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Inhibition of juvenile hormone biosynthesis in *Gryllus bimaculatus* by *Glycosmis pentaphylla* leaf compounds

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Abstract

The EtOAc fraction of *Glycosmis pentaphylla* leaf extract inhibits the juvenile hormone III-biosynthesis in vitro of corpora allata from 3 day old females of the field cricket *Gryllus bimaculatus*. The bioactive compound which is responsible for this activity was identified as the quinazolone alkaloid arborine. This alkaloid showed also a larvicidal activity against the mosquito *Culex quinquefasciatus*.

Keywords: Inhibition of juvenile hormone biosynthesis in crickets; *Gryllus bimaculatus*; Gryllidae; *Glycosmis pentaphylla*; Rutaceae; Arborine; Mosquito larvicide

1. Introduction

Consequent to the realization of the inimical effects of most synthetic insecticides and development of genetic resistance to them by insect pests, efforts have gained impetus in recent times to explore ecofriendly methods of insect control. A variety of plant products have been screened for their efficacy to control insect pests and vectors. Insecticidal activity of plant derived compounds such as nicotine, rotenoids, pyrethrins, precocenes, brassinosteroids and azadirachtin has been evaluated and a few compounds have been exploited commercially (Jacobson & Crosby, 1971; Berenbaum, 1989; Brooks & McCaffery, 1990; Duke, 1990; Mordue & Blackwell, 1993).

Manipulation of endocrine control of development, growth, metamorphosis and reproduction is one of the mechanisms of action of these secondary plant substances (Richter & Koolman, 1991; Couillaud & Peypelut, 1995). A few plant species such as *Calophyllum inophyllum* Linn. (Clusiaceae), *Glycosmis pentaphylla* Retz. (Rutaceae), *Rhinacanthus nasutus* Kurz. (Acanthaceae), *Solanum surratense* Burm. (Solanaceae) and *Solanum trilobatum* Linn. (Solanaceae) have been recently screened for their insecticidal activity in our laboratories (Pushpalatha, 1997; Muthukrishnan, Pushpalatha, & Kasthuribai, 1998). Exposure to partially purified leaf extract of *G. pentaphylla* in the medium extended the larval duration, inflicted very high larval mortality and induced developmental deformities in larvae of mosquitoes, *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Pushpalatha & Muthukrishnan, 1995). Oral administration of *G. pentaphylla* leaf extract to the penultimate and final instar larvae of the castor semilooper, *Achaea janata*, decreased the weight gain and inhibited larval–pupal and pupal–adult metamorphosis, respectively (Muthukrishnan & Ananthagowri, 1994). Besides, leaves of this plant are used to keep insects away from sweets and other edible items by natives in India, South Africa and Australia (Chopra, Nayar, & Chopra, 1956).

In order to identify the active principle in the leaf extract of *G. pentaphylla* and to identify the structure of at least one of the effective compounds, the extract was further purified by HPLC techniques accompanied with an in vitro bioassay which measures the rate of

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Table 1
In vitro inhibition (%) of JH III-biosynthesis in corpora allata from 3 day old adult females of *G. bimaculatus* by various frs. of the purification procedure. Mean values of 10 determinations ± S.E.

(A) EtOAc fr. of leaf extract									
Concentration (%)	0.1	0.05	0.01						
Inhibition (%)	83.4 ± 9.4	75.4 ± 3.2	41.5 ± 22.3						
(B) Sep-Pak frs. at a concentration of 0.1%									
MeOH (%)	0	20	40	60	80	100			
Inhibition (%)	19.8 ± 6.7	24.9 ± 3.7	36.5 ± 8.4	70.9 ± 4.7	67.4 ± 13.1	56.1 ± 26.9			
(C) Frs. from the first HPLC run at a concentration of 1% and 0.1%, respectively									
Fr. (R_f, min)	21	22	23	24	25	26	27	28	31
Inhibition (%) at 1% concentration	61.7 ± 6.1	75.0 ± 4.7	74.8 ± 3.2	70.3 ± 7.8	65.8 ± 4.1	54.1 ± 24.6	59.7 ± 14.2	33.7 ± 9.7	17.6 ± 13.4
0.1% concentration	–	62.3 ± 17.3	38.5 ± 21.7	28.2 ± 26.4	34.5 ± 29.7	–	–	–	–

juvenile hormone biosynthesis in the corpora allata of insects.

