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6β-Hydroxygedunin from *Azadirachta indica*. Its Potentiation Effects with Some Non-azadirachtin Limonoids in Neem against Lepidopteran Larvae

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The biological activity of 6β-hydroxygedunin isolated from *Azadirachta indica* A. Juss. was assessed using the gram pod borer, *Helicoverpa armigera* (Hubner), and Asian armyworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), alone and in combination with other limonoids, gedunin, salannin, nimbinene, and azadirachtin. The compound exhibited growth inhibitory activity in artificial diet bioassays, with 24.2 and 21.5 ppm, respectively, inhibiting growth by 50%. This efficacy was higher in comparison to gedunin (EC_{50} = 50.8 and 40.4 ppm), salannin (EC_{50} = 74.5 and 72.0 ppm), and nimbinene (EC_{50} = 391.4 and 404.5 ppm). Azadirachtin, however, remained the most active neem allelochemical against both insect species. Nutritional assays clearly demonstrated that, though relative consumption and growth rates of fourth instar larvae were reduced, gedunin-type compounds induced physiological toxicity, evident by reduced efficiency of conversion of ingested food (ECI) in feeding experiments. Salannin and nimbinene, on the contrary, induced concentration-dependent feeding deterrence only. In feeding experiments, combinations of the compounds revealed that when azadirachtin was present in a mixture, EC_{50} values did not deviate from the individual efficacy of azadirachtin (0.26 and 0.21 ppm, respectively) against *H. armigera* and *S. litura* larvae. However, a combination without azadirachtin did show a potentiation effect with potent EC_{50} values among structurally different molecules, i.e., when salannin or nimbinene was combined with 6β-hydroxygedunin or gedunin rather than structurally similar salannin + nimbinene or 6β-hydroxygedunin + gedunin. Obviously, azadirachtin being the most active compound in neem is not synergized or influenced by any other limonoid, but other non-azadirachtin limonoids were more potent in specific combinations vis-à-vis the structural chemistry of the compound. It is obvious from the present study that potentiation among non-azadirachtin limonoids having explicitly two different modes of action, such as feeding deterrence and physiological toxicity, may be playing a significant role in the potentiation effect.

KEYWORDS: ***Azadirachta indica*; gedunin; 6β-hydroxygedunin; salannin; nimbinene; azadirachtin; growth inhibitors; antifeedants; synergism; *Helicoverpa armigera*; *Spodoptera litura***

INTRODUCTION

Plant defense against insects is generally related to allelochemicals present, which are highly significant selective agents in the evolution of such defenses (*1*). About 2400 plant species have been reported to possess pesticidal properties, distributed in 189 families, among which about 22 families contain more than 10 plant species in each family that, in one way or the other, possess anti-insect properties (*2*). During the last two decades many such allelochemicals have been demonstrated to deter feeding in insects (*3*), are insecticidal (*4*), or are growth inhibitors (*5*) against insects.

Allelochemicals from the Indian neem tree *Azadirachta indica* A. Juss. are the classic examples of phytochemicals that are effective against pests of various crops. Among these, azadirachtin, a tetranortriterpenoid, has been extensively studied as an excellent antifeedant, a growth inhibitor, and a growth regulator for a wide variety of insect species (*6*−*8*). Apart from azadirachtin, several allelochemicals from this plant inhibit feeding in some specific insect pests (*9*). These studies have provided impetus for screening other neem compounds in detail to identify a potential phytochemical that could be used in commercial formulations.

