Bioefficacy and mode-of-action of some limonoids of salannin group from *Azadirachta indica* A. Juss and their role in a multicomponent system against lepidopteran larvae

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Biological activities of the salannin type of limonoids isolated from *Azadirachta indica* A. Juss were assessed using the gram pod borer *Helicoverpa armigera* (Hubner) and the tobacco armyworm *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). Inhibition of larval growth was concomitant with reduced feeding by neonate and third instar larvae. All three compounds exhibited strong antifeedant activity in a choice leaf disc bioassay with 2·0, 2·3 and 2·8 μg/cm^2 of 3-O-acetyl salannol, salannol and salannin, respectively deterring feeding by 50% in *S. litura* larvae. In nutritional assays, all three compounds reduced growth and consumption when fed to larvae without any effect on efficiency of conversion of ingested food (ECI), suggesting antifeedant activity alone. No toxicity was observed nor was there any significant affect on nutritional indices following topical application, further suggesting specific action as feeding deterrents. When relative growth rates were plotted against relative consumption rates, growth efficiency of the *H. armigera* fed diet containing 3-O-acetyl salannol, salannol or salannin did not differ from that of starved control larvae (used as calibration curve), further confirming the specific antifeedant action of salannin type of limonoids. Where the three compounds were co-administered, no enhancement in activity was observed. Non-azadirachtin limonoids having structural similarities and explicitly similar modes of action, like feeding deterrence in the present case, have no potentiating effect in any combination.

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1. Introduction

Allelochemicals from the Indian neem tree, *Azadirachta indica*, are classic examples of chemicals that impart protection to many crops against a variety of pests. Among these, only azadirachtin, a tetranortriterpenoid, has been extensively studied as an antifeedant, growth inhibitor and growth regulator (Koul 1992; Mordue and Blackwell 1993). Other than azadirachtin, several allelochemicals from neem have antifeedant or growth inhibition properties against some insect species (Koul *et al* 1996) but few detailed investigations have been carried out on these compounds. These studies have, therefore, provided the impetus for evaluating such non-azadirachtin type of compounds alone or in combination (Koul *et al* 2003, 2004) to identify their potential in commercial formulations. The salannin group is one such group of non-azadirachtin type of compounds, which are characterized by the two oxygen bridges C-6/28 and C-7/14. In fact, salannin was the first compound of this group from neem to show antifeedant properties against various insects (Yamasaki and Klocke 1989; Govindachari *et al* 1996; Koul *et al* 1996;

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Kraus 2002). Besides azadirachtin, salannin is the only constituent of neem for which a particular analytical isolation method has been developed (Yamasaki *et al* 1988). Significant antifeedant activity of salannin and other compounds of this group such as 3-deacetyl salannin (Kubo *et al* 1986; Kraus *et al* 1993; Ishida *et al* 1992), salannol (Kraus *et al* 1993), salannolacetate (Kraus 1984) and very moderately active salannolactames (Kraus *et al* 1987) has been recorded. Though salannin has been evaluated in detail against many insect species, the activity of other compounds of the salannin group has been mostly evaluated against *Epilachna varivestis*. The only efficacy data available for salannol against *E. varivestis* (Kraus 2002) indicates that this compound is more efficacious than even salannin against *E. varivestis* larvae (EC_{50} = 10 ppm against 14 ppm in case of salannin).

3-O-acetyl salannol, the most active compound of the salannin group against *E. varivestis*, has been isolated from *A. indica* (Kraus 1984; Rojatkar *et al* 1989), is also known to occur in *Melia volkensii* (Rajab and Bentley 1992) and has been partially synthesized (Yamasaki and Klocke 1989). Therefore, the present work was undertaken to study the efficacy and mode of action of this compound alone and also to determine its efficacy in combination with salannin and salannol to determine any significant combinatorial role of these compounds in the multi-component system of *A. indica* extracts.

