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Per os efficacy of *Ajuga* extracts against sucking insects

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Abstract: We studied the efficacy of water-soluble extracts from four *Ajuga* spp on the post-embryonic development of two exopterygota (sucking insect) species. To allow comparison between different *Ajuga* species, results are expressed in terms of quantity of plant extracted per litre of test solution. Crude methanolic extracts of all *Ajuga* plants tested, with the exception of *A genevensis*, showed considerable *per os* efficacy against larvae of both *Dysdercus cingulatus* F and *Acyrthosiphon pisum* (Harris) even at 1 g litre^{-1}. In the aphid tests the order of efficacy was *A bracteosa* Wallich ex Benth > *A chamaepitys* Schreber > *A reptans* L > *A genevensis* L. On *D cingulatus* the order of efficacy was: *A reptans* > *A bracteosa* > *A chamaepitys* > *A genevensis*. Extracts were fractionated on SepPak using a range of methanol/water mixtures. Results are expressed in terms of the initial weight of plant extracted. The 100% methanolic fraction of *A chamaepitys* was highly effective on *A pisum* (100% mortality at 1 g litre^{-1}) and less effective on *D cingulatus* (about 60% mortality at 5 g litre^{-1}). The entire 60 methanol + 40 water fraction was effective against test insects but showed different efficacies according to test species and concentration applied. 20-Hydroxyecdysone (20E), cyasterone (Cy) and ajugalactone (Ajl) were identified in the fractions from all *Ajuga* species, but the remaining phytoecdysteroid profile was quite different between *Ajuga* species. Capitasterone (Cap) and 28-*epi*-sengosterone (5Cy28′) were found only in *A reptans*, makisterone A (MaA) and 29-norcyasterone (29NCy) were only in *A chamaepitys*, while 22-acetylcyasterone (Cy22A), 3-*epi*-cyasterone (Cy′) and 3-*epi*-22-acetylcyasterone (Cy′22A) were only in *A bracteosa*. The total amount of phytoecdysteroids was 2053 mg kg^{-1} for *A bracteosa*, 1892 mg kg^{-1} for *A reptans* and 95 mg kg^{-1} for *A chamaepitys*.

Keywords: *Acyrthosiphon pisum*; *Dysdercus cingulatus*; *Ajuga* spp; phytoecdysteroids; bioassay; developmental disorders

1 INTRODUCTION

Among higher plants the *Ajuga* spp (Labiatae) are known to produce considerable amounts of ecdysteroids, neo-clerodane diterpenes and other secondary plant metabolites having both toxic and IDRD (insect developmental and reproduction disrupter) activity against insects.[1] Considerable numbers of phytoecdysteroids have been found and identified from different *Ajuga* species.[1–6] The phytoecdysteroid profile varies in amount and composition not only between plant species but also according to plant organs and season,[3] and can differ within one species according to its origin and the habitat where it is growing.[7] In the species of the *Ajuga* genus the most abundant phytoecdysteroids are 20-hydroxyecdysone (20E), cyasterone (Cy) and the most characteristic ajugalactone (Ajl). Among minor components polypodine-B (5,20E), sengosterone (5Cy), 29-norsengosterone (29N5Cy), 29-norcyasterone (29NCy) and various 22-dehydro- and acetylated derivates are known.[4,5]

Non-adapted insects are sensitive to ingested phytoecdysteroids[7–10] but Exopterygotes with sucking mouthparts are less targeted.[11,12] It was found that aphids do not accept *Ajuga* plants at the blooming period. Adults of whitefly (*Trialeurodes vaporariorum* Westwood) accepted it but could not colonise on *Ajuga* plants.[11,13] The polar methanolic SepPak fractions from extracts of *A reptans* L var *reptans* showed high activity against *Dysdercus cingulatus* F while apolar fractions were also active, but to a lesser degree.[14]

Our aim was to study the toxicity and IDRD activity of water-soluble parts of the crude and fractionated extracts from different *Ajuga* spp via oral uptake and

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to determine the main components responsible for this activity.

