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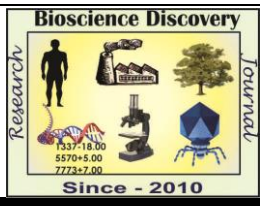

Haemocyte count on 5th day of 5th instar larvae of *Bombyx mori* L. inoculated with *Beauveria bassiana* infection and subsequent oral treatment of ehtanolic plant extract

Chavan J. A. [1*] and G. P. Bhawane[2]

[1]Department of Zoology, Government Vidarbha Institute of Science and Humanities, Amravati- 444 604
[2]Department of Zoology, Shivaji University, Kolhapur - 416 004
Email: jyotiachavan@gmail.com

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Abstract

In the present study, the plants having antifungal activity used against *Beauveria bassiana* inoculation of silkworm larvae. The results obtained in present study shows seven different types of haemocytes in each group. The decreased total haemocyte count (THC) was observed in inoculated group compared with their respective control group of both PM and CSR2 race. The differential haemocyte count shows changes according to the dose of plant extracts. The antifungal plants *Curcuma longa*, *Argemone mexicana* and *Clerodendrum multiflorum* showed more or less similar results when compared with control group.

INTRODUCTION

In insects circulating haemocytes or "blood cells" play very important physiological roles. Among the defense responses of insects, phagocytosis is considered to be the first barrier against pathogens and it has been described in the haemolymph of many insect species against biological agents (Gotz and Boman, 1985; Ratcliff, 1986; Chavan and Bhawane, 2017; Duresa *et al.*, 2014) and non-biological agents (Wiesner, 1991, 1992; Slovak *et al.*,1991; Narasimha Nayaka and Sharma 2014.).

The haemocytes performing various physiological functions in the body of insects and get migrated towards and engulf several targets such as apoptotic bodies, cell debris from damaged tissue and pathogens (Wood and Jasinto, 2007). In majority of insects Plasmatocytes and granulocytes are considered as the main cell types involved in all defense mechanisms (Ratcliffe *et al.*, 1985; Ratcliffe and Rowley, 1987; Wiesner and Gotz, 1993).

Nucleated haemocytes number varies with the insect species and the physiological state of the species. Insect haemocytes are classified as the granulocytes, prehaemocytes, plasmatocytes, spherulocytes, adipohaemocytes, coagulocytes and oenocytoides (Arnold and Hinks, 1976). Major function of the haemocytes is the encapsulation small particles and of the large foreign materials haemolymph, coagulation in storage and distribution of nutritive material.

Different studies were made on fungal and viral disease of silkworm but the information regarding the exact alteration in biochemical and physiological changes occurring inside the body of silkworm, throughout the progress of disease is limited. In the present studyefficacy of *B. bassiana* infection and simultaneous treatment of plant extracts and its effect on Total haemocyte count (THC) and Differential haemocyte count (DHC) was done on 5th day of 5th instars larvae.

MATERIAL AND METHODS

Rearing of silkworm: The silkworm larvae of the races, Pure Mysore (PM) mutivoltine and CSR2 Bivoltine were reared according to the standard method as described by Krishnaswami *et al.* (1978; 1979).

Preparation of plant extract: The shade dried selected plant material was powdered and kept in ethanol or extraction for 72 hours. After extraction ethanol was allowed to evaporate. The extract obtained was stored at 10ºC until further use (Alade and Irobi, 1993; Ahmed and Beg, 2001). Plant extract was prepared in distilled water with 6000ppm and used for the treatment against the *B. bassiana* inoculated silkworm larvae of both the races selected for the present study.

LD50 value for fungi *B. bassiana*: In *B. bassiana* infected larvae, LD50 value observed in PM racewas $1x10^6$spores/ml and in CSR2 was $1x10^5$spores/ml.

Inoculation of *B. bassiana* and plant extract treatment to silkworm larvae: The larvae were divided into 8 groups including control eachgroup containing 50 larvae. On the first day of fifth instar thelarvae were starved for 6 hours. Each larva from respectivetray was deeped individually in LC50 concentration(1x106spores/mL and 1x105spores/mL). After six hours the larvae were fed with ethanolic plant extract coated mulberry leaves for that 100 µL of 10 mg/mL solution of ethanolic plant extract. The plant extract was given for three days at the same time in morning only. Haemocyte count was done on 5th day of 5th instar larvae of *B. mori*.

