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Original article

Toxicity of some insecticides to the haemocytes of giant honeybee, *Apis dorsata* F. under laboratory conditions

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Abstract Quantitative studies concerning total and differential haemocyte counts and abnormalities were performed under laboratory conditions for larvae, pupae and adults collected from a wild *Apis dorsata* colony. Haemolymph samples were observed immediately, thirty and sixty minutes after field recommended concentration exposure of five different insecticides. Total haemocyte counts were significantly higher for larvae and pupae but less for adult bees, however, differential haemocyte counts insignificantly different. Exposure of insecticides showed variable response for tested insecticides with immediate increased change with ethofenprox, diafenthiuron and imidacloprid but decreased for all tested insecticides after sixty minutes. For differential haemocyte counts, plasmatocytes and granulocytes increased with exposure of insecticides. Immune response of haemocytes against insecticides showed different degree of abnormalities like agglutination, denucleation and cell shape distortion. Such studies may help for possible identification of insect defense mechanisms against their exposure to external hazards like insecticide exposure.

KEY WORDS: *Apis dorsata*; Insecticides; Haemocytes; Immunity response

1. Introduction

The giant honeybee, *Apis dorsata* Fabricius (Apidae: Hymenoptera) is one of the most important pollinators for crops of economic value. Pollinated crops used as food in Pakistan are 61, which include 26 fruit crops, 7 oilseed, 4 pulses, 19 vegetables, 2 spices and 3 nut trees (Irshad and Stephen, 2014). Recently the production value of pollinated dependent crop in Pakistan was quantified to be 1.59 billion US$. Of the total value, fruits are dominant with 0.98 billion, vegetables 0.32 billion, nuts 0.15 billion, oilseed 0.13 billion and spices 0.004 billion US $ (Irshad and Stephen, 2013). These crops partially or completely depend on honeybees or other insects for their pollination. A considerable decrease in yield happened if honeybee pollinators were missing (Klein et al., 2007).

The population of insect pollinators is influenced to a great extent by large scale use of chemical pesticides and changes in the environment during the recent past. Moreover, the insecticide residue on bee flora is an additional cause of bee mortality (Haq and Gardezi, 1983). Indiscriminate use of insecticides was considered major source responsible for the decrease in pollination by a loss of honeybees (Berry, 1987). Insecticides act as an instant killer of honeybees due to extensive damage to blood, besides other systems. The haematological studies have much importance in the field of insect physiology. The blood or haemolymph of the insect contains blood cells referred as haemocytes. These haemocytes which circulate are categorised into several types with their primary functions of coagulation, phagocytosis, encapsulation, detoxification, storage and distribution of nutritive materials (Sapcaliu et al., 2009).

Insect immune system is composed of humoral and cellular defense responses where later encompass encapsulation and phagocytosis (Lavine and Strand, 2002;

Schmidt et al., 2001). Insect haemolymph bathes and carries transmission of resources to different organs. Both internal and external factors affect the haemocyte counts depending on age, instar, sex, size and method of detection in different organs and systems depending upon their mode and functions (Jones, 1962). Nutrition, stage and instar may change them from one form to another (Jones, 1962). Main classification of haemocytes was of seven different types which later suggested as six (Gupta, 1979, 1993). Variation in cell count arose due to variance in studying haemocytes, their examination (Rosenberger and Jones, 1960), age difference and sex (Gilliam and Shimanuki, 1967; Mahmood and Yousuf, 1985). Response of haemocytes to different insecticides varied even with time and concentration (Shukla and Bahadur, 1986; Sabir, 1994; Ayub, 1996).

Such variation in haemocyte types and their count under insecticide stress urged this study to find out the total haemocytes count, differential haemocytes count and the abnormalities caused in *Apis dorsata*.

2. Materials and methods

2.1. Test Insects

A comb of giant honeybee, *Apis dorsata* F. was collected from field to study haemocytes count in worker bee larvae, pupae and adults and testing effect of insecticides to adult worker bees. Worker bees were collected in a ventilated flask provided with cotton swab moisten with 40% honey solution. These were immediately transferred in the laboratory to keep them at 25 ± 2^{o}C temperature and 50-60% RH.