2. Results and discussion

In our earlier studies (J. Muthukrishnan, unpublished results) it had been shown that EtOAc extracts of *G. pentaphylla* leaves significantly reduced hatchability of *C. quinquefasciatus* eggs, prolonged developmental duration of mosquito larvae, reduced the number of adults emerged and induced malformation during development and metamorphosis at sublethal concentrations (2–50 ppm). The EtOAc fr. of *G. pentaphylla* leaf extract also inhibits JH III-biosynthesis in vitro of corpora allata from 3 day old females of *Gryllus bimaculatus* at a concentration range of 0.1 to 0.01% (Table 1A). During the following purification procedure most of the JH-biosynthesis inhibiting material eluted from the Sep-Pak cartridges with 60 and 80% MeOH, respectively (Table 1B). Three successive HPLC separation steps (Table 1C shows the active fractions from the 1st HPLC run) led to a peak eluting at 13.7 min from the third column containing 2.78 mg of pure bioactive compound which was identified as arborine (2-benzyl-1-methyl-4(1H)-quinazolone, **1**). The structure of **1** was determined by means of EI-MS, ^{1}H NMR, ^{13}C NMR, APT and HMBC. **1** and 1-methyl-2-phenyl-4(1H)-quinazolone (**2**) were synthesized according to Naik, Amin, and Patel (1979).

Arborine (**1**) was tested on single corpora allata from 3 day old virgin females of *G. bimaculatus* at concentrations ranging from 10^{-7} to 10^{-2} M (Fig. 1). Fifty percent inhibition was achieved with ca. 4×10^{-5} M of **1**. In order to study reversibility of inhibition, after a 1.5 h of preincubation in medium with [^{14}C] methionine, single corpora allata were transferred to 20 µl of new radioactive medium and incubated for 2 h to establish the rates of synthesis by untreated glands; then the glands were transferred to medium

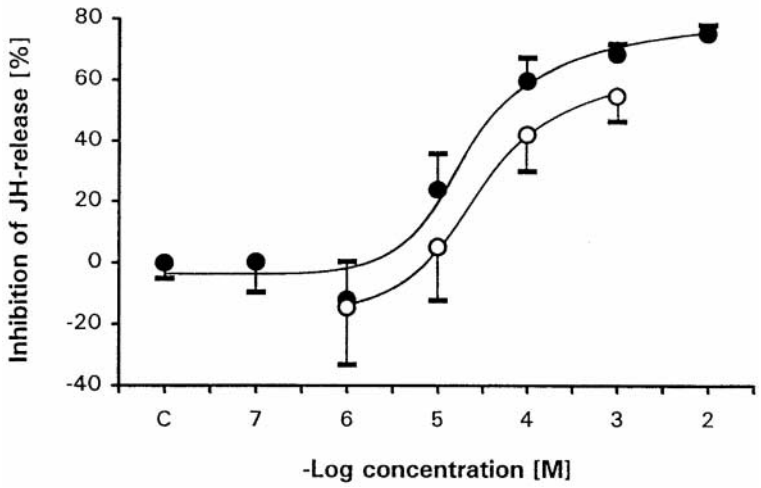

Fig. 1. Dose–response for inhibition of JH III-biosynthesis (release) by native arborine (**1**) (filled circles) and synthetic 1-methyl-2-phenyl-4(1H)-quinazolone (**2**) (open circles) for corpora allata from 3 day old adult virgin females of *Gryllus bimaculatus*. Percentage inhibition was calculated as [1 − (2nd incubation/1st incubation)] × 100. Data are mean values ± S.E. of 15 determinations. JH III-biosynthesis by untreated glands was ca. 35–40 pmol h^{-1} CA^{-1} in this experiment.