2. Materials and methods

2.1 *Chemistry*

The compound 3-O-acetyl salannol was isolated from the seeds of the neem tree, *Azadirachta indica* A. Juss by extracting seed powder with ethanol to produce a ethanol extract, which was chromatographed on silica gel using mixtures of Me_2CO-petrol since the elution was carried out in gradient mode enabling the collection of various fractions (Rajab and Bentley 1992). The active fraction confirmed through guided insect bioassay was subjected to repeated prep TLC using EtOAc-benzene and its final purification to produce an analytical sample was performed using a semi-prep HPLC column (30 cm × 0·8 cm) filled with 10 µ Nucleosil C-18 RP. The mobile phase was a mixture of acetonitrile, MeOH and water (58 : 5 : 37). The main peak was collected and evaporated to dryness leaving a residue, which was subjected to spectroscopic investigation. High resolution MS pointed to the molecular formula $C_{34}H_{46}O_9$. ^{1}HNMR spectrum of the compound showed characteristic signals of furan protons 7·33 m, 6·33 m, and 7·28 m as well as characteristic methyl group signals at 3·28 s (MeOCO), 2·06 s (CH_3CO), 1·64 d (H-18), 1·30 s (H-30), 1·25 s (H-29) s, 1·20 s, 0·94 s (H-19), and a pair of doublets 6H (5′ and 4′) $J_{3',4'}$ = 7Hz and $J_{5',3'}$ = 7Hz second $J_{5',2'}$ = 2Hz which pointed to a tetranortriterpenoid molecule. The comparison of ^{1}HNMR of 3-O-acetyl salannol led to the conclusion that it is almost the same as that of salannol except the signal of 3-H, which is shifted down field to 4·84 ppm. ^{13}CNMR data allowed us to confirm the molecular composition assignment from HRMS and indicated the amount and character of CH_2 and CH groups. All these data pointed to a structural formula of 3-O-acetyl salannol (figure 1), which was identical with that published by Rajab and Bentley (1992). The ^{13}CNMR data showed C-1 72·70 d; C-2 30·21 t; C-3 71·37 d; C-4 42·73 s; C-5 39·91 d; C-6 70·68 d; C-7 85·68 d; C-8 48·90 s; C-9 39·22 d; C-10 40·27 s; C-11 29·69 t; C-12 172·89 s; C-13 134·72 s; C-14 146·53 s; C-15 87·93 s; C-16 41·20 t; C-17 49·52 d; C-18 12·90 q; C-19 15·54 q; C-20 127·19 s; C-21 142·87 d; C-22 110·81 d; C-23 138·94 d; C-28 77·82 t; C-29 19·59 q; C-30 16·94 q; C-1′ 172·63 s; C-2′ 43·50 t, C-3′ 25·11 d; C-4′ 22·84 q; C-5′ 22·67 q; $\underline{C}H_3$ CO 21·17 q; $CH_3\underline{C}O$ 170·58 s.

Salannin and salannol (> 90% purity) were isolated from neem seed by known methods (Kraus and Cramer 1981; Yamasaki *et al* 1988).

2.2 *Insects*

Larvae of *H. armigera* and *S. litura* were taken from laboratory cultures maintained on an artificial diet prepared in the laboratory (Koul *et al* 1997). Larvae of *S. litura* were also maintained on castor leaves (*Ricinus communis*) for leaf-disc bioassays. The cultures were maintained at 27 ± 2°C and 16 : 8 L : D photoperiod. Generally neonate, and third- and fourth-stage larvae were used in our experiments.

2.3 *Growth assay*

3-O-acetyl salannol, salannol and salannin were individually mixed with the dry portion of the artificial diet at concentration ranges of 25–250 ppm for testing against neonates and 25–300 ppm for testing against fourth instars. The carrier solvent acetone was then evaporated. Control diet was treated with carrier alone.

Two 24 h old neonate larvae were placed on 1 g fresh weight of diet in an individual cup (30 ml) (Koul *et al* 1990). The cups were kept in a plastic tray lined with moistened filter paper to maintain humidity. The experiments were carried out in a growth chamber at 27 ± 2°C and 16 : 8 L : D photoperiod. Larval weight was assessed as a percentage of the controls after 7 d and larval mortality, if any, was also recorded. We used 40 larvae in each of three replicates to test each concentration. The concentrations inhibiting 50% growth relative to controls (EC_{50} values) were determined by probit regression analysis.

Similar methods were used to test materials against fourth-instar larvae.

2.4 *Leaf disc choice assay*

Antifeedant activity was assessed using a 5 h leaf-disc choice test. We punched 3·0 cm^2 discs from castor leaves and applied 10 µl of aqueous allelochemical solution emulsified with Triton-X-100 (0·1%) to each side using a micropipette. A treatment dose of 1–10 µg/cm^2 was used for each compound. Controls were treated with carrier alone. The leaf discs were dried at room temperature. One fourth-instar *S. litura* larva (12–24 h since the last moult) was placed into each 9 cm diameter arena containing one control and treated disc. To test each compound-concentration combination three replicates of 10 insects each were used. Consumption was recorded as described by Isman *et al* (1990) and Koul *et al* (1996). The index of feeding deterrence (FI) was calculated as $(C - T)/(C + T) \times 100$, where C = consumption of control discs and T = consumption of treated discs. FI_{50} was determined by probit analysis.