2 MATERIALS AND METHODS

2.1 Plant materials and plant extracts

The *Ajuga* plants (*A chamaepitys* Schreber, *A genevensis* L, *A reptans* L var *reptans*) were grown in field of the 'PPI HAS' experimental facility or in greenhouse (*A bracteosa* Wallich ex Benth). The plants (whole plant without roots) were collected in their blooming stage, and then air-dried under laboratory conditions.

Dried plant material (10 g) was ground and sonicated with methanol (2 × 190 ml) for 5 min. The extracts were combined and the solvent was evaporated at 50 °C under vacuum (Büchi Waterbath, B-480, Switzerland). Then the residue was dissolved in methanol (10 ml) and stored at −50 °C. The crude extract prepared this way contained the extract from 1 g of dried plant material ml^{-1}. It was tested in the first step of experiments to select among different *Ajuga* species according to their activity on test insects. Only the crude extracts showing at least 70% activity in these tests at the limits of 2.5 g $litre^{-1}$ against *D cingulatus* and 5 g $litre^{-1}$ against *A pisum* were further cleaned and fractionated as follows.

Crude extract (10 ml) was prepared from dried plant material (10 g) as above, water (10 ml) was added and centrifuged. Precipitate was removed and the solution was evaporated to dryness. The dry extract was dissolved in methanol (10 ml), and acetone (10 ml) was added to the solution. The precipitate was removed again by centrifugation. The methanol–acetone solution was evaporated to dryness and the residue was dissolved in water + methanol (1 + 1 by volume; 10 ml). To remove colouring material and apolar contaminants the mixture was partitioned against cyclohexane (30 ml). The aqueous methanol phase was dried under a stream of nitrogen, and the residue dissolved in distilled water (10 ml), sonicated for 5 min and fractionated on SepPak® Plus C_{18} cartridge (Waters Part No: 20 515) previously conditioned by vacuum filtration of methanol (10 ml) followed by water (10 ml). The extract was loaded onto the cartridge and eluted successively with 10 ml each of 10 + 90, 60 + 40 and 100 + 0 (by volume) methanol + water. The fractions were evaporated under vacuum (SpeedVac—Savant ISS 100, US) and stored at −50 °C. Samples for biological assays and further determinations were made by the same way from 10 g of dried plant material. The potentially active ingredients of the fractions were identified using HPLC.

2.2 Chemical analysis

The fractions from the SepPak C_{18} column were examined by HPLC on a Lichrocart 125 × 4 mm column, (packed with Lichrospher 100 RP-18, 5 µm) using a flow rate of 1.2 ml min^{-1} at 55 °C. The eluent was aqueous isopropanol (64 ml $litre^{-1}$) 0–30 min, followed by a gradient to 144 ml isopropanol kg^{-1} over 30–50 min and a final elution for 50–70 min with this eluent. Chromatograms were monitored at 242 nm and recorded with a data module,[3] and other physical properties were determined.[6] The whole procedure of chemical analysis was conducted in Barcelona at CID-CSIC. The 20E, MaA and 5,20E standards were obtained from the University of Szeged, Department of Pharmacognosy. Other standards were extracted from *A reptans* plants in Barcelona at CID-CSIC. All the extracted standards were fully characterized through their ^{1}H and ^{13}C NMR spectra and other physical properties.

2.3 Insect materials

Stocks of *A pisum* were reared from a field-collected population in 1996 and maintained continuously on broad bean (*Vicia faba* L) seedlings under greenhouse conditions in PPI HAS. The offspring of adult females, born on artificial diet, were used in tests.

Dysdercus cingulatus eggs were obtained from the Institute of Entomology, Czech Academy of Sciences and our stock rearing is based on it. The bugs are reared on cottonseeds in the laboratory at 26 (±2) °C and long day (16:8 h light:dark) conditions. The first-instar larvae of bugs were used in the experiments.