Haemocytes count:

Total haemocyte count and differential haemocyte count was done by using the method of Praful (1994).

For THC used the following formula,

$$THC = \frac{\text{No. of cells counted X 10}}{\text{Number of 1 sq. mm counted}}$$

For DHC Wright's stain was used dilution with 1:1 with phosphate buffer. The experiment was repeated for three times.

RESULTS

The results obtained on 5th day of experiment on the inoculation and subsequent treatment of plant extracts on THC and DHC are depicted in Table No. 1, different types of haemocytes which observed during study were showed in plate –I.

Total haemocyte count (THC):

The THC observed more in CSR2 race 15370.5THC/mm^3 than PM race 12584.0THC/ mm^3 in control group. The *B. bassiana* inoculation showed decreased THC in both races by 44.8% in PM and 47.5% in CSR2 as compared to control on 5th day of experiment. The treatment of ethanolic plant extract also showed the decrease by 14.9% and by 32.5% in PM and CSR2 race respectively. The treatment of *A. mexicana* shows decreased haemocyte count in both races by 17.6% and by 45.01% in PM and CSR2 race respectively. The decreased THC was notice after the treatment of *C. multiflorum* in both races 39.5% in PM and 41.04% in CSR2 race as compared to control group.

From the above results, it was cleared that the THC count decreased in all groups as compared to control but the plant extracts treated group showed minimum decreased percentage as compared to inoculated group of both races. It was probably due to the plants used in present study seems to have the antifungal property. In the CSR2 race showed maximum decrease of THC count than PM race when compared with control groups.

Differential haemocyte count (DHC):

Granulocyte:

In DHC study, granulocyte observed more in PM than in CSR2 race i.e. 47.1% and 31.4% respectively in their control groups. The *B. bassiana* fungal inoculation caused the decreased granulocyte percentage by 64.6% in PM and by 30.89% in CSR2 as compared to control. In *C. longa* treated group both the races showed decreased granulocyte count by 14.9% and by 32.5% in PM and CSR2 races respectively.

The *A. mexicana* treatment showed the decreased percentage of granulocyte in PM race by 23.56% but in CSR2 increased the granulocyte percentage observed by 6.14% as compared to control group. In *C. multiflorum* treatment showed decreased granulocyte percentage by 45.9% and by 5.41% in PM and CSR2 respectively as compared to control groups.

The above results showed that the *B. bassiana* inoculation causes the reduction in granulocyte percentage as compared to control group. The maximum reduction of granulocyte was observed in PM race than in CSR2 race. The *C. longa* and *C. multiflorum* showed the decreased granulocytes at significant level in both races. While the *A. mexicana* treated group showed no significant increased in CSR2 race and decreased in PM race when compared with control.

Prohaemocyte:

The prohaemocyte percentage observed more in CSR2 i.e. 27.8% than in PM i.e. 12.98% in their control groups. The inoculation of fungal spores of *B. bassiana* showed the increased prohaemocyte percentage by 28.35% and decreased prohaemocyte by 20.5% in PM and CSR2 respectively. After the inoculation in both the races subsequently treated with ethanolic plant extract showed the increased prohaemocyte in both races i.e. 28.35% and 12.23% in PM and CSR2 after the treatment with *C. longa* plant extract. The *A. mexicana* treatment showed decreased prohaemocyte percentage in both races by 37.59% in PM and 40.07% in CSR2 race. The treatment of *C. multiflorum* showed the decreased prohaemocyte percentage in PM by 60.7% and by 39.92% in CSR2 race as compared to control groups.

The above result noticed that *B. bassiana* affect the cell count percentage in CSR2 race but not in PM race. The subsequent treatment of ethanolic plant extracts shows the increase of prohaemocyte percentage in *C. longa* treated group. The maximum increase was observed PM race than CSR2. The maximum decrease was observed in *C. multiflorum* treated PM race as compared to control on 5^{th} day of experiment.

