Journal of Entomology

ISSN 1812-5670

Academic Journals Inc.

www.academicjournals.com

Journal of Entomology 9 (1): 23-31, 2012
ISSN 1812-5670 / DOI: 10.3923/je.2012.23.31

Effect of Some Botanicals on Hemocytes and Molting of *Papilio demoleus* Larvae

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ABSTRACT

The lemon butterfly, *Papilio demoleus* whose larvae are serious pest of citrus plant, causes potential threat to the citrus industry. Citrus trees are excellent source of vitamins and its fruits utilized directly by human being. Therefore, *P. demoleus* should be controlled in eco-friendly manner. Keeping hazardous effects of synthetic insecticides in mind the natural bio-pesticides are being tested here with reference to insects molting and hemocyte counts. In the present study, leaf extracts of *Eucalyptus globulus* and *Ageratum conyzoides* and clove extract of *Allium sativum* were evaluated for their bio-efficacy against V instar larvae of *P. demoleus*. The leaf and clove extract of aforesaid plants were prepared by macerating and extracting them in small quantity of Double Distilled Water (DDW). Total Haemocyte Count (THC) and Differential Hemocyte Count (DHC) were conducted and results revealed significant reduction in THC as well as great deal of variation in relative percentage of hemocytes in comparison to their control. The abnormalities in morphogenetic development were exhibited by the production of larval-pupal intermediates, pharate adults and different degrees of wing abnormalities in imagoes. Plant extracts also caused reduction in larval body weight showing their anti-feedant properties. Relatively more significant reduction in body weight was found in *Eucalyptus globulus* treated larvae. These results revealed that the botanicals used in this study are very economical and eco-friendly to non-target organisms and human being. But these botanicals/bio-pesticides are hazardous to lemon butterfly thus challenging its pest status in the crop field.

Key words: *Eucalyptus globulus*, *Ageratum conyzoides*, *Allium sativum*, *Papilio demoleus*, molting, botanicals, hemocyte

INTRODUCTION

The lemon butterfly, *Papilio demoleus* is an economically important pest whose larval forms cause serious damage to citrus family in the field by devouring large quantity of foliage during the later stages of their development (Bhutani and Jotwani, 1975; Srivastava, 1993). Nowadays, the use of synthetic pesticides due to their high efficacy and reliability of pest control has become popular. Besides, these pesticides have some negative effects also causing ecological damage and health hazards (Isman, 1999). Hence, most of the advanced countries have banned the practical use of few insecticides. Fortunately, investigation of paper factor by Slama and Williams (1966) was

a new indication to entomologists which gave a new trend to find out biodegradable, non-polluting and eco-friendly insecticides. In this context, a number of plants have been screened for their insecticidal properties (Choi *et al.*, 2003; Isman, 2000). The different degrees of developmental abnormalities caused by plant extracts are reported by several workers (Ahmed *et al.*, 2002; Rahman *et al.*, 2003; Khan and Kumar, 2003; Tiwari *et al.*, 2006; Mulungu *et al.*, 2007; Pandey *et al.*, 2007; Pandey and Tiwari, 2011). Ahmad *et al.* (2002) evaluated *Eucalyptus citriodora* for its insecticidal properties. The ovicidal and insecticidal effects of clove extract of *Allium sativum* were seen in *Dysdercus koenigii* by Katiyar and Srivastava (1984). Khoja and Gupta (1992) evaluated the toxicity of garlic extracts in some lepidopterous insects. Further, leaf extracts of *Ageratum conyzoides* were screened for their insecticidal properties (Katiyar and Srivastava, 1984; Singh and Rao, 2000). Keeping hazardous effects of synthetic insecticides in mind, the biocompatible and biodegradable natural bio-pesticides were used in the present study. The use of haemolymph as a medium for controlling insect pests has been made because the changes occurring in the haemolymph are quickly transferred to other portions of insect's body (Pugazhvendan and Soundararajan, 2009). Sahayarij *et al.* (2007) studied the cell mediated immune response of *Helicoverpa armigera* and *Spodoptera litura* against phytoecdysterone obtained from fern. While most of the studies till date have been made to study the effect of plant extracts on only one or two physiological aspects of insects, their effect on hemocytes, the cells constituting cellular immune system are very little studied. The insect pests may be controlled by disturbing their physiological activities i.e. feeding, molting, reproductive and immune systems. The present study was, therefore, initiated to investigate the effects of leaf extracts of *Eucalyptus globulus* and *Ageratum conyzoides* and clove extract of *Allium sativum* on hemocytes and molting of *Papilio demoleus*.

