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Effect of Neem gold on haemocytes of the tobacco armyworm, *Spodoptera litura* (Fabricius) (Lepidoptera; Noctuidae)

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Neem gold (0.15% azadirachtin) was evaluated for its effect on haemogram and ultrastructure of haemocytes of *Spodoptera litura* (Fab.). Oral treatment of Neem gold (500, 1000 and 1500 ppm) in artificial diet to the last instar larvae of *S. litura* caused a decrease in haemolymph volume; however, total haemocyte count (THC/mm^3), computed from five recorded types, viz. prohaemocytes (PRs), plasmatocytes (PLs), granular haemocytes (GRs), spherulocytes (SPs) and oenocytoids (OEs), decreased only after 48 to 72 h of treatment. At both scanning (SEM) and transmission electron microscopic (TEM) levels, PLs and GRs were the main cell types affected by the treatment, whereas in case of PRs, SPs and OEs the effect of Neem gold was negligible. SEM studies revealed Neem gold to cause a reduction or loss of filopods in PLs and that of cytoplasmic projections in GRs. TEM studies of PLs and GRs showed induction of vacuolization in their cytoplasm, degeneration of organelles and destruction of their plasma membrane.

INSECTS possess an open circulatory system which contains various types of haemocytes, the mesodermal cells which perform several physiological functions, including protection from pathogens. Due to this reason, the study of haemocytes has become an important area of research. In view of the environmental degradation by toxic pesticides, emphasis nowadays is being put on natural products for controlling the pest populations. The pathological effects of botanicals on haemocytes have been reported only in a few insect species[1–4], despite the plants being a source of non-toxic compounds utilized for insect control. The scanning electron microscopic (SEM) and transmission electron microscopic (TEM) studies of the haemocytes of *Spodoptera litura* have been reported[5,6]. The constituent of neem (viz. azadirachtin) possesses well-established anti-feedant and growth-inhibiting properties[7]. Azadirachtin inhibits feeding and growth in a wide variety of insect species. The developmental effects caused by it are attributed to disruption of endocrine events[7], although its principal site of action has not hitherto been identified. Because of lack of information on the effect of this important botanical on haemogram, i.e. total haemocyte count (THC), differential haemocyte count (DHC), haemolymph volume (HV) and ultrastructure of the haemocytes of a lepidopteran pest, the present investigations have been carried out.

A culture of *S. litura* (Lepidoptera; Noctuidae) was maintained in the laboratory on an artificial diet[8] (26 ± 2°C, 70% R.H., 16 : 8 h light : dark cycle). The 6th instar (last instar) larvae constituted the material for the present investigation. For field applications, azadirachtin is applied in the form of extracts from kernels of *Azadirachta indica*. Since Neem gold is one of the commercially available plant products from neem kernels containing azadirachtin, it was used in the present study. Neem gold (0.15% azadirachtin) was obtained from SPIC Science Foundation, Chennai, India. Stock solution (1%) of Neem gold was prepared in acetone and added to the dry ingre-

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dients of artificial diet of *S. litura*, with continuous stirring, so as to make 500, 1000 and 1500 ppm concentrations of treated diet after evaporation of acetone.

Freshly moulted (0 h) 6th instar larvae of *S. litura* of known weight were released in sterilized glass tubes having a piece of treated diet (1 larva/tube × 10 replicates) and after every 24 h, up to 72 h, the larval weight was recorded and a fresh piece of diet was given to the larvae. Controls of treated diet were run simultaneously.

The haemogram[9,10] (THC, DHC and HV) of the larvae of treated and control was performed at an interval of 24 h up to 72 h of age. For THC and DHC, heat fixation of larvae was carried out at 55–58°C (ref. 11). THCs were done by drawing 10 µl aliquot of haemolymph (using an automatic pipette) from the severed proleg of the larva and mixing immediately with 390 µl of physiological saline. THCs were conducted with a standard haemocytometer. DHCs were estimated by taking a drop of haemolymph on a clean glass slide and preparing the smear. The air-dried smears were exposed to vapours of acetic acid for five to ten minutes. The smears were then stained in dilute Giemsa's stain and differentiated wherever necessary in very dilute lithium carbonate or very dilute acetic acid. DHCs were performed by classifying 200 cells per smear[12]. HV was estimated by the exsanguination method[10]. Student's *t* test was used for statistical analysis. SEM and TEM studies of the haemocytes were conducted on control and 1500 ppm Neem gold-treated (the maximum growth inhibiting dose) larvae after 48 h of treatment.

