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Short Communication

Effect of neem limonoids on lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* (Guenée) (Insecta: Lepidoptera: Pyralidae)

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Abstract

Neem is derived from the neem tree *Azadirachta indica* A. Juss. (Meliaceae), and its primary insecticidal component is the tetranortriterpenoid azadirachtin and other limonoids. The effect of neem limonoids azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin on enzyme lactate dehydrogenase (LDH) activity of the rice leaffolder (RLF) *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae) larvae was investigated. There was a decrease in enzyme activity relative to the control at all concentrations tested. When fed a diet of rice leaves treated with neem limonoids in bioassays, gut tissue enzyme, LDH levels in rice leaffolder larvae are affected. These results indicate neem limonoids affect LDH activity. These effects are most pronounced in early instar larvae. Azadirachtin was the most potent in of all the limonoids in all experiments indicating strong enzyme inhibition. Clear dose–response relationships were established with respect to LDH activity.

Keywords: Azadirachtin; Salannin; Deacetylgedunin; Gedunin; 17-Hydroxyazadiradione; Deacetylnimbin; Enzyme activity; LDH; Toxicity

1. Introduction

Lactate dehydrogenase (LDH) (EC 1.1.1.28) is an important glycolytic enzyme present in virtually all animal tissues (Kaplan and Pesce, 1996). It is also involved in carbohydrate metabolism and has been used to indicate exposure to chemical stress (Wu and Lam, 1997; Diamantino et al., 2001). LDH is a parameter widely used in toxicology and in clinical chemistry to diagnose cell, tissue and organ damage. However, the potential of this enzyme as an indicative criterion in invertebrate toxicity tests has been scarcely explored (Ribeiro et al., 1999).

The Indian neem tree, *Azadirachta indica* A. Juss. (Meliaceae), has been found to be a promising source of natural pesticides. Several constitutions of its leaves

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and seeds show marked insect control potential and due to their relative selectivity, neem products can be recommended for many Integrated Pest Management (IPM) programs (Schmutterer, 1990). Limonoids are bitter tetranortriterpenes found predominantly in Meliaceae and Rutaceae (Champagne et al., 1989; Ishida et al., 1992). It is generally believed that bioactivity of neem is due to its azadirachtin (complex limonoids) content (Mordue and Blackwell, 1993; Senthil Nathan and Kalaivani, 2005; Senthil Nathan et al., 2005a,b,c). Azadirachtin is known to have adverse effects on more than 400 insect species (Mordue and Blackwell, 1993). During the last five decades, apart from the chemistry of the neem compounds, considerable progress has been achieved regarding the biological activity and medicinal applications of neem. Neem limonoids or their derivatives affect insect physiology in many different ways, but their modes of action on LDH are relatively unstudied. To examine the effects of neem limonoids on the insect gut, we conducted a study of the activity of LDH on an insect pest, the rice leaffolder (RLF), *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae).

2. Materials and methods

2.1. Laboratory mass culture of *Cnaphalocrocis medinalis*

Cnaphalocrocis medinalis larvae were collected from the paddy fields in and around Coimbatore district, Tamil Nadu, India, including the Paddy Breeding Station (PBS), Tamil Nadu Agricultural University. Larvae were reared in a greenhouse on potted rice plants covered with mesh sleeves at 27 ± 2 °C in a 14:10 light:-dark photoperiod and 85% RH. Rice plants were grown in earthenware pots, 18 cm tall with a 20 cm diameter top. Each pot held 15 plants and produced 62 tillers. The pots were placed in about 10 cm of water in a metal tray in the greenhouse (Senthil Nathan et al., 2004). The culture was initiated with partly grown larvae from the