MATERIALS AND METHODS

Eggs and early larval instars, collected from the lemon nurseries and lemon plants were raised in Environmental Chamber and maintained at 28±1°C temperature, 75-80% R.H. and 16 h photophase on fresh lemon leaves. Fifth instar larvae of desired age groups were sorted out and divided into two groups; experimental/test and control larvae. The test larvae were fed for varying periods on leaves treated with different concentrations of leaf and clove extracts of *E. globulus*, *A. conyzoides* and *A. sativum*, respectively whereas controls were raised on untreated fresh leaves.

Preparation of plant extracts and their application: Ten grams of fresh/dried leaves of *A. conyzoides* (bottle brush), *E. globulus* (safeda) and cloves of *A. sativum* (garlic) were macerated separately in all glass pestle and mortar and extracted with a small quantity of DDW. The extract was squeezed through a piece of sterilized muslin cloth and the final volume was made up to 10 mL and this was treated as stock solution. To keep the potentiality of extract ingredients for a longer time, the stock solution was kept in Environmental Chamber and maintained at 5±1°C temperature. Different concentrations to be used in present study (10, 20, 40, 50 and 100%) were prepared from stock solution by adding required quantity of double distilled water. Fresh lemon leaves to be given as food to experimental larvae were dipped in aforesaid crude extracts for 1-2 h and shade dried thereafter. Treated and untreated leaves were replaced at every 24 h interval in experimental and control groups. After feeding on treated leaves for a designated period, the larvae were fed on fresh untreated leaves till their survival. The weight of larvae was recorded daily

throughout larval development. To assess the toxic effect of botanical extracts: mortality, reduction in body weight, deformity in larvae, pupae as well as adults and molting abnormalities were recorded.

Hemocyte count: Ten to twelve haemolymph determinations, each from oozing cut prolegs of 4-5 larvae were drawn and diluted and hemocytes were counted on a standard blood cell counter under phase contrast microscope. The mean number of circulating hemocytes per mm^3 was calculated as per Jones (1962). Permanent preparations of haemolymph smear, staining and calculation of relative percentage of hemocyte types were similar to the methods adopted earlier (Pandey *et al*., 2008, 2010; Pandey and Tiwari, 2011).

Statistical analysis: The data were subjected to the statistical analysis by using Student's t' test. Microsoft Excel 2007 software was used to analyze the data.

RESULTS

Effect of feeding V instar larvae of *P. demoleus* on leaves treated with crude plant extracts on Total Hemocyte Count (THC), Differential Hemocyte Count (DHC) and molting are described as follows:

Effect on Total Hemocyte Count (THC): Feeding of V instar larvae for 24 h on fresh lemon leaves dipped in 50% crude fresh leaf extract of *A. conyzoides* caused about 24% reduction in THC 24 h after treatment as compared to their controls (Table 1). But a record of THC after 48 h of treatment (including 24 h feeding on untreated leaves) showed a decline of about 15% only. The effects of *E. globulus* and *A. sativum* on THC are more or less similar to effects of extract of *A. conyzoides*. Table 1 further showed 27% reduction in THC after 24 h feeding on 50% dry leaf extract of *A. conyzoides*. This reduction in cell count was raised to 38% if the larvae were fed on 100% dry leaves extract. The dry leaf extract of *E. globulus* is more effective in comparison to *A. conyzoides* as 40% decline in total cell number was noticed when the larvae were fed on leaves treated with 100% dry leaf extract for 24 h.

