Bioefficacy of toosendanin from *Melia dubia* (syn. *M. azedarach*) against gram pod-borer, *Helicoverpa armigera* (Hübner)

Opender Koul#, J. S. Multani, Gurmeet Singh and Seema Wahab*

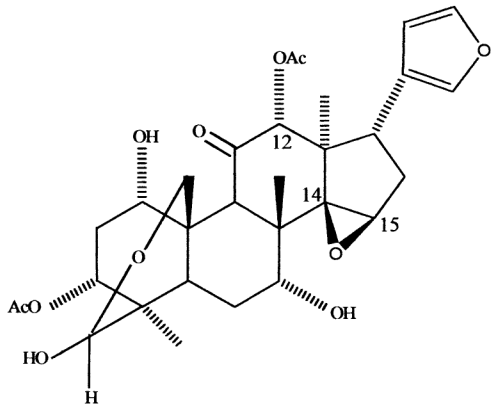
Insect Biopesticide Research Centre, 30, Parkash Nagar, Jalandhar 144 003, India
*Department of Biotechnology, CGO Complex, New Delhi 110 003, India

The antifeedant and growth-inhibitory activities of *Melia dubia* (syn. *M. azedarach*) methanol extract and the allelochemical toosendanin isolated from this fraction to *Helicoverpa armigera* were investigated. Artificial diet bioassay using neonate larvae showed the effect on growth in a dose-dependent manner. After seven days of feeding, EC_{50} value (concentration inhibiting larval growth by 50% relative to controls) for toosendanin (98% purity) was 26.8 ppm, and dose–response relationship was highly significant in linear regression analysis ($P < 0.05$). FI_{50} value (dietary concentration showing 50% feeding inhibition) for toosendanin in third-instar larvae was 56.6 ppm. The results from dietary utilization experiments on fourth-instar larvae revealed reduction in relative growth and consumption rates after oral administration of toosendanin, with a concomitant reduction in efficiency of conversion of ingested food (ECI) at higher concentration only. However, there was significant decrease in efficiency of conversion of digested food (ECD). Following topical treatment, there was significant decrease in relative growth rate and relative consumption rate, although in this case ECI, ECD and approximate digestibility were not significantly reduced. In view of the present findings, toosendanin seems to specifically induce feeding deterrence in *H. armigera* larvae and apparently stimulates deterrent receptor cells. What is obvious from our nutritional studies is that food intake in most of the larvae of *H. armigera* is suppressed due to toosendanin, which apparently reduces neural input from taste cells specialized to detect feeding stimulants. Particularly in topically-treated insects, no toxicity-mediated inhibition was recorded, and still the growth was inhibited in relation to reduced feeding. However, it is possible that toosendanin induces feeding inhibition in a much more intricate manner, which is subsequently responsible for the growth inhibition of insects.