In control larvae, plasmatocytes (PLs) show numerous pits (P), filopods (F) and granular haemocytes (GRs) which are characterized by cytoplasmic projections on their surface, as revealed by SEM (Figures 1 *a*, *b*). At both SEM and TEM levels, PLs (Figures 1 *c*, *d* and 2 *a*) and GRs (Figures 1 *d* and 2 *b*, *c*) were observed to be the main cell types affected by the treatment of Neem gold. The effect was negligible on the prohaemocytes (PRs), spherulocytes (SPs; Figure 2 *d*) and oenocytoids (OEs; Figure 2 *e*). PLs and GRs exhibited considerable reduction in their size besides a reduction or loss of filopods, and cytoplasmic projections (Figure 1 *c*, *d*). In some GRs,

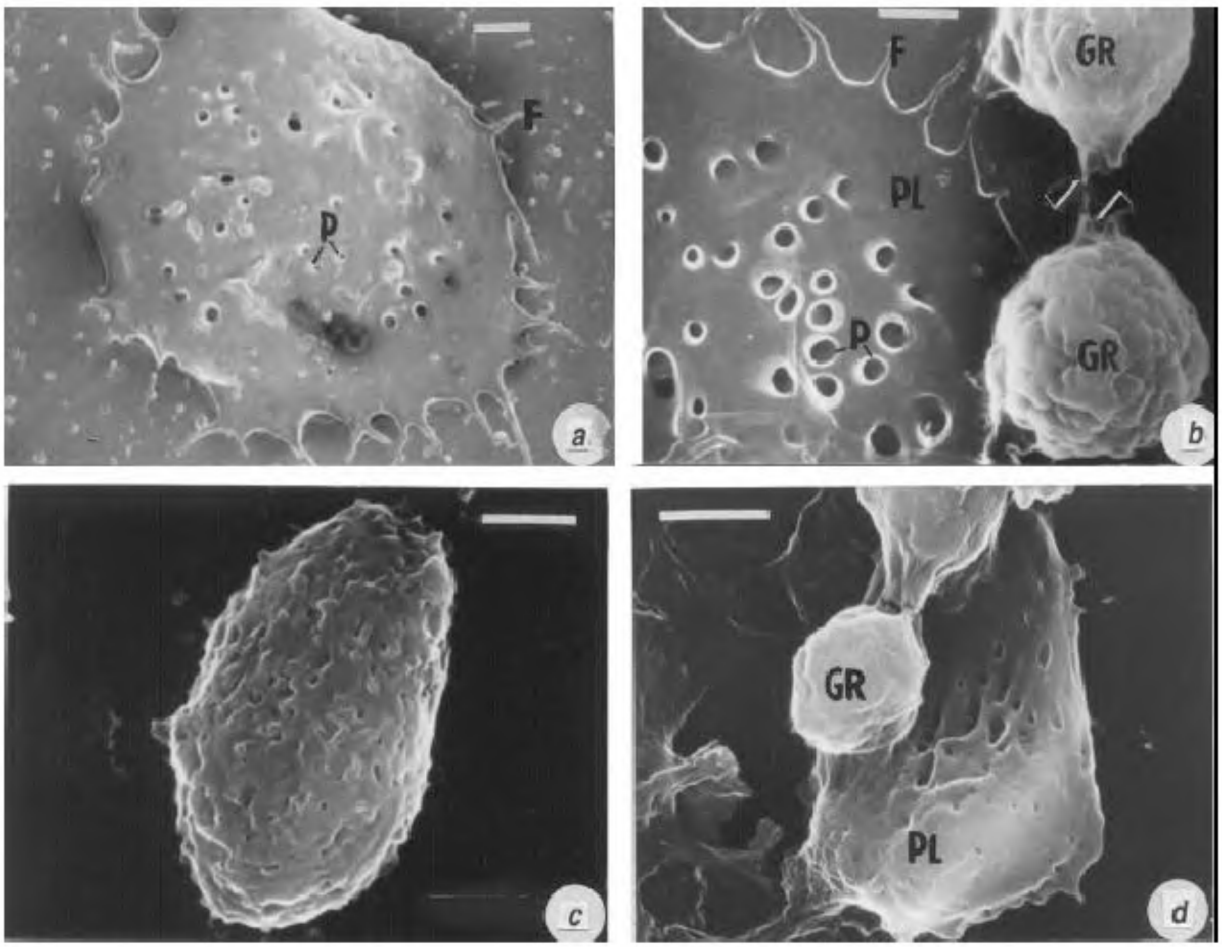

Figure 1 ***a*–*d***. SEM of haemocytes of 6th instar larvae of *Spodoptera litura* after 48 h. ***a***, ***b***, Normal haemocytes; ***c*–*d***, Haemocytes after Neem gold treatment. ***a***, Oval plasmatocyte (PL) showing numerous filopods (F) and surface pits (P). ***b***, PL enlarged to show surface pits (P) and filopods (F). Two granular haemocytes (GRs) with cytoplasmic projections (arrow heads) are also seen. ***c***, PL showing complete loss of filopods. ***d***, PL showing retracted filopods and GR showing absence of cytoplasmic projections (***a***, ***b***, Bar = 2 µm; ***c***, ***d***, Bar = 3 µm).

there was a close apposition along their boundaries (Figure 2 *b*). An extensive cytoplamic vacuolization was marked both in PLs and GRs (Figure 2 *a*, *c*).