Effect on Differential Hemocyte Count (DHC): The relative percentage of different hemocyte categories was found to vary greatly after feeding on leaves treated with aforesaid botanicals

Table 1: Effects of feeding on leaves treated with certain botanical extracts on Total Hemocyte Count (THC) of *P. demoleus* larvae

Hours after treatment	Conc. (%)	*A. conyzoides* (fresh leaf)	*A. conyzoides* (dry leaf)	*E. globulus* (fresh leaf)	*E. globulus* (dry leaf)	*A. sativum*	Control
24	50	5070±210.5** (-24%)	4890±190.5** (-27%)	4910±80.0** (-26%)	4685±200** (-30%)	5290±110** (-21%)	6670±580
	100	4630±180** (-31%)	4145±300** (-38%)	4540±110** (-32%)	4015±180 (-40%)	4730±90.5** (-29%)	6670±580
48	50	9130±300* (-15%)	8915±120* (-17%)	9095±110 (-16%)	8830±300 (-18%)	9315±180* (-14%)	10800±690
	100	8030±280** (-26%)	7055±180** (-35%)	7840±330** (-27%)	6540±180** (-39%)	8230±120.5** (-23.7%)	10800±690

Values represent Mean±SD of 10-12 haemolymph determinations, each drawn from 4-5 larvae. p-values: *<0.05; **<0.01. Values in parenthesis showed % decrease (-) and % increase (+) in number of THC in experimental larvae compared to controls

Table 2: Effects of feeding on leaves treated with 50% conc. of certain botanical extracts on Differential Hemocyte Count (DHC) of *P. demoleus* larvae

	Relative percentage of various haemocyte types							
	After 24 h				After 48 h			
Haemocyte types	Control	*A. conyzoides*	*Allium sativum*	*Eucalyptus globulus*	Control	*A. conyzoides*	*Allium sativum*	*Eucalyptus globulus*
PRs	4.8±0.5	4.0±0.3* (-17%)	4.2±0.6* (-13%)	4.3±0.6^{NS} (-10%)	4.9±0.5	3.9±0.4* (-20%)	4.1±0.5* (-16%)	4.6±0.7^{NS} (-6%)
PLs	36.7±1.9	32.9±1.2* (-10%)	33.1±1.3* (-9.8%)	33.0±1.7* (-10%)	38.3±3.0	34.2±2.0* (-11%)	33.9±2.5* (-11%)	33.8±2.1* (-12%)
GRs	24.2±1.9	19.6±1.2* (-19%)	20.3±1.5* (-16%)	21.3±1.5* (-12%)	29.4±2.6	20.3±1.8** (-31%)	20.7±2.1** (-30%)	26.6±1.6 (-10%)
SPs	21.2±2.5	28.3±2.0** (+33%)	27.4±2.4** (+29%)	26.3±1.6** (+24%)	11.8±0.5	22.3±2.0** (+89%)	22.6±1.8** (+92%)	15.3±1.2** (+30%)
ADs	9.3±1.5	10.6±1.4* (+14%)	10.9±1.0* (+17%)	10.6±1.0* (+14%)	10.8±0.8	12.2±1.2* (+13%)	11.9±1.5* (+10%)	12.5±1.2* (+16%)
OEs	3.8±0.4	4.6±0.6* (+21%)	4.1±0.8^{NS} (+8%)	4.5±0.5* (+18%)	4.8±0.4	7.1±0.6** (+48%)	6.8±0.8** (+42%)	7.2±0.6** (+50%)

Values represent Mean±SD of 10-12 haemolymph determinations, each drawn from 4-5 larvae. Values in parenthesis showed % decrease (-) and % increase (+) in number of DHC in experimental larvae compared to controls NS: Not significant; p values: *< 0.05; **<0.01