2.4 Experiments

Because of the differences in the amounts of materials extracted from the different species, results are expressed in terms of the amount of plant material extracted per unit volume of test solution. Thus, the stock solution contained the extract of 1 g of dry plant material ml^{-1} methanol, and this would be referred to as 1000 g $litre^{-1}$. For biological assays, the amount of stock solution required for a range of 1–10 g $litre^{-1}$ was dried under a stream of nitrogen and the residues were redissolved in water (10 ml). The mixture was sonicated for 5 min and incorporated in drinking water for *D cingulatus* larvae and in artificial diet for *A pisum* at 1, 2.5 and 5 g $litre^{-1}$.

The SepPak fractions (10 + 90, 60 + 40 and 100 + 0 methanol + water) were tested in similar way over a range of concentrations (mg kg^{-1}) in drinking water and artificial diet. We applied 20E as standard at 1 mg kg^{-1} concentration.

2.4.1 Dysdercus cingulatus

Twenty first-instar larvae (L_1) per replicate were collected and put into a plastic cup with 1.5 g of cottonseed as food and 4 ml of drinking water in a glass tube plugged with cotton wool. Development of larvae was monitored until the moult to adult. Every second day during the experiment the number of dead animals was recorded and the disorders were examined by stereomicroscope. The experiments were conducted under rearing conditions.

2.4.2 Acyrthosiphon pisum

Aphid-specific artificial diet was prepared[15,16] and injected between two Parafilm-M® layers.[17] The latter

were thinned by hand so that the aphid larvae could suck out the artificial diet through them. Five to six apterous virginoparae females of *A pisum* were collected and put into a plastic ring-cage (2.5 cm diameter × 0.5 cm high) with fine plane net on the bottom. This cage was covered by parafilm layers on the top prepared with diet as described above. Adults were removed after 24 h and their offspring were left inside the cages for a further day. The survivors (ten first-instar larvae per replicate) were used for experiments. Fresh diet was provided for the aphids every second day during experiments. The development of the larvae was monitored until the moult to adult. The number of dead insects was recorded every day and the disorders were collected and examined by stereomicroscope.

Percentage mortality was calculated by means of the Henderson–Tilton formula from the raw data obtained during the test procedures. The mean value of the mortalities so obtained is presented in the Results.

3 RESULTS AND DISCUSSION

3.1 Ecdysteroid profiles

From the 60 + 40 methanol + water SepPak fractions of all *Ajuga* species we have identified 20E, Cy and Ajl. The phytoecdysteroid profile was quite different between *Ajuga* species (Fig 1).

Ajuga bracteosa contained in a total of 2053 mg kg^{-1} phytoecdysteroids: 30 mg kg^{-1} ajugasterone B (AjB), 133 mg kg^{-1} 5Cy, 64 mg kg^{-1} 5,20E, 397 mg kg^{-1} 22-acetylcyasterone (Cy22A), 30 mg kg^{-1} 3-*epi*-cyasterone (Cy′), 237 mg kg^{-1} 3-*epi*-22-acetylcyasterone (Cy′22A), 574 mg kg^{-1} Ajl, 509 mg kg^{-1} Cy and 79 mg kg^{-1} 20E. For *A reptans* a total of 1892 mg kg^{-1} of phytoecdysteroids was found, comprising 71 mg kg^{-1} capitasterone (Cap), 122 mg kg^{-1} 28-*epi*-sengosterone (5Cy28′), 102 mg kg^{-1} 5Cy, 174 mg kg^{-1} 5,20E, 194 mg kg^{-1} AjB, 215 mg kg^{-1} Ajl, 185 mg kg^{-1} Cy and 829 mg kg^{-1} 20E. The phytoecdysteroid content was considerably less in *A chamaepitys*, a total of only 95 mg kg^{-1}: in addition to 25 mg kg^{-1} Ajl, 7 mg kg^{-1} Cy and 3 mg kg^{-1} 20E, we found 32 mg kg^{-1} makisterone A (MaA) and 28 mg kg^{-1} 29NCy.