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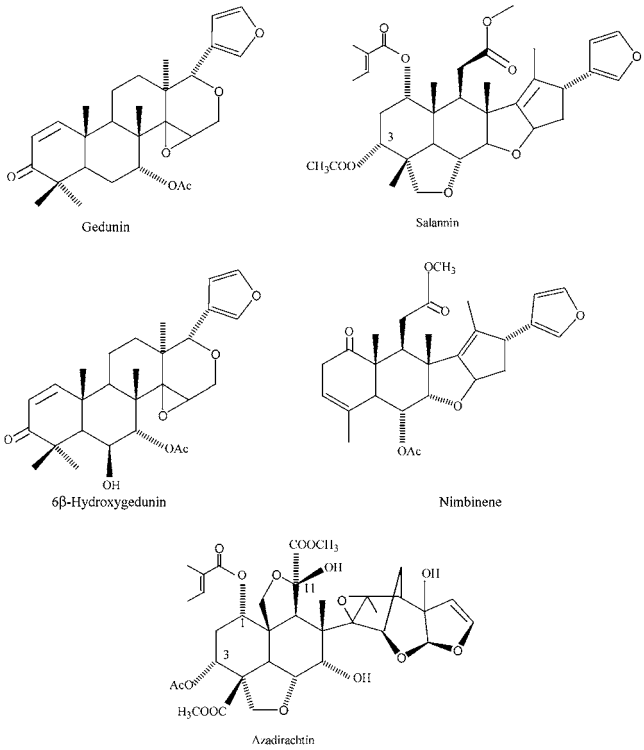
In nature, plant chemical defense against insect herbivory almost never depends on a single compound, but instead several compounds interact with pests, individually or in unison. The present work was undertaken as a part of our program to establish the multicomponent defense strategy of plants. The significance of mixtures lies in the fact that they equip the plant with a diverse arsenal against a variety of pests and the possibility exists that the mixtures may exhibit synergistic or additive effects. Accordingly, the isolation of gedunin-type compounds was taken up. In addition to gedunin, 6β-hydroxygedunin was isolated, which is a known synthetic compound (*10*) but has been isolated for the first time from *A. indica* and has not been evaluated against any specific insect species so far. Gedunin is known to induce feeding inhibition in *Ostrinia nubilalis* and *Epilachna varivestis* and insect growth inhibition in *Pectinophora gossypiella*, *Spodoptera frugiperda*, and *Helicoverpa zea* (*11*). The detailed study of gedunin and 6β-hydroxygedunin was carried out to demonstrate their efficacy independently and in combination with known active limonoids of neem, azadirachtin, salannin, and nimbinene, against gram



pod borer, *Helicoverpa armigera*, and tobacco armyworm, *Spodoptera litura*, larvae. An earlier effort in demonstrating the efficacy of neem allelochemicals such as azadirachtin, salannin, nimbinene, and nimbin individually against *S. litura* has revealed interference with growth via digestive enzyme impairment due to azadirachtin. However, other compounds only interfered at chemoreceptor/deterrent receptor levels (*9*), thereby showing that the compounds from lower biosynthetic levels primarily have antifeedant activity (*12*). In these studies, however, combination of the compounds was not studied.

MATERIALS AND METHODS

Chemistry. Neem seed kernel pressed oil (1 kg) was suspended in light petroleum and extracted with 80% MeOH. This solution was then diluted three times by volume with water and extracted with $CHCl_3$. The residue obtained after evaporation was chromatographed by the procedure of Lavie et al. (*13*) and eluted with benzene and increasing $CHCl_3$. Rechromatography of the fractions was done on acid-washed alumina using various proportions of benzene−EtOAc. Gedunin was isolated by a known procedure for crystallization from MeOH and compared with an authentic sample of gedunin. Along with gedunin, the fraction with R_f 0.60 ($CHCl_3$–acetone, 7:3) was collected, and the residue after evaporation of the solvent was crystallized from a benzene−hexane mixture. The compound gave physical data similar to those reported earlier for a synthetic compound (*10*). To produce an analytical sample, final purification was performed using a semi-preparative HPLC column (30 cm × 0.8 cm) filled with 10 μm of Nucleosil C-18 RP. The mobile phase was a mixture of acetonitrile, methanol, and water (58:15:27). The main peak was collected and evaporated to dryness, and the residue was subjected to spectral investigation. High-resolution MS pointed to the molecular formula $C_{28}H_{34}O_8$. The ^{1}HNMR spectrum showed characteristic signals similar to those reported for synthetic 6β-hydroxygedunin. ^{13}C NMR data confirmed the molecular composition assignment from HRMS and indicated the amount and character of CH_2 and CH groups.