Plasmatocyte:

In the control group of CSR2 race plasmatocytes count observed was 9.3%and in the PM race was 4.5%. The *B. bassiana* inoculated group showed the increased in palsmatocyte count in both race PM and CSR2 by 393.3% and by 130.2% respectively. The subsequent application of *C. longa* plant extract showesthat the increased plasmatocytes in both races by 22.22% in PM and by 204.3% in CSR2 race. In *A. mexicana* treated group showed the increased of the plasmatocyte by 142.2% and by 19.35% in PM and CSR2 respectively. The *C. multiflorum* plant extract showed decreased of plasmatocyte percentage in PM race by 51.11% while increased plasmatocyte percentage was observed in CSR2 race by 101.07% as compared to control groups.

All the above results showed that the inoculationof*B. bassiana* showes increased in the plasmatocyte content in both the races. The treatment of ethanolic plant extract in the entire group shows increased plasmatocyte percentage except the *C. multiflorum* treated PM race as compared to control.

Spherulocyte:

The higher spherulocyte percentage was observed in PM race i.e. 6.7% than in the CSR2 i.e 4.5% in control groups. The inoculation of *B. bassiana* showesthat increase in the spherulocyte percentage by 148.6% and by 255.55% in PM and CSR2 respectively as compared to control. The treatment of ethanolic plant extracts *C. longa* showed increased spherulocytes by 331.3% in PM and by 324.4% in CSR2 race. The *A. mexicana* treated group shows decrease in spherulocyte by 25.37% in PM race while increased in CSR2 by 93.33 as compared to control.

In *C. multiflorum* treated group sperulocyte percentage increased in both the races by 229.8% in PM and 157.7% in CSR2 as compared to control groups.

Adipohaemocyte:

The greater adipohemocyte percentage was observed in CSR2 race i.e. 21.7% than in the PM race i.e. 8.23% in their control groups. The inoculation of *B. bassiana* shows the decrease in adipohemocyte count by 31.95% in PM race and increase in adipohemocyte count by 48.38% as compared control. The treatment of *C. longa* shows the increase in adipohemocyte by 55.52% in PM race while decreased haemolymph percentage observed in CSR2 by 77.88%. In *A. mexicana* treated group increased percentage was observed in PM race by 168.52% while decreased percentage in CSR2 by 64.05% as compared to control. The treatment of *C. multiflorum* plant extract shows increase in adipohemocyte by 407.8% in PM race and decreased adipohemocytes in CSR2 by 37.32% as compared to control.

The above results prove that the *B. bassiana* inoculation shows decreased adipohemocyte in PM and increased in CSR2 race. The treatment of plant extracts in PM race shows that the significant increase wasobserved in adipohemocyte percents in all groups of ethanolic plant extracts treated group while the decreased adipohemocyte percentage was also observed in all group of CSR2 race. The maximum increased adipohemocyte percentage in PM race was observed in *C. multiflorum* treated group. While the maximum decrease was observedin adipohemocyte percentage in *C. multiflorum* treated group of CSR2 race as compared to control group.

Coagulocyte:

The greater coagulocyte percentage was observed in PM 20% than in the CSR2 race, which was 2.1% in their control groups. The inoculation of *B. bassiana* showsdecrease in PM race coagulocyte percentage by 81.0% while increased percentage of coagulocyte significant observed in CSR2 race by 938.09%. The treatment of ethanolic plant extract *C. longa* shows the decrease in the coagulocyte by 77.5% and increase by 71.42% in both PM and CSR2 race respectively. In *A. mexicana* treated groups PM race shows the decrease in coagulocyte percentage by 50% and increase in CSR2 race by 823.8% as compared with control group. The decreased coagulocyte percentage was observed in PM race while increase was observed in CSR2 race after the treatment with *C. multiflorum* plant extract.

The above results illutrate that all the groups of CSR2 (including fungus inoculated) shows increased coagulocyte percentage, the maximum percentage was observed in inoculated group of CSR2 and PM race shows the decrease in percentage of coagulocyte in all group of plant treated and in *B. bassiana* inoculated group the maximum decrease was observed in *B. bassiana* inoculated group. This result indicates that the plants contain antifungal activity which was used in present which were helpful in the recovery of disease in both races.