2.2. Test Chemicals

Five different insecticides, endosulfan (Thiodon® 35EC), bifenthrin (Talstar® 10EC), diafenthiuron (Polo® 500SC), imidacloprid (Confidor® 200SL) and ethofenprox (Trebon® 30EC) were used for this research in recommended concentration in the field condition as given below:

Insecticide used	Rate per acre (in 100 litres of water)	Percentage Concentration
Ethofenprox (Trebon®30EC)	125 ml	0.0375
Bifenthrin (Talstar®10EC)	250 ml	0.025
Diafenthiuron(Polo® 500SC)	250 ml	1.25
Endosulfan(Thiodon®35EC)	800 ml	0.28
Imidacloprid (Confidar® 200SL)	300 ml	0.6

Insecticides were applied at sub-lethal dose rates with the help of hand-operated micro-applicator (Burkard Scientific™). A five micro-litre droplet was applied at the thorax of each bee with five bees per treatment. Effect of insecticide application was observed immediately after application, after 30 minutes and 60 minutes in term of any change in total and differential haemocyte counts, and abnormalities caused by these insecticides.

2.3. Haemocytes count

Total and differential haemocyte counts were observed for larvae, pupae and adult worker bees under normal conditions and adult worker bees under insecticide treatment stress.

2.3.1. Differential haemocytes count

Sample of haemolymph were collected with the help of sterilized micro-needle syringe from thorax and metaleg in a Thoma white blood cell diluting pipette. Slide

for each sample was prepared with haemolymph smeared with Wright's stain for four minutes. Freshly prepared buffer solution (3.8 g Na_2HPO_4, 5.47 g KH_2PO_4 in one litre distilled water) of pH 6.6 was applied for 15 minutes to neutralize the haemocyte contents for differential staining. Oil immersion phase microscope (10X x 100X) was used for total and differential counts using tele-counter with 100 cells counted each time to determine various classes (Mahmood and Yousuf, 1985).

2.3.2. Total haemocytes count

Total haemocytes were counted with Neubauer haemocytometer. Haemolymph was drawn into Thoma white blood cell diluting pipette up to 0.5 mark and then diluted 20 times with Toissin's solution (1g NaCl, 8g Na_2SO_4, 30ml neutral glycerine, 0.025g methyl violet and 160ml distilled water) up to mark II. After discarding few drops, one drop was placed near edge of cover slip of Neubauer ruling and counting chamber automatically filled by capillary action. Four white-cell squares from both upper and lower chambers were counted. Total haemocytes were calculated by using formula suggested by Jones (1962). Five counts for each treatment were made as replication to observe change in increase or decrease in haemocyte count under normal conditions and after insecticide exposure with time intervals.

The experiment was repeated five times using Completely Randomized Design (CRD) and data were subjected to the standard statistical analysis using techniques of analysis of variance

3. Results

Based on shape and size of soma and nucleus, general appearance and staining of cytoplasm, degree of vacuolization, type, number, size and affinities of staining inclusions, five different haemocytes (prohaemocytes, plasmohaemocytes,

granulocytes, oenocytoids and spherulocytes) were identified in the haemolymph of honeybee.

3.1. Total haemocyte count (THC) in larvae, pupae and adult workers of *Apis dorsata* F. under normal conditions

Total haemocyte count in larvae of *A. dorsata* showed 45,875 blood cells/ mm^3. In pupae, THC was slightly less than larvae as 43,850 blood cells/ mm^3 whereas it was almost seven times less (6470 blood cells/ mm^3) in adult bee haemolymph than both larval and pupal stages (Table 1).

3.2. Differential haemocyte count (DHC) in larvae, pupae and adult workers of *Apis dorsata* F. under normal conditions

Differential haemocyte count in larvae revealed the highest percentage of prohaemocytes (41.6) followed by spherulocytes (33.8), plasmatocytes (13.2), oenocytoids (8.2) and granulocytes (3.2) respectively. For pupal DHC, prohaemocytes were the highest in percentage (43.2) followed by spherulocytes (22.2), oenocytoids (15.6), plasmatocytes (13.2) and granulocytes (5.8). For DHC in adult bees, prohaemocytes were higher in percentage (40) followed by spherulocytes (24.8), oenocytoids (19.2), plasmatocytes (12.2) and granulocytes (3.8). For overall DHC comparison in different life stages, pupae have more prohaemocytes and granulocytes than larvae and adults whereas oenocytoids were more in adults. Plasmatocytes are equal in immature stages but more than adults, however, spherulocytes were more in larvae (Table 2).

