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Cell Mediated Immune Response of *Helicoverpa armigera* Hubner and *Spodoptera litura* Fabricius to Fern Phytoecdysteron

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Abstract: The aim of this study to evaluat the immunological impact of the Crude Ecdysone Fractions (CEF) of 3 ferns such as *Christella parasitica* (L.) H.lev., *Pteridium aquilinum* (L.) Khun and *Hemionitis arifolia* (Burm) T. More. on two major polyphagous pests, *Helicoverpa armigera* Hubner and *Spodoptera litura* (Fabr.) (Lepidoptera: Noctuidae). The results revealed that the Haemosomic Index (HI) and the Total Haemocyte Count (THC) were higher in control than the experimental insects. Irrespective of the CEF treatment, HI increased gradually from the first day to the fourth day of observation. Similar trend was also observed for the THC. Among the three ferns, *C. parasitica* highly reduce the HI followed by *H. arifolia* and *P. aquilinum*. Six types of haemocytes viz., Prohaemocytes (PR), Plasmatocytes (PL), Granular cells (GR), Spherule cells (SP), Oenocytoids (OE) and Adipohaemocytes (AD) were observed in both pests. Among the six types of haemocytes observed, PL constituted highest percentage control categories of both *H. armigera* and *S. litura* population. In ferns treated categories, PR, SP and AD levels of both pests were increased. Among the six types of haemocytes, cellular mediated response was observed only on GR, SP, PR and PL cells, which either underwent lyses or aggregation. Lyses or aggregation were well pronounced in *H. armigera* than *S. litura*.

Key words: Phytoecdysone, ferns, fraction, polyphagous pests, haemosomic index, total haemocyte count, differential count, cellular meadiated immune response

INTRODUCTION

Helicoverpa armigera Hubner is one of the most important pests of filed crops worldwide. It is highly polyphagous and causes severe damage and loss to a wide range of food, fiber, oil, fodder, vegetable, horticultural, ornamental, aromatic and medicinal plants (Neoliya *et al*., 2007). It has developed resistance against most of the modern classes of insecticides like organophosphate, pyrethroid and carbomates (Yadav *et al*., 2006). *Spodoptera litura* (Fab.) is also one among economically important polyphagous pest causing damage to more than 50 crops (Venkateswarlu *et al*., 2006). This is the first lepidopteran to develop insecticide resistance in India during 1965. Development of insecticide resistance in this pest has been faster in the last two decades (Arms *et al*., 1997; Kranthi *et al*., 2002; Radhika *et al*., 2006). Very recently Srivastava and Srinivas Reddy (2006) reported that this pest could be controlled using neem based insecticides. Misuse and excessive use of synthetic pesticides and also the hazards associated with these chemicals forced the pest management practitioners to explore alternative and ecofriendly means of crop protection. Phytochemical pesticides gained considerable impportance throughout the world, as they are environmentally safe and much safer to higher animals including humans.

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Ferns are members of a large and diverse group of plants commonly referred to as the lower plants. Ecdysones have also been isolated from ferns and are believed to deter feeding by mimicking insect molting hormones (Markham *et al*., 2006; Meepagala *et al*., 2006). Ecdysone analogues or phytoecdysone were identified from ferns phytoecdysones are being suggested as suitable biopesticide for the pest control since insects may not be able to develop resistance to these compounds (Selvarj, 2002; Selvarj and Sahayarij, 2005; Selvarj *et al*., 2005). The insect immune system is different from vertebrate's immune system as they lack an antigen-antibody complex, though they are capable of responding very effectively against the various foreign invaders. Tiwari *et al*. (1999), Tiwari and Shukla (2000) and Neoliya *et al*. (2007) reported the hematological changes of insects in response to the exogenous administration of ecdysone has been reported. Impact of metyrapone and nucleopolyhedro virus on the morphology of hemocytes of *Spodoptera littoralis* (Boisd.) and *Helicoverpa armigera* (Hubner) has studied by Gelbic *et al*. (2006) and Kalia *et al*. (2001) respectively. However no information was available for the immunological response of *H. armigera* and *S. litura* against phytoecdysone except Sharma *et al*., (2003). Moreover, Anitha and Subramanyam (1998) studied the juvenoid induced of *S. litura*. Therefore we evaluated the effect of fern phytoecdysone on total count (THC) and differential count of haemocytes (DHC), Haemosomic Index (HI) and cell mediated of *H. armigera* and *S. litura*.