Oenocytoid:

The oenocytoid percentage wasobserved in CSR2 race and PM race was 10.3% and 3.6% respectively. The increased oenocytoid haemocyte was observed in both races after the *B. bassiana* inoculation in PM by 397.2% and in CSR2 by 6.79%. The application of ethanolic plant extract *C. longa* shows the increased percentage in both the races by 38.88% amd 65.0% in PM and CSR2 races respectively. The treatment of *A. mexicana* showed the increased oenocytoid haemocytes by 112.7% in PM race and decreased by 6.79% in CSR2 race as compare to control. In *C.multoflorum* treated group PM race showed the decreased oenocytoid haemocytes by 66.66% and increased by 13.59% in CSR2 race as compared to control group.

The above observation showed that the *B. bassiana* causes the increased oenocytoid haemocytes than control. In *C. longa* treated both races shows increase in haemocytes percentage. The maximum percentage increase was observed in CSR2 than in PM race.*A. mexicana* treated group shows increased haemocyte percentage in PM and decrease in CSR2 oenocytoid haemocytes. In *C. multiflirum* treated group PM race shows decrease in oenocytoid haemocytes and in CSR2 race increased oenocytoid percentage as compared to control.

DISCUSSION

In the present study seven types of haemocytes were observed in silkworm *B. mori* i.e. granulocytes (GRs), prohaemocytes (PRs), Plasmatocytes (PLs), Spherulocytes (Spls), Adipohaemocytes (Ads), Coagulocytes (COs),Oenocytes (OEs), similar observations on haemocyte types were made by earlier workers in lepidopteron insects including silkworm *B. mori* (Raina, 1976; Beeman *et al.*, 1953; Ribeiro *et al* ., 1996; Riebeiro, and Brehlin, 2006).

The haemocytes count was more in 5th day old larvae than the other day. The THC was more in CSR2 race than the PM race. The similar results reported by Mallikarjuna *et al*., (2002), that the THC were increased in initial stage of infection and decreased in the later stage of infection i.e. on the 5th day. These present results agreed with these findings. Once the entomopathogenic fungus have penetrated in host integument and gained access to the nutrient rich haemocoel, they are confronted with the host humoral and cellular defense mechanism (Butt *et al.*, 1988).

The phagocytosis, encapsulation, and nodule formation is the main reaction for clearance of pathogen and other foreign particles reported by (Carton and Nippi (1997) and Ractcliffe (1993). The treatment of plant extracts of these plants in *B. bassiana* inoculated groups showed the decreased THC in a comparable manner when compared with their control on 5th day in PM and CSR2 races. The maximum increase was observed in *C. longa* and *C. multiflorum* treated groups as compared to their control groups.

Haemocytes are extremely efficient in removing pathogen by accomplishing a series of reaction designated as phagocytosis, nodule formation or encapsulation. The obtained data agreed with thefindings of the earlier investigation that their number may increase (Balavankatasubbaiah *et al.*, 2001; Al-Attar, 2010).

The effect of aqueous extracts of *A. barbedensis* , *P. corylifolia* and *B. spectabilis* used against the CPV infection and studied the THC showed that the plant extract treated group observed more or less similar results as observed in the present study by Kirankumar *et al.*, (2012) which supports the present findings. He reported in inoculated group, the THC get increased after CPV infection upto 2nd day of infection. Then there was decrease in the later 3-8 days.

In the control group they have reported that the THC was significantly low as compared to the treatment because the increase may represent the defense response of silkworm, *A. mylitta* against the invading pathogen. The observed data agreed with observations of the earlier workers. They have made investigations that once entomapathogenous fungi have penetrated in the host integument and gained access the nutrient rich haemocoel (Kirankumar *et al*. 2012; Lakshmi Devi and Yellamma 2013).

From the above study, it is clear that the plant extracts of *C. longa, A .mexicana,, C. multiflorum* enhance the defense response probably by enhancing the cellular, immunity, which is actively participate in the elimination of invadingpathogen like *B. bassiana* in PM and CSR2 race, due to which when the inoculated larvae with *B. bassiana* subsequently treated with plant products reduce the disease incidence due to the increase THC and DHC which is helped for silkworm to eliminate the invaded microbes and help for survival.