Nimbinene and salannin were obtained from neem seeds as reported earlier (*9*). Azadirachtin (>95% purity by HPLC) was isolated from seeds of Indian neem, *A. indica*, by the modified method of Nakanishi (*14*). The results of the spectral investigation of isolated azadirachtin were identical to reported data (*15*) achieved on the basis of detailed ^{1}H NMR and ^{13}C NMR spectroscopic analyses. All three compounds were used for comparisons and as multicomponent combinations in this study.

Insects. The gram pod borer, *H. armigera*, and tobacco armyworm, *S. litura*, were taken from laboratory cultures maintained on an artificial diet prepared in the laboratory (*16*). *S. litura* larvae were also maintained on castor leaves (*Ricinus communis*) for leaf disk bioassays. The cultures were maintained at 27 ± 1 °C at 16:8 LD photoperiod. Generally, neonate, third- and fourth-stage larvae were used in various experiments.

Growth Evaluation. 6β-Hydroxygedunin and gedunin isolated from *A. indica* A. Juss. seed pressed oil were individually mixed with the dry portion of the artificial diet at a concentration range of 20−200 ppm in acetone. The carrier solvent was evaporated, and the control diet was treated with carrier alone.

Upon hatching, two 24-h-old neonate larvae were placed on 1 g of fresh weight diet in an individual Solo cup (1 oz) as described earlier (*17*). 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 at 16:8 LD photoperiod. Larval growth was assessed as a percentage of the controls after 7 days on the basis of larval weight. Larval mortality, if any, was also recorded. Forty larvae were used for each concentration. The concentration inhibiting 50% growth relative to the controls was determined by regression analysis. This procedure was also followed for the evaluation of salannin, nimbinene, and azadirachtin, and EC_{50} values were determined.

Early fourth instar larvae (average weight 25 ± 2 mg) were also used to determine EC_{50} values in artificial diet for this stadium as mentioned above. However, the treatment range of various compounds was 100−800 ppm, except for azadirachtin, where the treatment level was between 0.2 and 1.4 ppm. The dose inhibiting 50% growth (ED_{50}) of these larvae via topical application of the limonoids was also determined in the range of 2−10 μg/larva, except for azadirachtin, where the dose range was 0.02−0.08 μg/larva.

Choice Feeding Assay. Antifeedant activity was assessed using a short-term (5 h) leaf disk choice test. The 3.0 cm^2 disks were punched out from castor leaves (*R. communis*) and treated on each side with 10 μL of aqueous solution emulsified with Triton-X-100 (0.1%) of the above-mentioned compounds, except azadirachtin, in the range of 1−10 μg/cm^2. In the case of azadirachtin the concentration range was 0.005−0.02 μg/cm^2. The controls were treated with carrier alone. The leaf disks were dried at room temperature, and 12−24-h-old fourth instar *S. litura* larvae were introduced into each arena (9 cm diameter) containing one control and one treated disk. Experiments were carried out with one larva per Petri dish with 10 replicates for each treatment. Consumption was recorded, and 50% feeding inhibition (FI_{50}) in each case was calculated as described earlier (*9*, *18*).

Nutritional Analysis. To segregate the behavioral effects from toxicity, 6β-hydroxygedunin was subjected to nutritional analysis. The

Table 1. Effective Concentrations (ppm) of 6β-Hydroxygedunin, Gedunin, and Other Limonoids of *A. indica* Inhibiting Growth of *H. armigera* and *S. litura* (Neonates) in a Dietary Assay ($n = 40$)

	H. armigera			S. litura		
compd	EC_{50} (95% CI)	EC_{95} (95% CI)	slope ± SE	EC_{50} (95% CI)	EC_{95} (95% CI)	slope ± SE
6β-hydroxygedunin	24.2 (19.5–27.1)	54.2 (43.8–84.5)	1.8 ± 0.41	21.5 (18.1–25.5)	78.6 (55.2–112.0)	2.9 ± 0.30
gedunin	50.8 (42.2–61.2)	141.4 (94.9–210.8)	3.7 ± 0.68	40.4 (32.8–49.8)	125.6 (85.2–185.0)	3.3 ± 0.62
nimbinene	391.4 (373.6–410.0)	521.2 (474.2–572.9)	13.2 ± 2.03	404.5 (385.0–424.9)	531.5 (478.9–589.8)	13.8 ± 2.40
salannin	74.5 (59.5–87.6)	174.6 (114.3–270.2)	4.3 ± 0.98	72.0 (56.2–87.8)	190.8 (113.8–322.7)	3.8 ± 0.94
azadirachtin	0.26 (0.15–0.36)	1.46 (0.43–4.13)	2.2 ± 0.65	0.21 (0.14–0.31)	1.29 (0.42–3.81)	2.1 ± 0.63

