Research Article

Received: 5 October 2017 Revised: 5 December 2017 Accepted article published: 2 February 2018 Published online in Wiley Online Library: 25 March 2018

(wileyonlinelibrary.com) DOI 10.1002/ps.4880

Larvicidal efficacy of Adiantobischrysene from *Adiantum latifolium* against *Oryctes rhinoceros* through disrupting metamorphosis and impeding microbial mediated digestion

Ravindrannair Pradeep Kumar,[a] Anil John,[b] Praveen Kumar,[c] Kaleekkal Vasupillai Dinesh Babu[d] and Dasammal Asirvadam Evans[a*]

Abstract

BACKGROUND: *Oryctes rhinoceros* Linn. (Coleoptera: Scarabaeidae) is a serious pest of coconuts and other palms. Symbiotic gut bacteria play significant roles in the digestion of cellulosic materials as well as in some other physiological processes essential for the existence of *O. rhinoceros* larvae. The study was undertaken to isolate a compound with antibacterial and larvicidal activities from the leaves of *Adiantum latifolium* Lam. following a bioassay-guided method.

RESULTS: Methanol extract (ME) of dry leaf powder of *A. latifolium* showed larvicidal activity against third-instar *O. rhinoceros* (LD_{50}, 5018 mg/kg) with antibacterial activity on its gut microbiota. An *in vitro* study showed the bacteria *Bacillus cereus*, *Micrococcus lylae*, *Stenotrophomonas maltophilia*, *Kocuria rosea*, *Burkholderia mallei*, *Staphylococcus epidermidis*, *S. arlettae* and *Corynebacterium afermentans* identified from the larval gut were sensitive to ME. Bioactivity-guided isolation of the compound by liquid–liquid extraction and column chromatography resulted in Adiantobischrysene which showed antibacterial and larvicidal activity (LD_{50}, 8.4 mg/kg) and led to weight loss and precocious metamorphosis in larvae. An enzyme immunoassay showed a large peak in 20-hydroxyecdysone that commits larvae to precocious metamorphosis.

CONCLUSION: This study demonstrated that the antibacterial and metamorphosis disrupting activity of Adiantobischrysene make it a natural pesticidal compound against *O. rhinoceros*.

Keywords: *Adiantum latifolium*; *Oryctes rhinoceros*; gut microbiota; Adiantobischrysene; 20-hydroxyecdysone

1 INTRODUCTION

The holometabolous insect rhinoceros beetle, *Oryctes rhinoceros* Linn. (Coleoptera: Scarabaeidae) is a serious pest of coconuts and other palms throughout tropical regions of the world. Adult beetles bore into the centre of the crown, feed on exuding tissue juices and damage the meristematic tissues, stunting plant development. Pest attack leads to a reduction in the photosynthetic activity of the palm and yield. The holes made by the beetle serve as entry points for the lethal secondary attacks by other pests or pathogens.[1]

Decaying organic matter and cattle dung are breeding sites for the adult beetle. The larval stages with three instars are more vulnerable, feed on structural polysaccharides of plant tissues present in decaying organic matter or cow dung and take ~ 100–250 days to reach the adult stage.[2] Third-instar larvae are voracious feeders, and grow for 60–165 days before entering a non-feeding prepupal stage of 8–13 days. The enzyme cellulase is absent in larval and adult *O. rhinoceros*.[3] They rely on the metabolic versatility of microorganisms as symbionts in the digestion of lignocellulosic food.[4]

Plant extracts in general have been recognized as important natural sources of insecticides.[5] Biopesticides are considered safe to the ecosystem and free from any residual effect on crops.[6] *Adiantum latifolium* Lam. (Polypodiales: Adiantaceae), a fern, is valued for its medicinal uses and antibacterial activity.[7] Phytochemical studies show the presence of various classes of compounds such as steroids, triterpenoids, phenolics, saponins, tannins and alkaloids.[8]

Colonization by symbiotic gut bacteria is inevitable in *O. rhinoceros* larvae, which possess a large fermentation chamber in the gut that assists in symbiotic digestion and the number of cellulolytic bacteria increases with each instar along with volume of food consumed.[9] Identification of these bacteria in their insect

* *Correspondence to: DA Evans, Department of Zoology, University College, Thiruvananthapuram, Kerala, 69503, India. E-mail: drevansda@gmail.com*

a *Department of Zoology, University College, Thiruvananthapuram, India*

b *Division of Phytochemistry, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Thiruvananthapuram, India*

c *Department of Zoology, Government College for Women, Thiruvananthapuram, India*

d *Department of Chemistry, Government College for Women, Thiruvananthapuram, India*

host and the novel strategy of killing a pest by inhibiting symbiotic bacterial growth will pave the way for a method of pest control without developing insect resistance against phytocompounds.

The growth and development of insects are under the control of various intrinsic and extrinsic factors such as hormones and nutrition.[10] In many insect species, the availability of food during larval development is critical for metamorphosis.[11] Third-instar larvae of *O. rhinoceros* may undergo precocious metamorphosis on prolonged starvation provided they have reached a critical weight of 12.17 g for pupation and fail to pupate if the reduction in body weight is found to be beyond a critical level.[12] Plants are also a natural source of anti-ecdysone and anti-juvenile hormones which are more useful in controlling pest insects as they induce precocious metamorphosis and larval damage.

The larvicidal efficacy of methanol extracts (ME) of *A. latifolium* as an eco-friendly agent against *O. rhinoceros*, its antibacterial activity via ingestion through food, identification of the bioactive compound and an assay of the 20-hydroxyecdysone (20E) titre in haemolymph form the subject matter of this study.

2 MATERIALS AND METHODS

2.1 Experimental organism

Third-instar larvae of *O. rhinoceros* (body weight 8.5 ± 1 g) were collected from dung pits in and around Thiruvananthapuram District, Kerala State, India and maintained on sterilized cow dung until use.[3]

2.2 Plant material

Leaves of *A. latifolium* were collected from different locations in Thiruvananthapuram. The plant was identified by KP Rajesh (Sreekrishna College, Guruvayoor, India). A voucher specimen (no. 107) has been deposited in the herbarium of the Government College for Women, Thiruvananthapuram. Plant leaves were dried in the shade and ground to a fine powder.