Damage to the plasma membrane was prominent in PLs and GRs (Figure 2 *a*, *c*). Both the structured and the structureless granules of GR were reduced in size and number, and with the advancement of the effect, it became difficult to make a distinction between the two types, viz. the structured and structureless granules due to their highly damaged condition. The mitochondrial cristae, rough endoplasmic reticulum and Golgi bodies of PLs became swollen, prominent and ultimately underwent degeneration due to lysis (Figure 2 *a*). Lysis was also observed in the cytoplasm of GRs and, with advancement of vacuolization, all the organelles lost their defined structure and were destroyed in the extremely affected cells. PLs and GRs are known to give out cytoplasmic processes in retaliation to any invading foreign material[13], and suppression of such processes in the present study in *S. litura* implies the weakening of cellular defence reactions by Neem gold.

Treatment of Neem gold in 6th instar larvae of *S. litura* exhibited a dose-dependent increase in THC after 24 h. This resulted in a percentage increase of 4.20, 12.72 and 21.10 at concentrations 500, 1000 and 1500 ppm respectively. The botanical caused a dose-dependent decrease in the THC after 48 h of treatment. At the highest concentration (1500 ppm) of Neem gold, the decrease was recorded as 23,185 ± 663 (in controls 34,618 ± 923). The percentage decrease for 500, 1000 and 1500 ppm of Neem