The main phytoecdysteroid components of *A reptans* reared in Spain have been found to be Ajl, Cy, 29NCy and 29N5Cy, comprising on average 92% of the total phytoecdysteroid content.[3] A different ratio of phytoecdysteroids was earlier reported from Hungarian stocks of *Ajuga* species: 25 mg kg^{-1} Ajl, 30 mg kg^{-1} Cy, 8 mg kg^{-1} 29 NCy, along with 42 mg kg^{-1} 20E from *A reptans* var *reptans*, and 14–21 mg kg^{-1} Ajl together with small amount (2–9 mg kg^{-1}) of 20E from *A chamaepitys*.[8]

3.2 Effects on *Acyrthosiphon pisum*

The screening of *Ajuga* crude extracts on *A pisum* revealed the order of efficacy as follows: *A bracteosa* > *A chamaepitys* > *A reptans* > *A genevensis* (Fig 2).

The extracts from *A bracteosa* and *A chamaepitys* caused high (80–100%) mortality even at 1 g litre^{-1} concentration (data not shown), while the activity of *A genevensis* did not reach the limit (70% final mortality at 5 g litre^{-1}) for further testing. The fractionated crude extracts showed different activity. Among the 10 + 90, 60 + 40 and 100 + 0 methanol + water SepPak fractions the 60 + 40 fráction (mainly containing phytoecdysteroids) showed activity similar to the crude extracts against aphids and the two other fractions had no effect. The only exception was the 100% methanol fraction of *A chamaepitys*, which produced 100% mortality at day 10 (Table 1). This less polar fraction contains neo-clerodanes from *A reptans* var *reptans* with high activity on mosquito (*Aedes aegypti* L) larvae but less or not active on *D cingulatus*.[14] This is the first time that activity of this fraction against aphids has been described, and it seems to be unusual, indicating the presence of new, less polar compound(s) with high activity during the last moulting process on *A pisum*.

Whitefly (*T vaporariorum*) larvae have a feeding habit similar to aphids (ie they feed on phloem). The

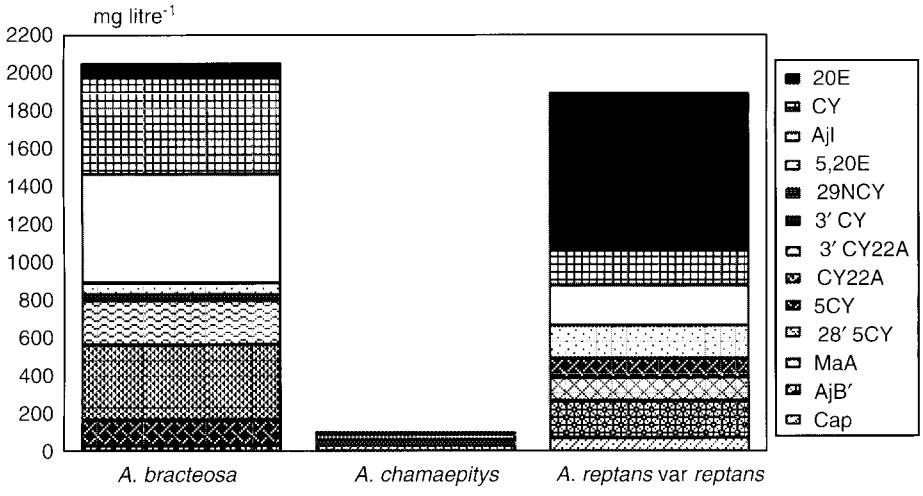

Figure 1. Phytoecdysteroid profile of tested *Ajuga* species.