Table 2. Feeding Inhibition (FI) of Fourth Instar *S. litura* Larvae after Oral Administration of 6β-Hydroxygedunin and Other Neem Limonoids in a Leaf Disk Choice Assay

compd	FI_{50} (μg/cm^2)	95% confidence interval	compd	FI_{50} (μg/cm^2)	95% confidence interval
6β-hydroxygedunin	0.6	0.53–0.74	salannin	3.0	2.1–4.4
gedunin	3.9	3.6–4.3	azadirachtin	0.015	0.005–0.019
nimbinene	17.6	11.7–24.5			

experiment was carried out using both *H. armigera* and *S. litura* early fourth instar larvae. In this experiment 20 larvae were provided with 6β-hydroxygedunin at a dietary concentration of 110 ppm based on EC_{50} values determined against this stage of larva. The relative growth per unit weight of the insect at the outset of the experiment (RGRi) and relative consumption rate at the outset of the experiment (RCRi) were calculated on a dry weight basis after 3 days of feeding. The index of food conversion efficiency (ECI) was calculated as described earlier (*16*). The dietary utilization experiment was also carried out in a similar fashion using gedunin (150 ppm), salannin (150 ppm), nimbinene (600 ppm), and azadirachtin (0.4 ppm). Concentrations used for these compounds were also based on the predetermined EC_{50} values of each compound. In another set of experiments the compounds were applied to larvae topically at predetermined levels where about 50% growth inhibition was achieved. 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, NV) attached to a repeating dispenser (PB-600, Hamilton Co.). The controls were treated with acetone alone. In the case of azadirachtin treatments (being a very effective antifeedant) care was taken to avoid any contact with the mouthparts of the larvae during topical application. The larvae were then allowed to feed on the untreated diet.

Combination Evaluation. To establish the multicomponent strategy of plants as a defense mechanism, combinations of other compounds with 6β-hydroxygedunin were tested. In each case, the compounds were combined in equal proportions (2 mg per compound) and final concentrations were made on the basis of EC_{50} values of the most active component in the combination. This was done for two reasons: (i) due to considerable variation in the natural concentration of limonoids in seeds from various ecotypes and (ii) to make sure that at least 50% inhibition of the most active component, in any case, is achieved. Nine combinations were tested: 6β-hydroxygedunin + gedunin, 6β-hydroxygedunin + azadirachtin, 6β-hydroxygedunin + nimbinene, 6β-hydroxygedunin + salannin, nimbinene + salannin, 6β-hydroxygedunin + salannin + nimbinene, gedunin + salannin + nimbinene, 6β-hydroxygedunin + gedunin + salannin + nimbinene, 6β-hydroxygedunin + gedunin + salannin + nimbinene + azadirachtin. These combinations were given to 48-h-old neonate larvae of *H. armigera* and *S. litura* on artificial diets for 7 days in a fashion similar to that mentioned above. The EC_{50} values for each combination were calculated.

RESULTS

The initial diet bioassay against neonate larvae of *H. armigera* and *S. litura* using 6β-hydroxygedunin, gedunin, salannin, nimbinene, and azadirachtin assessed growth inhibition in both insect species. The effective concentration to inhibit 50% of growth due to 6β-hydroxygedunin was 24.2 and 21.5 ppm, respectively. Gedunin was less efficacious (50.8 and 40.4 ppm) followed by salannin (74.2 and 72.0 ppm) and nimbinene (391.4 and 404.5) against these young larvae (**Table 1**). As usual azadirachtin was the most potent with an EC_{50} of 0.26 ppm against *H. armigera* and 0.21 ppm against *S. litura* larvae.