3.3. Total haemocyte count (THC) and differential haemocyte count (DHC) in adult workers of *Apis dorsata* F. under insecticide exposed conditions

3.3.1. Effect of Ethofenprox on THC and DHC of adult worker honeybees

THC slightly increased immediately after insecticide exposure (7635 cells/mm^3), increased dramatically after half an hour (20410 cells/mm^3) but drastically decreased after one hour exposure (5490 cells/mm^3) when compared to normal (6470 cells/mm^3). The percentage of prohaemocytes, plasmatocytes and granulocytes increased from the normal, i.e., 40, 12.2, 3.8 to the percentage of 44.8, 33 and 10.8 respectively after exposure to ethofenprox whereas the percentage of oenocytoids and spherulocytes decreased from the normal, i.e., 24.8 and 19.2 to the percentage of 3 and 8.4 respectively (Tables 3 and 4).

3.3.2. Effect of Bifenthrin to THC and DHC of adult worker honeybees

THC decreased immediately after insecticide exposure (4740 cells/mm^3), increased after half an hour (8170 cells/mm^3) but drastically decreased after one hour exposure (3615 cells/mm^3) when compared to normal (6470 cells/mm^3). The percentage of plasmatocytes and granulocytes increased from the normal, i.e., 12.2 and 3.8 to the percentage of 57.4 and 7.2 respectively after exposure to bifenthrin while the percentage of prohaemocytes, oenocytoids and spherulocytes decreased from the normal (Tables 3 and 4).

3.3.3. Effect of Diafenthiuron on THC and DHC of adult worker honeybees

THC slightly increased immediately after insecticide exposure (8215 cells/mm^3) and after half an hour (8780 cells/mm^3) but dropped after one hour (5286 cells/mm^3) when compared to the normal (6470 cells/mm^3). The percentage of plasmatocytes and granulocytes increased from the normal, i.e., 12.2 and 3.8 to the percentage of 31.2 and 42.6 respectively after exposure to diafenthiuron whereas prohaemocytes, oenocytoids and granulocytes decreased from the normal (Tables 3 and 4).

3.3.4. Effect of Endosulfan on THC and DHC of adult worker honeybees

THC slightly decreased immediately after insecticide exposure (5310 cells/mm^3) but increased after half an hour (8580 cells/mm^3). It again dropped after one hour exposure (5270 cells/mm^3) when compared to the normal (6470 cells/mm^3). The percentage of plasmatocytes and granulocytes increased from the normal, i.e., 12.2 and 3.8 to the percentage of 55 and 7.8 respectively after exposure to endosulfan while the percentage of prohaemocytes, oenocytoids and spherulocytes decreased from the normal (Tables 3 and 4).

3.3.5. Effect of Imidacloprid on THC and DHC of adult worker honeybee

THC slightly increased immediately after insecticide exposure (8105 cells/mm^3) but dropped after half an hour (5320 cells/mm^3) and one hour exposure (4485 cells/mm^3) when compared to the normal (6470 cells/mm^3). The percentage of prohaemocytes, plasmatocytes and granulocytes increased from the normal, i.e., 40, 12.2 and 3.8 to the percentage of 46.4, 26.6 and 13.6 respectively after exposure to imidacloprid whereas the percentage of oenocytoids and spherulocytes decreased from the normal, i.e., 24.8 and 19.2 to the percentage of 2.4 and 11 respectively (Tables 3 and 4).

Overall results for THC showed immediate decrease with bifenthrin and endosulfan but increased for other insecticides. THC decreased only for imidacloprid after half an hour and decreased for all after an hour exposure (Fig 1). For DHC, percentages of plasmatocytes and granulocytes increased after application of all five insecticides but decreased for oenocytoids and spherulocytes. Percentage of prohaemocytes increased from normal after application of ethophenprox and imidacloprid but decreased for bifenthrin, diafenthiuron and endosulfan (Fig 2).

3.4. Statistical analysis

Statistical analysis of the data regarding the total haemocyte count after the application of different insecticides, endosulfan bifenthrin, diafenthiuron, imidacloprid and ethofenprox, at different time intervals is presented in Table 5. It is evident that the insecticides, various time intervals and interaction among insecticides and time intervals are highly significant.

3.5. Abnormalities in the haemocytes after the application of different insecticides

Certain abnormalities were observed after exposure of all the tested insecticides to the haemocytes. These included enlargement and agglutination of cells, rupturing of cell wall, displace of nuclei to one side of the cells, distortion of cell shape, abnormal staining of haemocytes and de-nucleation of the cells (Plates 1-7).