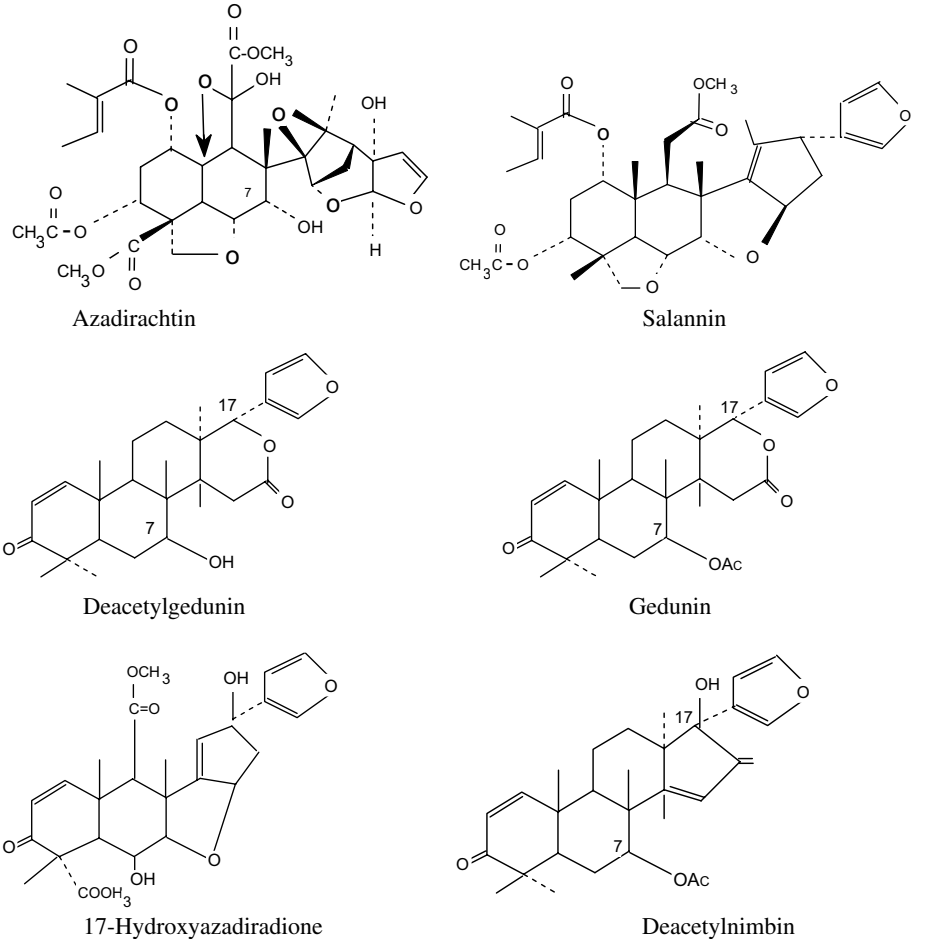

Fig. 1. Structure of neem limonoids tested against *C. medinalis*.

field. Thereafter, newly hatched larvae were placed on ca. 60-day-old plants of the rice variety 'TN1'.

After pupation, adults emerged on plants in the sleeves. To maintain the culture, 12 female and 13 male moths were placed in an oviposition cage containing one potted plant. The moths were fed with 10% sucrose solution fortified with a few drops of vitamin mixture (Multidec drops®, Ashok Pharmaceuticals, Chennai- 24, India) to enhance oviposition. After two days, the potted plants were removed from the oviposition cage. Leaf portions containing eggs were clipped and placed on moist filter paper in Petri dishes. These eggs were used to establish the culture of *C. medinalis*.

2.2. Neem limonoids

Six neem limonoids (Fig. 1) (purity > 99%) namely azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin were sent from M. Ishida, Central Research Laboratories, Taiyo Kagaku Co. Ltd., Japan. They were dissolved in isopropanol and different concentrations were prepared by dilution with isopropanol.

2.3. Bioassays

Bioassays were performed with third to fifth instars of *C. medinalis* using concentrations ranging from 0.10 to 1 ppm. Control leaves were treated with isopropanol and air dried. A minimum of 10 larvae/concentration were used for each experiment and experiments were replicated five times. The effective concentration (EC_{50}) was calculated using probit analysis (Finney, 1971).

2.4. Treatments

The fresh rice leaves (*Oryza sativa* L.) were coated with different concentration of limonoids and air-dried. Control leaves were treated with isopropanol and air-dried. Third to fifth larval instars were starved for 4 h, and were individually fed with different concentrations of limonoids. Uneaten leaves were removed every 24 h, and replaced with fresh treated leaves. A minimum of 10 larvae/concentration were used for all the experiments and experiments were replicated five times.

