because
361 they could not complete their life cycle. The histological observations of the gut showed
362 that *S. littoralis* is very sensitive to *A. iva* crude leaf extract and the mixture of the three
363 fractionated phytoecdysteroids (20-hydroxyecdysone, makisterone A and cyasterone).
364 The larval gut cells showed histolysis with clear signs of apoptosis. The gut epithelium
365 showed massive deterioration, there was destruction of the microvilli of the columnar
366 cells, and formation of vacuoles. In smaller larvae, mortality occurred during molting
367 between instars, whereas in bigger larvae, most mortality was at the prepupal stage. Our
368 results of gut cell destruction support the notion that the effect on pupation could be a
369 consequence of disruptions in hormonal balance effected by internal levels of ecdysone.
370 External ecdysteroid detoxification is one of the main ways in which insects overcome the
371 toxic effects of these compounds. The transition from one stage in ovarian development
372 to another, such as from previtellogenesis to vitellogenesis and then chorionogenesis, is
373 governed by the actions of several pathways that respond to different titers of 20-
374 hydroxyecdysone (Swevers and Iatrou 2003).

375 Feeding on phytoecdysteroids such as ecdysterone, polypodine B and
376 ponasterone A induces ecdysial failure associated with the appearance of larvae
377 having two head capsules and developmental anomalies during metamorphosis in
378 *Acrolepiopsis assectella* (Arnault and Slama 1986). Since the reduced growth rate (Figs. 3,
379 4) suggests that larvae are adversely affected by ingestion of the crude leaf extract and
380 of the mixture of three phytoecdysteroids from *A. iva*, we assume that the effect observed
381 on LWG and RGR reflects another possible mode of action of phytoecdysteroids.
382 Abnormal gut development can lead to reduced LWG, leading to mortality. In the present
383 study, disruptions in *S. littoralis* gut morphology and disappearance of microfilament
384 structures in actin (Fig. 6) could be a consequence of the phytoecdysteroid titers in *A. iva*
385 crude leaf extract. Actin microfilaments in particular have been associated with the
386 rounding and loss of adhesion that frequently occur with viral infection or
387 transformation in response to secondary metabolites (Carley et al. 1981; Meyer et
388 al. 1981), with the intracellular transport of viral structural proteins and viral
389 particles (Bohn et al. 1986), with the budding process of many enveloped viruses

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390 (Mortara and Koch 1989), and with the assembly of virions in the cytoplasm
391 (Jackson and Bellett 1989) and in the nucleus (Wang and Goldberg 1976).
392 In conclusion, our data suggest that the phytoecdysteroids and clerodanes of *A.*
393 *iva* may be useful for the management of economically important insect pests such as *S.*
394 *littoralis*, while reducing the risks to human health and the environment.
395

396 ## Acknowledgments

397

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400 fruitful advice and comments.
401

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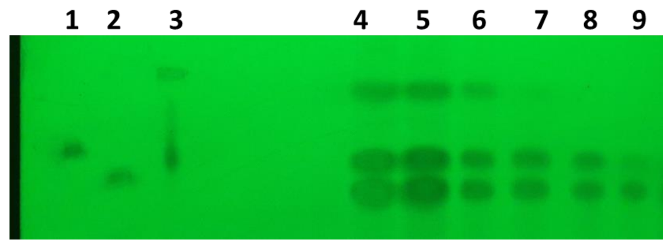

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554 Fig. 1 Identification of *A. iva* phytoecdysteroids by TLC. TLC plate shows the separation of
555 three phytoecdysteroids (20-hydroxyecdysone, makisterone A and cyasterone) (4–6),
556 commercial ecdysterone standards (1–3): makisterone (1), 20-hydroxyecdysone (2) and
557 cyasterone (3). Fractions (7–9) show the presence of only 20-hydroxyecdysone and
558 cyasterone. Fractions 4–6 were used in the bioassays
559