4. Discussion

Haemocyte classification shows variable types which vary from insect species and even nutrition, stage and instar may change them from one form to another (Jones, 1962) with seven different types previously suggested as six (Gupta, 1979, 1993). We identified five different types of haemocytes in larvae, pupae and adults of *A. dorsata*. One cell type was observed in contrast to present finding by some authors previously. Instead of spherulocytes, they observed adipohaemocytes in test insects (Zaidi and Khan, 1976; Mall and Gupta, 1979; Barduco et al., 1988). These adipohaemocytes in place of granulocytes were identified in different life stages of *Apis mellifera* worker (Zapol' Shikh, 1976). Masconi et al. (1989) noted cystocyte instead of oenocytoids but Ali and Ilyas (1986) and Pelc (1986) mentioned cystocyte as additional. Prohaemocytes were observed maximum in different test insects as we found for *A. dorsata* larvae and pupal stages (Bhatti, 1993; Ayub, 1996), however, it contradicted

to plasmatocytes as dominant haemocytes for some other insect species (Al-Hariri and Suhail, 2001; Rizwan-ul-Haq et al., 2005; Fatima et al., 2014).

Variation in cell count arise depending on method of studying haemocytes, examination of fixed and unfixed haemocytes (Rosenberger and Jones, 1960) and age difference (Gilliam and Shimanuki, 1967). Total haemocyte count (THC) ranging from 15000-275000 cells/mm^3 were observed in *Gryllus assimilis* and 15000-60000 cells/mm^3 in *Periplanata* (Tauber and Yeager, 1934, 1935, 1936) which were later counted as 26050 to 42250 cells/mm^3 in males and 29000 to 46600 cells/mm^3 in females of *Gryllus bimaculatus* (Mahmood and Yousuf, 1985). Gupta and Sutherland (1968) later counted THC in *P. americana* from 7996 to 27796 cells/mm^3. Total haemocyte count in untreated larvae of *A. dorsata* were 45,875 blood cells/ mm^3 slightly less in pupae and almost seven times less (6470 blood cells/ mm^3) in adult bee haemolymph with prohaemocytes and spherulocytes being most common haemocytes. Such high number of haemocytes in insects shows their ability to withstand external environmental stresses (Mahmood and Yousuf, 1985; Gupta and Sutherland, 1968).

Exposure of insecticides to test insect in most of the cases resulted in sudden increase in haemocyte count immediately after application with decrease within half and hour and then again increase after one hour of exposure. Similar change of increase with decline in between was previously observed which might be the result of immune system for recovery to hazards of insecticide exposure (Ayub, 1996; Rizwan-ul-Haq et al., 2005; Fatima et al., 2014). However, there existed observation of smooth decline in haemocyte counts (Shukla and Bahadur, 1986) which might be due to difference in test insect, insecticide and concentration tested. Variable responses of different haemocyte increase such as prohaemocytes and plasmatocytes

than other types existed under exposure to different test insecticides (Al-Hariri and Suhail, 2001; Fatima et al., 2014). Contrastingly, oenocytoids and adiphohaemocytes were major part of count in *Dysdercus koenijii* than that of granular haemocytes and plasmatocytes (Sexena and Tikku, 1990). In *Acanthaspis pedestris*, application of endosulfan and quinalphos increased prohaemocytes but decreased granular ones (Ambrose and George, 1996). Prohaemocytes and plasmatocytes divide actively; however, their presence in haemolymph circulation primarily depends on their continued division (Gardiner and Strand, 2000). This trend for such prohaemocytes and plasmatocytes existed for the test insect with slight changes in counts of cystocyte and oenocytoids which decreased in other test insects. H. Kwon et al., (2014) described that among haemocyte types, plasmatocytes and granulocytes are considered as key players in cell-mediated immunity, although it is likely that other haemocyte types interact with plasmatocytes and granulocytes and contribute towards the immune response. Both granulocytes and plasmatocytes carry out immune functions associated with encapsulation and phagocytosis in most Lepidoptera and some Coleoptera (Lavine and Strand, 2002; B. Manachini et al., 2011). Most of the previous researchers (Bhatti, 1993; Ayub, 1996; Al-Hariri and Suhail, 2001) confirmed haemocyte abnormalities under insecticide stress.