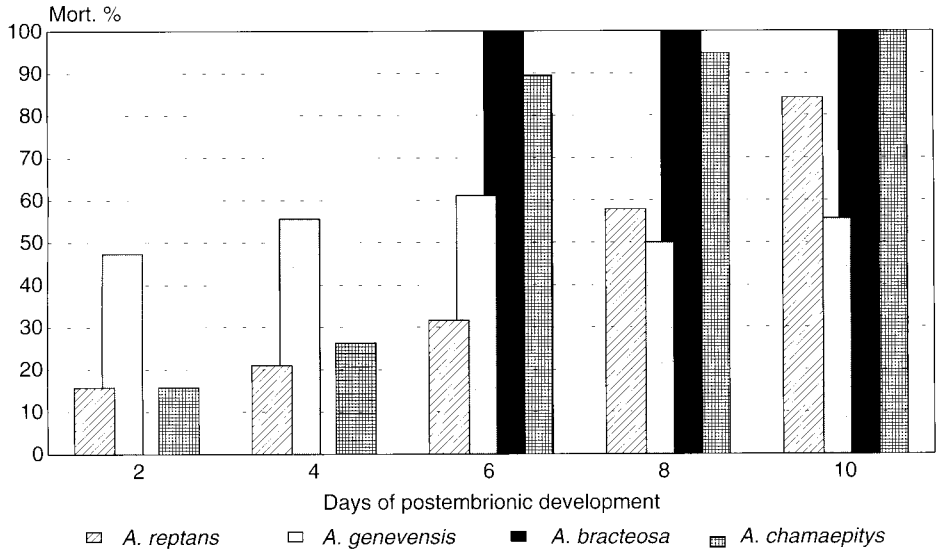

Figure 2. Effect of *Ajuga* crude extracts (5 g kg^{-1}) on *Acyrthosiphon pisum* larvae during post-embryonic development.

Table 1. Effect of SepPak fractions at 1 g kg ^{1} on *Acyrthosiphon pisum*

Plant species	Fraction methanol +water	Mortality at day 4 (%) (±SE)[a]	Mortality at day 10 (%) (±SE)[a]
A reptans	60 + 40	21.1 (±5.3)	52.6 (±15.8)
A bracteosa	60 + 40	37.5 (±12.5)	100
A chamaepitys	60 + 40	26.3 (±10.5)	84.2 (±5.2)
A chamaepitys	100 + 0	10.0 (±5.0)	100
20E (1 mg kg^{-1})		50.0 (±10.0)	55.6 (±9.1)

[a] Corrected by Henderson–Tilton formula. Data are reported as means (±SE) of four replicates.

Figure 3. Head of *Acyrthosiphon pisum* L_4 larvae with double cuticle at the basal segments of antennae.

effect of phytoecdysteroids in a 30 mg kg^{-1} aqueous solution applied through the culture medium has been tested on whitefly. It was found that 29N5Cy and Ajl had a strong inhibitory effect on first-instar larvae of whitefly, 29NCy and 5,20E showed slight effects, while 20E was almost inactive.[13] In our experiments, 20E showed high activity on early instars of *A pisum* even at 1 mg kg^{-1} concentration. The dynamics of aphid mortalities caused by SepPak 60 + 40 methanol + water fractions show low amounts of active compounds or slow beginning of activity, the compounds behaving differently from 20E used as standard (Table 1). Among the dead aphids during the moulting process the most typical abnormality was the double cuticle (Fig 3). Poorly developed or curled wings were also observed in some adult survivors. These symptoms are similar to those observed earlier when RH 5849, a non-steroidal ecdysteroid agonist, was applied topically to parasitised aphid (*Myzus persicae* Sulz) larvae.[18] This indicate that ingested phytoecdysteroids can provoke similar symptoms and may act on the moulting process of *A pisum*. In certain cases a high number of secondary rhinaria (olfactory sensilla) on the third segment of antennae occurred in wingless (apterous) adult females, such as is typical for winged (alate) forms. Apterous females of *A pisum* have 2–3 rhinaria on the third segment of the antennae, while alates have 15–17 rhinaria on the same segment. We have found disorders in apterous adults with an abnormally high number (7–11) of rhinaria on the third segment (Fig 4), which is more than that for a normal apterous adult but less than for a normal alate.