MATERIALS AND METHODS

Fern Collection Andphytoecdysone Extracts Preparation

C. parasitica, *P. aquilinum* and *H. arifolia* were collected from Kothiyar Hills and identified by Dr. V.S. Manickam, Center for Biodiversity and Biotechnology, St. Xavier's College, Palayamkottai. The voucher specimens have been preserved in our laboratory. Laboratory experiments were conducted in Crop Protection Research Centre, St. Xavier's College in 2002. The plant materials were washed thrice in tap water and once with distilled water. They were shade dried for two weeks. The dried plant materials were ground with domestic grinder and stored in refrigerator for further use. The phytoecdysone fraction (PEF) was extracted using the standard method of Horn and Bergamasco (1985). From the PEFs, 0.1% solutions prepared with ethanol and used for the experiment.

Collection and Maintenance of Pests

Different larva of *H. armigera* and *S. litura* were collected from the groundnut fields of Tirunelveli and Kanyakumari districts, Tamil Nadu and were reared on groundnut leaves under laboratory conditions on fresh groundnut leaves (29±1°C; 65-70% RH and 11L and 13D photoperiod). *S. litura* were reared in plastic trough (21.0×28.0×9.0 cm) whereas *H. armigera* larvae were reared individually in plastic containers (100 mL volume) to avoid cannibalism. Laboratory emerged ten days old larva (225-250 mg for *S. litura* and 200-225 mg for *H. armigera*) was used for the experiment.

Experimental Design

Five grams of fresh groundnut leaves (TMV 7 Variety) were taken and were immersed in PEF (10 mL) separately for experiments and ethanol for control for 5 min. The immersed leaves were then shade dried for 10 minutes and provided to the laboratory emerged 6 h. starved 10 day old 4th instars *S. litura* and *H. armigera* larvae separately in plastic containers (250 mL capacity). Sixty larvae were maintained for each treatment as well as for control categories separately. The containers were covered with muslin cloth and the larvae were allowed to feed the PEF (0.1%) treated groundnut leaves continuously for four days. Cleaning and feeding were done for every 24 h. Ten replication was maintained for each plant separately. After every 24 h of treatment 6 larvae were selected randomly

from each categories and their initial weight was recorded. Forelegs of the larvae were amputated using fine sterilized scissors and the haemolymph was collected. Again the larval final weight was recorded. By using the initial and final weight of the above haemosomic index (HI) was calculated using the following formula:

$$HI\ (\%) = \frac{\text{Initial weight of the animal-Final weight of the animal}}{\text{Initial weight of the animal}}$$

The haemolymph collected from the insect was directly drawn into the WBC pipette up to 0.5 marking and mixing immediately diluted with an acidified physiological saline (NaCl-4.65 g, KCl-0.5 g, $CaCl_2$-0.11 g, Gentian violet -0.005 g and acetic acid -0.125 mL and made up into 100 mL with distilled water) mixed thoroughly. THCs were conducted with a standard haemocytometer (Naubauer's) and the haemocytes population per mm^3 of haemolymph was calculated by multiplying the average haemocytes per count with a depth and a dilution factors.

For haemocyte profile, one or two drops of 4% formal in were placed on a clean micro slide. Then place one or two drops of haemolymph on to the formal in solution. They were mixed well and a thin film was smeared on the slide and then the film was air dried for few minutes. The slides were stained with Giemsa's stain for about 10-15 min, allowed them to dry and observe under the microscope. The haemocytes of different kinds (DHC) were counted and expressed in percent (Sharma *et al*., 2003). Microscopic studies of the haemocytes were conducted on control and all fern treated larvae after 96 h of treatment. Correlation and multiple regressions' analyses were performed between control to fern extracts and also within the extracts.

RESULTS

Haemosomic index of both the experimental and control categories were increased as the exposure time increased (Table 1 and 2). The results also revealed that the HI was lower in the experimental animals than the control larvae at different intervals of the exposure. Even though the experimental categories showed a gradual increase, they did not achieve the increase as in control. Among the experimental categories of *H. armigera*, highest and lowest HI was recorded in *P. aquilinum* and *C. parasitica* treated categories, respectively (Table 1). In control *S. litura* larvae, HI was 40, 41, 46 and 53% for 24, 48, 72 and 96 h, respectively.