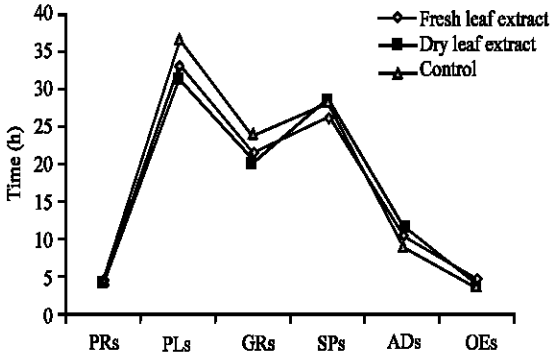

Fig. 1: Effects of leaf extract of *Eucalyptus globulus* (50% conc.) on DHC after 24 h of treatment

(Table 2). The count of Prohemocytes (PRs), Plasmatocytes (PLs) and Granulocytes (GRs) declined to 17, 10 and 19%, respectively after feeding on treated leaves of fresh leaf extract of *A. conyzoides*. Whereas, feeding on leaves treated with clove extract of *A. sativum* for 24 h caused a reduction of 13, 9.8 and 16% in their counts. The percentage of Spherocytes (SPs), Adipohemocytes (ADs) and Oenocytoides (OEs), on the other hand, increased in experimental group but this rise in their count varied greatly after 24 h feeding on untreated leaves. A relatively similar result was found in the larvae treated with fresh leaf extract of *E. globulus* while dry leaf extract caused more reduction in the number of PRs, PLs and GRs (Fig. 1, 2).

Effect on morphogenetic development: The effects of clove extract of *A. sativum* and leaf extract of *A. conyzoides* on morphogenetic development of *P. demoleus* are shown in Table 3. The feeding of early V instar larvae on 10% clove extract of *A. sativum* treated leaf for two days resulted

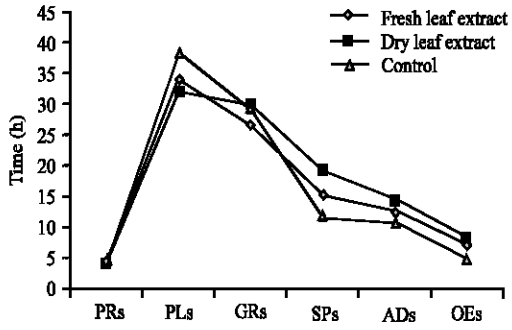

Fig. 2: Effects of leaf extract of *Eucalyptus globulus* (50% conc.) on DHC after 48 h of treatment

Table 3: Effects of feeding on leaves treated with botanicals on morphogenetic development of *P. demoleus* *

Feeding on leaves treated with botanicals	5th instar larvae	Conc.(%)	Feeding (days) on treated leaves	Effects on Moulting		Morphogenetic development
				Larval-pupal	Pupal-imaginal	
Allium sativum	Early	20	4	Occurred	-	LPI
(clove extract)	Early	10	2	Occurred	Did not occur	Pharate adult
	Late	20	1	Occurred	Occurred	Eclosion complete, wings small sized with slight deformity
	Early	50	1	Occurred	Occurred	Eclosion more, wings severely crumpled
	Early	100	1	Occurred	-	LPI
Ageratum conyzoides	Early	20	1	Occurred	Occurred	Eclosion complete, wings crumpled
(fresh leaf extract)	Early	10	1	Occurred	Occurred	Eclosion complete
	Late	10	1	Occurred	Occurred	Normal butterfly
	Early	50	1	Occurred	Occurred	Eclosion more, wings more crumpled
	Early	100	1	Occurred	Occurred	Pharate adult
Eucalyptus globulus	Early	10	1	Occurred	Occurred	Eclosion complete, small sized adult
(fresh leaf extract)	Late	10	1	Occurred	Occurred	Normal butterfly
	Early	20	1	Occurred	Occurred	Eclosion more, wings more crumpled
	Early	50	1	Occurred	Occurred	Partial eclosion
	Early	100	1	Occurred	-	LPI
Control (acetone treated)	-	-	-	Occurred	Occurred	Normal butterfly

*10-12 insects were used in each experimental and corresponding control groups separately

in pharate adults while feeding on 20% for four days and 100% for one day, both produced Larval-pupal Intermediates (LPIs). Late V instar larvae fed on 20% concentration for a day though molted into adults but with slightly deformed wings whereas 50% concentration for a day yielded adults with severely crumpled wings.