3.3 Effects on *Dysdercus cingulatus*

In the experiments with *D cingulatus*, crude extracts of the same *Ajuga* species showed high activity, as was found in aphid tests. However, in contrast to the aphid test, *A reptans* showed similar activity to *A bracteosa* and *A chamaepitys* at 2.5 and 5 g litre^{-1}. Most survivors and

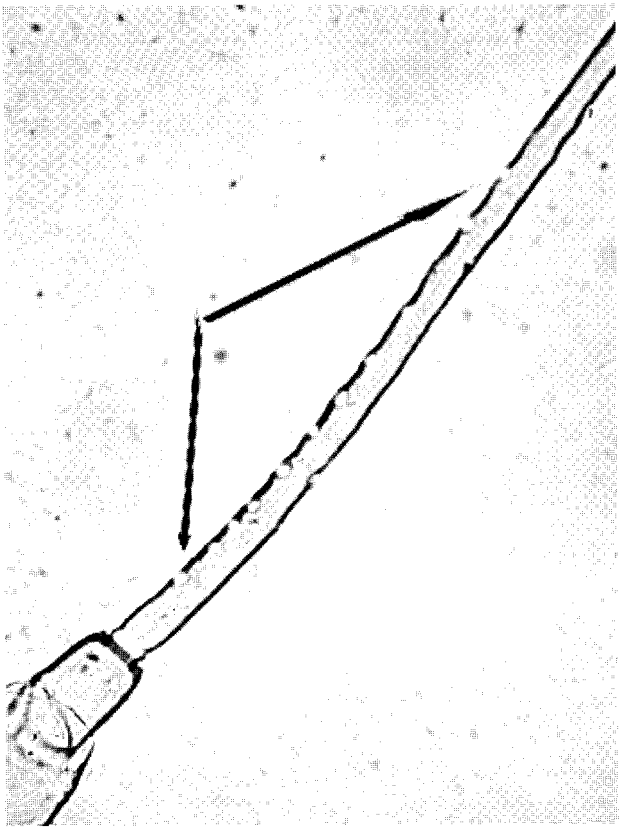

Figure 4. Third segment of antenna of adult apterous *Acyrthosiphon pisum* with 11 rhinaria.

least moulting disorders occurred in the treatments with *A genevensis*. The efficacy of *A genevensis* crude extract was reduced dramatically at 1.0 g litre^{-1}, and it was therefore excluded from further testing. The effects were dose-dependent in each *Ajuga* extract, and, beside larval mortality, various moulting disorders were observed among adults, especially at lower concentrations (Fig 5). The moulting abnormalities showed symptoms similar to those found with the azole-analogue moulting inhibitors.[19]

The effect of SepPak fractions is shown in Table 2. We used a higher concentration (5 g kg^{-1}) of SepPak fractions (SP) for testing than in the aphid test because the ecdysteroids were incorporated into the occasionally used drinking water of cotton bugs while aphids feed continuously on the treated artificial diet. The 60 + 40 methanol + water fractions of all *Ajuga* species showed similar final efficacy but different mortality dynamics. The 100% methanol extract of *A chamaepitys* showed a remarkable effect on the early instars of *D cingulatus* but this remained at a low level until the end (21st day) of the experimental period. A similar low efficacy of apolar fractions has been observed in other experiments with *A reptans*.[14]

4 CONCLUSIONS

The high biological activity of *A chamaepitys* extracts is associated with a nearly twenty times lower amount of phytoecdysteroids in its 60 + 40 methanol + water fraction than is found in other *Ajuga* extracts. This indicates that other compounds than those identified here are responsible for the activity. Among 100% methanol fractions, only that from *A chamaepitys* was active against both species tested. The apolar neo-clerodanes, ajugapitins and chamaepitin have also been detected in *A chamaepitys*.[20] The neo-clerodanes are known as diterpenoids with insect-antifeedant activity, so we suggest that neo-clerodanes from *A chamaepitys* cause the high insecticidal activity against *A pisum* and lesser activity against *D cingulatus*.