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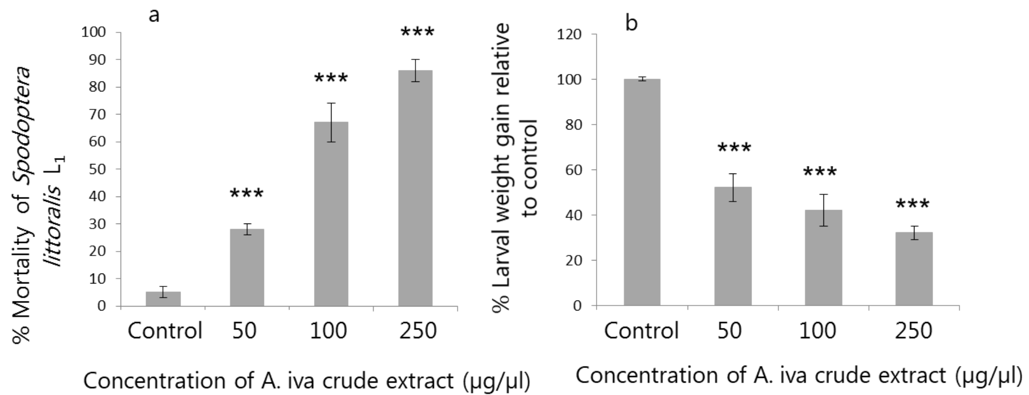

562

563 Fig. 2 Effect of different concentrations of *A. iva* crude leaf extract on *S. littoralis* first instar
564 (L_1) larval (n = 110) mortality (mean ± SEM) (a), and larval weight gain (%) (mean ± SEM)
565 of *S. littoralis* third instar (L_3) larvae (b). Asterisks above columns indicate significant
566 difference ($p \leq 0.05$) by t- test (t_{108} = 6.105, 4.308 and 3.220 for 50, 100 and 250 µg/µl,
567 respectively); $p < 0.001$ for all treatments, Levene's test $p = 0.326$ (a), and by repeated
568 measures ANOVA ($F_{3.104}$ =20.334, 17.246 and 13.007 for 50, 100 and 250 µg/µl,
569 respectively); $p < 0.001$, Mauchly's test $p = 0.152$ (b) between treatments and the control

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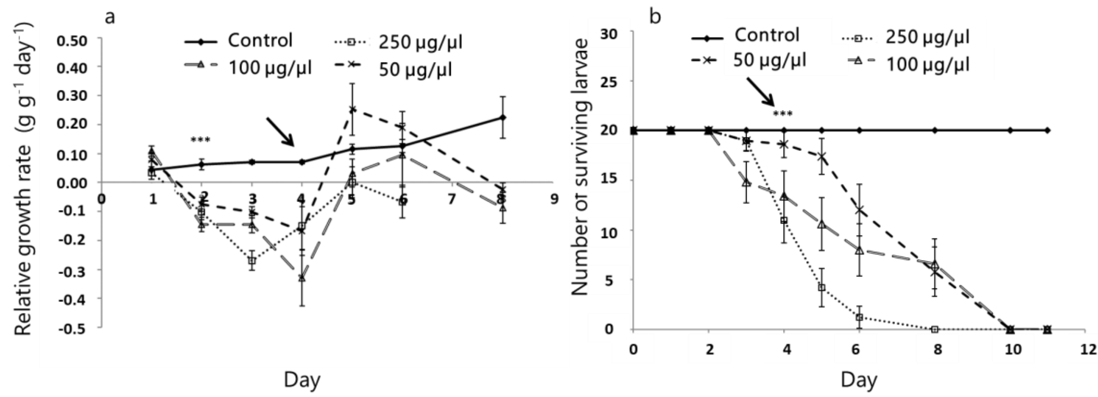

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573 Fig. 3 Effect of different concentrations of *A. iva* crude leaf extract on *S. littoralis* third

574 instar larval relative growth rate (mean ± SEM) (a) and survival (b); n = 20. Asterisks above

575 points indicate significant difference ($p \leq 0.05$) between treatments and the control