2.5. Preparation of enzyme extract

Second to fifth instars of treated *C. medinalis* were used to quantify enzyme activities. Enzyme extract were prepared by the method of Applebaum (1964) and Applebaum et al. (1961). Individuals were anaesthetized with 5 mm × 5 mm cotton pads soaked in ether and the entire digestive tract was dissected out in ice cold insect Ringer's solution. The Malpighian tubules, adhering tissues and gut contents were removed. The gut was split into regions (foregut, midgut and hindgut), weighed and each region was homogenized for 3 min at 4 °C in citrate–phosphate buffer (pH 6.8) using a tissue grinder. Homogenized gut sections were suspended in 4 °C buffer and diluted to 1 ml. The homogenate was centrifuged at 500 rpm for 15 min and the supernatant was used as the enzyme source.

2.6. Estimation of lactate dehydrogenase (LDH) (EC 1.1.1.27)

To standardize volumes, 0.2 ml of NAD + solution was added to the 'test' and 0.2 ml of water was added to the control test tubes, each containing 1 ml of the buffered substrate; 0.01 ml of the sample was also added to the 'test'. Test tube samples were incubated for exactly 15 min at 37 °C and then arrested by adding 1 ml of colour reagent (2,4-dinitrophenyl hydrazine reagent) to each tube and the incubation was continued for an additional 15 min. After the contents were cooled to room temperature, 10 ml of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline to maximize development of hydrazine. At exactly 60 s after the addition of alkali to each tube, the intensity of colour was measured at 440 nm. Replicated blanks with standards were run through the same procedure. Inclusion of the calculated amount of reduced co-enzyme in the standard makes allowance for the chromogenicity of $NADH_2$ formed in the test. The enzyme activity is expressed as multi International Units (mIU) per mg protein per minute (King, 1965).

A mIU is defined as the amount of enzyme that is required to catalyze the conversion of 1 μm of lactate to pyruvate or pyruvate to lactate3 per minute per ml of the sample under the prescribed assay conditions.

2.7. Statistical analysis

The effective concentration was calculated using probit analysis (Finney, 1971). Data from enzyme activity were subjected to analysis of variance (ANOVA of arcsine square root transformed percentages). Differences between the treatments were determined by Tukey's multiple range test ($P \leqslant 0.05$) (Snedecor and Cochran, 1989; SAS Institute, 2001).

3. Results and discussion

The efficacy of neem limonoids were evaluated in this study. Our results show that neem limonoids affected the physiology of *C. medinalis* at several doses. Treatment with neem limonoids significantly decreased the activity of LDH. The maximal suppression of LDH activity was obtained by azadirachtin treatment at 1 ppm (62%, 59% and 55% in second, third and fourth instar, respectively).

Changes in metabolism and decreases in the enzyme activity of neem treated individuals may be expected to affect enzyme titers and activities (Schmutterer, 1990; Mordue and Blackwell, 1993; Senthil Nathan et al., 2004, 2005d) Our data support this hypothesis (Table 1 and Fig. 2), because higher LDH activity in control insects is most probably due to consumption as well as utilization of large quantities of food.

An EC_{50} value of neem limonoids against rice leaffolder is shown in Fig. 3. Azadirachtin was most potent in all experiments with at least EC_{50} (0.043 ppm, 0.057 ppm and 0.063 ppm for third, fourth and fifth instars, respectively). Change in LDH activities after treatment with neem limonoids toxin indicate that changing the physiological balance of the midgut might affect these enzymes (Senthil Nathan et al., 2004). In the present study, the decrease in the activity of these enzymes after neem limonoids were fed to *C. medinalis* suggests that limonoids are affecting gut physiological events (i.e. ion transport) that might influence the enzyme (Senthil Nathan et al., 2005a). The chemical structures of the assayed compounds are shown in Fig. 1. In this study, the potent enzyme inhibitors were azadirachtin, salannin, deacetylgedunin and gedunin. These four compounds have some common structural features such as furan ring and an α,β-unsaturated ketone in their A-ring. Azadirachtin is by far the most potent enzyme inhibitor among all the limonoids, being more than five times as effective as the least potent enzyme inhibitor, gedunin.