Total Haemocytes Count (THC) of *H. armigera* and *S. litura* is presented in Table 2. From the results, it is very clear that the THC increased with the increase of exposure period. Both *H. armigera* and *S. litura* THC population was higher and lower in CP and PA respectively than the control. THC

Table 1: Haemosomic index (in %) of *S. litura* and *H. armigerain* in relation to phytoecdysone fraction of *C. parasitica* (CP), *P. aquilinum* (PA), *H. arifolia* (HA) and Control (CO)

Time (h)	CO	CP	PA	HA
Helicoverpa armigera				
24	40	32	37	35
48	41	36	40	38
72	46	45	47	47
96	53	46	50	50
Spodoptera litura				
24	35	33	30	32
48	46	38	46	45
72	49	40	47	45
96	54	45	50	48

Table 2: Total haemocytes count THC (10^4/cu mm^3) of *H. armigera* and *S. litura* treated with crude phytoecdysone fraction of *C. parasitica*, *P. aquilinum* and *H. arifolia*

Ferns	24 h	48 h	72 h	96 h
H. armigera				
C. paracitica	1.48±0.08	1.85±0.15	2.14±0.06	2.47±0.08
P. aquilinum	1.42±0.10	1.87±0.05	2.19±0.15	2.82±0.15
H. arifolia	1.41±0.11	1.79±0.14	2.19±0.25	2.76±0.11
Control	1.37±0.13	1.74±0.13	2.18±0.11	2.69±0.14
S. litura				
C. paracitica	1.82±0.04	2.04±0.11	2.35±0.06	2.63±0.06
P. aquilinum	1.55±0.05	1.98±0.03	2.49±0.07	2.76±0.06
H. arifolia	1.65±0.06	1.99±0.03	2.51±0.09	2.65±0.05
Control	1.64±0.06	2.01±0.087	2.51±0.07	2.65±0.04

in HA is either equal for *S. litura* or higher for *H. armigera* than control category. The correlation analyses between CP to PA were significant at 5% level ($r = 0.97$; $p<0.05$). The correlation was slightly reduced between CP and HA ($r = 0.95$; $p<0.05$) treated *H. armogera*. Correlation coefficient value between the control and CP was 0.99 and 0.95 for *H. armigera* and *S. litura*, respectively. For the both the pests, we obtained perfect Correlation coefficient value ($r = 1$) between *P. aquilinum* and *H. arifolia*. Multiple regression analysis between exposure time and ferns treatments showed that the result was insignificant for *H. armigera* ($F = 395.10$; $p = 0.0025$; $p<0.0046$) than *S. litura* ($F = 44.88$; $p = 0.021$; $p<0.0118$).

We found haemocytes to be composed of prohaemocytes (PR), plasmatocytes (PL), granular cells (GR), spherule cells (SP), oenocytoids (OE) and adipohaemocytes (AD) in both *H. armigera* and *S. litura* (Table 3 and 4). Proportion of PL population was higher in control *H. armigera*, followed by SP, GR, PR, OE and AD. In experimental categories, highest PR count was recorded for *P. aquilinum* treatment followed by *H. arifolia* and *C. parasitica* at 24 h. The PL count was decreased in all the three ferns PEF treated groundnut leaves fed *H. armigera* (Table 3). For instance, highest reduction was observed in *P. aquilinum* followed by *H. arifolia* (Table 3) and *C. parasitica*. Even though PL count of the control gradually decreased form 24-72 h. of exposure, its population increased at 96 h. of observation. The reduction in PL count was more pronounced in the experimental categories and it was extended up to the 72 h of observation. Similar trend was also observed in GR. However in *C. parasitica* treated category, the GR count was gradually decreased up to 96 h. Same trend was observed for OE with an exception of *P. aquilinum* at 24 h SP cell count of the control showed marked increase from 24 h up to 96 h. of observation. This increasing trend was more pronounced in the experimental categories. For instance, the order of increase was 31.2, 31.4, 35.5 and 39.0% from 24-96 h for *P. aquilinum*.