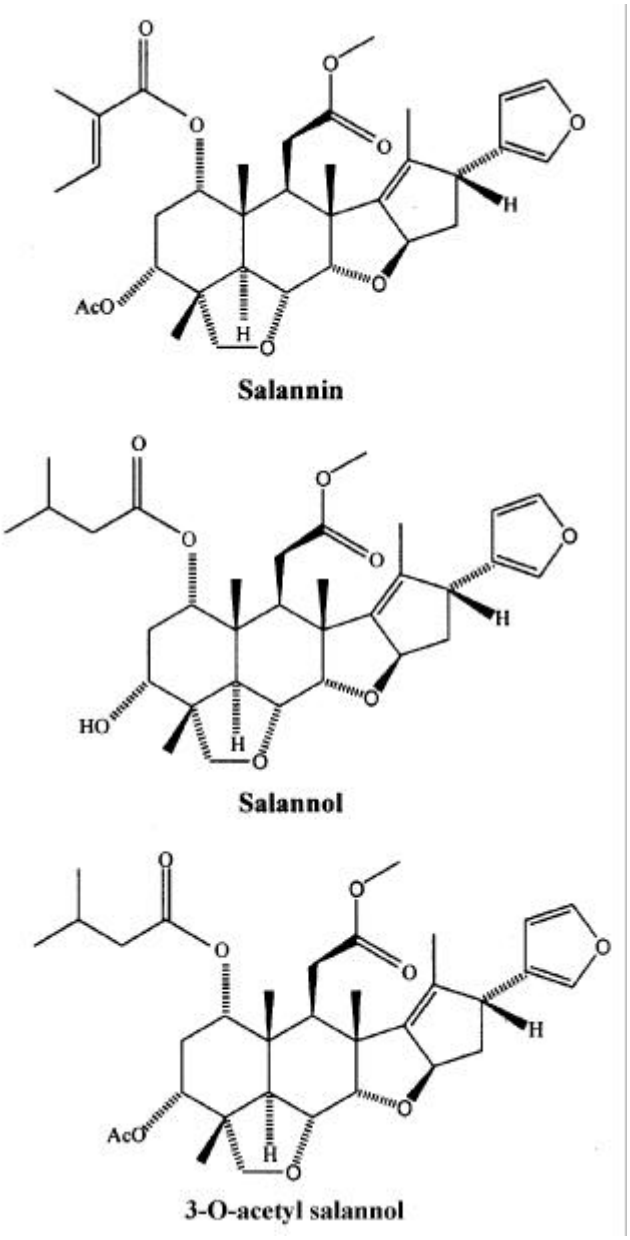

Figure 1. Structures of salannin type of compounds isolated from *A. indica*.

2.5 *Nutritional analysis*

To segregate behavioural effects from toxicity-mediated effects, the three compounds were subjected to nutritional analysis using early fourth-instar *H. armigera* and *S. litura* larvae. We provided three replicates each of 10 larvae with either 3-O-acetyl salannol, salannol or salannin (150 ppm) in artifical diet. Relative growth per unit weight of the insect at the outset of experiment (RGR_i) and relative consumption rate at the outset of experiment (RCR_i) were calculated on dry-weight basis after 3 d of feeding as G/I (G = change in larval dry weight/day and I = starting larval dry weight) and C/I(C = change in diet dry weight/day and I = starting larval dry weight), respectively. After termination of the experiment (72 h) the larvae, remaining food and frass were dried at 60°C to constant weight and nutritional indices calculated. The index of food conversion efficiency (ECI) was calculated as $100 \times G/C$, where G = dry weight gain of the insect and C = dry weight of food consumed, efficiency of conversion of digested food (ECD) as [weight gained/(food ingested – frass weight) × 100] and approximate digestibility (AD) as [(food ingested – frass weight)/food ingested × 100] (Koul *et al* 1997). Concentrations used for these compounds were based on the pre-determined EC_{50} values of each compound.