LDH is involved in the production of energy, being particularly important when a considerable amount of additional energy is required immediately. A negative correlation between LDH activity and ambient oxygen levels for some aquatic organisms were suggesting a possible biochemical adjustment in response to the lowered

Table 1
Lactate dehydrogenase activity (mIU/mg/protein/min) of *C. medinalis* after treatment with neem limonoids

Treatments (ppm)	Larval instar		
	III*	IV*	V*
Control	14.68 ± 1.82^{a}	26.37 ± 2.51^{a}	37.14 ± 4.21^{a}
Azadirachtin 1.00	5.63 ± 0.95^{c}	10.94 ± 1.23^{c}	16.54 ± 1.95^{bc}
Salannin 1.00	7.32 ± 0.90^{b}	14.83 ± 1.54^{b}	20.43 ± 2.15^{b}
Deacetylgedunin 1.00	8.15 ± 1.13^{b}	16.68 ± 1.60^{b}	24.65 ± 2.75^{ab}
Gedunin 1.00	10.18 ± 1.45^{b}	20.72 ± 2.14^{ab}	31.19 ± 3.26^{ab}
17-Hydroxyazadiradione 1.00	9.42 ± 1.28^{b}	18.27 ± 2.00^{b}	27.92 ± 3.12^{ab}
Deacetylnimbin 1.00	10.05 ± 1.45^{b}	18.56 ± 2.00^{b}	28.54 ± 3.36^{ab}

* Means within columns followed by the same letter are not significantly different (Tukey's test, $P \leqslant 0.05$) ± standard error.

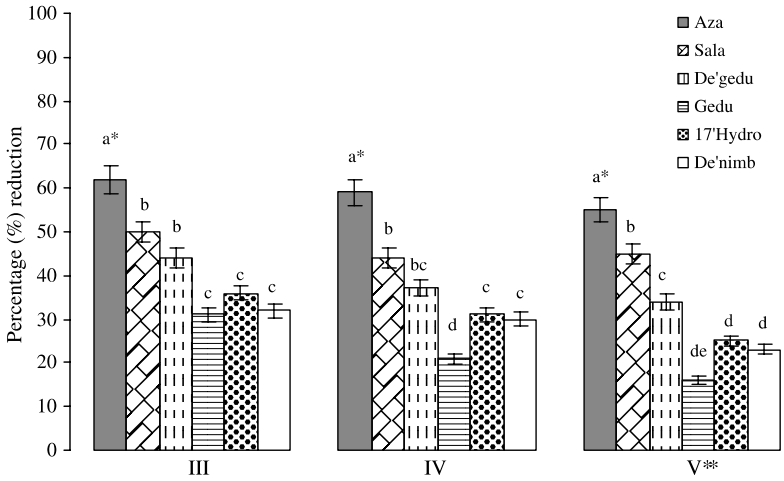

Fig. 2. Percent reductions of LDH activities in third to fifth instar larvae of *C. medinalis* after treatment with neem limonoids. *Means (±(SE) standard error) followed by the same letters above bars indicate no significant difference ($P \leqslant 0.05$) in a Tukey test. **Larval instars. Azadirachtin (Aza); Salannin (Sala); Deacetylgedunin (De′gedu); Gedunin (Gedu); 17-Hydroxyazadiradione (17′Hydro); Deacetylnimbin (De′nimb).

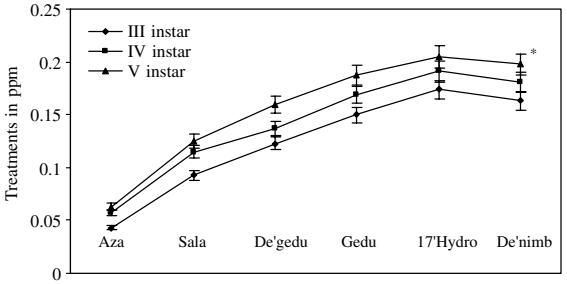

Fig. 3. Effective concentrations (EC_{50}) of neem limonoids against third to fifth instar larvae of *C. medinalis*. *Values are means of five replicates; ± standard error. Azadirachtin (Aza); Salannin (Sala); Deacetylgedunin (De′gedu); Gedunin (Gedu); 17-Hydroxyazadiradione (17′Hydro); Deacetylnimbin (De′nimb).

oxygen levels. This probably occurs also in situations of chemical stress. Therefore, this enzyme may be a sensitive criterion in laboratory (Wu and Lam, 1997; Diamantino et al., 2001; Senthil Nathan et al., 2005d). After treatment with neem limonoids a decrease in LDH activity denotes reduced metabolism in the insect and may be due to the toxic effects of neem derivatives on membrane permeability, especially of the gut epithelium (Smirle et al., 1996; Senthil Nathan et al., 2004, 2005a). In conclusion, neem limonoids had significant effects on larval RLF and they caused reduction of LDH enzyme activity. These effects were more pronounced in early instars. The adult physiology is thus impaired after larvae were exposed to neem limonoids.

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