The results of *S. litura* DHC showed that after 24 h of exposure, percentage of individual haemocyte type increased in all the experimental categories except GR. Maximum GR was recorded in *C. parasitica* followed by *P. aquilinum* treated categories. Whereas, SP and PR cells counts were increased steadily in *H. arifolia* treated category when compared to the control. At 48 h of exposure, PL count was decreased in *P. aquilinum* and *H. arifolia* treated categories. When compared to the control PR, SP, AD and OE counts were increased up to 48 h observation, a reduction was observed in GR count. The highest reduction of GR was recorded in *P. aquilinum* followed by *H. arifolia*. The highest PR count was recorded in *P. aquilinum* followed by *H. arifolia*. The SP cell count was higher in *H. arifolia* followed by *P. aquilinum*. AD count was 1.8% in *H. arifolia* and the highest OE was recorded in *P. aquilinum* and *H. arifolia* treated categories. After 72 h of exposure a steady increase of both PR and SP were recorded in *P. aquilinum* treated category while the highest percentage of PL and GR were recorded in *C. parasitica* treated category. In the experimental categories the OE

Table 3: Differential haemocytes count THC (10^4/cu mm^3) of *H. armigera* treated with crude phytoecdysone fraction of control (CO), *C. parasitical*, *P. aquilinum* and *H. arifolia*

Time (h)	Extracts	Prohaemocyte	Plasmatocyte	Granulo cyte	Sperule cell	Adipohaemocyte	Oenocytoids
24	CO	4.2	38.1	26.2	28.7	0.9	1.9
	CP	4.8	35.2	27.0	30.0	1.0	2.0
	PA	7.0	31.8	27.0	31.2	1.8	1.2
	HA	6.5	33.4	26.8	30.8	1.0	1.5
48	CO	4.4	35.4	26.4	28.8	1.7	3.3
	CP	5.2	33.6	26.0	29.5	1.2	4.5
	PA	7.4	29.2	25.0	31.4	2.0	5.0
	HA	7.0	30.5	25.4	30.5	1.8	4.8
72	CO	6.0	31.9	24.7	31.7	1.5	4.2
	CP	6.5	30.0	25.7	32.0	1.6	4.2
	PA	6.5	29.0	22.1	35.5	2.2	4.8
	HA	7.0	29.3	24.1	33.0	2.0	4.6
96	CO	3.2	32.7	25.0	34.3	1.4	3.4
	CP	4.4	30.6	24.0	36.8	1.2	3.0
	PA	4.0	29.5	23.3	39.0	1.2	3.0
	HA	4.2	29.4	24.5	37.5	1.4	3.0

Table 4: Differential haemocytes count DHC of *S. litura* treated with crude phytoecdysone fraction of Control (CO), *C. parasitica*, *P. aquilinum* and *H. arifolia*

Time (h)	Extracts	Prohaemocyte	Plasmatocyte	Granulocyte	Sperule cell	Adipohaemocyte	Oenocytoids
24	CO	10.2	36.5	34.6	15.0	1.2	2.5
	CP	10.5	34.2	35.4	16.2	1.5	2.2
	PA	10.5	34.0	35.0	17.0	1.5	2.0
	HA	10.8	34.0	34.5	17.0	1.5	2.2
48	CO	10.0	35.0	36.3	15.5	1.2	2.0
	CP	11.2	35.3	33.6	16.2	1.5	2.2
	PA	12.5	34.6	31.3	17.5	1.6	2.5
	HA	12.0	34.2	31.5	18.0	1.8	2.5
72	CO	10.6	34.8	35.9	16.2	1.0	1.5
	CP	12.2	35.0	30.3	18.2	1.5	2.8
	PA	13.2	34.2	27.6	20.2	1.8	3.0
	HA	13.0	34.0	27.5	20.5	2.0	3.0
96	CO	11.3	35.6	32.1	16.0	2.0	3.0
	CP	10.0	35.2	28.6	21.0	2.0	3.2
	PA	11.6	34.8	30.3	19.0	1.5	2.8
	HA	11.2	34.6	30.2	19.5	1.5	3.0

population at 96 h was maintained as observed at 72 h exposure. But in the control an increase up to 3% was observed. The AD counts of *P. aquilinum* and *H. arifolia* treated categories recorded a decline but an opposite trend was observed in the control and *C. parasitica* treated categories. The PR count in the control was increased from 72-96 h. An opposite trend has been observed in the experimental categories. The increased of PL and GR counts were compensated by the decline of SP and AD cells (Table 4).