The effective concentration to induce 50% (EC_{50}) growth inhibition in early fourth instar larvae due to 6β-hydroxygedunin treatment was about 110 ppm. Gedunin and salannin were similar to each other in their efficacy (148.6 and 150.5 ppm). Nimbinene was the least active ($EC_{50} = 600$ ppm). Azadirachtin, in contrast, induced similar effects at 0.4 ppm. The effective dose for 50% inhibition (ED_{50}) via topical application also followed a similar trend, with azadirachtin inhibiting growth by 50% at 0.05 μg/larva in *H. armigera*, while the other limonoids were only effective between 3.5 and 10.0 μg/larva. These predetermined levels of treatment to induce 50% inhibition were used to analyze and compare the effects of all the allelochemicals on dietary utilization by the larvae.

In the leaf disk choice assay there was substantial reduction in feeding (**Table 2**) when azadirachtin was provided to *S. litura* larvae with an $FI_{50} = 0.015$ μg/cm^2 followed by 6β-hydroxygedunin (0.6 μg/cm^2), salannin (3.0 μg/cm^2), gedunin (3.9 μg/cm^2), and nimbinene (17.6 μg/cm^2). However, as with most other antifeedants, the activity cannot compare with that of azadirachtin.

Nutritional analyses after administration of 6β-hydroxygedunin in the diet of *H. armigera* larvae indicated reductions in RGRi, RCRi, and ECI in comparison to the controls. At an avearge EC_{50} value of 110 ppm, reduction in growth (1.37 (mg/mg)/day) due to 6β-hydroxygedunin correlated with reduced efficency of the conversion of ingested food (35.3%). Gedunin also exhibited similar results with significant reduction in ECI in comparison to the controls (**Table 3**). On the contrary, there was reduction in growth relative to the reduced consumption rate after oral treatment of azadirachtin without any significant effect on ECI. Similarly, salannin and nimbinene failed to show any significant difference in ECI values when compared to the controls (**Table 3**). ECI was reduced after topical application of 6β-hydroxygedunin, gedunin, and azadirachtin with a significant decrease in relative growth rate, but not in the case of salannin or nimbinene (**Table 4**). In the case of salannin and nimbinene none of the parameters were different from those of the controls in topical treatments (**Table 4**). The results obtained in nutritional experiments with *S. litura* larvae were in no way

Table 3. Feeding, Growth, and Efficiency of Conversion of Ingested Food by Fourth Instar *H. armigera* Larvae ($n = 10$) Fed an Artificial Diet Containing Various Limonoids from *A. indica* at Concentrations Based on EC_{50} Values[a]

	nutritional index (mean ± SE)		
compd (concn, ppm)	RGRi ((mg/mg)/day)	RCRi ((mg/mg)/day)	ECI (%)
6β-hydroxygedunin (110)	1.37 ± 0.04b	3.83 ± 0.2c	35.3 ± 3.8b
gedunin (150)	1.59 ± 0.08c	4.02 ± 0.8c	39.2 ± 4.6b
nimbinene (600)	1.83 ± 0.03c	3.41 ± 0.5c	53.6 ± 3.1a
salannin (150)	1.62 ± 0.06c	3.13 ± 0.3bc	51.8 ± 4.7a
azadirachtin (0.4)	1.43 ± 0.04b	2.86 ± 0.3b	50.9 ± 5.8a
control	2.69 ± 0.3a	5.03 ± 0.7a	53.5 ± 4.9a

[a] Means within a column followed by the same letter are not significantly different, $P > 0.05$, on the basis of Duncan's multiple range test.

Table 4. Feeding, Growth, and Efficiency of Conversion of Ingested Food by Fourth Instar *H. armigera* Larvae ($n = 10$) after Topical Application of Various Limonoids from *A. indica* at Concentrations Based on ED_{50} Values[a]

	nutritional index (mean ± SE)		
compd (concn, μg/larva)	RGRi ((mg/mg)/day)	RCRi ((mg/mg)/day)	ECI (%)
6β-hydroxygedunin (3.5)	1.57 ± 0.08bc	4.83 ± 0.7b	32.9 ± 4.8b
gedunin (6.5)	2.02 ± 0.1b	5.04 ± 0.5ab	40.1 ± 3.4b
nimbinene (10)	3.08 ± 0.8a	5.66 ± 0.9a	54.7 ± 5.1a
salannin (5)	3.05 ± 0.7a	5.56 ± 0.8a	55.0 ± 5.8a
azadirachtin (0.05)	1.33 ± 0.04c	5.21 ± 0.7a	25.5 ± 1.8c
control	3.13 ± 0.3a	5.71 ± 0.7a	54.5 ± 3.5a

[a] Means within a column followed by the same letter are not significantly different, $P > 0.05$, on the basis of Duncan's multiple range test.

different from those of *H. armigera* treatments; therefore, they have not been included herein.