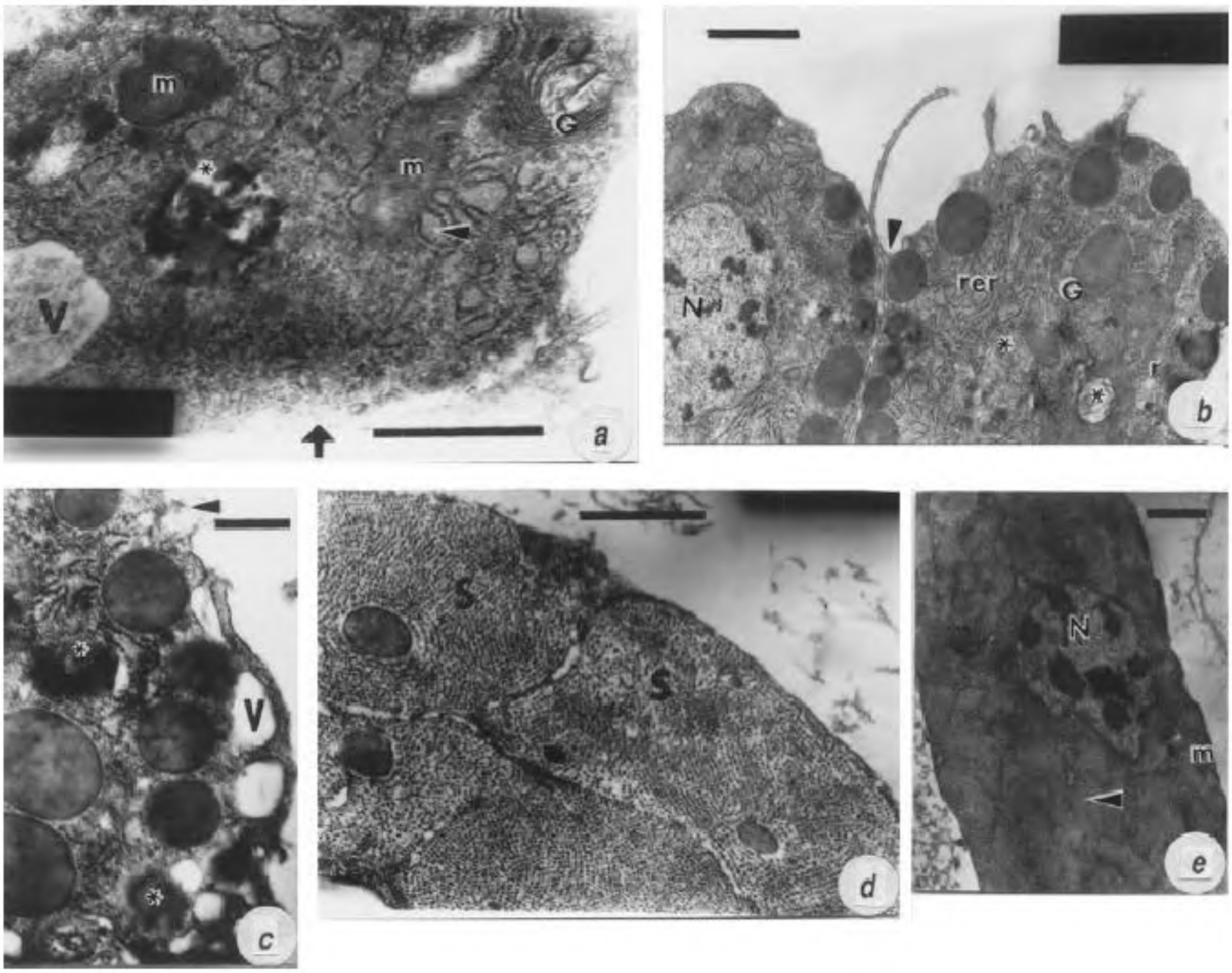

Figure 2 *a*–*e*. TEM of haemocytes of 6th instar larvae of *Spodoptera litura* after Neem gold treatment for 48 h. ***a***, Plasmatocyte (PL) showing absence of cell membrane (arrow), vacuolization (V) and swelled rough endoplasmic reticulum (arrow head), mitochondria (m), Golgi bodies (G). Asterisk shows degenerating organelles. ***b***, Two granular haemocytes (GRs) showing close adherence (arrow head), destruction of structured compactness within granules (asterisk). Nucleus (N), Golgi body (G), rough endoplasmic reticulum (rer) and ribosomes (r) are also seen. ***c***, Highly affected GR showing vacuolization (V), shrunken granules (asterisk) and damaged plasma membrane (arrow head). ***d***, Spherulocyte (SP) showing absence of any effect (S, normal spherule). ***e***, Oenocytoid (OE) showing absence of any effect. Hyaline areas (arrow head), nucleus (N) and mitochondria (m) are seen (***a***, ***b***, ***d***, ***e***, Bar = 1 μm; ***c***, Bar = 0.5 μm).

gold treatments was found to be 22.39, 32.97 and 33.02 respectively. THC remained low compared to controls, 72 h after treatment. Decrease in the THC at 1500 ppm of Neem gold treatment was 14,528 ± 670 (in controls, 29,031 ± 1011) and the per cent decrease at its 500, 1000 and 1500 ppm concentrations was 23.59, 46.03 and 49.95 respectively. Table 1 lists the effect of Neem gold on THC.

The effect of varied concentrations of Neem gold on the DHC during 24 to 72 h after treatment has been studied (Table 2–4). A decrease in PRs, PLs, SPs and increase in GRs and OEs has been observed, which was more pronounced after 48 to 72 h treatment at a concentration of 1500 ppm of Neem gold (Table 4).