Among the haemocytes, cytotoxic responses were observed in PL, GR, PH and SP cells. They showed either lyses or aggregation behaviour (Fig. 1 and 2). Among the two pests, both the cell lyses and cell aggregation was more pronounced in *H. armigera* (Fig. 1) than *S. litura* (Fig. 2). In *H. armigera* the above said impacts were mainly caused by *P. aqulinum* (Fig. 1b, f, I and m). *P. aqulinum* induce all types of cells such as, PL, SP and PH. In addition to the cell lyses and cell aggregation *H. arifolia* caused pseudopodia formation in GR (Fig. 1e). In *S. litura* maximum cell aggregation was more pronounced in *H. arifolia* treatment than *P. aquilinum*. All the three ferns caused SP cell lyses. It was more pronounced in *P. aquilinum* followed by *C. parasitica* and *H. arifolia* (Fig. 2).

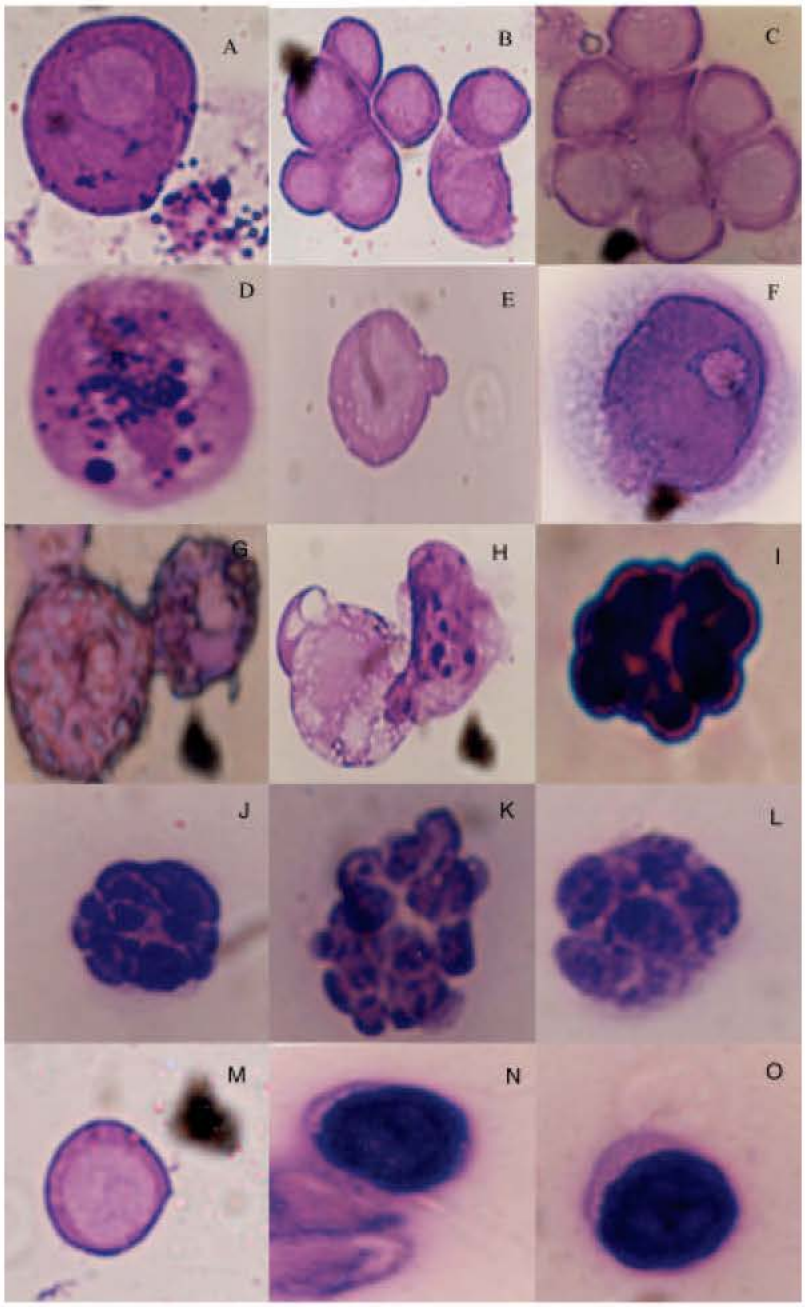

Fig. 1: Haemyocytes of control *H. armigera* larvae plasmatocytes (A) and its aggregation caused by *P. aquilinum* (B) *H. arifolia* (C) granulocytes normal (D) and *H. arifolia pseudopodia* (E) cytolysis caused by *P. aquilinum* (F) and *C. parasitica* (G) normal spherule calls (H) and its cells lysis caused by *C. parasitica* (I) *P. aquilinum* (J) and *H. arifolia* (K), proheamocytes (L) normal and its cell lysis *P. aquilinum* (M) *H. arifolia* (N) and (O)

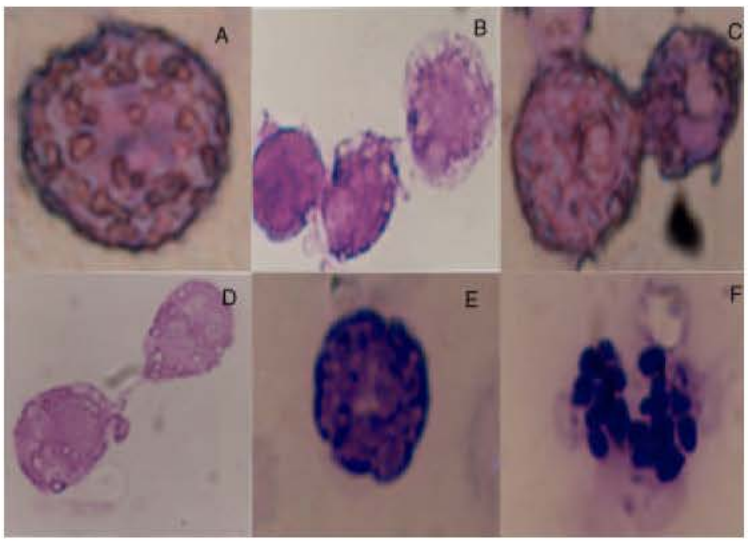

Fig. 2: Haemyocytes of control *S. litura larvae* Granulocytes (A) and its cell aggregation and lysis caused by C. paracitica (B and C) *P. aquiilinum* (D) *H. arifolia* (E) ruptured spherile cells treated with *P. aquiilinum* (F) showing disintegration of lipid droplet

DISCUSSION