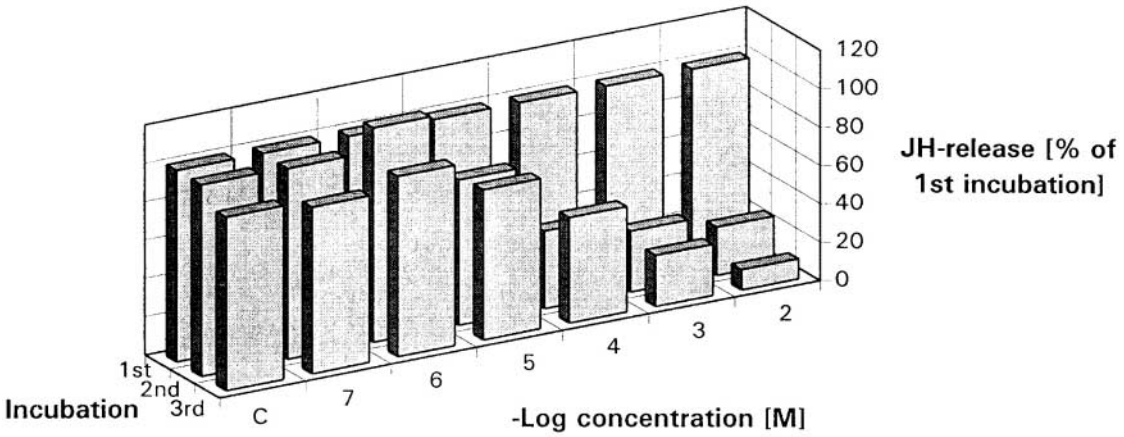

Fig. 2. Test for reversibility (third incubation) of inhibition of JH III-biosynthesis (release) by synthetic arborine (**1**) for corpora allata from 3 day old adult virgin females. The second incubation was carried out in medium containing 10^{-7}–10^{-2} M of arborine (**1**). Controls (C) were incubated in medium without addition during each incubation period. Rates of JH III-biosynthesis during the first incubation (between 35 and 45 pmol h^{-1} CA^{-1}) were set to 100%. Data are means of 10 determinations. For clarity, standard error is not shown.

containing 10^{-7}–10^{-2} M **1** and incubated for another 2 h. Finally, they were transferred to medium without addition of **1** and incubated again for 2 h. Up to a concentration of ca. 10^{-4} M, glands slightly recovered from inhibiting rates, but there was no reversibility of inhibition at higher concentrations (Fig. 2). With compound **2**, fifty percent inhibition of JH III-biosynthesis was achieved at around 8×10^{-4} M (Fig. 1).

In the present study we also followed the in vivo effect of HPLC frs on mosquito larval mortality. Results on the mortality of fourth instar larvae of *C. quinquefasciatus* are shown in Table 2. The bioassay in vivo revealed two peaks (A_1, A_2) eluting at 11–12 min from the first HPLC column (Table 2) as the most potent ones. Addition of 50 ppm of these frs to the rearing H_2O of larvae resulted in a mortality of 30–50% after 48 h of incubation. Compounds eluting in these fractions, however, were not effective in the JH-bioassay in vitro (less than 30% inhibition of JH-release).

Addition of 10^{-4}–10^{-5} M of **1** to H_2O resulted in only low mortality of mosquito larvae after 48 h (3–5%), but 83–100% of the larvae died after 216–240 h of incubation (Table 3). Similar results were obtained at 10^{-4}–10^{-5} M of **2** in H_2O. In both situations, none of the surviving larvae pupated. The results demonstrate that partially purified *G. pentaphylla* leaf extract, in addition to **1**, contains strong insecticide activity against dipteran species. Efforts to isolate and identify this mosquito larval development inhibiting compound(s) are in progress.