A dose-dependent decrease has been observed in the larval body weight and HV, 24 h after treatment of Neem gold (Table 5). After 24 h the percentage growth inhibition (GI) and percentage reduction in HV caused by treatment were observed to be 5.48 GI, 5.24 HV at 500 ppm; 16.34 GI, 12.59 HV at 1000 ppm, and 22.98 GI, 19.06 HV at 1500 ppm. Percentage GI and percentage reduction in HV brought about by treatment were 6.91 GI, 6.62 HV at 500 ppm; 24.71 GI, 24.05 HV at 1000 ppm, and 33.98 GI, 29.73 HV at 1500 ppm after 48 h. In comparison to controls, after 72 h the percentage GI and percentage reduction in HV were 23.70 GI, 23.21 HV at 500 ppm; 39.14 GI, 36.51 HV at 1000 ppm, and 42.37 GI, 37.07 HV at 1500 ppm.

The internal ultrastructural details of the affected haemocytes further revealed destruction of plasma membrane in many PLs, sometimes leading to a complete loss of these cells from the haemolymph, and the haemocyte counts, both differential and total, confirmed this fact. The rapid disintegration of GRs, initiated by vacuolization and loss of compactness of organelles leading to degranulation and a degenerative transformation within a period of 48 h, further emphasize upon the collapse of immunity-building mechanism of *S. litura* under the influence of Neem gold. These observations on PLs and GRs are

Table 1. Total haemocyte count (THC/mm^3) in Neem gold oral treatment to 6th instar larvae (0 h) of *S. litura*, 24 to 72 h post-treatment

Concentration diet (ppm)	Duration after treatment (h) 24 THC/mm^3 (mean ± SE)*	48 THC/mm^3 (mean ± SE)*	72 THC/mm^3 (mean ± SE)*
0	23,033 ± 1004	34,618 ± 923	29,031 ± 1011
500	24,001 ± 967^{ns} (4.20)	26,866 ± 999^c (22.39)	22,180 ± 645^c (23.59)
1000	25,965 ± 909^a (12.72)	23,202 ± 982^c (32.97)	15,666 ± 646^c (46.03)
1500	27,894 ± 1099^b (21.10)	23,185 ± 663^c (33.02)	14,528 ± 670^c (49.95)

$n = 10$; ns, Not significant; a, Significant, $P < 0.05$; b, Significant, $P < 0.01$; c, Significant, $P < 0.001$; *Values in parentheses are % increase in THC; *Values in parentheses are % decrease in THC.

Table 2. Differential haemocyte count in Neem gold-treated 6th instar larvae (0 h) of *S. litura* Fab. 24 h post-treatment

Haemocyte type (%)	Control	Neem gold concentration in diet (ppm) (mean ± SE) 500	1000	1500
PR	4.90 ± 0.41	4.20 ± 0.25^{ns}	3.80 ± 0.55^{ns}	3.50 ± 0.42^a
PL	44.10 ± 2.47	42.90 ± 2.89^{ns}	41.70 ± 2.35^{ns}	39.00 ± 2.69^{ns}
GR	35.80 ± 2.04	38.00 ± 2.68^{ns}	39.50 ± 2.54^{ns}	43.70 ± 2.74^a
SP	14.20 ± 1.84	13.00 ± 1.35^{ns}	12.80 ± 0.95^{ns}	11.00 ± 1.05^{ns}
OE	1.00 ± 0.31	1.90 ± 0.64^{ns}	2.20 ± 0.61^{ns}	2.80 ± 0.53^b

PR, Prohaemocyte; PL, Plasmatocyte; GR, Granular haemocyte; SP, Spherulocyte; OE, Oenocytoid; $n = 10$; a, Significant, $P < 0.05$; b, Significant, $P < 0.01$; ns, Not significant.

Table 3. Differential haemocyte count in Neem gold-treated 6th instar larvae (0 h) of *S. litura* 48 h post-treatment

Haemocyte type (%)	Control	Neem gold concentration in diet (ppm) (mean ± SE) 500	1000	1500
PR	4.00 ± 0.55	4.00 ± 2.61^{ns}	3.30 ± 0.77^{ns}	3.10 ± 0.86^{ns}
PL	46.80 ± 2.37	45.10 ± 2.88^{ns}	41.50 ± 3.02^{ns}	34.9 ± 2.81^a
GR	33.40 ± 2.30	36.20 ± 3.02^{ns}	40.20 ± 2.99^{ns}	47.00 ± 3.03^a
SP	14.20 ± 1.27	13.00 ± 0.98^{ns}	12.80 ± 1.94^{ns}	12.70 ± 0.95^{ns}
OE	1.60 ± 0.36	1.70 ± 0.17^{ns}	2.2 ± 0.55^{ns}	2.30 ± 0.47^{ns}

PR, Prohaemocyte; PL, Plasmatocyte; GR, Granular haemocyte; SP, Spherulocyte; OE, Oenocytoid; $n = 10$; a, Significant, $P < 0.01$; ns, Not significant.