2.3 Determination of larvicidal activity

Larvicidal activities were tested against *O. rhinoceros* larvae maintained for this purpose. Each group included six larvae reared in plastic containers with cow dung in the presence and absence of the test material. Various extracts of plant powder were thoroughly mixed with cow dung after dissolving in 25 mL of distilled water with 1% Tween 80, at various concentrations and served as food. The experiment was repeated six times and included a control with an appropriate concentration of Tween 80. Larval body weights and mortality were recorded each day for 3 weeks. The medium was changed every 3 days. A series of dose–response bioassays was carried out including the control to determine LD_{50} values.

2.4 Gut microbial load analysis

Treated and control larvae were dissected in 0.9% sterile saline on day 10 after cleaning the body surface with 70% ethanol in a UV laminar air flow. The mid- and hindgut regions of each larva were removed aseptically, homogenized in sterile saline and centrifuged at 3000 rpm. Microbial load was analysed by performing a surface agar culture. The supernatant suspension was serially diluted to concentrations of 10^{-1} and 10^{-5}. 100 µL of solution was taken using a sterile pipette and plated on nutrient agar medium (Himedia) in Petri dishes. The dishes were incubated at 37 °C for 48 h and the number of colony-forming units (CFU)/mL was calculated. The experiments were repeated three times for each replicate.

2.5 Isolation and identification of gut bacteria

Bacterial colonies were identified primarily by their morphological characteristics which include colour, consistency, surface texture, appearance and opaqueness. These colonies were picked and purified by repeated streaking on fresh nutrient agar plates, identification at species level was done by using a Biomérieux Vitek 2 system.

2.6 *In vitro* study of antibacterial activity

Antibacterial activity was studied using the agar well diffusion method. Plates were prepared by pouring 20 mL of molten nutrient agar medium into the sterile Petri dishes. The agar left to solidify for 5 min, then the medium surface was impregnated with 24 h grown strains. Wells (8 mm diameter, 2 cm apart) were made on each of these plates using a sterile cork borer. Then 100 µL extracts were added to the wells and the plates were incubated at 37 °C for 48 h. The diameter of any clear zone was measured against the test culture.

2.7 Bioactivity-guided isolation from *A. latifolium* leaves

Dried leaf powder of *A. latifolium* (2 kg) was extracted with methanol using soxhlet apparatus, filtered and concentrated to dryness under vacuum and subjected to bioactivity studies. The ME (210 g) was suspended in distilled water and then partitioned successively with ethyl acetate and *n*-butanol. The ethyl acetate (29 g), *n*-butanol (64 g) and water fraction (106 g) thus obtained were again subjected to bioactivity studies. The ethyl acetate fraction showed larvicidal and antibacterial activity. This active fraction was subjected to column chromatography over silica gel (200–400 mesh size, Merck) initially eluted with hexane/chloroform then chloroform/methanol gradients to give eight fractions. The bioactive fraction obtained from eight fractions (1.56 g) was subjected to reverse phase column chromatography over Cosmosil 75C 18-OPN (using a methanol/water mixture (90: 10 v/v) as the mobile phase) to obtain pure compounds. Structural characterization of the bioactive compound (210 mg) was carried out by infrared (IR) spectroscopy, ^{1}H NMR, ^{13}C NMR, high-resolution mass spectrometry (HRMS) and thin-layer chromatography (TLC). TLC spots were detected either by spraying anisaldehyde–sulphuric acid reagent or Hanessian's stain.

2.8 Enzyme–immunoassay measurement of the level of ecdysteroids in haemolymph

The ecdysteroid level in haemolymph was measured as described by Porcheron *et al.*[13] Haemolymph samples from control and treated larvae were collected in pre-labelled sterile Eppendorf tubes by cutting the third thoracic proleg of the larvae and subsequent squeezing of its body, and centrifuging at 10 000 ***g*** for 10 min at 4 °C. A 100-µL aliquot of the supernatant was mixed with methanol (1: 9), and centrifuged at 10 000 ***g*** for 10 min. The resultant supernatant was evaporated to dryness in a vacuum and resuspended in 50 µL EIA buffer. Ecdysone concentrations were measured using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's procedures. Ellman's reagent was used for the chromogenic reaction and the absorbance was read at 405 nm on a plate reader.

2.9 Statistical analysis

The statistical significance of data for control and treated groups was assessed by analysis of variance (ANOVA) using SPSS 13 for Windows. Statistical significance was accepted when $P \leq 0.05$.

Table 1. Effect of crude extracts of *Adiantum latifolium* against third instar larvae of *Oryctes rhinoceros*

Extract	Treatment concentration (%, w/v)	Weight loss/gain (%)*	Day of significant weight loss†	Mortality rate (%)‡	Percent transformation into prepupae
Methanol extract	Control	16.26	–	00 ± 00^{a}	
	0.25	−12.72	10	35.00 ± 4.28^{b}	20
	0.5	−14.18	9	53.33 ± 3.33^{c}	–
	1	−17.14	8	93.33 ± 2.10^{d}	–
Ethyl acetate fraction of methanol extract	Control	15.34	–	00 ± 00^{a}	–
	0.025	−11.84	9	16.66 ± 2.10^{b}	30
	0.05	−15.28	9	43.33 ± 2.10^{c}	–
	0.1	−18.67	9	88.33 ± 2.10^{d}	–

*After 10 days.
†Day on which loss of body weight appeared significant at 0.01 level compared to control.
‡After 3 weeks. Values are means ± SE of six replicates. Significance at 0.01 level.

Table 2. Effect of methanol extract of *Adiantum latifolium* on number of bacterial colonies in mid gut and hind gut of larvae *of Oryctes rhinoceros*

Treatment concentration (%, w/w)	CFU*(mean ± SE)	
	Mid gut	Hind gut
Control	$3.86 \times 10^{5} \pm 0.08^{d}$	$8.26 \times 10^{5} \pm 0.24^{c}$
0.25	$2.93 \times 10^{5} \pm 0.08^{c}$	$6.23 \times 10^{5} \pm 0.24^{b}$
0.50	$1.38 \times 10^{5} \pm 0.13^{b}$	$2.38 \times 10^{5} \pm 0.11^{a}$
1.00	$0.59 \times 10^{5} \pm 0.04^{a}$	$1.79 \times 10^{5} \pm 0.08^{a}$

All values represent an average of three replicates.
*Significance at 0.001 level.