INTEREST in the application of natural products in integrated pest management (IPM) remains high. During the last decade ample emphasis has been placed on the reduced use of synthetic pesticides and the use of biopesticides less disruptive to the environment. Among botanical biopesticides, the Meliaceae family in general and the genus *Melia* in particular has shown great potential for pest management in terms of secondary plant chemistry or the presence of allelochemicals in its various species. In recent past *Melia azedarach*, *M. toosendan* and *M. dubia* have been characterized by the production of a group of modified triterpenes, the limonoids derived from the precursor 4,4,8-trimethyl-17-furanyl-steroid skeleton. It was also observed that all the three species having taxonomic similarities on one hand, have many similarities in chemical profile as well. Accordingly, both *M. dubia* and *M. toosendan* have now been confirmed as synonyms of *M. azedarach*[1–3]. All the three species, as of today being considered as same species now, produce a limonoid toosendanin, which was first isolated from the bark of the tree *M. toosendan* in 1980 (ref. 4). However, toosendanin has been isolated from *M. azedarach* var. *japonica* by the name of 12-acetoxyamoorastatin[5] and identified by comparisons of its spectra with earlier literature data[6]. Since 1980, research into the use of this limonoid as a biopesticide has been carried out[7–12], but there are scanty reports about mode-of-action due to species-specific variations. The compound acts as a growth inhibitor, stomach poison and antifeedant to a number of insect pests. Its activity has also been shown to get synergized by dillapiol, another natural phytochemical from dill oil that is non-persistent in nature[13]. In evaluating the bioactivity against insects of refined bark extracts containing 60–75% toosendanin, it has also been observed that on a weight-to-weight basis, the extracts were more inhibitory to growth of some insects than pure toosendanin, which suggests the activity of other constituents in the extracts[8]. Toosendanin is closely related to meliatoxins, the other group of anti-insect compounds[14] produced in *M. azedarach*. The significant differences between meliatoxins and toosendanin are the absence of C-12 OAc function in most of meliatoxins and lack of C-14/15 epoxide in series 'B' meliatoxins. During the last decade the use of toosendanin as a biopesticide has remained restricted to China, where refined bark extract containing approximately 3% toosendanin (racemic mixture) as the active ingredient is used. The exploitation of this compound remained in the background in view of the notion of *M. toosendan* being specifically native to China. However, our recent success in isolating toosendanin from *M. dubia* (syn. *M. azedarach*) prompted us to undertake the present study and to quantitate the antifeedant and growth-inhibitory effects of pure toosendanin. The study has been conducted against the economically important polyphagous gram pod-borer, *Helicoverpa armigera* (Hübner) using a series of different bioassays, with the aim of determining the overall role of this compound in pest management.

Twigs with bark of *M. dubia* (syn. *M. azedarach*) were collected from Ganga Lake area of Arunachal Pradesh in

*For correspondence. (e-mail: koul@jla.vsnl.net.in)



Scheme 1. Toosendanin.

the eastern part of India during May and June. The plant material was shade-dried and subsequently pulverized to fine powder. The powder was extracted in a sequential manner with petroleum ether, dichloroethane and methanol. The methanol extract was partitioned with EtOAc and water. The EtOAc soluble fraction was chromatographed on silica gel and elution started with a gradient of MeOH in CH_2Cl_2. A fraction obtained with 100% MeOH was rechromatographed to give toosendanin (Scheme 1). The toosendanin concentration was measured by high performance liquid chromatography and compared with a pure sample of toosendanin 98% purity (courtesy M. B. Isman, University of British Columbia, Canada). The spectral data were identical to the literature data[5].

The gram pod-borer, *H. armigera* (Hübner) was taken from laboratory cultures maintained on artificial diet prepared in the laboratory[15]. The cultures were maintained at $27 \pm 2°C$ at 16 : 8 LD photoperiod. Generally neonate, third and fourth stage larvae were used in various experiments.

Methanol extract of *M. dubia* was mixed with the dry portion of the artificial diet, i.e. before gelling, at initial concentrations of 0.5 to 2.5 mg g^{-1} dry weight of diet (100 to 500 ppm) in MeOH. The carrier solvent was evaporated and control diet was treated with carrier alone.

Upon hatching, two neonate larvae were placed on 1 g fresh-weight diet in individual solo cups (1 oz), as described earlier[14]. The cups were kept in a plastic tray lined with moist filter paper to maintain humidity. The experiments were carried out in a growth chamber at $27 \pm 2°C$ at 16 : 8 LD photoperiod. Larval growth was assessed as a percentage of the controls after seven days based on larval weight. Larval mortality, if any, was also recorded. Forty larvae were used for each concentration. The concentration inhibiting 50% growth relative to controls was determined by regression analysis.

Toosendanin was subjected to similar evaluation against neonate larvae to provide final concentrations of 20 to 140 ppm. Control diets were prepared with carrier alone. Larval weight was recorded after seven days of feeding and the concentrations inhibiting 50 and 95% growth were recorded as above.