576

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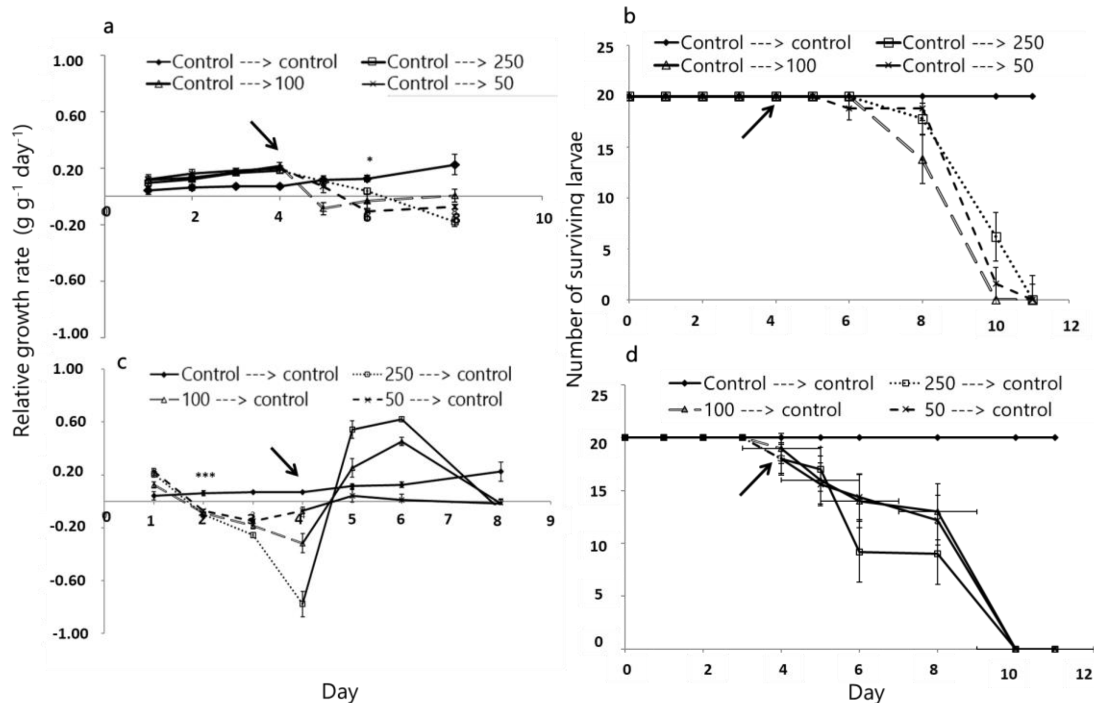

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578

579 Fig. 4 Effect of *A. iva* crude leaf extract on *S. littoralis* third instar larval relative
580 growth rate (mean ± SEM) and survival. Larval relative growth rate (a) and survival
581 (b) when fed on control leaf (treated with water) until day 4, and then fed on
582 leaves treated with crude leaf extract at three concentrations until the end of the
583 experiment (a). Relative growth rate (c) and survival (d) of the larvae after feeding
584 on crude leaf extract at three concentrations until day 4 and then control leaves
585 until the end of the experiment; n = 20. Asterisks above points indicate significant
586 difference ($p \leq 0.05$) between treatments and the control. Arrow points to day 4

587

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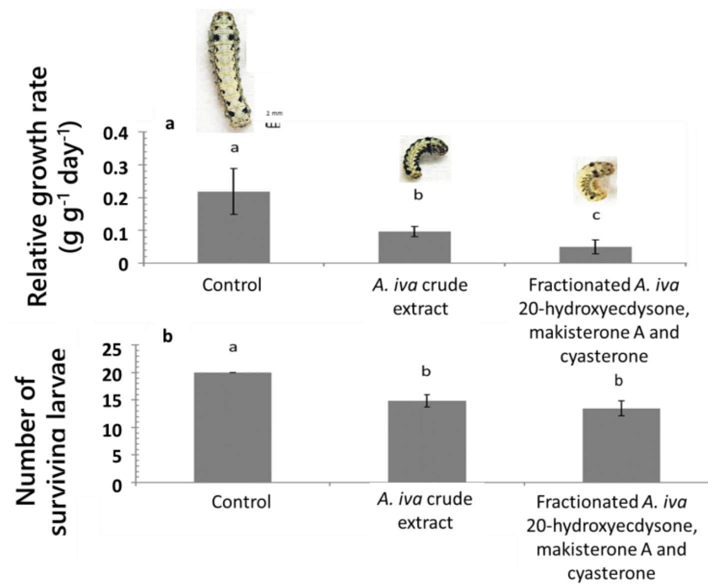