To determine the multiple-component effects of various neem limonoids against both *H. armigera* and *S. litura* (**Table 5**) in feeding experiments, combinations of the compounds were tested. When azadirachtin was present in a mixture, it dominated in its efficacy, and EC_{50} values were similar to that of azadirachtin (0.26 and 0.21 ppm for *H. armigera* and *S. litura* larvae, respectively) alone against the larvae. None of the concentrations of 6β-hydroxygedunin, gedunin, salannin, or nimbinene seemed to influence the activity of azadirachtin. However, combinations without azadirachtin did show a potentiation effect with potent EC_{50} values in specific combinations (**Table 5**). While 6β-hydroxygedunin or gedunin when combined with salannin or nimbinene showed more effect, the combination of 6β-hydroxygedunin with gedunin or salannin with nimbinene exhibited a reduced activity against both species tested. Obviously, there was potentiation between the compounds with two different modes of action, i.e., the feeding deterrence and the physiological toxicity. The combination of antifeedant compounds (salannin + nimbinene) or the compounds inducing only toxicity (6β-hydroxygedunin + gedunin) did not exhibit any potentiation (**Table 5**).

DISCUSSION

It has been demonstrated that limonoids from Rutales show some level of antifeedant or growth inhibitory activity against insects (*11*). 6β-Hydroxygedunin-treated *H. armigera* and *S. litura* larvae gained less weight when fed on treated diets, and the activity was similar to that observed for gedunin, salannin, and azadirachtin or in earlier reports for nimbocinol and azadiradione (*19*). These effects were concentration dependent, with azadirachtin being the most active compound. 6β-Hydroxygedunin was more active than gedunin followed by salannin and nimbinene. As the first report of 6β-hydroxygedunin evaluation against *H. armigera* and *S. litura* larvae, the difference seen in efficacy could be due to the presence of ring-D epoxy and 6-OH functions, which are absent in gedunin. Gedunin remains more effective than salannin against younger larvae, but not against older larvae. Gedunin-type compounds have originated due to oxidative expansion of the D-ring, which is a Baeyer−Villiger type of oxidation of nimbocinol and azadiradiones. According to the results obtained in the present study, such modifications in the gedunin type of structures have made them more potent than nimbocinol and azadiradone, which also act as chronic toxins (*19*) against lepidopteran larvae. Apparently, activity increases further with additional OH substitution at C-6, as observed in 6β-hydroxygedunin. It is well-known that 7-deacetyl-17β-hydroxyazadiradione is a chronic growth-inhibiting limonoid that has additional 17β-OH function compared to nimbocinol. Its EC_{50} of 240 ppm against *H. zea* (*20*) makes it more active than azadiradione but less active than nimbocinol that has a C-7 OH function, possibly the important functional group for growth inhibitory activity. This, however, does not conform with earlier findings which showed that alteration in C-7 substitutions resulted in diminished bioactivity, e.g., gedunin having a C-7 OAc function (*21*). Azadiradione, however, seems to be general less efficacious in inhibiting growth in lepidopteran larvae such as *H. zea* ($EC_{50} = 250$ ppm), *S. frugiperda* ($EC_{50} = 130$ ppm), and *Heliothis virescens* ($EC_{50} = 560$ ppm), requiring higher levels of treatment (*20*, *22*).