Antifeedant activity was determined by modified diet choice test[16]. Two small, pre-weighed cubes of artificial diet containing one concentration of toosendanin (20 to 100 ppm) and two pre-weighed control diet cubes were placed in alternating positions in 9-cm diameter petri dishes. Single, 2–8-h-old third-instar larva, pre-starved for 2 h, was placed in the centre of each dish. Single larva was used to avoid cannibalism that is prevalent in these larvae. There were ten replicates per concentration. Consumption by the larvae from each diet cube was recorded only after 6 h, as the short duration evaluation gives the specific assessment of behavioural response. Feeding deterrence index (FI) was calculated as $(C-T)/(C+T) \times 100$, where C is consumption of larvae on control diet and T the consumption of larvae on treated diet cubes[14].

In order to eliminate the behavioural effects from toxicity-mediated effects, toosendanin was subjected to nutritional analysis. The experiment was carried out using early fourth-instar larvae. In this experiment ten larvae per concentration were provided with toosendanin concentration of 20 and 40 ppm in diet. Relative growth per unit weight (RGRi) of the insect at the outset of experiment and relative consumption rate (RCRi) at the outset of experiment were calculated on dry-weight basis after three days of feeding as G/I (G is the change in larval dry weight per day, and I the starting larval dry weight) and C/I (C is the change in diet dry weight per day, and I the starting larval dry weight). Index of food conversion efficiency (ECI) was calculated using a procedure $100 \times G/C$, where G is the dry weight gain of the insect and C the dry weight of food consumed, along with efficiency of conversion of digested food (ECD) and approximate digestibility (AD), as described earlier[15]. Another set of experiments was also carried out in similar fashion with a difference that the toosendanin treatment was given to larvae topically at the rate of 1.0 and 2.0 µg per larva. Third-instar *H. armigera* larvae (av. weight 10 ± 1 mg) were used. For each dose, ten larvae were treated on the dorsal surface with a single 1.0 µl drop of toosendanin 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

Table 1. Growth inhibitory effect of methanol fraction of *M. dubia* on neonate *H. armigera* larvae

Concentration (ppm)	Growth (% of control ± SE)
100	69.5 ± 5.4a
200	31.2 ± 2.8b
500	10.5 ± 1.8c

Means within a column followed by different letters are significantly different; based on Tukey's test.

Table 2. Growth inhibitory effect of toosendanin on neonate *H. armigera* larvae

Concentration (ppm)	Growth inhibition (% of control)	EC_{50} (95% *CI*)	EC_{95} (95% *CI*)	Slope ± SE
20.0	41.2 ± 3.6			
50.0	68.7 ± 4.6			
80.0	83.9 ± 8.7	26.8 (21.5–33.4)	183.9 (136.4–248.2)	1.97 ± 0.11
110.0	86.3 ± 6.8			
140.0	92.5 ± 7.3			

with acetone alone. The larvae were then allowed to feed on untreated diet.

From 2.0 kg of dry plant material 40 g of crude extract with petroleum ether, 38 g with dichloroethane and 135 g with methanol was obtained, which was equivalent to 2.0, 1.9 and 6.75% of dry weight respectively. Methanol fraction of the *M. dubia* extract inhibited larval growth of neonate *H. armigera* larvae in a dose-dependent manner, when added to artificial diet (Table 1) in the range of 100 to 500 ppm of the extract. The extract inhibited larval growth by 50% at 147.5 ppm (95% *CI* = 126.8–171.2) with a slope value of 2.62 ± 0.5.