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591 Fig. 5 Effect of *A. iva* crude leaf extract (250 µg/µl), and of the three fractionated and
592 purified phytoecdysteroids (250 µg/µl) on *S. littoralis* third instar larval relative growth
593 rate (mean ± SEM) (a) and survival (b). The phytoecdysteroid fraction contained 20-
594 hydroxyecdysone, makisterone A and cyasterone; n = 20. Development of *S. littoralis*
595 larvae shown above the columns after 4 days feeding on control leaves, or leaves treated
596 with 250 µg/µl *A. iva* crude leaf extract or 250 µg/µl of the three fractionated
597 phytoecdysteroids. Different letters above columns indicate significant difference ($p \leq$
598 0.05) between treatments and the control

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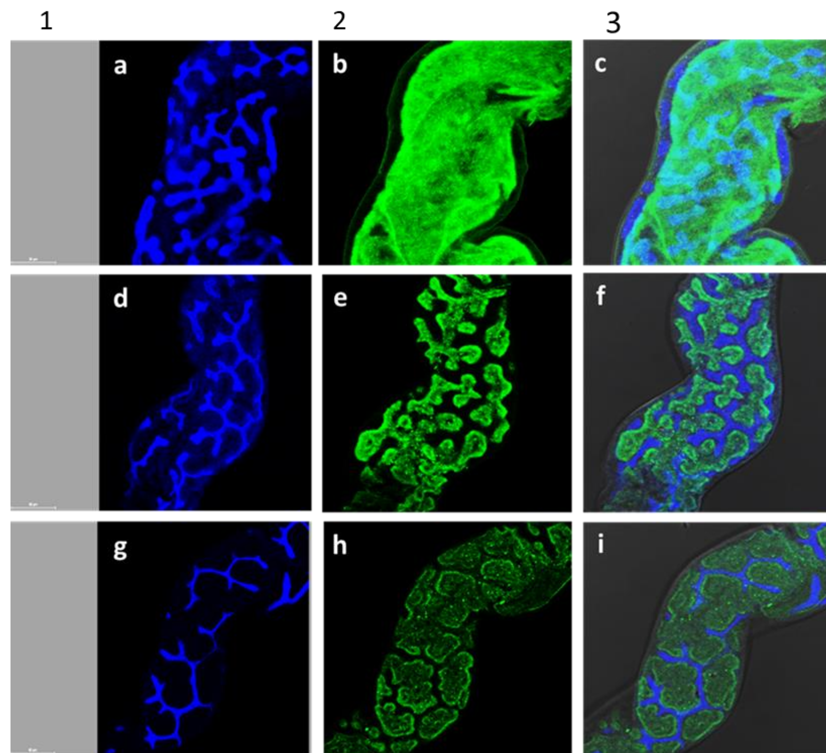

603

604 Fig. 6 Gut morphology of *S. littoralis* third instar larvae after feeding on treated or non-
605 treated leaves: control castor bean leaves treated with water (a–c), leaves treated with *A.*
606 *iva* crude leaf extract (250 μg/μl) (d–f), and leaves treated with 250 μg/μl of three
607 fractionated and purified phytoecdysteroids from *A. iva* leaf extract (20-hydroxyecdysone,
608 makisterone A and cyasterone) (g–i) for 8 days (60 μm, respectively). Blue: DAPI staining
609 of the nuclei under dark field (1); green: phalloidin staining of actin filaments under dark
610 field (2), and double DAPI staining of the nuclei and phalloidin staining of actin filaments
611 under dark field (3)

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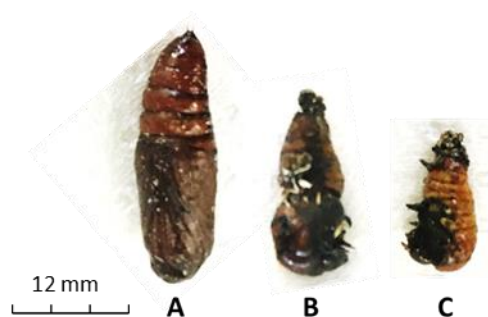