Table 3 further showed that the feeding on leaves treated with 10% extract (fresh leaf) of *A. conyzoides* did not prevent larval-pupal or pupal-imaginal molting of both early and late V instars. It is also revealed that feeding with 20-50% conc. yielded butterflies with different degrees of wing deformities. An increase in conc. up to 100% produced pharate adults.

Feeding on 50% conc. of dry leaf extract of *E. globulus* caused 100% mortality immediately after feeding. But larvae died the next day after feeding on same concentration of *A. conyzoides*.

Table 4: Effects of feeding of leaves treated with botanical extracts on body weight of *P. demoleus* larvae

Treatments	Conc. (%)	Body weight (g) in V instar larvae at 24 h intervals						
		0	24	48	72	96	120	144
Ageratum conyzoides	25	0.383±0.015 ^{NS}	0.560±0.025 ^{NS}	1.185±0.040*	1.210±0.060*	1.305±0.080*	1.420±0.090**	0.920±0.035*
(fresh leaf extract)	50	0.384±0.010 ^{NS}	0.500±0.030*	1.065±0.035**	1.190±0.070**	1.210±0.050**	1.315±0.090**	0.900±0.030*
Eucalyptus globulus	25	0.382±0.015 ^{NS}	0.520±0.030*	1.160±0.045*	1.200±0.070*	1.300±0.055*	1.410±0.060**	0.910±0.015*
(fresh leaf extract)	50	0.387±0.020 ^{NS}	0.490±0.025**	1.100±0.040*	1.180±0.060**	1.205±0.065**	1.305±0.020**	0.890±0.020*
Allium sativum	25	0.380±0.010 ^{NS}	0.540±0.025*	1.175±0.040*	1.285±0.080*	1.390±0.060*	1.430±0.060**	0.895±0.025*
(clove extract)	50	0.386±0.015 ^{NS}	0.510±0.030*	1.150±0.040*	1.295±0.085*	1.310±0.075*	1.340±0.080**	0.915±0.020*
Control (acetone treated)		0.385±0.010	0.572±0.030	1.232±0.070	1.474±0.080	1.545±0.090	0.970±0.070 (pre-pupa formation)	-

Values represent Mean±SD of 10-12 haemolymph determinations, each drawn from 4-5 larvae. NS: Not significant; p values: *<0.05; **<0.01. -: Body weights were recorded only up to prepupal stage

While 20% conc. of *E. globulus* produced pharate adults, 40% conc. induced formation of LPIs. Forty per cent conc. of *A. conyzoides* also yielded LPIs. These LPIs were completely devoid of prolegs in comparison to those produced after feeding on fresh leaf extract. The feeding of early V instar larvae on leaves treated with 10% conc. of dry leaf extract of *E. globulus* for a day though did not prevent molting but emerging butterflies possessed crumpled wings.

Effect on body weight: Besides causing a delay of one day in pupation, feeding on leaves treated with *E. globulus* extract resulted in less consumption of food and hence reduction in body weight of larvae in comparison to their controls (Table 4). Further, dry leaf extract caused more reduction in body weight than fresh leaf extract.