Haemocytes as sensitive entities in insect haemolymph have variable response to different insecticides. Their number can fluctuate up and down under insecticide stress and response to immune system against external exposure. They vary in their mitotic division in normal conditions and destruction by insecticide application for survival and protection of organism strengthening immune system.

Conclusions

Quantitative analysis for total and differential haemocyte counts and abnormalities resulted for different life stages of *Apis dorsata* when observed immediately, thirty and sixty minutes after different insecticides showed variable response. There existed immediate increase in response to ethofenprox, diafenthiuron and imidacloprid, however, decreased for all tested insecticides after sixty minutes for total haemocyte counts. For differential haemocyte counts, plasmatocytes and granulocytes increased with exposure of all five insecticides. Different abnormalities like agglutination, de-nucleation and cell shape distortion were observed.

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Table 1 Total haemocytes count (THC) in larvae, pupae and adult workers of *Apis dorsata* F. under normal conditions

No. of observations	THC in Cells/mm^3		
	Larvae	Pupae	Adult
1	46475	42875	6525
2	44575	41650	6450
3	46725	45650	6425
4	46600	43375	6575
5	45000	45700	6375
Average	45875	43850	6470

Table 2 Differential haemocytes count (DHC) in larvae, pupae and adult workers of *Apis dorsata* F. under normal conditions

Haemocytes Av. %	Larvae	Pupae	Adult
Prohaemocytes	41.6	43.2	40
Plasmatocytes	13.2	13.2	12.2
Spherulocytes	33.8	22.2	24.8
Oenocytoids	8.2	15.6	19.2
Granulocytes	3.2	5.8	3.8

TABLE 3 Total haemocytes count (THC) in adult workers of *Apis dorsata* F. under different insecticide exposure

Name of insecticide	Time interval	Average no. of cells/mm^3
Ethofenprox	T_1	7635
	T_2	20410
	T_3	5490
Bifenthrin	T_1	4740
	T_2	8170
	T_3	3615
Diafenthiuron	T_1	8215
	T_2	8780
	T_3	5286
Endosulfan	T_1	5310
	T_2	8580
	T_3	5270
Imidacloprid	T_1	8105
	T_2	5320
	T_3	4485
Control		6470

T_1 = Just after application, T_2 = After 30 minutes, T_3 = After 60 minutes

Table 4 Differential haemocytes count (DHC) in adult workers of *Apis dorsata* F. under different insecticide exposure

Haemocytes Av. %	Ethofenprox	Bifenthrin	Diafenthiuron	Endosulfan	Imidacloprid	Control
Prohaemocytes	44.8	21.8	19.2	26.4	46.4	40
Plasmatocytes	33	57.4	31.2	55	26.6	12.2
Spherulocytes	8.4	10.6	5	7.4	11	19.2
Oenocytoids	3	3	2	3.4	2.4	24.8
Granulocytes	10.8	7.2	42.6	7.8	13.6	3.8

Table 5 Analyses of variance regarding the effect of different insecticides on the total haemocytes count of the adult workers of the *Apis dorsata* F.

S.O.V	**d.f.**	**S.S.**	**M.S.**	**F.Cal.**
Treatment	14	1127147207	80510514.74	3674.18**
Insecticides (I)	4	313063883.333	78265970.833	3571.75**
Time interval	2	376602706.667	188301358.333	8593.3307**
I x T interaction	8	437480616.667	54685077.083	2495.611**
Error	60	1314750.0	21912.5	
Total	74	1128461966.667		