A second set of experiments was carried out with compounds applied to larvae topically. A dose of 10 µg/insect was used. Larvae were treated on the dorsal surface with a single 0·5 µl drop of each compound in acetone using a fine 25 µl syringe (7105 series syringe, Hamilton Co., Reno, Nevada, USA) attached to a repeating dispenser (PB-600, Hamilton Co.). Controls were treated with acetone alone. The larvae were then allowed to feed on untreated diet.

Comparison of RGR_is and RCR_is in each case was made using the procedure followed by Wheeler and Isman (2001). A standard curve, relating RGR_is and RCR_is was constructed by feeding larvae different amounts of food (0, 50, 100, 200 mg and unlimited, $n = 20$ for each weight). Larvae were weighed and placed on the diet, one per cup.

The assay trays were stored as mentioned above. After 3 days, larvae, frass and diet were separated, dried and weighed. For the determination of effect of compounds, diets were incorporated with different concentrations of 3-O-acetyl salannol, salannol and salannin (25, 50, 75, 100, 250 ppm, $n = 20$ in each case). The diet was weighed (ca. 1 g per larva) and the experiment carried as above. Both experiments were carried out simultaneously at the same time and for same duration. RGR_is and RCR_is were calculated and linear regression analysis performed, correlating control RGR_is and RCR_is and test RGR_is and RCR_is. Difference between regression coefficients was used to test for differences between the two regressions.

2.6 *Evaluation of combinations*

For each combination, the compounds were combined in equal proportions (2 mg per compound) and final concentrations were made in the range of EC_{50} values of the most active component in the combination. This was done to ensure that at least 50% inhibition by the most active component in all cases was achieved. Combinations were: 3-O-acetyl salannol + salannin, 3-O-acetyl salannol + salannol, 3-O-acetyl salannol + salannin + salannol; salannin + salannol. These combinations were fed to 48 h old neonate larvae of *H. armigera* in artificial diets for 7 d as above. The EC_{50} values for each combination were calculated.

3. Results

The initial diet assay against neonate larvae of *H. armigera* and *S. litura* assessed how effectively the salannin group of compounds reduced the growth. 3-O-acetyl salannol was most effective (EC_{50} = 64·2 ppm) in comparison with salannol and salannin and the same trend was observed in both species (table 1).

The leaf disc choice bioassay, however, showed similar antifeedant properties (overlap of confidence intervals) for all three compounds in *S. litura* larvae. The feeding inhibition index (FI_{50}) ranged between 2·0 and 2·8 μg/cm^2 determined from a line of best fit (table 2).

Nutritional analysis revealed that the compounds specifically act as antifeedants. The compounds, when incorporated into artificial diet reduced RGR_i and RCR_i without any significant change in the ECI, ECD or AD values. However, reduction in growth was significantly correlated with dietary concentrations ($P < 0·05$). When the compounds were applied topically to the larvae, no significant effects were seen at any dose for any of the indices (tables 3 and 4).

Plotting relative growth rates against consumption rates was used to differentiate deterrent and toxic effects of the compounds. Two lines were generated for each, one calibration curve, where a range of RCR_is were generated and correlated to RGR_is and one test line, where larvae were fed diets containing a range of compound concentrations (25–250 ppm). RGR_i and RCR_i for each set of larvae were subjected to linear regression analysis (as shown for 3-O-acetyl salannol in figure 2, and similar results were obtained for salannol and salannin). The slope (regression coefficient) of the regression line represents the growth efficiency of the larvae. The two regression coefficients were compared by calculating the variance of the difference between the two estimates of the regression coefficients. This test showed that the growth efficiency of *H. armigera* fed on diet containing 3-O-acetyl salannol, salannol or salannin did not differ significantly from that of the control larvae.

Thus, the reduction in growth of larvae fed on diet containing salannin type of compounds is entirely due to reduced food intake. Salannin compounds did not differ in their activity when combined in different mixtures (table 5). None of the concentrations of 3-O-acetyl salannol, salannol or salannin showed significantly increased activity over single compounds. This indicated that structurally similar compounds did not increase efficacy when

Table 1. Effective concentrations (ppm) of various salannin type limonoids inhibiting growth (neonates) of *H. armigera* and *S. litura* in a dietary assay ($n = 120$).