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PLATE - I

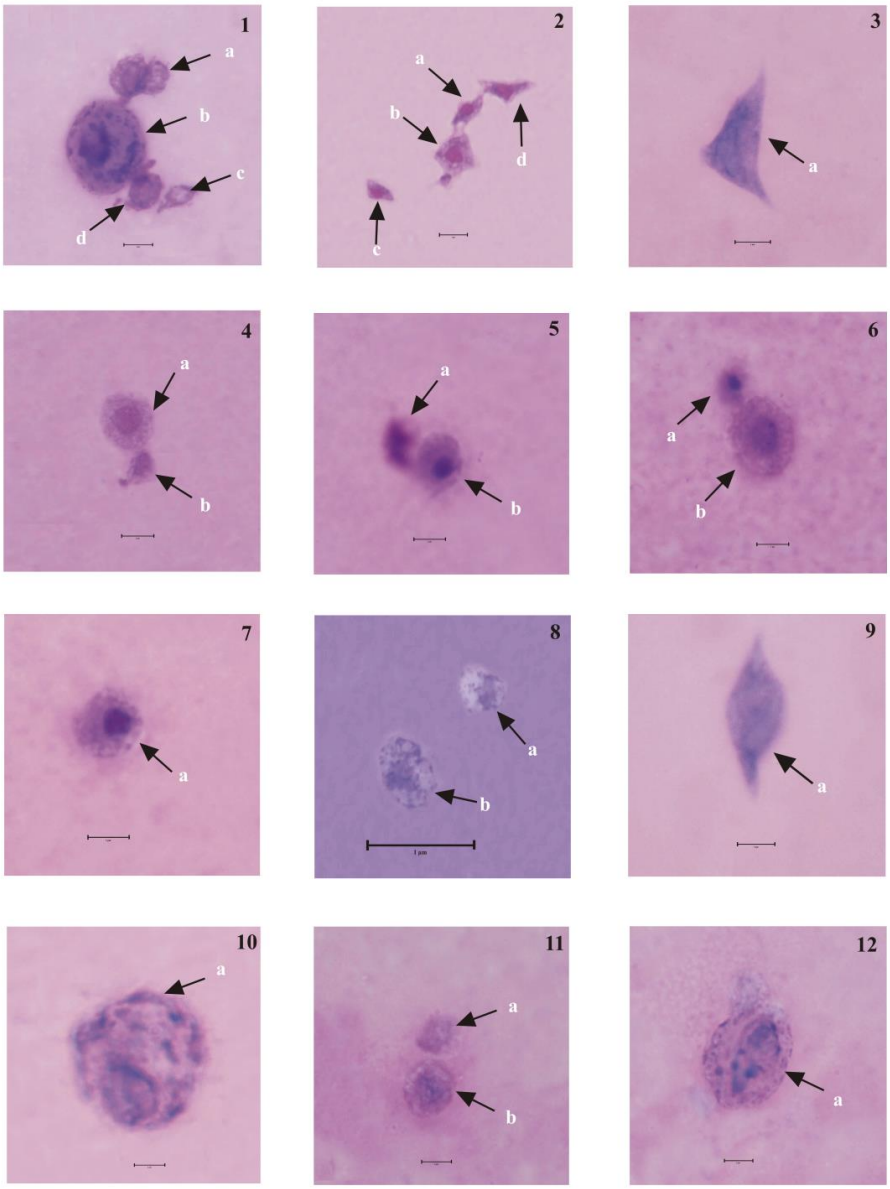

PLATE -I

Types of haemocytes

Fig. 1	**a. c.** and **d.** Prohaemocyte **b.** Granulocyte
Fig. 2	**a. - d.** Plasmatocytes
Fig. 3	Plasmatocyte
Fig. 4	**a.** Coagulocyte and **b.**Spherulocyte
Fig. 5	**a.** Prohaemocyte and **b.** Oenocytoid
Fig. 6	**a.** Oenocytoid **b.** Spherulocyte
Fig.7	Oenocytoid
Fig. 8	**a.** and **b.** Adipohaemocyte
Fig. 9	Plasmatocyte
Fig. 10	Granulocyte
Fig. 11	Adipohaemocyte
Fig. 12	Coagulocyte

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