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In vitro and *in vivo* inhibition of haemolymph juvenile hormone esterase activity by the ethanol extract of *Clerodendrum inerme* in fifth instar larva of castor semilooper, *Achaea janata* (L.)

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Juvenile hormone (JH) is a unique hormone in insects that controls growth, development, metamorphosis and reproduction. It has been well established that JH esterase is an enzyme involved in JH regulation. Results of both *in vitro* and *in vivo* studies revealed that ethanol leaf extract of *Clerodendrum inerme* significantly inhibits haemolymph JH esterase activity affecting growth and normal development of the test insect, castor semilooper (*Achaea janata*), a serious pest of oil seed crops like castor and groundnut.

Keywords: *Achaea janata*, *Clerodendrum inerme*, ethanol leaf extract, juvenile hormone.

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SINCE its discovery by Wigglesworth[1], insect juvenile hormone (JH) is known to be critical for the regulation of growth, development and metamorphosis as well as reproduction in insects[2]. Haemolymph JH titre is controlled by its synthesis in corpora allata and degradation in haemolymph and other peripheral tissues[3]. The primary route of JH degradation is hydrolysis of its ester by the highly specific JH esterase (JHE). Another route of JH degradation is hydration of the 10,11-epoxide by JH epoxide hydrolase. The importance of JHE in the metabolism of JH, control of JH titre and induction of metamorphosis has been well recognized by enzymatic inhibition of JH using transition state analogue inhibitors and manipulation of JHE gene expression using baculovirus expression systems or transgenic insects[4–6]. That JHE expression is induced by JH has been shown in several insect species[7–10].

The possibility of ecologically important interactions between insects and plant natural products that mimic JH action led to the discovery of numerous phytojuvenoids (sesamin, sesamolin, juvocimene, etc.) of modest to very high JH activity isolated from several plant families. The discovery of juvocimene that disrupted the insect hormonal process has paved the way for the development of a second generation of JH-active commercial products, such as fenoxycarb[11]. The effectiveness of these hormone-based insect growth regulators appears to be stable against several insects that are pests of agricultural crops.

Clerodendrum inerme L. Gaertn (Lamiaceae), commonly known as Kashmir bouquet, is a biennial, hardy plant which is widely grown as a hedge plant along home gardens. The leaf extract as well as sun-dried leaf powder of this plant have been shown to contain growth disruptive properties against a few insect species[12,13]. However, the induction of growth disruptive action of plant secondary metabolites on JH level has not been demonstrated. The only route of JH metabolism in insects is haemolymph ester hydrolysis. The correlation of high levels of haemolymph JHE activity with low levels of JH during development in several lepidoptera suggests that JHE is an important regulator of JH titre, especially in the regulation of metamorphosis[14]. The present study was designed to investigate both *in vivo* and *in vitro* effects of ethanol leaf extract of *C. inerme* on JHE titre in the early and late fifth instar larvae of castor semilooper, *Achaea janata*, a serious pest of castor plant as well as other oil seed plants like peanut and groundnut crops.

In the present study, late fourth instar larvae exhibiting head capsule slippage were separated from the culture maintained in the Department of Zoology, Karnatak University, Dharwad, India. Those undergoing ecdysis were individually maintained on fresh castor leaves supplied *ad libitum*. Larvae with uniform body weight (250 ± 10 mg) were used throughout the experiments. The effect of *C. inerme* extract on haemolymph JHE activity was examined following both *in vitro* and *in vivo* experiments.

Developmental profile of haemolymph JHE activity was determined from the pooled sample from 2 to 3 larvae at an interval of 8 h during fifth instar larval development from D1 to D5 (L5D1 to L5D5). Haemolymph was collected from the cut end of the abdominal pro-legs into 10 × 75 mm test tubes held in ice and containing a few crystals of phenylthiourea (PTU). Haemolymph was centrifuged for 5 min at 1000 *g*. The supernatant was diluted in standard sodium phosphate buffer (ionic strength = 0.2 M, pH 7.4, 0.01% PTU).