	H. armigera			*S. litura*		
Compounds	EC_{50} (95% CI)	EC_{95} (95% CI)	Slope ± SE	EC_{50} (95% CI)	EC_{95} (95% CI)	Slope ± SE
3-O-acetyl salannol	64·2 (56·1–73·4)	166·9 (141·1–236·5)	3·38 ± 0·4	65·6 (57·5–74·8)	169·1 (136·2–244·6)	3·41 ± 0·4
Salannol	79·7 (67·5–94·1)	219·7 (151·2–319·2)	3·73 ± 0·7	77·4 (66·0–90·9)	220·8 (154·1–316·3)	3·61 ± 0·6
Salannin	86·5 (77·1–96·9)	187·4 (147·4–238·2)	4·89 ± 0·7	87·7 (76·5–100·4)	197·3 (149·8–274·4)	4·51 ± 0·8

Table 2. Feeding inhibition (FI) of 4th instar *S. litura* larvae after oral administration of various limonoids in a leaf disc choice assay.

Compound	FI_{50} (μg/cm^2)	Confidence interval (95%)
3-O-acetyl salannol	2·0	1·9–3·0
Salannol	2·3	2·0–4·4
Salannin	2·8	1·5–3·5

compared to their individual EC_{50} values (table 5) in the case of both *H. armigera* and *S. litura* larvae.

4. Discussion

Limonoids from the Rutales are known to show varying levels of antifeedant or growth inhibitory activity against insects (Champagne *et al* 1992). Our tests confirm this.

Table 3. Feeding, growth and dietary utilization by 4th instar *H. armigera* larvae after oral and topical administration of salannin type limonoids.

	Nutritional indices (Mean ± SE)				
Treatment	RGR_i (mg/mg/d)	RCR_i (mg/mg/d)	ECI (%)	ECD (%)	AD (%)
Oral feeding (ppm)					
3-O-acetyl salannol (150)	$1·49 \pm 0·3^c$	$2·90 \pm 0·9^c$	$52·3 \pm 5·0^a$	$57·3 \pm 7·6^a$	$91·8 \pm 8·9^a$
Salannol (150)	$1·72 \pm 0·2^b$	$3·32 \pm 0·7^b$	$51·8 \pm 6·3^a$	$55·6 \pm 8·8^a$	$93·2 \pm 9·2^a$
Salannin (150)	$1·73 \pm 0·07^b$	$3·43 \pm 0·8^b$	$51·6 \pm 5·3^a$	$56·6 \pm 5·3^a$	$90·6 \pm 9·6^a$
Control	$2·79 \pm 0·3^a$	$5·23 \pm 0·9^a$	$53·5 \pm 6·0^a$	$55·9 \pm 6·2^a$	$92·4 \pm 9·3^a$
Topical application (μg/insect)					
3-O-acetyl salannol (10·0)	$2·87 \pm 0·6^a$	$5·38 \pm 1·0^a$	$52·7 \pm 7·3^a$	$58·3 \pm 7·7^a$	$90·4 \pm 8·0^a$
Salannol (10·0)	$3·01 \pm 0·4^a$	$5·66 \pm 0·9^a$	$52·9 \pm 6·6^a$	$58·0 \pm 5·9^a$	$91·4 \pm 9·7^a$
Salannin (10·0)	$2·98 \pm 0·6^a$	$5·40 \pm 0·8^a$	$53·4 \pm 4·8^a$	$59·8 \pm 8·3^a$	$88·8 \pm 9·3^a$
Control	$3·00 \pm 0·3^a$	$5·61 \pm 0·7^a$	$54·0 \pm 3·5^a$	$59·3 \pm 7·8^a$	$91·2 \pm 9·8^a$

Means within a column followed by the same letter are not significantly different ($P > 0·05$) based on Tukey's test.

Table 4. Feeding, growth and dietary utilization by 4th instar *S. litura* larvae after oral and topical administration of salannin type limonoids.