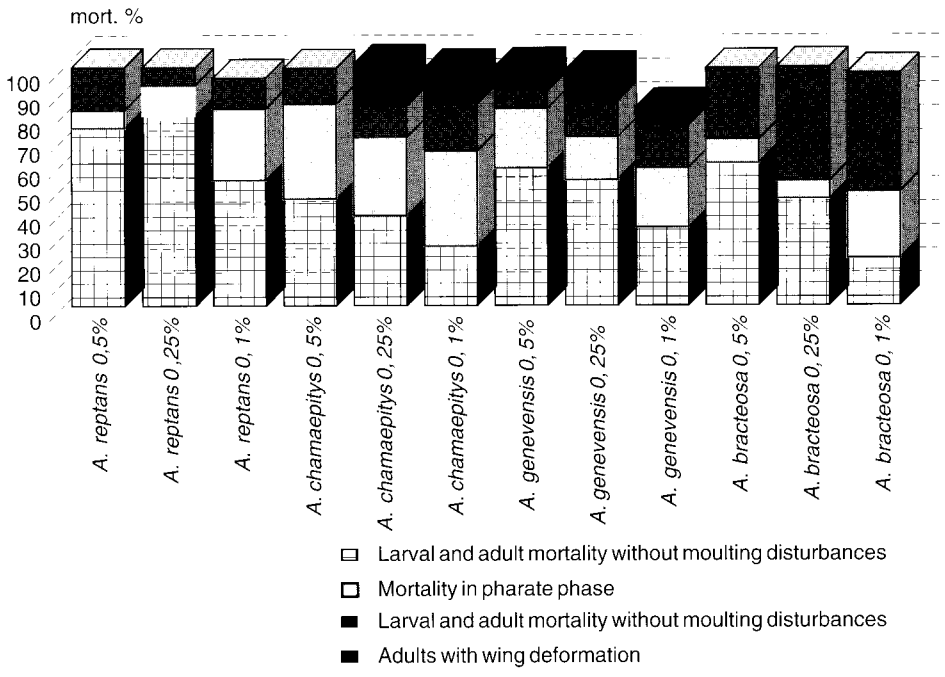

Figure 5. Effect of *Ajuga* crude extracts during post-embryonic development of *Dysdercus cingulatus*.

Table 2. Effect of SepPak fractions at 5 g kg^{-1} on *Dysdercus cingulatus*

Plant species	Fraction methanol + water	Mortality at day 4 (%) (±SE)[a]	Mortality at day 10 (%) (±SE)[a]	Mortality at day 21 (%) (±SE)[a]
A reptans	60 + 40	16.1 (±6.1)	100	100
A bracteosa	60 + 40	34.8 (±13.1)	88.9 (±11.1)	100
A chamaepitys	60 + 40	16.2 (±8.1)	65.0 (±15.0)	88.9 (±11.1)
A chamaepitys	100 + 0	29.7 (±5.4)	58.6 (±13.8)	59.4 (±14.5)
20E (1 mg kg^{-1})		8.7 (±4.3)	63.2 (±5.3)	66.7 (±6.7)

[a] Corrected by Henderson–Tilton formula. Data are reported as means (±SE) of four replicates.

Both *A bracteosa* and *A reptans* have a considerable (*ca* 2000 mg kg^{-1}) phytoecdysteroid content, but in different ratios. While in *A reptans* 20E, AjI and AjB are the main components, in *A bracteosa* the amount of AjI, Cy, Cy22A and 3′Cy22A was above 200 mg kg^{-1}. Comparison with the efficacy of the internal standard (1 mg kg^{-1} 20E) suggests that AjI and different cyasterone-based compounds are more active against *A pisum* than is 20E. In the case of *D cingulatus* AjI, AjB and 20E may be responsible the high activity of the extracts. To determine the real activity of different components further testing is required with each pure compound rather than the mixtures used here.

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