JHE activity was determined by the partition assay following the procedure described by Hammock and Roe[14]. Haemolymph diluted in standard sodium phosphate buffer (1 : 9 v/v), was incubated at 30°C containing recemic [H]3-JH III (12Ci/nmol, tritiated at C-10, New England, Nuclear, Boston, MA) mixed with unlabelled recemic JH III. The final JH III concentration was 5 µM/reaction, and haemolymph dilution and incubation times were chosen to produce linear increase in JH metabolism with time. At the end of the incubation period, the reaction was stopped by adding NaCl until saturation and extracted three times with ethyl acetate. The pooled ethyl acetate volume was reduced to 100 µl under nitrogen gas and 20 µl of ethyl acetate containing JH metabolites was chromatographed along with standards in separate lanes with [H]3-JH-III, JH acid, JH diol and JH acid–diol on Brinkmen poly gram SIL G, 0.25 mm thick plastic plates (activated 30 min at 100°C). The TLC plate was developed using hexane : ethyl acetate : glacialacetic acid (88 : 33 : 1, v/v/v) as solvent system. Each lane was cut into 4 mm fractions and the fractions assayed separately by liquid scintillation. Activity recovered was >90% of that applied to the plate. The developmental profile of haemolymph JHE activity of castor semilooper is shown in Figure 1.

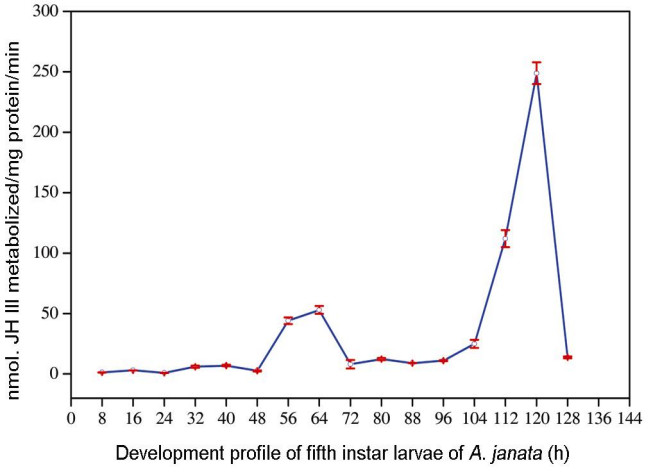

Figure 1. Haemolymph JH III esterase activity profile during 5th instar development of *Achaea janata*.

Table 1. *In vitro* inhibition of juvenile hormone esterase activity by *Clerodendrum inerme* leaf extract in fifth instar *Achaea janata*

Concentration of extract (μg) in 10 μl ethanol	JHE activity (nmol JH III metabolized/mg protein/minute)	Inhibition (%)
Control	280.0 ± 11.3	–
20	281.0 ± 6.6	–
40	259.0 ± 7.0	7.5
60	257.0 ± 4.8	8.21
80	213.0 ± 4.0	23.92
100	201.0 ± 6.0	28.21
OTFP	195.0 ± 2.34	30.35

Results are mean ± SE of five independent experiments. OTFP: 3-Octylthio-1,1,1-trifluoropan-2-one, a known inhibitor of JHE. The enzyme was incubated with OTFP at the concentration of 4×10^{-9} for 15 min and then the substrate was added.

Table 2. *In vivo* inhibition of JHE activity by *C. inerme* leaf extract in fifth instar *A. janata*

Concentration of extract (μg) in 10 μl acetone applied topically	JHE activity (nmol JH III metabolized/ mg protein/minute)		Inhibition (%)
	Control	Treated	
25	310.6 ± 6.6	310.00 ± 8.2	–
50	298. 8 ± 6.0	293.03 ± 7.0	1.7
75	300.0 ± 6.8	275.90 ± 8.0	8.3
100	315.0 ± 8.0	250.00 ± 9.8	20.6

Results are mean ± SE of five independent experiments.

The ethanol extract of *C. inerme* leaf powder was prepared using all-glass Soxhlat apparatus using 500 ml of full-proof ethanol for 24 h. Later the solvent was removed using rotary flash evaporator under reduced pressure. The weight of dried residue was determined and stored at 4°C until further use. The residue was re-dissolved in known volume of ethanol or acetone and used as test compound. Tissue protein concentration was determined according to the procedure of Lowry *et al.*[15].

The results of haemolymph JHE activity profile (Figure 1) revealed two peaks during fifth instar larval development. The first peak appeared prior to the wandering stage (56–64 h) and the second peak appeared during late larval instar.