	Nutritional indices (Mean ± SE)				
Treatment	RGR_i (mg/mg/d)	RCR_i (mg/mg/d)	ECI (%)	ECD (%)	AD (%)
Oral feeding (ppm)					
3-O-acetyl salannol (150)	$1·89 \pm 0·3^c$	$3·63 \pm 0·4^c$	$52·0 \pm 6·0^a$	$64·7 \pm 7·6^a$	$80·3 \pm 8·9^a$
Salannol (150)	$2·16 \pm 0·1^b$	$4·22 \pm 0·7^b$	$51·8 \pm 6·3^a$	$64·6 \pm 8·8^a$	$80·2 \pm 9·7^a$
Salannin (150)	$2·23 \pm 0·07^b$	$4·45 \pm 0·6^b$	$50·1 \pm 6·3^a$	$62·6 \pm 5·3^a$	$80·5 \pm 8·6^a$
Control	$3·49 \pm 0·6^a$	$6·75 \pm 0·8^a$	$51·8 \pm 6·5^a$	$65·8 \pm 6·2^a$	$78·9 \pm 8·3^a$
Topical application (μg/insect)					
3-O-acetyl salannol (10·0)	$3·87 \pm 0·7^a$	$6·48 \pm 1·0^a$	$59·7 \pm 6·8^a$	$69·8 \pm 7·7^a$	$85·6 \pm 7·5^a$
Salannol (10·0)	$3·88 \pm 0·4^a$	$6·59 \pm 0·8^a$	$57·9 \pm 6·6^a$	$68·0 \pm 6·0^a$	$85·1 \pm 8·6^a$
Salannin (10·0)	$3·90 \pm 0·7^a$	$6·68 \pm 0·9^a$	$58·3 \pm 4·8^a$	$69·8 \pm 7·3^a$	$83·5 \pm 9·7^a$
Control	$3·80 \pm 0·4^a$	$6·66 \pm 0·7^a$	$56·9 \pm 5·5^a$	$68·3 \pm 7·3^a$	$83·3 \pm 7·8^a$

Means within a column followed by the same letter are not significantly different ($P > 0·05$) based on Tukey's test.

Larvae of *H. armigera* and *S. litura* treated with 3-O-acetyl salannol, salannol or salannin gained less weight when fed on treated diets and the activity was concentration dependent, with 3-O-acetyl salannol being the most active compound. Interestingly, except for salannin, this group of compounds has been mostly evaluated against *Epilachna varivestis* (a coccinellid beetle) and is active in the range of 10–20 ppm. Therefore, the present study is the first one to determine in detail the efficacy of these natural compounds against lepidopterans. Some synthetic derivatives such as desacetyl and detigloyl salannin compounds have been evaluated against *Spodoptera* and *Heliothis* species to demonstrate that desacetylation or detiglation reduces the activity of salannin significantly (Blaney *et al* 1990; Simmonds *et al* 1990). While the three compounds were active against *H. armigera* and *S. litura* in the range of 60–90 ppm (EC_{50} against neonates), the EC_{50} for salannin in *Heliothis virescens* has been reported to be higher (170 ppm) (Kubo *et al* 1986).

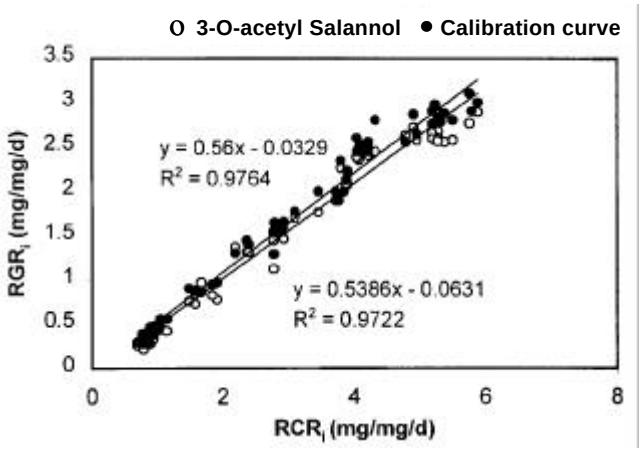

Figure 2. Relationship between relative consumption rates and relative growth rates of *H. armigera* larvae fed on different quantities of artificial diet (calibration curve) and larvae fed on diet containing varying amounts of 3-O-acetyl salannol.

The leaf-disc choice assay showed feeding deterrence in all three compounds. The assay resulted in 50% feeding inhibition in fourth-instar *S. litura* larvae for 3-O-acetyl salannol (FI_{50} = 2·0 μg/cm^2) that was comparable to salannol (FI_{50} = 2·3 μg/cm^2), but slightly superior to salannin (table 2). Previously, salannin had been reported to deter feeding in a leaf-disc bioassay against *S. frugiperda* (FI_{50} = 13 μg/cm^2) third-instar larvae (Rajab *et al* 1988). However, salannol or 3-O-acetyl salannol have not been evaluated against any lepidopteran insect.