Previously, neem limonoids were reported to deter feeding in a leaf disk bioassay against *S. litura* in a long-term bioassay (24 h) against third instar larvae (*23*). However, we believe that this inhibition with a potent EC_{50} value is possibly due to the

Table 5. Combined Efficacy of 6β-Hydroxygedunin with Gedunin, Nimbinene, Salannin, and Azadirachtin against 48-h-Old Neonate *H. armigera* and *S. litura* Larvae[a]

	H. armigera		S. litura	
combination (concn range, ppm)	EC_{50} (ppm)	95% confidence interval (slope value)	EC_{50} (ppm)	95% confidence interval (slope value)
6β-hydroxygedunin + gedunin (10–50)	33.2	27.4–40.3 (3.9 ± 0.8)	28.1	24.0–32.8 (4.9 ± 0.9)
6β-hydroxygedunin + azadirachtin (0.1–0.5)	0.2	0.25–0.32 (4.0 ± 0.6)	0.22	0.19–0.26 (3.7 ± 0.5)
6β-hydroxygedunin + nimbinene (10–50)	20.8	18.7–23.3 (3.8 ± 0.4)	19.6	17.9–21.5 (3.5 ± 0.4)
6β-hydroxygedunin + salannin (10–50)	17.7	15.9–19.6 (3.2 ± 0.4)	18.5	16.8–20.4 (3.4 ± 0.3)
nimbinene + salannin (40–140)	130.4	117.6–144.7 (3.4 ± 0.3)	118.6	106.9–130.5 (3.1 ± 0.1)
6β-hydroxygedunin + salannin + nimbinene (10–50)	16.8	14.7–21.4 (3.6 ± 0.1)	17.0	15.3–20.0 (4.0 ± 0.5)
gedunin + salannin + nimbinene (20–80)	37.9	34.6–41.7 (3.6 ± 0.5)	30.9	27.9–34.2 (3.7 ± 0.6)
6β-hydroxygedunin + gedunin + salannin + nimbinene (10–50)	14.7	11.4–18.9 (2.6 ± 0.5)	14.9	11.2–19.8 (2.9 ± 0.6)
6β-hydroxygedunin + gedunin + salannin + nimbinene + azadirachtin (0.1–0.5)	0.27	0.23–0.32 (3.9 ± 0.7)	0.23	0.18–0.29 (3.6 ± 0.9)

[a] Individual EC_{50} (ppm) against two species evaluated: azadirachtin, 0.26 and 0.21; 6β-hydroxygedunin, 24.2 and 21.5; gedunin, 50.8 and 40.4; salannin, 74.5 and 72.0; nimbinene, 391.4 and 404.5.

long duration of exposure and chronic toxicity rather than a purely antifeedant effect. In fact, if the compounds interact at chemoreceptor/deterrent receptor levels, absolute antifeedant effects are depicted effectively in short-duration treatment exposures. 6β-Hydroxygedunin, though 40-fold less active than azadirachtin as a feeding deterrent, is potentially much better in its efficacy than other limonoids (**Table 2**) due to its physiological toxicity. Gedunin appears to be a week antifeedant against *S. litura* as has been shown previously against *Epilachna* species (*21*). It has been established that most of the alterations to ring-A and at C-7 of gedunin resulted in diminished antifeedant bioactivity against the European corn borer (*21*) that may be specifically true for the antifeedant mode of action but needs to be confirmed through electrophysiological assays.