Tropical plants are of great promise from the point of view of discovering and developing new botanical insecticides (Berenbaum, 1989). Members of the families Meliaceae, Rutaceae, Asteraceae, Labiatae and Canellaceae seem to possess the most promising botanicals for use at the present (Jacobson, 1989). For successful application of plant compounds in insect biocontrol, it is obligatory to understand the mechanisms of their action in the target insects as well as the spectrum of insects affected by them.

The idea of using plant compounds in insect control which influence the hormonal control of insect development, metamorphosis and reproduction was advanced for the first time by Slama and Williams (1965). Hormone-mimetics and antihormones which

Table 2
In vivo effects of partially purified *G. pentaphylla* leaf extract against fourth instar larvae of *C. quinquefasciatus*. Results are expressed in % mortality. Each test was started with 20 freshly molted fourth instar larvae. Frs. from 1st HPLC run at a concentration of 50 ppm. C, control

	Fr.													
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	C
R_t (min)	11.08–11.40	11.85–12.27	16.05–16.30	16.72–16.90	17.58–17.72	17.72–18.10	18.28–18.43	18.57–18.73	18.97–19.33	19.45–19.62	19.97–20.25	20.70–20.98	28.50–28.75	
Mortality (%)														
after 24 h	40	20	20	15	5	0	0	0	0	0	0	10	5	2
after 48 h	50	30	35	20	5	0	10	5	10	15	0	10	15	3
after 72 h	65	50	40	30	15	20	25	20	20	40	25	45	40	5

Table 3
Treatment of *C. quinquefasciatus* fourth instar larvae with 10^{-4} and 10^{-5} M arborine (**1**) and 1-methyl-2-phenyl-4(1H)-quinazolone (**2**), respectively. Results are expressed in % mortality. Each test was started with 20 freshly molted fourth instar larvae

	Exposure time (h)									
	24	48	72	96	120	144	168	192	216	240
1										
10^{-5} M	2	3	10	–	–	60	68	82	83	88
10^{-4} M	5	5	15	–	–	72	77	82	83	100
2										
10^{-5} M	0	0	15	–	–	72	75	82	92	95
10^{-4} M	5	5	20	–	–	73	80	82	97	100
Control	0	0	0	7	–	–	12	13	15	–

disturb developmental processes in insects are commonly known as insect growth regulators (IGR). Meanwhile, several juvenoids and antijuvenile compounds have been identified which may be of practical importance in insect control (Cymborowski, 1992; Bowers, 1976). Recently, we demonstrated that female adult crickets quickly recovered from treatment in vivo with the antijuvenile hormone precocene, whereas the corpora allata were irreversibly inhibited by in vitro exposure to 10^{-3} M precocenes (Wennauer, Kassel, & Hoffmann, 1989). Less attempts have been made to find ecdysoids and antiecdysteroids (Cymborowski, 1992). The most efficient plant substance with molt inhibiting activity is azadirachtin, a triterpenoid plant limonoid from the neem tree (*Azadirachta indica*) (Mordue & Blackwell, 1993). Azadirachtin significantly inhibits the ecdysteroid biosynthesis in vitro in ovaries and abdominal integument from female adult crickets (Lorenz, Lenz, & Hoffmann, 1995).

About 60 alkaloids containing a quinazoline nucleus have been found in taxonomically unrelated families of higher plants, but also in microorganisms and in animals (Mothes, Schütte, & Luckner, 1985). Two quinazolones, glomerine (1,2-dimethyl-4(1H)-quinazolone) and homoglomerine (2-ethyl-1-methyl-4(1H)-quinazolone), have been identified in the European milliped *Glomeris marginata* (Schildknecht, Wenneis, Weiss, & Maschwitz, 1966). These quinazolones are closely related to the plant alkaloids **1** (Chakravarti, Chakravarti, & Chakravarti, 1953) and glycorine (1-methyl-4(1H)-quinazolone) which were formerly isolated from *Glycosmis arborea* (Pakrashi & Bhattacharyya, 1962). In the present study we isolated and identified **1** as a major compound from the EtOAc soluble fr. of a leaf extract of *G. pentaphylla*. This alkaloid was shown to inhibit juvenile hormone III-biosynthesis in vitro in the corpora allata from 3 day old virgin females of *G. bimaculatus* in a dose-dependent manner. The JH III-biosynthesis inhibiting effect remained preserved when the benzyl group attached in position 2 to the quinazolone structure of **1** had been replaced by a phenyl group in compound **2**. This study at first demonstrates the natural plant alkaloid **1** with an allatostatic potential. Studies on the capability of **1** to inhibit JH III-biosynthesis in the corpora allata from other (pest) insect species are in progress.