Table 3. Bacterial strains isolated and identified from the gut of *Oryctes rhinoceros* larvae

Bacterial types		
Gram positive	Rod shaped	*Bacillus cereus*
		Corynebacterium afermentans
	Coccus	*Micrococcus lylae*
		Kocuria rosea
		Staphylococcus epidermidis
		Staphylococcus arlettae
Gram negative	Rod shaped	*Stenotrophomonas maltophilia*
		Burkholderia mallei

Table 4. Growth inhibition by Adiantobischrysene against Gram-positive and Gram-negative gut bacteria

Bacterial type		Inhibition zone (mm)*	
		Control	Adiantobischrysene
Gram negative	*Bacillus cereus*†	00 ± 0.00	17.00 ± 0.57
Gram positive	*Stenotrophomons maltophilia*†	00 ± 0.00	16.67 ± 0.57

*Repeated the experiments three times for each replicate, mean ± SE of zone of inhibition of bacteria, concentration 0.25 mg/mL.
†Significant at 0.001 level.

Post-hoc testing was carried out using Duncan's new multiple range test (MRT). LD_{50} values were calculated by probit analysis. Bar graphs were plotted in Microsoft Excel 2007 and tables in Microsoft Word 2007.

3 RESULTS

3.1 Larvicidal activity

The ME of *A. latifolium* caused significant dose-dependent mortality in *O. rhinoceros* larvae (Table 1). Intoxicated larvae at the initial phase of toxicity stopped feeding and showed a highly dilated gut, which led to an assumption that indigestion may be the reason for flatulence (Fig. 1). Dose-dependent weight loss was observed. The duration of feeding leading to a significant reduction in body weight decreased as the concentration of ME in food increased, despite the larvae being in the actively feeding and growing stage. At a low concentration (0.25%), 20% of larvae entered prepupal stage, but failed to pupate. The reduction in body weight at a high ME dose (0.5%, 1%) is beyond the critical weight and the larvae failed to prepupate. These effects were then measured in the ethyl acetate fraction of ME and the bioactive compound Adiantobischrysene was isolated from the bioactive sub-fraction of the ethyl acetate fraction (Table 1, Fig. 2). LD_{50} values were 5018, 583 and 8.4 mg/kg for ME, ethyl acetate fraction and Adiantobischrysene, respectively.

3.2 Gut microbial load analysis, isolation and identification of bacteria

Microbial analysis of gut contents showed sharp decrease in CFU/mL of bacteria in the midgut and hindgut (Table 2). Eight strains of bacteria in seven genera, six Gram positive and two Gram negative were isolated and identified from the larval gut (Table 3). All strains were highly sensitive to the ME and ethyl acetate fraction of *A. latifolium*, Adiantobischrysene showed antibacterial activity against the Gram-positive and Gram-negative gut bacteria (Table 4, Figs 3,4).

3.3 Levels of ecdysteroids in haemolymph

Decreased body weight and early precocious pupation were also caused by Adiantobischrysene, suggesting that the ecdysteroid

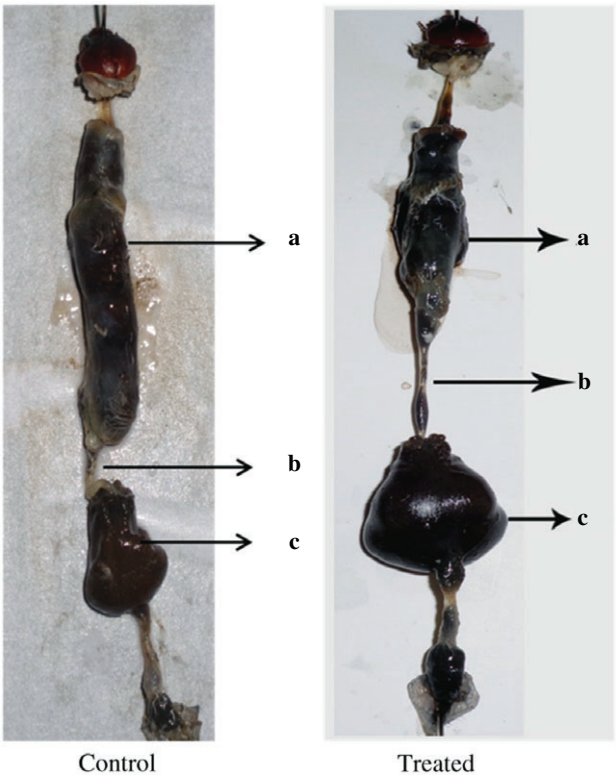

Figure 1. Gut of *Oryctes rhinoceros* larvae. (a) Mid gut, (b) anterior sphincter, (c) hind gut.

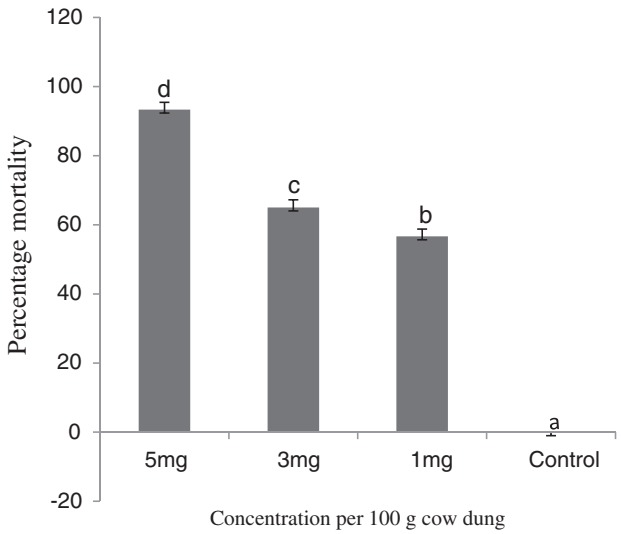

Figure 2. Larval mortality caused by Adiantobischrysene against the larvae of *Oryctes rhinoceros*. Each value is the mean ± SE of three replicates. Values marked with different letters indicate significant differences between groups ($P \leq 0.05$).

changes occur in the larval haemolymph. To address this, we measured ecdysteroid concentrations in the haemolymph from day 4 to day 12 of treatment (Fig. 5). In the control larvae, ecdysteroid levels in the haemolymph showed a passive increase from 250 pg/mL on day 4 to 810 pg/mL on day 12. In the 1 mg treated larvae, levels changed from 350 pg/mL on day 4 to 1833 pg/mL on day 12; in 3 g treated larvae, levels changed from 420 pg/mL on day 4 to 2466 pg/mL on day 12; and in 5 mg treated larvae, levels changed from 453 pg/mL on day 4 to 5266 pg/mL on day 12. A sudden decrease in ecdysteroid concentration occurred on day 10 followed by a sudden surge in the ecdysone titre. This induced prepupation even in larvae weighing 9.14 g, but there was no further development.