Table 4. Differential haemocyte count in Neem gold-treated 6th instar larvae (0 h) of *S. litura* 72 h post-treatment

Haemocyte type (%)	Control	Neem gold concentration in diet (ppm) (mean ± SE) 500	1000	1500
PR	3.60 ± 0.42	3.00 ± 0.26^{ns}	2.80 ± 0.57^{ns}	2.70 ± 0.47^{ns}
PL	44.40 ± 2.62	33.50 ± 3.07^{a}	27.50 ± 2.84^{b}	20.30 ± 2.56^{b}
GR	31.60 ± 2.25	43.70 ± 3.13^{c}	50.40 ± 2.77^{b}	59.30 ± 2.29^{b}
SP	17.00 ± 1.18	16.00 ± 1.82^{ns}	15.30 ± 2.16^{ns}	13.60 ± 1.42^{ns}
OE	3.40 ± 0.88	3.80 ± 0.77^{ns}	4.00 ± 0.44^{ns}	4.10 ± 0.64^{ns}

PR, Prohaemocyte; PL, Plasmatocyte; GR, Granular haemocyte; SP, Spherulocyte; OE, Oenocytoid; $n = 10$; a, Significant, $P < 0.02$; b, Significant, $P < 0.001$; c, Significant, $P < 0.01$; ns, Not Significant.

Table 5. Larval weight and haemolymph volume in Neem gold oral treatment to 6th instar larvae (0 h) of *S. litura*, 24 to 72 h post-treatment

	Duration after treatment (h)					
	24		48		72	
Concentration in diet (ppm)	Larval wt (mg) (mean ± SE)♣	Haemolymph volume (µl) (mean ± SE)*	Larval wt (mg) (mean ± SE)	Haemolymph volume (µl) (mean ± SE)	Larval wt (mg)(mean ± SE)	Haemolymph volume (µl) (mean ± SE)
0	344.50 ± 11.53	86.24 ± 3.25	547.70 ± 17.37	145.59 ± 7.13	746.20 ± 17.89	198.84 ± 8.36
500	325.60 ± 10.40^{ns} (5.48)	81.72 ± 2.02^{ns} (5.24)	508.90 ± 19.47^{ns} (6.91)	135.95 ± 5.99^{ns}	569.30 ± 16.38^{c} (23.70)	152.68 ± 4.97^{c} (23.21)
1000	288.20 ± 10.21^{a} (16.34)	75.38 ± 2.71^{b} (12.59)	411.60 ± 20.58^{c} (24.71)	110.57 ± 5.15^{c} (24.05)	454.10 ± 18.15^{c} (39.14)	126.24 ± 4.73^{c} 36.51
1500	265.30 ± 13.92^{c} (22.98)	69.80 ± 2.61^{c} (19.06)	360.90 ± 13.00^{c} (33.98)	102.30 ± 4.23^{c} (29.73)	430.00 ± 18.58^{c} (42.37)	125.12 ± 6.72^{c} (37.07)

$n = 10$; a, Significant, $P < 0.01$; b, Significant, $P < 0.02$; c, Significant, $P < 0.01$; ns, Not Significant; ♣Values in parentheses are % growth inhibition; * Values in parentheses are % reduction in haemolymph volume.

in conformity with those described for plumbagin on the haemocytes of *Dysdercus koenigii*[3,4].