In vitro effect of test compound on haemolymph JHE activity was tested as follows. Known quantity of dried ethanol extract was re-dissolved in known volume of ethanol (as carrier solvent) such that the final concentration of the test compound should be in 10 μl of the carrier solvent, and the test compound was used in the range 20–100 μg. A 100 μl diluted haemolymph (1 : 9, v/v), mixed with 10 μl test compound was pre-incubated at 30°C for 15 min with constant shaking and control tubes received 10 μl of ethanol only. After the incubation period, recemic [H]3-JH III (12 Ci/nmol, titrated at C-10, New England, Nuclear, Boston, MA) mixed with unlabelled recemic JH III was added to the experimental and control tubes and incubated at 30°C for 30 min. The reaction was stopped by the addition of methanol and iso-octane (1 : 1; v/v) mixture. Then each tube was vortexed vigorously and aqueous phase was separated by centrifugation at 1000 *g*. The aqueous phase was analysed for JH metabolites using TLC procedure described earlier. The results are summarized in Table 1.

It is evident from the present *in vitro* experiments that the test compound inhibited JHE activity in graded response in relation to its concentration. As much as 28.21% inhibition of JHE esterase activity was observed in larval groups treated with 100 μg of test material. It was significant to note that the inhibitory effect was almost equal to the inhibition of JHE activity by synthetic 3-octylthio-1,1,1-trifluoropan-2-one (OTFP), (30.23%) a proven and potential JHE esterase inhibitor[16–18].

For *in vivo* studies, a known quantity of ethanol leaf extract residue was re-dissolved in a known volume of acetone and various concentrations of test compound in 10 μl acetone solution were prepared. Ten microlitre of each concentration of the solution was topically applied on the dorsal inter-segmental region of un-anaesthetized late L5 D5 (128 h old) larvae. The control group of larvae received 10 μl carrier solvent alone. At the end of 8 h, the haemolymph from experimental and control groups of larvae was collected and analysed for JH metabolites by TLC procedure described earlier; the results are presented in Table 2. The results of the *in vivo* studies in respect of *C. inerme* leaf extract on haemolymph JHE activity are similar to those of the *in vitro* studies. *In vivo* inhibition of JHE at 100 μg of test compound was 20.6%.

The behavioural studies of both control and treated groups (10 larvae in each group) revealed that only controlled group of larvae on day-3 entered into wandering behaviour (movement of larvae in all directions on the leaf surface, particularly towards tender and fresh parts of the leaf), whereas none of the larvae treated with test compound underwent wandering stage. Since the larvae were fed with known number of castor leaves each time, it was observed that the food consumption was less in treated groups of larvae compared to the untreated ones. These observations were further supplemented by our findings that the fifth instar larvae (L5 D1 to D5) of *A. janata* treated topically with *C. inerme* leaf extract demonstrated reduced larval body size (Figure 2 *a*) and those pupated were also small and exhibited morphological deformities when compared with the corresponding control groups (Figure 2 *b*). The adults that emerged from the experimental group showed several morphological deformations (Figure 2 *c*). Several lines of evidence indicate that JH plays an important and significant role in regulating pre-metamorphic behaviour of insects, in particular holometabolus insects[19–21].

The correlation between larval size and wandering duration is based on JH effects and wandering can be induced in any larval instar following the elimination of JH[19]. The results of the present *in vivo* and *in vitro* studies suggest that *C. inerme* extract appeared to influence the JH titre during fifth instar larval development of *A. janata.* These results also support earlier observations[2]. Two dynamic events are apparently involved in the regulation of JH titre – JH biosynthesis and JH degradation. Evidences suggest that in Lepidoptera, haemolymph ester hydrolysis by JHE is a major player responsible for the metabolism of JH[17,22,23]. Several lines of evidence indicate that JH plays an important role in inducing wandering behaviour, as well as regulating pre-metamorphic behaviour, such as application of JH causes aberrant cocoon spinning[2]. Two peaks in JHE activity occur during the last instar development of castor semilooper (Figure 1) the first prior to wandering and the second prior to ecdysis to the pupa. This has been demonstrated in a number Lepidoptera species. It has been observed in the present study that haemolymph JHE activity is minimal during early larval development (L5 D1 to D3) and increases significantly in the late fifth instar on day-5 (L5D5, Figure 1). It is pertinent to mention in this context that in the fifth instar tobacco hornworm, *Manduca sexta* disappearance of JH from the blood as well as from other tissues is a pre-metamorphic change in the epidermis[20]. Further, the titre of 20-hydroxyecdysone (moulting hormone) rises in the absence of JH and also causes release of ecdysone[2]. It may be argued from the observations and results of the present study that the second peak activity of JHE which appears in late L5 D5 larva induces final ecdysis leading to pupal stage in castor semilooper.