3.4 Structural elucidation of bioactive compound

The bioactive compound had needle-like crystals, m.p. > 250 °C, HRMS spectrum (M+) peak at *m/z* 890.74 (100%), (M + 1) + peak at 891.74 (64.1%), (M + 2) + peak at 892.74 (20.7%). Elemental analysis gave the molecular formula of the compound as $C_{58}H_{98}O_6$. IR spectrum (KBr) showed a hydroxyl group (3410 cm^{-1}) and an aliphatic C–H stretching group (2920 cm^{-1}).

^{1}H NMR (400 MHz, $CDCl_3$) shows δ ppm 0.80 (3H,s), 0.87 (6H,s), 0.92 (3H,s), 0.99 (3H,s), 1.16 (3H,s), 1.18–2.0 (24H,m), 2.94 (1H,m), 3.26 (3H,s), 3.7(2H,s).

^{13}C NMR (400 MHz, $CDCl_3$) shows δ ppm 16, 25.2, 86.2, 76.9, 42, 34.6, 17.8, 49.2, 37.8, 52.6, 36.2, 28.7, 39.2, 40.4, 29.3, 35.8, 42.8, 52, 20.1, 28.65, 21.6, 17.7, 20.8, 16.1, 15.8, 16.3, 57.9, 63.2, 80.2, 92.

^{1}H NMR (400 MHz, $CDCl_3$) spectrum showed the presence of six singlet methyl signals at δ 0.80, 0.87 (2× CH_3), 0.92, 0.99 and 1.16 in addition to a singlet methine proton at 3.7 and methoxy proton at δ 3.26. ^{13}C NMR (400 MHz, $CDCl_3$) spectrum showed the presence of 30 carbon signals for 30 types of carbon. Its fragmentation gives peaks at *m/z* 890.74, 876.72, 862.71, 858.67, 824.67, 701.53, 675.55, 469.33 and 393.32 (Figs 6,7). Comparison with authentic data indicates that the compound is a dimer of triterpenoid.[14] It is a newly reported compound named Adiantobischrysene with the IUPAC name (3aR,5aR,7aS,8S,11bR,13bR)-3-(1,2-dihydroxy-1-((3aS,5aS,7aR,8R,11bS,13bS)-8-hydroxy-9-methoxy-3a,5a,7a,8,11b,13b-hexamethyl-icosahydro-1H-cyclopenta(*a*)chrysen-3-yl)ethyl)-9-methoxy-3a,5a,7a,8,11b,13b-hexamethyl-icosahydro-1H-cyclopenta(*a*)chrysen-8-ol (Fig. 8).

4 DISCUSSION

Larvae of Scarabaeidae spend their immature stages in decaying matter rich in cellulose.[2] Microorganisms like bacteria, protozoa and fungi are the most efficient cellulose and hemicellulose degraders in nature. Insects have symbiotic associations of gut bacteria including cellulolytic and non-cellulolytic strains, that aid in the digestion of lignocellulosic food in the absence of gut cellulase enzyme, providing nutrition, and the synthesis of vitamins and sterols. The bacteria also protect the host from other potentially harmful microbes and detoxification.[15–18]

Carbohydrate fermentation and by-product absorption were studied in *O. nasicornis* larvae where cellulose digestion is brought about by hindgut bacteria. Eight species of bacteria are present in the midgut of *O. monoceros*.[4,19] In *O. rhinoceros* larvae, the middle region of hind gut has an enlargement called proctodeal dilation in which fermentation of food takes place and gut bacteria have a profound role in cellulose digestion.[12,20,21] In this study, we identified eight species from seven genera of bacteria in the gut of *O. rhinoceros* larvae. The presence of these bacteria in many organisms and their cellulolytic activity have been reported previously. *B. cereus* has been reported from the gut of *O. rhinoceros* larvae with cellulolytic and hemicellulolytic activity, *M. lylae* and *C. xerosis* in the midgut of adult *O. monoceros* and *M. lylae* in the intestine of the ornamental fish *Parachromis managuensis*.[19,20,22] *S. maltophilia* is present in the midgut of *Lutzomyia longipalpis*, *Burkholderia*

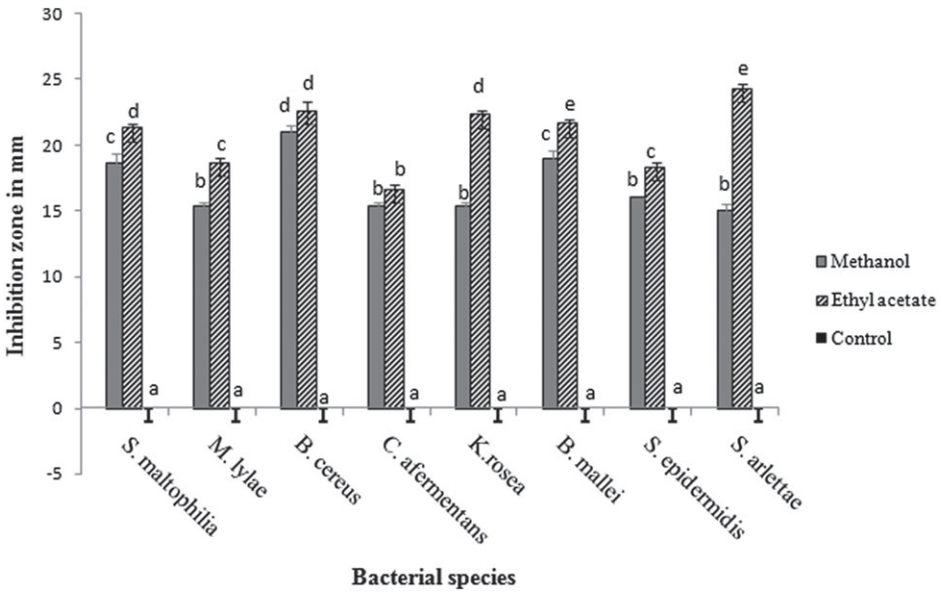

Figure 3. Growth inhibition of bacteria by methanol extract (concentration 25 mg/mL) and ethyl acetate fraction (concentration 5 mg/mL) of *Adiantum latifolium*. Values marked with different letters indicate significant differences between groups ($P \leq 0.05$).