**Highly significant

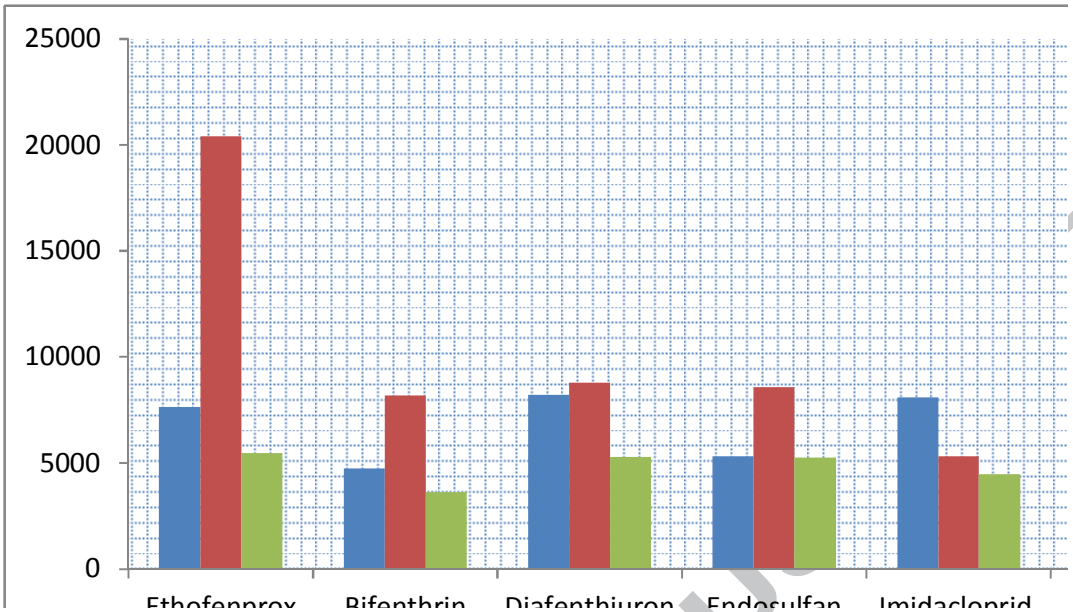

Fig 1 Effects of different insecticides on total haemocytes count (THC) of adult workers of *Apis dorsata* F.

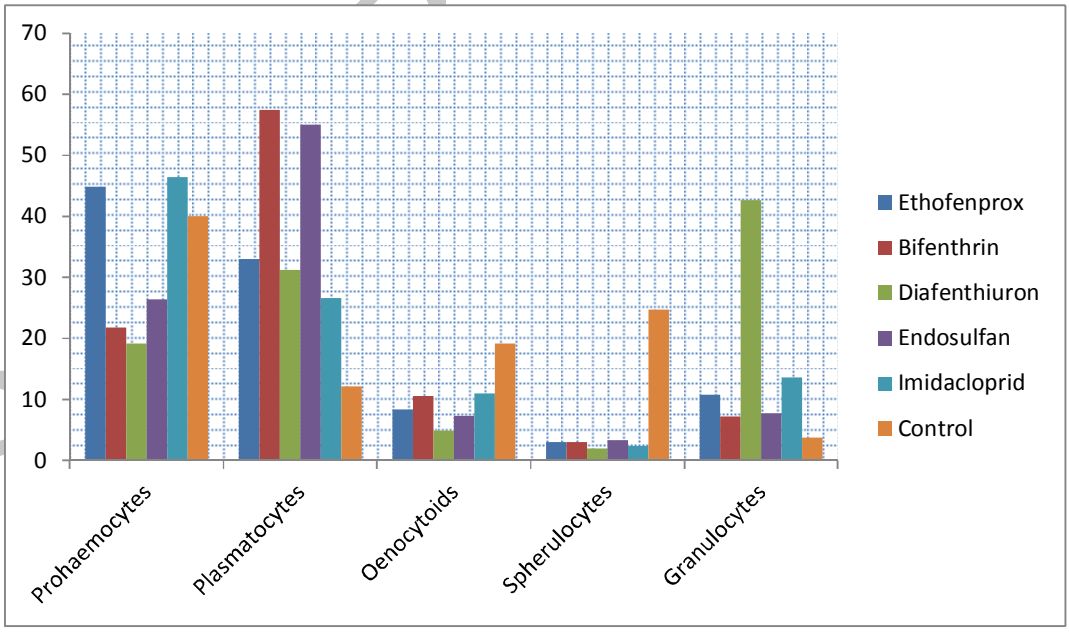

Fig 2 Effects of different insecticides on differential haemocyte count (DHC) of adult workers of *Apis dorsata* F.