DISCUSSION

The developmental abnormalities seen in present insect caused by plant extracts have been reported earlier (Schmutterer, 1988; Singh, 1996; Sahayarij and Paulraj, 2001; Ahmed *et al.*, 2002; Rahman *et al.*, 2003; Khan and Kumar, 2003; Tiwari *et al.*, 2006; Mulungu *et al.*, 2007; Pandey *et al.*, 2007; Pandey and Tiwari, 2011). The factors for these abnormalities are seen to lie in the endocrine system and the metamorphosis hormones secreted by them (Koul and Isman, 1991). Pandey *et al.* (2007) found that the dietary treatments of *Danaus chrysippus* larvae with extract of *A. conyzoides* and *A. sativum* caused production of LPIs, pharate adults and deformed butterflies with crumpled wings. Similar effects were also seen in *P. demoleus* larvae fed on leaves treated with fresh/dry leaf extracts of *A. conyzoides*, *E. globulus* and clove extracts of *A. sativum*. Dry leaf extracts are more effective than extracts of fresh leaves because of more concentration of insecticidal ingredient.

Ageratum sp. as a source of precocene and the role of latter as anti-allatin are well known (Bowers, 1976; Khan and Kumar, 2003). In *Pericallia ricini*, Khan and Kumar (2003) reported that the precocene exhibited two categories of effects- JH (Juvenile hormone)-antagonistic (precocious pupation) and JH-agonistic (delayed molting, ecdysial failure, pupal-adult intermediates and deformed adults). Observations made in present study resemble with the second category of result seen in *P. ricini*. It is thus presumed that the plant extracts used in present study are having JH-agonistic and not the JH-antagonistic effect on *P. demoleus* larvae. Abahussain (2006) showed effect of the non-steroidal ecdysone agonist, RH-5849, as a control agent against the

false stable fly. Further, reduction in THC after feeding on leaves treated with different crude botanical extracts in *P. demoleus* larvae substantiates the finding of other workers (De Azambuja *et al.*, 1991; Saxena and Tikku, 1990; Sharma *et al.*, 2003). The reduction in THC is reported to be due to 1, toxic effects of botanicals 2, inhibitory effects of plant extracts on endocrine glands and their secretions and 3, formation of nodules (Sharma *et al.*, 2003; Sabri and Tariq, 2004; Pandey *et al.*, 2007). It is reported that production, multiplication and differentiation of hemocytes are controlled by ecdysone (Tiwari and Shukla, 2000). Since the nodules are not seen in experimental larvae of *P. demoleus*, it is assumed that these botanicals exert their effects by inhibiting the secretion of ecdysone via brain. Further, the reduction in PRs number may be attributed either to the inhibition of their mitotic division, their conversion to other types of cells or to the inhibition of activity of haematopoietic organs responsible for their production. More such studies are needed to corroborate this assumption. Being phagocytic in nature, PLs and GRs are readily attracted to any foreign substance (Sharma *et al.*, 2003) and therefore, are not freely available in the haemolymph for their count leading to their reduction. George and Ambrose (2004) recorded reduction in PLs of adult *Rhynocoris kumarii* due to toxic effect of organo-phosphates. The OEs being thick (Gupta, 1979) may resist penetration of botanicals and remain unaffected.

The reduction in body weight of treated larvae showed the antifeedant property of botanicals. Such a loss in body weight has also been reported after topical application of leaf extract of *A. conyzoides* in *Spodoptera litura* by Singh and Rao (2000) and in *D. chrysippus* larvae by Pandey *et al.* (2007).

CONCLUSIONS

The botanicals used in present study exert their effect directly upon the Neurosecretory Cells (NSCs) of the brain. They cause reduction in phagocytic response of hemocytes and reduction in body weight. Furthermore, the altered release of Brain Hormone (BH) and other two metamorphosis hormones seem to cause developmental abnormalities in present insect. The over-all effects of botanicals offer a novel approach to the insect pest management. Unlike other insecticides that kill both pest and predators, botanical extracts are relatively economical, biodegradable and eco-friendly to non-target organisms and human being as well. But these botanicals/bio-pesticides are hazardous to lemon butterfly thus challenging its pest status in the crop field.

ACKNOWLEDGMENTS

Authors are thankful to the Vice-chancellor, Nehru Gram Bharati University (NGBU), Allahabad and Principal, K. N. Govt. P.G. College Gyanpur for providing laboratory facilities.

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