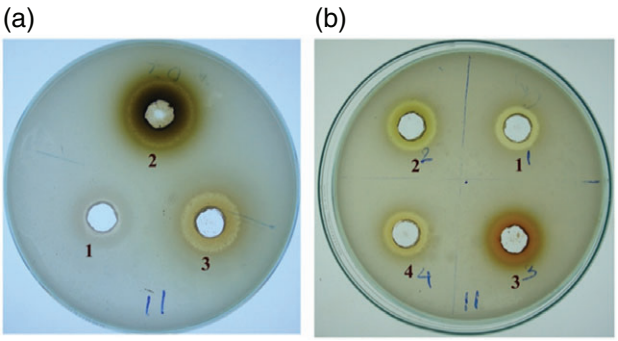

Figure 4. Antibacterial activity of different extracts of *Adiantum latifolium*. Plate a: 1, negative control; 2, methanol extract; 3. ethyl acetate fraction. Plate b: 1, 2, 4, duplicates of Adiantobischrysene; 3, bioactive sub-fraction of ethyl acetate fraction.

symbionts in *Riptortus clavatus* and *Leptocorisa chinensis*, *B. fungorum* in *Harpalus pensylvanicus* and *Anisodactylus sanctaecrucis*, and *Staphylococcus* sp. in gut of third-instar larvae of *Helicoverpa armigera*.[23–26] The enzyme activities, degradation of organic compounds, growth on NaCl, production of acid from carbohydrates, susceptibility to antibiotics and other physiological characteristics of the genus *Kokuria* have been reported previously.[27] These studies support our findings that the bacteria identified here play significant roles in the digestion of cellulosic materials and in other physiological processes essential for the existence of the *O. rhinoceros* larvae.

The multitrophic and multifunctional roles of symbiotic bacteria provide a new strategy for pest control by disrupting the symbiotic interaction, thereby causing a deleterious effect on host. The potential of antibiotics to manipulate the mutualistic interaction between a host and its bacteria has been studied widely in insects. Feeding norfloxacin to adult *Eurygaster integriceps* significantly impaired the growth and development of offspring in a dose-dependent manner.[28] Weight loss and mortality was seen in *O. rhinoceros* larvae on feeding an antibiotic-treated cow dung mixture because it eliminates microbes.[12] In the present study, *A. latifolium* was investigated for its larvicidal activity via inhibiting the growth of bacterial colonies in the gut of third-instar larvae of *O. rhinoceros*. The antibacterial activity of *A. latifolium* extracts against the bacterial strains *S. aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Escherichia coli* is well known.[7,29] In the present study, ME of the plant showed larvicidal effect and a decrease in bacterial load in the gut measured as CFU/mL. An *in vitro* study of the ME and its ethyl acetate fraction showed significant activity against all the identified gut bacteria.

Adiantobischrysene isolated from the active sub-fraction showed antibacterial activity against Gram-positive and Gram-negative gut bacteria. Nutritional deficiency, starvation as well as disturbance of the digestion process, caused by the compound reduced the larval growth rate and body weight. Significant weight loss and dilation of gut in larvae occurred within 10 days leading to an inability of the larvae to take food and gradual death at higher treatment doses. Adiantobischrysene is a dimer of triterpenoids with a molecular weight of 891.4 g/mol. Terpenoids have been found to possess antioxidant and antimicrobial properties in various studies.[30–32] Plants usually produce triterpenes in tissue, organ and developmental specific manners, and in response to environmental disturbance, pest and pathogen attacks.[33] Clerodane diterpenes present in *Detarium microcarpum* and the triterpenoid of *Lantana camara* showed insecticidal activity against *Reticulitermes speratus* and *Odontotermes obesus* respectively.[34–36] *A. latifolium* is a rich source of triterpenes, valued for their various uses in traditional medicine, for example, as an anxiolytic, analgesic and anti-inflammatory agent.[7,29] These findings reinforce the antibacterial and larvicidal activities of Adiantobischrysene as in our study.

At lower concentrations (0.25% methanol, 0.025% ethyl acetate extracts and 1 mg Adiantobischrysene) larvae showed a decrease in body weight and underwent precocious metamorphosis to prepupation within 3 weeks, even at a low weight of 9.48 g, and subsequent death. Previous studies indicated a critical weight of 12.17 g for precocious pupation of *O. rhinoceros* on prolonged starvation.[12] Any change in the composition and quality of its feed at an early third instar stage can increase or decrease larval

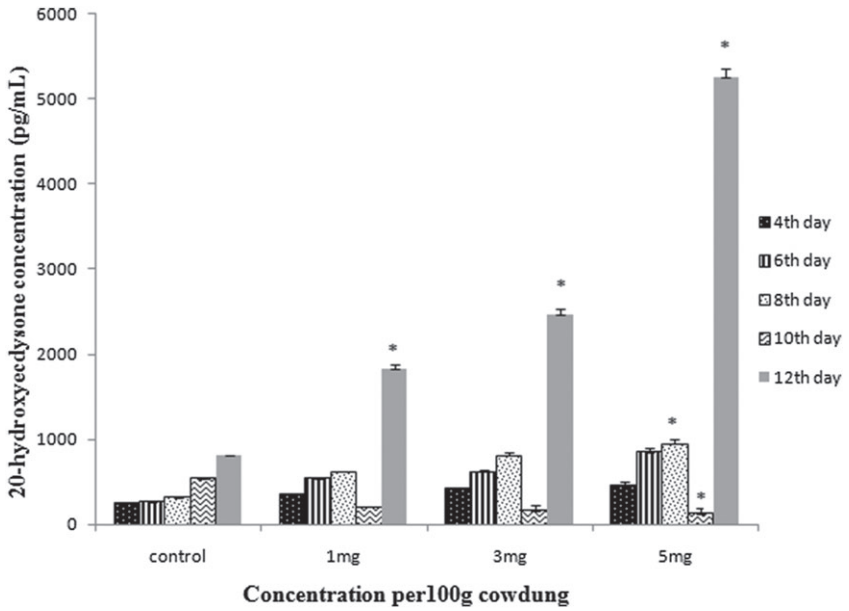

Figure 5. Effect of Adiantobischrysene on 20-hydroxyecdysone titre in the haemolymph of third instar larvae of *Oryctes rhinoceros*. *Significant at 0.05 level. Each value is a mean value of three separate determinations with error bars.