After seven days of feeding, EC_{50} value (concentration inhibiting larval growth by 50% relative to controls) for toosendanin isolated from methanol fraction (98% purity) was 26.8 ppm (Table 2) and dose–response relationship was highly significant in linear regression analysis ($P < 0.05$). Inhibition of larval growth in these bioassays could arise either through behavioural (antifeedant) or physiological (post-ingestive) effects. However, it was obvious from the experiment that there was significant decrease in consumption during the course of development of the larvae. A dose-dependent decrease in consumption from 13.5 to 42.2% within seven days in comparison to controls was recorded. This implied that after toosendanin treatment at various concentrations, the antifeedant effect was responsible for the depletion of growth. Interestingly, toosendanin was not lethal to the gram pod-borer *H. armigera* at the levels of evaluation in any experiment.

In the diet-choice test of short duration of 6 h, toosendanin inhibited feeding of insects. FI_{50} value (dietary concentration showing 50% feeding inhibition) for toosendanin in third-instar larvae was 56.6 ppm (95% *CI* = 46.3–69.1, Slope = 3.05 ± 0.64). In this test, larvae increasingly avoid feeding on treated diet cubes in a dose-dependent manner (Figure 1).

The results from dietary utilization experiments on fourth-instar larvae are shown in Table 3. Relative growth and consumption rates were reduced after oral administration, with a concomitant reduction in ECI at higher concentration only. At highest dose used (40 ppm) RGRi was reduced to 1.09 mg/mg/d in relation to the reduction in RGRi of 3.05 mg/mg/d, which was about 46% reduction within three days. Although ECI values were not influenced to a significant extent, there was significant decrease in ECD. Following topical treatment there was

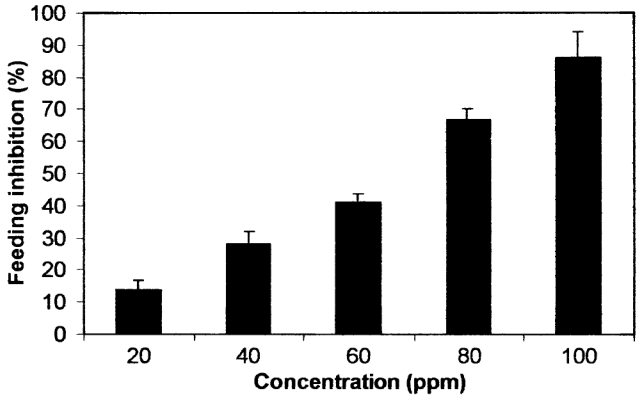


Figure 1. Feeding inhibition action of toosendanin on third-instar *H. armigera* larvae in diet-choice test.

significant decrease in RGRi and RCRi, although in this case ECI, ECD and AD values were not significantly reduced (Table 3).

Toosendanin from *M. dubia* (now synonymous to *M. azedarach* and *M. toosendan*) is a compound with C-19/28 oxygen bridge and a member of amoorasatatin group[5]. It is closely related to meliatoxins, also known insect feeding and growth inhibitors[14]. Earlier studies with toosendanin suggest that this limonoid acts as an antifeedant, growth inhibitor and stomach poison[7,8,17–19]. Variation in the activity of toosendanin also seems to be related to the purity of the compound. It has been established that toosendanin exists as an equilibrium mixture of two compounds (C-28 epimers) and when tested as racemic mixtures, the activity is significantly reduced[9]. Obviously, in our studies 98% pure toosendanin was used, and feeding deterrence was the specific behavioural mode-of-action against *H. armigera* that was responsible for the subsequent depletion of growth among larvae. Our study indicates that toosendanin is a reasonably effective antifeedant against *H. armigera* larvae, with EC_{50} of 26.8 ppm against neonate larvae in feeding diet no-choice tests and 56.6 ppm against third-instar larvae in diet-choice test. Thus it is significantly more efficacious than close relatives, the meliatoxins, which are active in the range of 300–500 ppm[14]. It seems that meliatoxins are less active than toosendanin due to the absence of C-12 OAc function in meliatoxin 'A' series of compounds, and lack of C-12 OAc and C-14/15 epoxide in meliatoxin 'B' series of compounds. However, azadirachtin from neem, *Azadi-*