3. Experimental

3.1. General

EI-MS: Varian MAT-8500 at 70 eV. NMR: 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C), reverse probehead, δ in ppm, solvent (CD_3OD–pyridine-d_5 (1:1)). For the analyt. and semi-prep. HPLC a Jasco HPLC system equipped with two pumps and a variable wavelength monitor (detection at 220 nm) together with Super Pac Spherisorb ODS (250 × 4 mm, 25 μm, Pharmacia), Capcell Pak C1, SG 120 (125 × 8 mm, 5 μm, Shiseido) prepacked columns were used.

3.2. Isolation

Leaves of *G. pentaphylla* were collected in 1995 from fields in the provinces Kottayam and Kerala in India. *G. pentaphylla* was identified by Prof. D. Padmanaban, Madurai. A voucher specimen of the plant is deposited in the Herbarium of the Department of Environmental Biology, Madurai, India.

Dried powdered leaves (100 g) of *G. pentaphylla* were extracted with MeOH × 3. The methanolic residue was redissolved in MeOH and defatted with an equal volume of petrol in a separating funnel. The defatted methanolic fr. was evapd and the residue dissolved in EtOAc. The EtOAc soluble fr. was then washed with equal volume of H_2O, dried, weighed (4.58 g) and redissolved in 458 ml EtOAc to get a 1% soln. Aliquots (1 × 0.2 ml, 5 × 0.5 ml) were dried, redissolved in water and loaded onto Sep-Pak C_{18} cartridges (Waters) which had been rinsed with 10 ml of MeOH and equilibrated with 10 ml of H_2O. The Sep-

Pak cartridges were eluted with stepwise MeOH/H_2O gradients (MeOH%) of 0, 20, 40, 60, 80, 100, each 4 ml (×6). Further purification of the bioactive material from the 60% Sep-Pak fr. was carried out with RP-HPLC. First HPLC run (×17): Super Pac Spherisorb ODS, solvent A: 0.115% TFA in H_2O; solvent B: 0.1% TFA in MeCN; gradient: 0–2 min of 10% B, 2–52 min of 10–60% solvent B (linear gradient, 1% solvent B per min), followed by a 10 min rinse at 100% solvent B; flow rate: 1 ml/min. For further purification of the 80% Sep-Pak methanolic fr. a linear gradient of 10–80% solvent B, 2–72 min, 1% solvent B per min was used. Second HPLC run (×23): Bioactive material from the first HPLC runs was fractionated in a second run on the same column as above, but using 20 mM NH_4OAc in H_2O as solvent A (pH 7.0) and 20 mM NH_4OAc in 80% MeCN as solvent B. Gradient: 0–2 min at 16% B, 2–52 min linear gradient of 16–75% solvent B, followed by a 10 min rinse at 100% solvent B; flow rate: 1 ml/min. Bioactive material from this HPLC step was loaded onto a third HPLC column (×9) Capcell Pak C1, SG 120, solvent A: 0.115% TFA in H_2O; solvent B 0.1% TFA in MeCN; gradient: 0–2 min at 30% B; 2–20 min of 30–48% solvent B (linear gradient, 1% solvent B per min), followed by a 3 min rinse at 100% solvent B; flow rate 3 ml/min. Rt 13.7 min, 2.78 mg **1**.