In vitro studies with haemolymph JHE from last instar cabbage looper, *Trichoplusia ni* showed that when OTFP-sulfone that mimics the backbone of JH, was topically applied, it selectively inhibited >90% of JHE activity, while having no effect on 1-naphthylacetate esterase activity. This juvenizing effect of OTFP-sulfone provides direct evidence of the importance of JHE in the regulation of JH. The present study on the effect of *C. inerme* leaf extract on the haemolymph JHE activity demonstrates a dose-dependent inhibition of JHE (Tables 1 and 2).

In the light of these observations, it may be argued that the inhibition of JHE activity appears to create a JH environment that causes inhibition of wandering behaviour of the larvae, which might have caused nutritional imbalance in late fifth instar larvae of *A. janata.* These pre-metamorphic changes affected larval growth and size that resulted in morphological deformities during pupal and adult developments (Figure 2 *b* and *c*). However, further research is required to identify juvenomimetic factor(s) present in the *C. inerme* leaf and work is in progress.

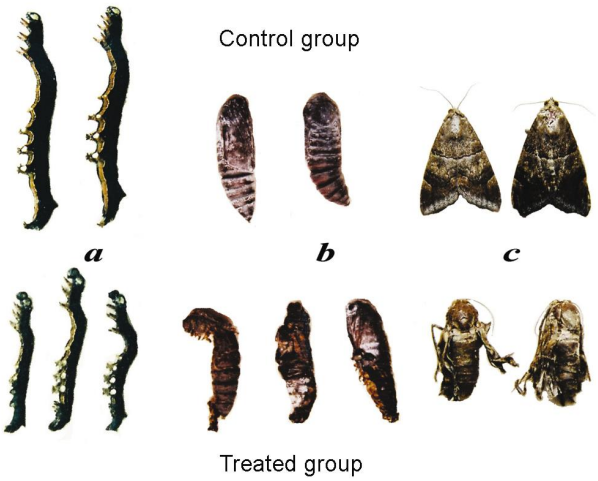

Figure 2. Fifth instar larvae treated with *Clerodendrum inerme* leaf extract exhibit growth inhibition in larvae and deformities in pupae and adults of *A. janata.*

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Molecular characterization of viroid associated with tapping panel dryness syndrome of *Hevea brasiliensis* from India

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Tapping panel dryness (TPD) disease is the most economically important malady affecting the quality and quantity of yield of rubber latex. The etiological agent of the disease has not yet been characterized. In the present communication, we report the association of a viroid belonging to potato spindle tuber viroid group (PSTVd). The isolates cloned from TPD-affected rubber samples and tomato leaves infected by the extract from TPD-affected rubber showed more than 95% identity with PSTVd.

Keywords: Rubber latex, tapping panel dryness disease, tomato leaves, viroids.

VIROIDS are unique plant pathogens consisting of low molecular weight (LMW), non-encapsidated, autonomously replicating single-stranded RNA molecules (~246 to 425 nucleotides (nt)) without any functional open reading frames (ORFs) in their genome[1]. Ever since the first report of a viroid on potato[2], about 32 viroid species and more than 340 viroid variants have been recorded. In the 'Subviral RNA Database' (http://subviral.med.uottawa.ca/), more than 1700 viroid sequences are available. They are the etiologic agents of diverse diseases affecting food, industrial and ornamental herbaceous and lignaceous plants. Viroid-induced symptoms range from necrosis to less severe developmental disorders, including leaf chlorosis, necrosis, stunting, flowering alterations, and fruit and seed deformations. They are more prevalent in vegetatively propagated plants and in high-yielding clones and varieties of plants developed.

Potato spindle tuber viroid (PSTVd) belonging to the family Pospiviroidae, has a wide host range and has been reported from a wide variety of ornamental and horticultural crops; to name a few, capsicum[3], potato[4,5], gooseberry[6–8], *Brugmansia* spp[9], *Solanum jasminoides*[9], petunia[10], tomato[11], cestrum[7] and dahlia[12]. Avocado is by far the only tree species where PSTVd has been

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