Table 3. Feeding, growth and dietary utilization by fourth-instar *H. armigera* larvae after oral and topical administration of toosendanin

Treatment (ppm)	RGRi (mg/mg/d)	RCRi (mg/mg/d)	ECI (%)	ECD (%)	AD (%)
	Nutritional indices (mean ± SE)				
			Oral feeding		
0.0	2.02 ± 0.3[a]	4.77 ± 0.9[a]	42.3 ± 5.0[a]	57.3 ± 7.6[a]	73.8 ± 8.9[a]
20.0	1.72 ± 0.2[b]	4.02 ± 0.7[b]	40.8 ± 6.3[ab]	54.8 ± 8.8[b]	73.6 ± 9.2[a]
40.0	1.09 ± 0.3[c]	3.05 ± 0.8[c]	35.6 ± 4.8[b]	43.6 ± 5.3[c]	77.2 ± 9.0[a]
µg/insect			Topical application		
0.0	2.23 ± 0.6[a]	4.88 ± 1.0[a]	45.7 ± 7.3[a]	58.3 ± 7.7[a]	78.4 ± 8.0[a]
1.0	2.01 ± 0.4[b]	4.36 ± 0.9[ab]	45.9 ± 6.6[a]	58.0 ± 5.9[a]	79.1 ± 9.7[a]
2.0	1.78 ± 0.5[c]	4.01 ± 0.8[b]	44.8 ± 4.7[a]	56.8 ± 8.3[a]	78.8 ± 6.3[a]

Means within a column followed by the same letter are not significantly different, $P > 0.05$; based on Tukey's test.

rachta indica, is the most potent, active principal with EC_{50} of 0.23 ppm and 0.4 ppm respectively, against *H. armigera* neonate and third-instar larvae, and remains almost 10 to 15-fold more active[21].

The antifeedant activity of toosendanin is further confirmed by the dietary utilization experiments. When incorporated into artificial diet at 20 and 40 ppm levels, toosendanin reduces the growth rate by about 46% within three days, with significant effect on the relative consumption rate (36% reduction). Index of dietary utilization (ECI), however, was moderately reduced by about 15.8% at 40 ppm and 3.5% at 20 ppm concentration (Table 3). This shows that the relative growth rate of fourth-instar larvae was reduced concomitant with the reduction in relative consumption rate. Obviously, these results do implicate antifeedant effect and seem to be the primary mode of action of toosendanin. The nutritional experiments lend further support to this hypothesis, as after topical application of toosendanin the effect on relative consumption rate was observed *vis-à-vis* the growth rate and the ECI, ECD and AD were not significantly inhibited (Table 3). However, an interesting observation in oral nutritional experiments was a significant reduction in ECD values, which signifies the inability of the larvae to convert digested food into biomass. This implies that the candidate compound that was consumed by the larvae slowly interfered with conversion of digested food in the gut, and justifies the earlier observations of toosendanin being called a stomach poison[20].