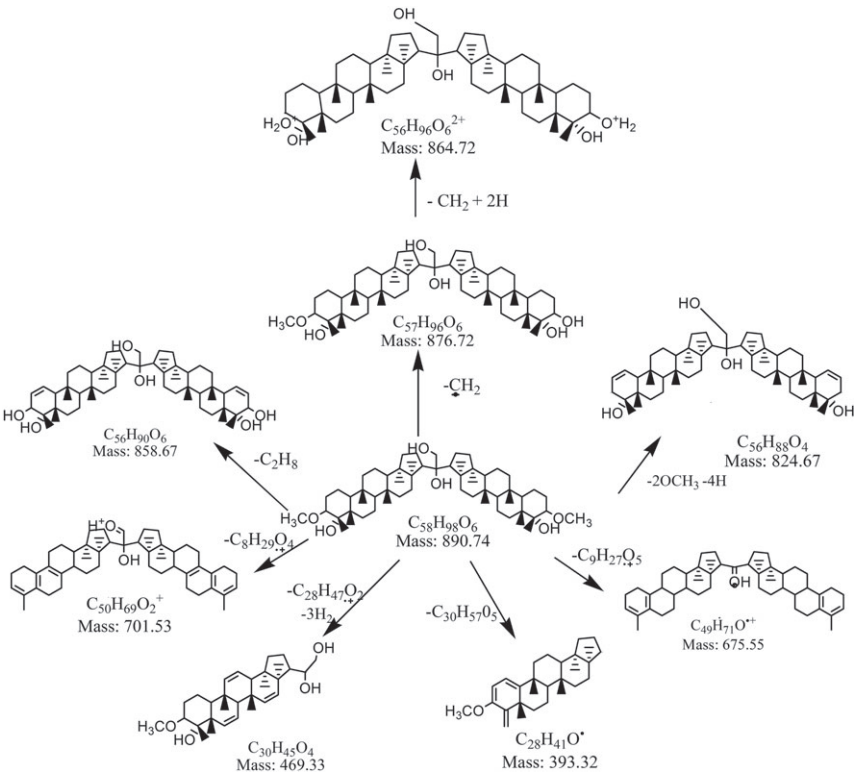

Figure 6. Fragmentation of the compound Adiantobischrysene.

development and survival rate. Because third-instar larvae are voracious feeders of decaying matter, during pupation the adult structures are formed and replace larval structures. The pupa does not feed, and its energy must come from foods it ingested as a larva. The increase in cellulolytic bacterial count with increased larval stage can be attributed to the increased volume of food consumed. At higher doses (0.5% and 1% methanol, 0.05% and 0.1% ethyl acetate extract, and 3 and 5 mg Adiantobischrysene) the antibacterial activity of the compound caused indigestion of food in early third-instar larvae that prevented it from attaining the critical weight of 12.17 g necessary for starvation-induced metamorphosis.

The growth and development of an insect is under the control of various intrinsic and extrinsic factors such as hormones and nutrition.[10] Ecdysone and its active metabolite 20E, collectively known as ecdysteroids, are essential for controlling development, for example moulting, metamorphosis and diapause.[37] Surpassing a critical weight results in the initiation of an endocrine cascade for

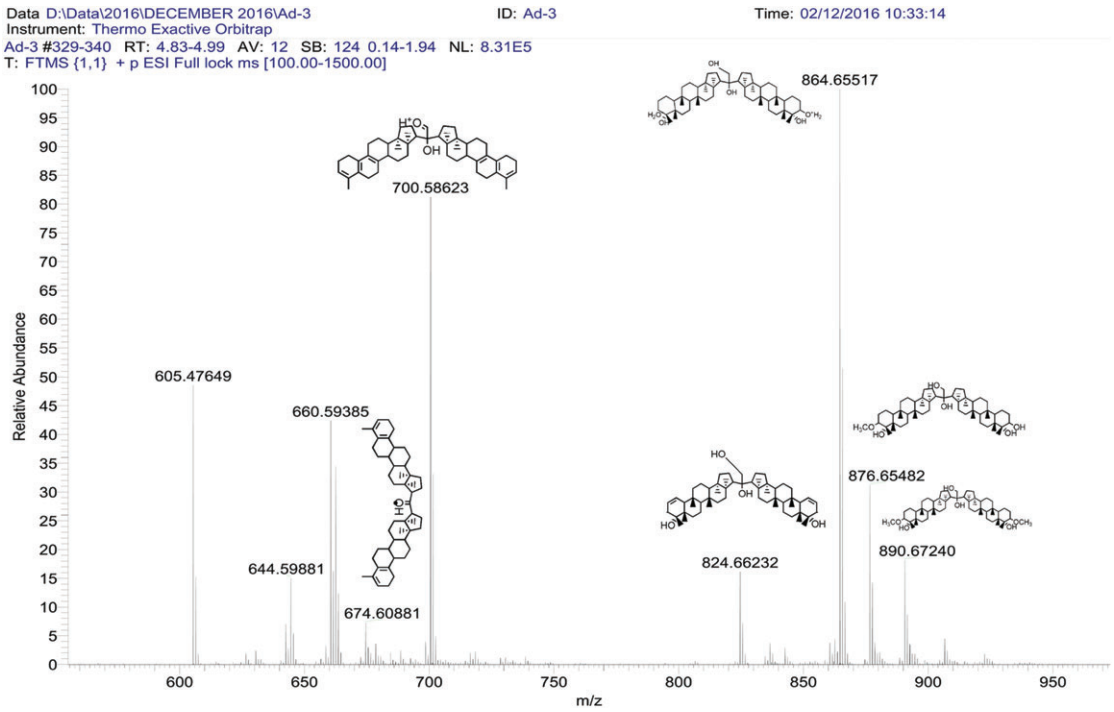

Figure 7. HRMS spectrum showing fragmentation of Adiantobischrysene.