Haemocytes appears to be an important indicator for the growth and metamorphosis of insects. Chapman (1998) stated that the haemosomic index and the total haemocytes count increases as the concentration of ecdysone increases in the haemolymph. It helps the animal to digest the dead cells and tissues during the process of metamorphosis and to cast the molt skin during the process of molting (Karlson, 1996; Kalia *et al*., 2001). The results of this study also support their view. The process of molting could be triggered by the exogenous administration of ecdysone hormonal analogues like fern PEFs. It may interfere with the normal developmental process and the animals prepare to molt but they failed to complete the task (Kalia *et al*., 2001; Hadapad *et al*., 2001; Falleiros *et al*., 2003; Gelbic *et al*., 2006).

We observed a distinct decrease HI of *H. armigera* and *S. litura* when they were fed with PEF treated leaf throughout the experimental period. These changes may be due to the interactions of beta ecdysone in CP and HA and alpha and beta ecdysone in PA (Selvarj *et al*., 2005). Furthermore, this might be the result of feeding deterrence as well as the unpalatability of the food or due to the prevention from feeding and locomotion (Kubo and Hanke, 1986). In lepidopteran caterpillars, water comprises 50% of the haemolymph volume. It reflects the importance of hydrostatic function of haemolymph (Chapman, 1998). He further added that the increased volume of haemolymph is associated with an increased activity of an antidiuretic hormone. However the reduction in HI of the experimental insects might be due to starvation too. Previously Wigglesworth (1972) reported that the haemocytes transport ecdysone to the epidermis. But though the larva consumed ecdysone through food, it could not be reached into the epidermis. However Kubo and Hanke (1986) and Keyserlingk (1986) reported that precocious molting occurred in the treatment categories of their experiment and hence they were in doubt that whether the ecdysone is transported through the haemocytes or through some other carrier proteins.