It is well known that azadirachtin incorporated into the artificial diet of fourth-instar larvae of *H. armigera* significantly reduced the consumption (up to 76% reduction) and relative growth rate (up to 80% reduction) of larvae compared to controls[21]. Efficiency of conversion of ingested food into biomass was not significantly reduced. The disparity in consumption rate and growth rate is, therefore, not seen in experiments involving azadirachtin-treated diets, as both parameters decrease with increasing azadirachtin concentration, as would be expected from the involvement of chemoreceptors[22,23]. Azadirachtin has a marked antifeedant effect on most insect species, which is regulated through the chemoreceptors located in mouthparts. However, the degree of deterrence varies from species to species depending upon the deterrent chemoreceptor sensitivity to the compound, so that incorporation of azadirachtin into the diet will involve a multiple effect of starvation, growth inhibition and growth regulation. Of course, this has correlation with the mode of treatment and the stage of the stadium at the time of treatment[24]. Toosendanin seems to follow this pattern that might involve the chemoreceptors. In view of the present findings, toosendanin seems to specifically induce feeding deterrence in *H. armigera* larvae, as has been shown in the case of *Pieris brassicae*. By using electrophysiological techniques, it has been established that toosendanin stimulates a deterrent receptor cell located in the medial maxillary sensillum styloconicum in *P. brassicae*. Toosendanin also inhibits response of both the sugar and glucosinolate receptor cell, which is localized in the lateral sensillum stylonicum[25]. This points to a complex perception process, involving a combination of several different sensory mechanisms. For instance, when taste cells of army worm, *Mythimna separata* were exposed to toosendanin for some time, bursting activity was observed[26]. What is obvious from our nutritional studies is that food intake in most of the larvae of *H. armigera* is suppressed due to toosendanin, which apparently reduces neural input from taste cells specialized to detect feeding stimulants. Particularly, in topically-treated insects, no toxicity-mediated inhibition was recorded and still the growth was inhibited in relation to reduced feeding. However, it is possible that toosendanin induces feeding inhibition in a much more intricate manner. This conclusion is based on behavioural evidence that insects can distinguish between different feeding deterrents[27] and also perceive qualitative differences, as is obvious from the variation in the activity if we compare toosendanin with azadirachtin in case of *H. armigera*.

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Regeneration status and population structure of Rudraksh (*Elaeocarpus ganitrus* Roxb.) in relation to cultural disturbances in tropical wet evergreen forest of Arunachal Pradesh

Putul Bhuyan, M. L. Khan* and R. S. Tripathi#

Department of Forestry, North-Eastern Regional Institute of Science and Technology, Nirjuli 791 109, India
#Department of Botany, North Eastern Hill University, Shillong 793 022, India

Density, population structure and regeneration status of Rudraksh (*Elaeocarpus ganitrus* Roxb.) were recorded in four stands of a tropical rainforest exposed to varying magnitude of disturbance. The population was discontinuous as a few size classes of the species were absent. The density of adult trees is 21 individuals per hectare in the undisturbed stand, 19 in the mildly-disturbed stand, 14 in the moderately-disturbed stand and 12 in the highly-disturbed stand. The regeneration was recorded in the undisturbed (two saplings and 200 seedlings/ha), mildly-disturbed (four saplings and 200 seedlings/ha) and moderately-disturbed (100 seedling/ha) stands, while no regeneration (saplings and seedlings were absent) was recorded in the highly-disturbed stand. The highest basal area was recorded in the undisturbed stand (4.2 m^2/ha), intermediate (2.8 and 2.6 m^2/ha) in the mildly- and moderately-disturbed stands and least (1.9 m^2/ha) in the highly disturbed stand. Seedling survival and growth were more in the undisturbed stand. No cut stump was recorded in the undisturbed- and highly-disturbed stands. Sprouting ability of the cut stumps was more in the natural stands compared to the plantation. As the Rudraksh showed sporadic occurrence, its biological conservation is necessary.

ELAEOCARPUS, a genus with about 360 species of the family Elaeocarpaceae, contains hard and highly ornamental stony endocarp commonly known as 'Rudraksh'. The stony endocarp (nut) is used as religious jewellery in the form of beads throughout India and Southeast Asia. Out of about 120 species of *Elaeocarpus* reported from different parts of Asia, including Nepal, Bhutan, Sikkim, Tibet, Java, Indonesia, foothills of the Himalayas and various parts of India, 25 are found in India. The Rudraksh (*Elaeocarpus ganitrus*) tree is common along the foothills of all districts of Arunachal Pradesh, except Tawang and Upper Subansiri and some other high-altitude areas. The Rudraksh is found in tropical evergreen forests, which are characterized by three-tier forest structure. Rudraksh is usually present in the second storey.

*For correspondence. (e-mail: mlk@nerist.ernet.in)