3.3. Arborine (*1*)

Mp 161–162°C. EI-MS *m/z* (rel. int.): 249 $[M-1]^+$ (100), 104 (8). ^1H NMR: δ 3.73 (s, CH_3–N), 4.25 (s, CH_2), 7.27 (m, H-4′), 7.31 (m, H-2′, H-6′), 7.33 (m, H-3′, H-5′), 7.55 (ddd, $J_{5,6} = 8.0$ Hz, $J_{6,7} = 7.8$ Hz, $J_{6,8} = 0.9$ Hz, H-6), 7.67 (d, $J_{7,8} = 8.6$ Hz, H-8), 7.83 (ddd, $J_{7,8} = 8.6$ Hz, $J_{6,7} = 7.8$ Hz, $J_{5,7} = 1.5$ Hz, H-7), 8.25 (dd, $J_{5,6} = 8.0$ Hz, $J_{5,7} = 1.5$ Hz, H-5). ^{13}C NMR: δ 36.0 (CH_3-N), 43.3 (CH_2), 117.1 (C-8), 127.6 (C-6), 128.5 (C-4′), 128.7 (C-5), 129.4 (C-2′, C-6′), 130.2 (C-3′, C-5′), 135.9 (C-7), 136.1 (C-1′), 165.0 (C-2), 171.4 (C-4).

3.4. Animals

Eggs of *C. quinquefasciatus* Say (Diptera: Culicinae) were obtained from the Centre for Research in Medical Entomology, Indian Council of Medical Research, Madurai, India, and were hatched in plastic bowls (40 cm in diameter) containing chlorine-free boiled and cooled (27°C ± 2°C) tap H_2O. The juveniles were fed ad libitum with powdered yeast and dog biscuits (3:1). H_2O was changed on every alternative days. To ensure synchronous molting and emergence all the newly molted larvae belonging to an instar were transferred to a new tray. The pupae were sucked up using a glass pipette and transferred in finger bowls containing H_2O to standard mosquito cages (35 × 35 × 35 cm) for emergence at 27°C. The freshly emerged males were provided with 10% (w/v) sucrose soln through cotton buds, whereas females were provided with blood meal from an immobilized hen, *Gallus domesticus*, during night. Small plastic bowls with H_2O were placed inside the cages for oviposition. The eggs laid were used for maintaining the colony and to obtain fourth instar larvae for the bioassay experiments.

Mediterranean field crickets, *G. bimaculatus* de Geer (Ensifera: Gryllidae) were reared at 27°C under a long day (16 h light–8 h dark) photoregime at 30–40% relative humidity on mixed rabbit (2021), rat/mouse (1311) and cat (5031) Altromin® standard diet and H_2O ad libitum. On the day of adult emergence sexes were separated and staged according to their chronological age. Corpora allata were taken from 3 day old adult virgin females. Dissections were carried out in modified cricket Ringer (Lorenz, Lorenz, & Hoffmann, 1997).

3.5. Bioassays

Freshly molted fourth instar larvae of *C. quinquefasciatus* were tested to determine the larvicidal activity of the different frs of plant extracts and formulations. To 100 ml of 0.08% saline H_2O (Muthukrishnan et al., 1998) taken in 150 ml glass bowls appropriate volume of the frs/compounds (in MeOH) was added and 20 larvae were released into the bowls. In the control, larvae were exposed to 100 ml saline H_2O containing the same volume of MeOH as above. Yeast powder and dog biscuits (3:1) were provided as nutrients for the larvae. Mortality of the larvae was monitored for 240 h and the number of adults emerged was noted. Two replicates were maintained for each tested concentration.

The rapid partition assay for determination of juvenile hormone III-biosynthesis/release (Feyereisen & Tobe, 1981) by corpora allata from 3 day old virgin females of *G. bimaculatus* was performed as recently described by Lorenz et al. (1997) and Lorenz, Kellner, and Hoffmann (1995). All results are means ± S.E. for the number of individual measurements indicated in the legend of each figure. The Welch *t*-test was used in the statistical treatment of the data.

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