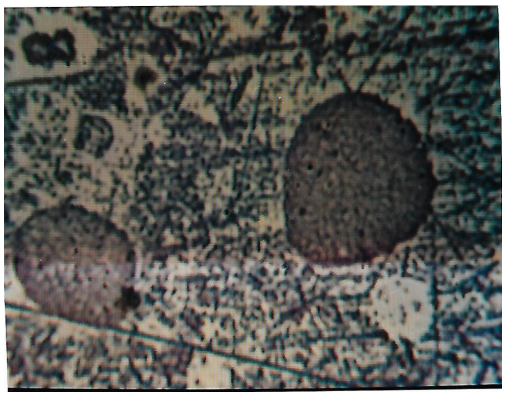

Plate 1 Enlargement of the Cells

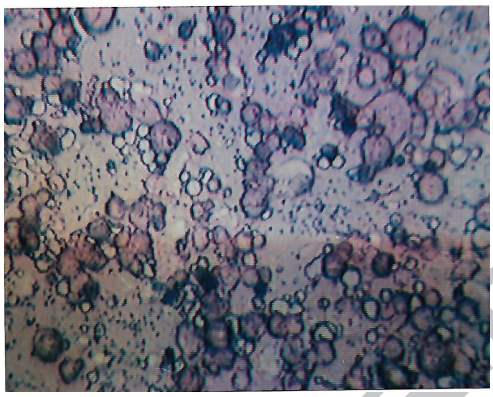

Plate 2 Agglutination of the Cells

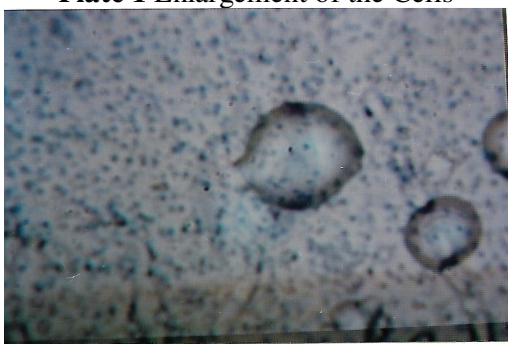

Plate 3 Rupturing of the Cell Wall

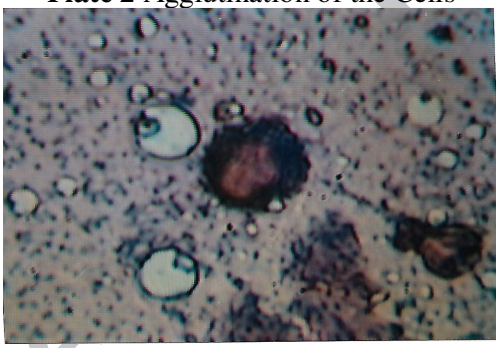

Plate 4 Displacement of Nuclei on one side of the Cells

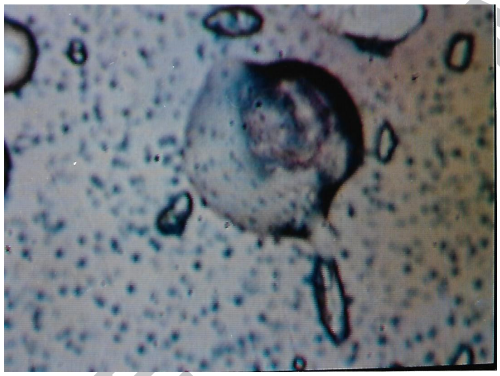

Plate 5 Distortion of the shape of the cells

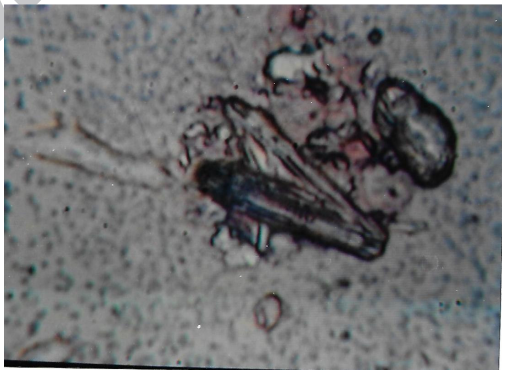

Plate 6 Abnormal Staining of the Haemocytes

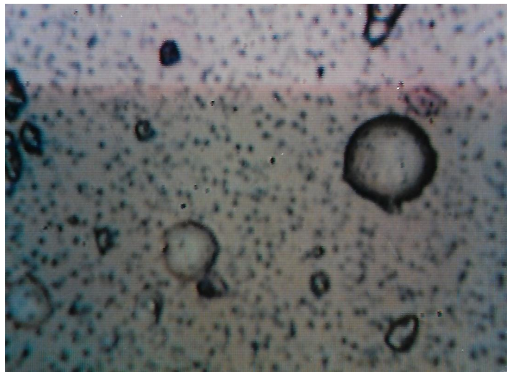

Plate 7 De-nucleation of the Cells

Plates 1-7 Abnormalities observed in haemocytes of adult workers of *Apis dorsata* after application of different insecticides