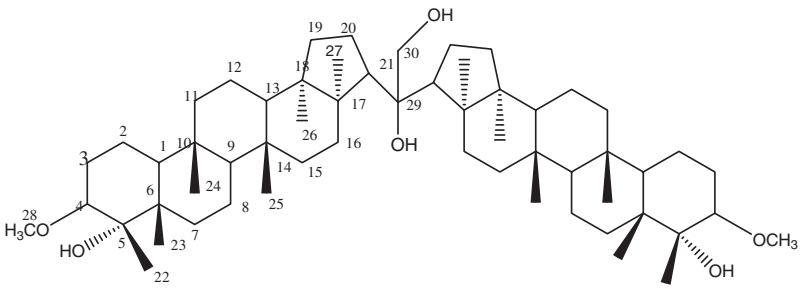

Figure 8. Structure of Adiantobischrysene.

metamorphosis, as originally described for the tobacco hornworm, *Manduca sexta*.[38] Once larvae reach the critical weight, juvenile hormone titres decline, allowing the release of prothoracicotropic hormone, which is produced in the brain and secreted from the corpora allatum.[39] This acts on the prothoracic gland leading to synthesis of the moulting hormone ecdysone and 20E. In this study, the enzyme immunoassay showed high levels of 20E in the haemolymph of the larvae when compared with controls, and an ecdysone surge occurred on day 10. This was because indigestion of food caused a nutritional shortage and simultaneous release of 20E in exponential quantities that might have induced precocious prepupation even at a very low weight of 9.48 g. A similar observation of elevated 20E leading to precocious pupation was observed in *Drosophila*.[40] Starvation signals from the gut induce an increase in ecdysteroid concentration in the haemolymph to above the threshold level via activation of the ecdysone synthesis pathway.

We found very high levels of 20E in the haemolymph of larvae treated with 1 mg of the insecticidal compound Adiantobischrysene. However, precocious pupation did not occur at higher concentrations of 3 and 5 mg. Triterpenoids in plants act as natural analogues of insect juvenile hormones disrupting normal metamorphosis.[41] Two triterpenoids, cucurbitacin B and cucurbitacin D have been isolated from seeds of *Iberis umbellata* and shown to be responsible for the antagonistic activity of a ME of this species in preventing the 20E-induced morphological changes in the *Drosophila*.[42] These triterpenoids are generally detrimental to insect development and are toxic at higher concentrations. Cucurbitane-type triterpenoid compounds from the bitter gourd *Momordica charantia* showed anti-estrogenic activity on their enzyme receptor-modulating potential.[43] Dimeric triterpenoid glycoside from *Rubus rigidus* strongly enhanced the antioxidant activity of its monomers.[44] We therefore assume that Adiantobischrysene may also possess anti-ecdysone activity preventing larvae from precocious metamorphosis at doses >1 mg.

These findings showed that larvicidal activity of Adiantobischrysene against *O. rhinoceros* may be via multiple effects. One target is the symbiotic gut microbes because microbial cellulolysis is essential for larval growth and metamorphosis. Physiological starvation due to Adiantobischrysene caused a hormonal imbalance by elevating the level of ecdysone hormone in haemolymph, inducing precocious metamorphosis. At higher concentrations, the compound also showed antagonistic activity preventing ecdysone hormone from inducing morphological changes. Most conventional synthetic insecticides are rapid-acting neurotoxins

with non-selective poisoning on all the fauna of ecosystem. Therefore, natural compounds with selective toxicity via ingestion of food and having multifaceted effects on the target are of prime importance because they are eco-friendly.

ACKNOWLEDGEMENTS

The first author is very grateful to the University Grants Commission, India for providing research grant under Faculty Development Programme. Authors are grateful to Kerala State Council for Science, Technology and Environment for laboratory infrastructure development in Zoology Department, University College, Thiruvananthapuram. Authors are thankful to Mahatma Gandhi University, Kerala, India for providing facilities for spectral analysis of the phytocompound.