As mentioned above the present study has revealed that both gedunin and 6β-hydroxygedunin are chronic toxins rather than absolute antifeedant compounds. Dietary utilization experiments indicate that 6β-hydroxygedunin induces toxicity; nutritional indices were used to determine whether the effect was due to feeding or growth inhibition. When fourth instar *H. armigera* larvae were fed 6β-hydroxygedunin in their diet, the growth rate decreased with the decrease in consumption rate. However, interestingly, the efficiency of conversion of ingested food (ECI) also decreased. ECI is an overall measure of an insect's ability to utilize the food that it ingests for growth. Therefore, a decrease in ECI indicates that more food is being metabolized for energy but less is converted into body growth. This is further evidenced from dietary utilization experiments where 6β-hydroxygedunin was applied topically to fourth instar larvae wherein there was a drop in ECI, though the consumption rate was not affected significantly. Similar results were obtained after gedunin treatment, which suggests a similar mode of action. The reduction in ECI in the case of 6β-hydroxygedunin and gedunin was about 40% and 27%, respectively, with a reduction in growth equal to 49% and 35% at these intermediate levels of treatment. Azadirachtin, however, induced strong physiological toxicity by reducing the growth by about 58% and ECI by 53% after topical treatments. In comparison to azadirachtin, therefore, the other two compounds were moderate in their activity but definitely induced chronic toxicity in *H. armigera* larvae. The dietary analyses show that salannin and nimbinene are absolute antifeedant allelochemicals. In fact, many limonoids can be antifeedants without having chronic toxicity (*12*), such as salannin and nimbinene in our study and limonin, epilimonol, nomilin, meliantriol, melianol, trichillins, pedonin, toonacilins, etc. in other studies (*9*, *11*, *24*). Azadirachtin is a strong antifeedant and growth regulator, and both activities are independent of each other, which is evident from the different ECI values in oral and topical treatments in *H. armigera* (**Tables 3** and **4**). Electrophysiological assays with lepidopteran larvae have demonstrated that the antifeedant effect, at least for azadirachtin, is mediated by stimulation of the sensitive peripheral maxillary styloconic sensilla (*25*, *26*). Growth inhibitory/regulatory effects, on the other hand, are mediated through various enzyme blockages or direct effects on tissues and organs (*9*). Obviously, limonoids can have different primary modes of action depending on the test insect species, and they can exhibit both antifeedant and toxic modes of action (*27*). Oral administration, topical application, and injection of limonoids have been shown to interfere with growth via secretion of trypsin-type proteinases from gut epithelial cells and other digestive and catabolic enzymes (*9*, *28*, *29*). It is, therefore, possible that gedunin-type limonoids act in a similar fashion and damage the insect's digestive tract. However, at this stage it is not clear whether this damage is irreversible. This requires a further detailed investigation via the effect on enzymatic activity.

Commercial neem formulations based on neem seed extracts containing various limonoids are being used for pest management; however, there is no report available to show the interaction of these compounds, if any, that could influence the efficacy of the products. The present study shows that some combinations of limonoids influence the activity of mixtures. Non-azadirachtin-type limonoids do not influence the most potent compound, azadirachtin, and the EC_{50} values of the mixtures did not deviate from the individual efficacy of azadirachtin. On the contrary, a combination without azadirachtin, i.e., 6β-hydroxygedunin, salannin, and nimbinene in various specific mixtures, did show potentiation with higher efficacy values as compared to the individual EC_{50} values (**Table 5**) determined for *H. armigera* and *S. litura* neonate larvae. Surprisingly, potentiation did not occur between the compounds among non-azadirachtin compounds that possessed a similar mode of action, such as chronic toxins (6β-hydroxygedunin + gedunin) or antifdeedants (salannin + nimbinene), and the action was rather antagonistic. This could be due to the compatibility of two compounds for each other because of extreme similarity in structures (for instance, the only difference is an additional C-6 OH function in 6β-hydroxygedunin compared with gedunin) and, therefore, possible competitive failure of absolute interaction at target sites. This confirms our recent hypothesis that potentiation among non-azadirachtin limonoids having explicitly two different modes of action, such as feeding deterrence and chronic toxicity, may be playing a significant role in the potentiation effect (*19*). Obviously, azadirachtin being the most potent compound in neem does not seem to be influenced by any other neem allelochemicals, but moderately active non-azadirachtin-type limonoids do show potentiation effects and could be useful in developing potential formulations for pest management as mixtures. This strategy, in particular, will be advantageous for those raw materials having low levels of azadirachtin content. It is an established fact that the level of azadirachtin content varies in neem ecotypes in relation to the climate, soil type, and altitude of the region of procurement (*30*) and some extracts, though having low levels of azadirachtin, are rich in non-azadirachtin-type limonoids. Multicomponent mixtures having several candidate compounds will also help in having a unique complex pest control agent with a variety of toxic, growth inhibitory, and antifeedant effects. Such complexes are desirable in that the anti-insect spectrum of action is increased, because different species have different responses to individual compounds. These are also likely to be more durable with respect to insects evolving resistance and developing behavioral desensitization as shown in some binary mixtures of plant essential oil allelochemicals (*31*). Such mixtures will also help in making efficacious neem preparations without azadirachtin or when the level of azadirachtin is very low vis-à-vis the specific neem ecotypes.

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