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EFFECT OF ANDROGRAPHOLIDE ON THE PROTEIN CONTENT OF *TRIBOLIUM CONFUSUM* (DUVAL)

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ABSTRACT

The post harvest losses and quality deterioration of food grain caused by insect pests during storage is the major problem in assuring food security in developing countries. Synthetic insecticides and fumigants have proved very effective in the control of these stored product pests. However, the problems associated with chemical insecticides such as genetic resistance by pest species, residual toxicity, increasing cost of application, pollution of the storage environment and hazard from handling directed a need for effective and biodegradable alternatives. Hence an attempt was made to control the stored product pest, *Tribolium confusum* using Andrographolide, the leaf extract of medicinal herb *Andrographis paniculata.* Its influence on the protein changes in the haemolymph, fat body and ovaries was studied. The protein content in the haemolymph, fat body and ovaries increased gradually in the larvae, pupae and the adults of *Tribolium confusum*, where as in Andrographolide treated resultant larvae there was a significant decrease in the protein content when compared with the controls. This sharp decline in protein content resulted in the development of various abnormalities such as larval-pupal intermediates, pupal- adult intermediates and abnormal adults.

Keywords: Andrographolide, post harvest losses, *Tribolium confusum*, protein content, morphological abnormalities.

INTRODUCTION

Safety of food grains is one of the most important challenges confronting the grain handling agencies and stored product entomologists of the world. Protection of food requires as much attention as is required for its best production. It is supported by the fact that "Post harvest losses are directly proportional to the backwardness of a nation" (Ashfaq *et al*., 2001).

India is basically an agro-based country and more than 70% of Indian population depends on agriculture for its livelihood (Vasudha Lingampally *et al*., 2013). The Indian economy is largely determined by agricultural productivity. Increased agricultural productivity is required in the future to supply the needs of an increasing population. The success of agricultural operations depends on effective storage of food grains.

Post harvest losses primarily due to insect pests represent a major economic constraint to worldwide food security. Their presence in stored foods directly affects both the quantity and quality of the commodity (Farjana Nikkon *et al*. 2009). In India losses caused by insect pests account for 6.5% of stored grain (Akinneye *et al.,* 2006).

Tribolium confusum is one of the most serious cosmopolitan pests in stored grain and related products. It is considered a secondary pest, which can easily infest damaged or broken kernels and apart from grain, it is particularly destructive to flour and other processed grain products. It is also often recorded attacking oil seeds, dried fruits and tree nuts.

It is constantly found in granaries, mills, warehouses and in grain shipments. It gets into all parts of milling machinery and is the insect most commonly found in flour after it leaves the mill. This species is resistant to several traditional insecticides which are commonly used as grain protectants (Boussaada *et al.,* 2008).

Control of storage pests by synthetic insecticides has serious drawbacks (Akinneye *et al.,* 2006). Repeated use of synthetic pesticides for pest management has disrupted natural biological control systems and led to pest resistance, pest resurgence and secondary pest outbreaks. They are also highly persistent accumulating themselves at various concentrations in different levels of the ecosystem (Jagajyothi and Martin, 2010).

Moreover synthetic insecticides penetrate into stored grain and often become toxic (Ogendo *et al*. 2004) to the consumer. Kabeh and Jalingo (2007) reported that some of these insecticides are carcinogenic and mutagenic in action. Therefore safe and efficient stored product pest management is essential to protect the grains from infestation by insects and other agencies (Kiruba *et al*., 2008).

Natural plant products are presently in the focus of research efforts to develop alternatives to conventional insecticides for the protection of agricultural commodities because of their mammalian safety and efficacy (Schmutterer, 1989; Othira *et al.,* 2009).

Plant derived extracts are reported to have the ability to influence the proportion of various biochemical components (carbohydrates, lipids, proteins etc.) in the body of insects, thus disturbing the internal metabolism of the insect, causing their reduced activity or mortality (Vijayaraghavan *et al*., 2010).

On this backdrop Andrographolide a terpenoid isolated from the leaves of the medicinal plant *Andrographis paniculata* is chosen. *Andrographis paniculata* is a herbaceous plant in the family Acanthaceae, native to India and Srilanka. It is widely cultivated in southern Asia, where it is used to treat infections and some diseases, often being used before antibiotics were created. Mostly the leaves and roots were used for medicinal purposes.

Andrographis paniculata is used in traditional Siddha and Ayurvedic systems of medicine as well as tribal medicine in India and some other countries for multiple clinical applications. Its therapeutic value is due to its mechanism of action which is perhaps by enzyme induction (Meenatchi Sundaram *et al.,* 2009).

Andrographis paniculata plant extract is known to possess a variety of pharmacological activities. Andrographolide, the major constituent of the extract is implicated towards its pharmacological activity (Rajgopal *et al.,* 2003).

The enormity of the work conducted on Andrographolide is large but only a handful of data is available with regard to its bio-pesticide control. Therefore the present study has been undertaken to assess the effect of Andrographolide on the protein content of haemolymph, fat body and ovaries of *Tribolium confusum.*

Material and methods

Tribolium confusum were reared on mixed flour of wheat and jowar and maintained at 27 ± 1^0C

temperature and 60 ± 5% relative humidity. Freshly moulted fifth instar, sixth instar larvae and zero hour pupae were treated topically on the abdominal region with 1µg of Andrographolide dissolved in 1µl of acetone with the help of a Hamilton micro syringe. Thirty fifth instar, sixth instar larvae and zero hour pupae were treated each time and the experiments were replicated five times. Parallel controls treated with 1µl of acetone were maintained. After the treatment a suitable gap of time was given for the total absorption of the extract and they were transferred into the diet. The treated larvae and pupae were observed daily and the changes were recorded.