REFERENCES

1 Bedford GO, Biology, ecology and control of palm rhinoceros beetles. *Annu Rev Entomol* **25**:309–339 (1980).
2 Nirula KK, Investigations on the pest of coconut palm. Part 2. *Oryctes rhinoceros* L. *Indian Coconut J* **8**:161–179 (1955).
3 Sreekumar S and Prabhu VKK, Digestive enzyme secretion during metamorphosis in *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). *Proc Anim Sci* **97**:67–71 (1988).
4 Bayon C and Mathelin J, Carbohydrate fermentation and by-product absorption studied with labelled cellulose in *Oryctes nasicornis* larvae (Coleoptera:Scarabaeidae). *J Insect Physiol* **26**:833–840 (1980).
5 Rahuman AA and Venkatesan P, Larvicidal efficacy of five cucurbitaceous plant leaf extracts against mosquito species. *Parasitol Res* **103**:133–139 (2008).
6 Mukherjee U and Singh HN, Eco-friendly approaches to manage diamond-back moth, *Plutella xylostella* (L.) in cauliflower. *J Appl Zool Res* **17**:57–60 (2006).
7 Antonysamy J, Antibacterial potentials of *Adiantum* species against the UTI pathogens. *J Microbiol Exp* **4**:2–4 (2017).
8 De Britto AJ, Gracelin DHS and Kumar PBJR, Phytochemical studies on five medicinal ferns collected from southern western ghats, Tamilnadu. *Asian Pac J Trop Biomed* **2**:S536–S538 (2012).
9 Raman KVA, Magadum SB and Datta RK, Feed efficiency of the silkworm *Bombyx mori* hybrid (NB4D2 × KA). *Insect Sci. Applic* **15**:111–116 (1994).
10 Keshan B, Thounaojam B and Kh SD, A comprehensive study of the changes in ecdysteroid levels during the feeding phase of fifth instar larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Eur J Entomol* **112**:632–641 (2015).
11 Munyiri FN and Ishikawa Y, Endocrine changes associated with the starvation-induced premature metamorphosis in the yellow-spotted longicorn beetle, *Psacothea hilaris*. *Gen Comp Endocrinol* **144**:150–155 (2005).
12 Rani R, Jaya S and Chandran D, Effect of antibiotics on larval growth and metamorphosis in *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). *J Plant Crop* **30**:63–65 (2002).
13 Porcheron P, Moriniere M, Grassi J and Pradelles P, Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. *Insect Biochem* **19**:117–122 (1989).
14 Ibraheim ZZ, Ahmed AS and Gouda YG, Phytochemical and biological studies of *Adiantum capillus-veneris* L. *Saudi Pharm J* **19**:65–74 (2011).
15 Dillon RJ and Dillon VM, The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol* **49**:71–92 (2004).
16 Genta F, Dillon R, Terra W and Ferreira C, Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. *J Insect Physiol* **52**:593–601 (2006).
17 Feldhaar H, Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol Entomol* **36**:533–543 (2011).
18 Ferrari J and Vavre F, Bacterial symbionts in insects or the story of communities affecting communities. *Phil Trans R Soc B Biol Sci* **366**:1389–1400 (2011).
19 Desai A and Bhamre P, Diversity of gut bacterial fauna of *Oryctes monocerus* Linnaeus (Coleoptera: Scarabaeidae). *Bionano Front* **5**:142–144 (2012).
20 Sari SLA, Cellulolytic and hemicellulolytic bacteria from the gut of *Oryctes rhinoceros* larvae. *Biodiversitas* **17**:78–83 (2016).
21 Taylor E and Crawford C, Microbial gut symbionts and desert detritivores. *Sci Rev Arid Zone Res* **1**:37–52 (1982).
22 Sivakumar K, Janani D and Shree Rama M, Analysis of microbial biodiversity in intestine of ornamental fishes gut. *Int J Fish Aquat Stud* **2**:232–234 (2015).
23 Oliveira SM, Moraes BA, Gonçalves CA, Giordano-Dias CM, D'Almeida JM and Asensi MD, Prevalence of microbiota in the digestive tract of wild females of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae). *Rev Soc Bras Med Trop* **33**:319–322 (2000).
24 Kikuchi Y, Meng XY and Fukatsu T, Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). *Appl Environ Microbiol* **71**:4035–4043 (2005).
25 Lundgren JG, Lehman RM and Chee-Sanford J, Bacterial communities within digestive tracts of ground beetles (Coleoptera : Carabidae). *Ann Entomol Soc Am* **100**:275–282 (2007).
26 Mishra PK and Tandon SM, Gut bacterial flora of *Helicoverpa armigera* (Hub.) (Lepidoptera: Noctuidae). *Indian J Microbiol* **43**:55–56 (2003).
27 Han SK, Nedashkovskaya OI, Mikhailov VV, Kim SB and Bae KS, *Salinibacterium amurskyense* gen. nov., sp. nov., a novel genus of the family Microbacteriaceae from the marine environment. *Int J Syst Evol Microbiol* **53**:2061–2066 (2003).
28 Kafil M, Bandani AR, Kaltenpoth M, Goldansaz SH and Alavi SM, Role of symbiotic bacteria in the growth and development of the Sunn pest, *Eurygaster integriceps*. *J Insect Sci* **13**:99 (2013).
29 Babu C, Irudayaraj V and Punitha SMJ, Phytochemical and antibacterial activity of *Adiantum latifolium* Lam. *Pteridol Res* **1**:10–12 (2012).
30 Singh B and Singh S, Antimicrobial activity of terpenoids from *Trichodesma amplexicaule* Roth. *Phyther Res* **17**:814–816 (2003).
31 Iwu M, Ezeugwu C, Okunji C, Sanson DR and Tempesta MS, Antimicrobial activity and terpenoids of the essential oil of *Hyptis suaveolens*. *Int J Crude Drug Res* **28**:73–76 (1990).
32 Sauerwein M and Becker H, Growth, terpenoid production and antibacterial activity of an in vitro culture of the liverwort *Fossombronia pusilla*. *Planta Med* **56**:364–367 (1990).
33 Ghosh S, Biosynthesis of structurally diverse triterpenes in plants: the role of oxidosqualene cyclases. *Proc Indian Natl Sci Acad* **82**:1189–1210 (2016).
34 Lajide L, Escoubas P, and Mizutani J, Termite antifeedant activity in tropical plants. 3. Termite antifeedant activity in *Detarium microcarpum*. *Phytochemistry* **40**:1101–1104 (1995).
35 Verma S and Verma R, Termiticidal triterpenoid from leaves of *Lantana camara* var. *aculeata*. *J Inst Chem* **77**:23–25 (2005).
36 Verma RK and Verma SK, Phytochemical and termiticidal study of *Lantana camara* var. *aculeata* leaves. *Fitoterapia* **77**:466–468 (2006).
37 Fielenbach N and Antebi A, *C. elegans* dauer formation and the molecular basis of plasticity. *Genes Dev* **22**:2149–2165 (2008).
38 Shafiei M, Moczek AP and Nijhout HF, Food availability controls the onset of metamorphosis in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Physiol Entomol* **26**:173–180 (2001).
39 Gilbert L and Goodman W, Chemistry, metabolism, and transport of hormones controlling insect metamorphosis. *Metamorphosis*. Springer, New York, pp. 139–175 (1981).
40 Terashima J, Takaki K, Sakurai S and Bownes M, Nutritional status affects 20-hydroxyecdysone concentration and progression of oogenesis in *Drosophila melanogaster*. *J Endocrinol* **187**:69–79 (2005).
41 Amos T, Williams P and Guesclin P, Compounds related to juvenile hormone: activity of selected terpenoids on *Tribolium castaneum* and *T. confusum*. *J Econ* **67**:474–476 (1974).
42 Dinan L, Whiting P, Girault JP, Lafont R, Dhadialla ST, Cress DE *et al.*, Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. *Biochem J* **327**:643–650 (1997).
43 Hsu C, Hsieh C, Kuo Y and Huang C, Isolation and identification of cucurbitane-type triterpenoids with partial agonist/antagonist potential for estrogen receptors from *Momordica charantia*. *J Agric* **59**:4553–4561 (2011).
44 Nguelefack TB, Mbakam FHK, Tapondjou LA, Watcho P, Nguelefack-Mbuyo EP and Ponou BK, A dimeric triterpenoid glycoside and flavonoid glycosides with free radical-scavenging activity isolated from *Rubus rigidus* var. *camerunensis*. *Arch Pharm Res* **34**:543–550 (2011).