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615 Fig. 7 Metamorphosis of control and treated *S. littoralis* larvae. Pupation of *S. littoralis*
616 larvae after 15 days of exposure (feeding for 4 days) on *A. iva* crude leaf extract. Control
617 (treated with water) (a), 250 µg/µl *A. iva* crude leaf extract (b) and 250 µg/µl of three
618 fractionated and purified phytoecdysteroids from *A. iva* leaf extract fractions (20-
619 hydroxyecdysone, makisterone A and cyasterone) (c). Deficient development of pupation
620 in (b) and (c) is due to lower levels of the ecdysteroids responsible for molting
621
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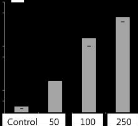
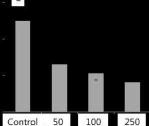

a
Control
50
100
250
Concentration of A. iva crude extract (µg/µl)


b
% Larval weight gain relative to control
Control
50
100
250
Concentration of A. iva crude extract (µg/µl)

a

Control
250 μg/μl
100 μg/μl
50 μg/μl

***

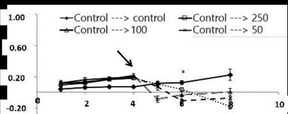
0.50
0.40
0.30
0.20
0.10
0.00
-0.1
-0.2
-0.3
-0.4
-0.5

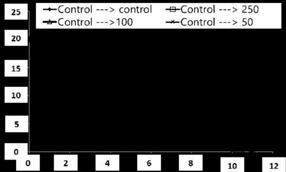
0 1 2 3 4 5 6 7 8 9

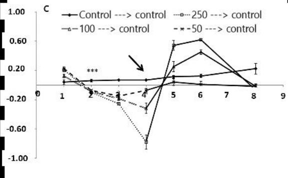
Control
250 μg/μl
50 μg/μl
100 μg/μl

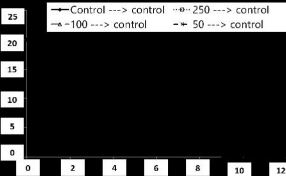
30
25
20
15
10
5
0

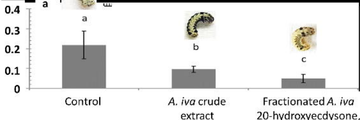
0 2 4 6 8 10 12

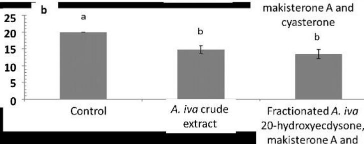

Control ---> control
Control ---> 250
Control --->100
Control ---> 50
1.00
0.60
0.20
-0.20
-0.60
-1.00
0
2
4
6
8
10
*

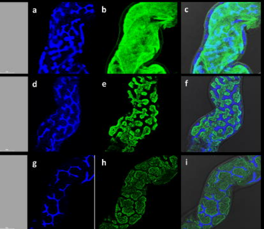

Control ---> control
Control ---> 250
Control --->100
Control ---> 50
25
20
15
10
5
0
0
2
4
6
8
10
12


C
Control ---> control
250 ---> control
100 ---> control
50 ---> control
1.00
0.60
0.20
-0.20
-0.60
-1.00
***
0
1
2
3
4
5
6
7
8
9


Control ---> control
250 ---> control
100 ---> control
50 ---> control
25
20
15
10
5
0
0
2
4
6
8
10
12


a
Relative growth rate
(g g⁻¹ day⁻¹)
0.4
0.3
0.2
0.1
0
a
b
c
Control
A. iva crude extract
Fractionated A. iva 20-hydroxyecdysone, makisterone A and cyasterone


b
Number of surviving larvae
25
20
15
10
5
0
a
b
b
Control
A. iva crude extract
Fractionated A. iva 20-hydroxyecdysone, makisterone A and


a
b
c
d
e
f
g
h
i