Starvation effect has also been reported to increase the THC in *Prodenia eridania*[11], which may probably be due to the fall in HV. The main active constituent of Neem gold is azadirachtin, a tetranortriterpenoid which is a strong antifeedant and also acts as a potent growth inhibitor at microgram levels[7]. Ayyangar and Rao[14] recorded a steep fall in THC/mm^3 (percentage reduction 64) after 72 h of injection of azadirachtin to the last instar larvae of *S. litura* Fab., whereas reduction in THC was observed to be 49.95% after 72 h of oral feeding of 1500 ppm of Neem gold (present study). The difference in percentage of THC fall in two cases is due to the fact that Ayyangar and Rao[14] injected azadirachtin which is a sole active ingredient, while during the present study Neem gold was orally fed to the insect. Azadirachtin has been reported to cause an overall reduction of 31 to 41% in THC in *Cyrtacanthacris tatarica*[3]. In *Periplaneta americana*[2], injection of azadirachtin to the last instar nymphs results in 20–25% decrease in haemocytes after 24 h of treatment. These findings clearly bring forth the efficacy of the botanical in drastically lowering down the counts. The initial triggering in THC after 24 h of treatment was due to the starvation effect. The decrease in THC was observed after 48 to 72 h due to the biological effects induced by Neem gold. The THC came down from 27,894 to 14,528 at 1500 ppm of Neem gold after 72 h of treatment.

DHCs in the larvae under the influence of Neem gold revealed a dose-dependent decrease of PRs, PLs and SPs and an increase in GRs and OEs at all concentrations of Neem gold (500 to 1500 ppm)-treated 6th instar larvae of *S. litura*. The increase or decrease of different cell types became more significant with the passage of time, especially after 72 h of treatment. In the adults of the bug, *Dysdercus koenigii*[4], PRs and PLs were seen to decrease and OEs to increase, after treatment with the plant product plumbagin. The present study on the effect of botanical on haemocyte ultrastructure and haemogram in polyphagous pest *S. litura*[15], will supplement the knowledge of cellular defence mechanisms in insects so as to make use of botanicals for control of insects by suppressing their defence reactions[16]. The question whether the haemocytes are affected directly or via some physiological or endocrinological pathway is yet to be answered, in

spite of reports that developmental effects caused by azadirachtin are attributed to disruption of endocrine events[7].

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The odds of a seismic source near Dwarka, NW Gujarat: An evaluation based on proxies

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The Kachchh–Saurashtra region is part of an old rift basin, featuring a number of linear structures, some of them holding potential for occasional $M > 7$ earthquakes. A challenging question is to identify probable sources of large earthquakes in this region. Recognizing earthquake-generated structures preserved within the geological formations is an effective method to constrain the seismic sources in space and time. During the recent excavations at Bet Dwarka, in the Gulf of Kachchh, we identified a seismically generated sand blow that has disrupted an ancient cultural horizon. The age data suggest that the causative earthquake may have occurred about 2000 years ago. While the source of this earthquake remains to be constrained, its timing seems to be singular; our studies elsewhere in Kachchh have not disclosed any feature of comparable age, as yet. This find opens the possibility for another surprise source, adding a new dimension to the seismic hazard of this region.

TWO large earthquakes have occurred within an interval of 200 years at two different parts of the Kachchh basin, a Mesozoic rift in northwestern India (Figure 1). Of these, the 1819 earthquake (M 7.5) occurred in the northern flank of the basin and the event of 2001 (M 7.6) was sourced at its southern margin[1–3]. Such a short span of time between the earthquakes as observed here is quite exceptional for a region that is considered to be 'slow-deforming', and this naturally calls for an assessment of its potential sources[4,5]. The traditional approach has been to use historical information for generating database on past seismicity. In the recent years, archaeo- and paleoseismological techniques have developed as useful tools for reconstructing earthquake history[6,7]. Identifying seismically induced liquefaction features, deducing their stratigraphic context, and dating various sedimentary layers to constrain the age of the earthquakes are common approaches adopted in the palaeoseismological researches[8]. Archaeoseismology concerns with the disruptions to cultural settlements, which are particularly useful because they often contain datable material like pottery, bones and organic matter, and the combined use of both these techniques provides better age constraints[9,10].

In this paper, we focus on Bet Dwarka (Figure 1; inset), a locale of ancient habitation that flourished since Harappan times (~ 3000 yr BP). A major fault is located close to this area (Figure 1), but no significant seismic activity has been reported from here[11]. One tremor is reported to have occurred on 12 May 1962 near Dwarka and according to local sources, it lasted for a few seconds (Pushkar Gokani, private commun.). Although some archaeological investigations have been conducted in Dwarka earlier, these sites have not been examined from the point of understanding the past earthquakes. Thus, when the National Institute of Oceanography (NIO) excavated some trenches in Bet Dwarka, as part of their archaeological investigations, we chose to examine them for proxy indicators of earthquakes (see Figure 1 for loca-

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