Using nutritional indices, it was established that the reduction in consumption accounted for the majority of the decrease in growth rate, as there was no reduction in ECI and ECD values. ECI is an overall measure of an insect's ability to utilize the food that it ingests for growth. Change in ECD also indicates the overall increase or decrease of the proportion of digested food metabolized for energy. Therefore, no change in ECI and ECD values indicate that ingested allelochemicals do not exhibit any chronic toxicity. This gets further support from topical treatment experiments where none of the parameters showed a reduced trend. This implies that the two species used in the present study were remarkably similar in their responses to the candidate compounds. Therefore, we conclude that salannin type compounds act primarily as antifeedant compounds against insects.

Another approach used to confirm this was comparison of RGR_i and RCR_i values with one set of insects being fed varying amounts of control diet (0 to excess) and one set of treated diets (25 to 250 ppm) (Wheeler and Isman 2001). Interestingly the regression coefficients of the two regression lines were not significantly different ($P > 0·01$), with the insects fed in two different cohorts growing equ-

Table 5. Efficacy of 3-O-acetyl salannol, salannol and salannin in various combinations, against 48 h old neonate *H. armigera* and *S. litura* larvae*.

	H. armigera		*S. litura*	
Combinations (ppm range)	EC_{50} (ppm)	95% confidence interval (slope value ± SE)	EC_{50} (ppm)	95% confidence interval (slope value ± SE)
3-O-acetyl salannol + salannol (25–200)	70·8	56·8–90·2 (4·08 ± 1·06)	71·6	53·6–89·9 (4·6 ± 0·9)
3-O-acetyl salannol + salannin (25–200)	78·5	63·3–92·0 (5·7 ± 1·1)	77·8	57·7–96·3 (5·9 ± 0·7)
Salannol + salannin (25–200)	80·8	61·9–102·5 (4·6 ± 0·5)	81·1	54·6–97·7 (5·5 ± 0·6)
3-O-acetyl salannol + salannol + salannin (25–200)	71·9	58·2–98·9 (4·7 ± 0·3)	70·7	56·6–89·5 (5·8 ± 0·5)

*Individual EC_{50} (ppm) of 3-O-acetyl salannol = 64·2 and 65·6; salannol = 79·7 and 77·4 and salannin = 86·5 and 87·7 against the two species evaluated, respectively.

ally for a given RCR_i. This corroborates our conclusion that the reduced growth of larvae under the influence of salannin type compounds is entirely due to starvation and not because of the toxic effect of the ingested salannins. Previous reports, for instance on citrus limonoids, also demonstrate that limonoids act principally as antifeedants and not as toxins against *Spodoptera frugiperda* larvae (Mendel *et al* 1993) or *Leptinotarsa decemlineata* beetles (Liu *et al* 1990). However, the same limonoids can be post-ingestive toxins for other insect species (Mendel *et al* 1991; Chen *et al* 1995). Although the present study clearly demonstrates that the salannin type of compounds act as antifeedants and not post-ingestive toxins, limonoids, on the whole, could have different primary modes of action depending on the test insect species (Koul *et al* 2002). Azadirachtin is a potential example of this as its oral administration reduces RGR and RCR, but not ECI and ECD i.e. antifeedant activity. However topical application results in reduction of RGR, ECI and ECD, but not RCR, indicating toxicity, where energy is diverted from biomass production to detoxification, i.e. increase in costs (Koul and Isman 1991; Koul *et al* 1996).

In our earlier studies we demonstrated that azadirachtin, being the most potent compound in neem and efficacious at very low concentrations, is not influenced by other neem allelochemicals. Secondly, mixtures of moderately active non-azadirachtin limonoids do show increased activity if they are different structurally, as well as have different modes of action, i.e. compounds with very close stereochemistry must compete for the same target site (Koul *et al* 2003, 2004). The present findings further support these conclusions, in that feeding deterrence induced by salannin type compounds is not enhanced when in various combinations as they possess the same type of activity (feeding deterrence) and are structurally similar with minor changes in functional groups. From our earlier studies (Koul *et al* 2003, 2004) and subsequently our present findings, it is obvious, therefore, that various combinations could be useful in developing mixed formulations for pest management. Mixtures of several compounds will also ensure that the formulations have a variety of toxic, growth inhibitory and antifeedant effects. Such complexes are desirable in that the target spectrum is widened, because different species respond differently to individual compounds. These mixtures are also likely to reduce the potential for development of genetic resistance or development of behavioural desensitization.

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