Treatment of PEF of CP in 10 days old *H. armigera* and *S. litura* exhibited decreased in the THC. It was well pronounced (8%) in *H. armigera* than in *S. litura* (1%). These observations are in

conformity with those described for neem gold on the THC of *S. litura* (Sharma *et al*., 2003). These findings clearly bring forth the efficacy of the CP in drastically lowering down the heamocyte counts. In this experiment *P. aquilinum* treated *S. litura* and *H. armigera* recorded lowest haemocyte count at 24 h exposure. After 24 h the haemocyte population was increased. This suppression at the initial stage might be due to the high deterrence of these plants. Sharma *et al*. (2003) found that the neem gold treatment decreased THC after 48 h. Followed by an increase might be due to the consumption of the treated food due to prolonged starvation. Tiwari *et al*., (1999) and Tiwari and Shukla (2000) also recorded similar results in *Papilio demoleus* larvae treated with 20-hydroxy ecdysone.

There is often a disagreement with the nomenclature and classification of haemocytes due to variability in morphological characteristics of haemocytes. Based upon the morphology we recorded 6 types of haemocytes in both the insects (PR, PL, SP, GR, AD and OE). Kalia *et al*. (2001) reported all these six types of haemocytes in *H. armigera*. However, previously only five types of haemocytes such as PR, SP, PL, CO (coagulocyte) and OE were observed in the same species. Structural organizations and morphology of these cells are similar in *H. armigera* and *S. litura*. The production and differentiation of haemocytes have been shown to be regulated either by prothorasic glands (PTG) or by changes in hormone levels which influence the mitotic index and size of the haemocyte population either directly or indirectly (Tiwari *et al*., 1999). DHCs in the larvae under the influence of PEFs of ferns reveled that changes in the differential counts after phytoecdysone treatment and these changes simulated acceleration in the aging process. Pech and Strand (1996) reported that both plasmatocytes and granulocytes are the two sub-classes of heamocytes adhere to foreign surface and involved in encapsulation. Present study reveled that population of both plasmatocytes and granulocytes decreased after 24 h of fern treatment indicates; these cells are not involved in encapsulation process. Similar reduction in plasmatocytes and granulocytes number was also observed by Sharma *et al*. (2003); Gelbic *et al*. (2006). Tiwari and Shukla (2000) added that GR was played an important role in encapsulation reaction ultimately it decrease the level of GR. Along with the GR, PL also involved in the immune response of the insects.

Rajkumar *et al*. (2000) and Sharma *et al*. (2003) reported that the plant biopesticide treatment generally decrease the PR, PL, SP population and increase the GR and OE population. In the present experiment, the highest response was observed in PEF of *P. aquilinum* treated category. This response coincides with the immunological response during ecdycial events. But no clear-cut pathological response was observed in this experiment, as observed by Kalia *et al*. (2001) in *H. armigera* treated with NPV. However incidence of cytolysis was observed in case of GR and SP and clumping behaviour in PL. Gupta (1994) stated that both PL and GR are involved in the cell-mediated immune response to invading foreign materials and suppression of such process. In addition GR and SP are also involved in this cellular defense, which is evident from its higher degree of cytolytic response. Williams (1956) stated that since the ecdysones are the hormonal factors of insects, the pests might not develop any defensive strategies against these compounds. We also agree with the statement of Williams and we believe that the increasing haemocyte count in the experimental categories might be the result of the immuno stimulant effect triggered by the exogenous administration of fern phytoecdysone as suggested by Fomovskaia (1992) and Tiwari *et al*. (1999). It can also be assumed that the cuticular disruption as observed in the fern phytoecdysone treated insects (Selvarj, 2002) might lead to microbial infection through the wound. So that the haemocytes showed some kind of cell mediated immune response. The structural details of the affected haemocytes further revealed destruction of plasma membrane in many PL there by the PL count drastically decreased. Furthermore, PL and GR are the major immune responding cells in the insect so phytoectysone first to attack the PL and GR and their population either decreased undergoes lyses sometime GR showed budding or encapsulation. The rapid lyses of GR may be initiated by vacuolization and loss of compactness of organelles leading to degranulation and a degenerative transformation (Sharma *et al*., 2003). From the results it is very clear that if an insect

take molting hormone mimicking phytoecdysone through their food, it may change the heamolymph as well as their constituents both by number and structure, the use of these substances for integrated pest control seems to have good potential.

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