Tissues studied biochemically were haemolymph, fat body and ovaries. Haemolymph from the larvae, pupae and adults was collected using the rapid centrifugation method of the Nation and Thomas (1965). Fat body was dissected and rinsed free of haemolymph with Ringers solution. 10% homogenate was prepared for protein estimation. The late sixth instar larvae, pupae and adults were dissected under a binocular microscope in freshly prepared Ringer solution. The fat body adhering to the ovaries was completely removed. Equal quantities of distilled water were added after the imagination of the tissue. The ovarian extracts were centrifuged at 2500 rpm for 10 minutes. The supernatant was collected and used for protein estimation. Estimation of proteins was done by the method of Lowry *et al.* (1951). The experimental data recorded was analyzed statistically (Mean and Standard deviation) and the graphs were plotted using Microsoft Excel.

RESULTS

1. Protein content in control insects

1.1. Haemolymph proteins

Larval stages: A gradual rise has been observed in the haemolymph protein level of the fifth instar larvae. The first day of the fifth instar i.e., 15^{th} day of the life cycle recorded a protein value of 0.982 ± 0.008 mg/ml of haemolymph. It gradually increased from1.358 ± 0.004 mg/ml of haemolymph on the second day to 1.678 ± 0.008 mg/ml of haemolymph on the last day of the fifth instar i.e., 17^{th} day of the life cycle.

The concentration of total soluble protein in the haemolymph of the sixth instar recorded a rapid increase from 1.91 ± 0.014 mg/ml on the first day of the sixth instar to a maximum of 2.798 ± 0.008 mg/ml of haemolymph on the final day of this instar i.e., 21^{st} day of the life cycle (Figure 1).

Pupa: The increase in the haemolymph protein concentration in the sixth instar was followed by a sudden decline during the early pupal period. The haemolymph protein content of the early pupa was 1.896 ± 0.005 mg/ml. There was a decline of 0.902 mg/ml from the final day of sixth instar to the first day of the pupa. This is due to selective accumulation of haemolymph proteins in the fat body during larval pupal transformation. There is a steady decrease in the pupal concentration from 1.896 ± 0.005 mg/ml to 0.984 ± 0.005 mg of protein/ml of haemolymph (Figure 1).

Adult: The haemolymph protein concentration of the freshly emerged adult recorded a value of 0.662 ± 0.016 mg/ml and it declined to 0.294 ± 0.005 mg of protein/ml of haemolymph on the fourth day of the adult life (Figure 1).

1.2. Fat body proteins

Larval stages: A gradual rise has been observed in the fat body protein concentration of the third instar larvae. The first day of the fifth instar recorded a protein content of 0.99 ± 0.01 mg/gm weight of the tissue. It gradually increased from 1.248 ± 0.08 mg/gm weight of the tissue on the second day to 1.418 ± 0.008 mg/gm weight of the tissue on the third day of the fifth instar (17 day old) larvae. The protein content of the fat body increased further to 1.662 ± 0.008 mg/gm weight of the tissue on the first day of the sixth instar (18 day old) larvae. The second, third and fourth days showed a further increase of 1.89 ± 0.01, 2.06 ± 0.014 and 2.294 ± 0.005 mg protein /gm weight of the fat body tissue respectively (Figure 2).

Pupa: The fat body protein content showed a further increase during early pupal period. The fat body protein content of the early pupa i.e., on the 22nd day of the life cycle was 2.542 ± 0.008 mg/gm weight of the tissue. There was a slight decrease in the pupal fat body protein concentration from 2.382 ± 0.008 mg/gm weight of the tissue on the second day (23rd day of the life cycle) to 1.516 ± 0.016 mg/gm weight of the tissue on the last day of the pupal life (26th day of the life cycle) (Figure 2).

Adult: The fat body protein content of the freshly emerged adult was 1.206 ± 0.008 mg/gm weight of the tissue and decreased to 0.834 ± 0.013 mg/gm weight of the tissue on the second day of the adult life. On the third and fourth days the fat body protein content further decreased to 0.518 ± 0.013 and 0.272 ± 0.008 mg/gm weight of the tissue respectively (Figure 2).

1.3. Ovarian proteins

Larval stages: The ovaries on the first day of the sixth instar (18 day old larvae) recorded a protein content of 0.364 ± 0.005 mg/gm weight of the tissue. It further increased to 0.53 ± 0.01 mg/gm weight of the tissue on the third day. The last day of the sixth instar recorded a value of 0.686 ± 0.013 mg/gm weight of the tissue (Figure 3).

Pupa: There was a steady increase in the protein concentration of the ovary during the pupal period. The first day of the pupal period recorded a value of 1.268 ± 0.010 mg/gm weight of the tissue. It gradually increased from 1.644 ± 0.013 mg/gm weight of the tissue on the second day to 1.932 ± 0.019 mg/gm weight of the tissue on the third day of the pupal period i.e., 26th day of the life cycle. The fourth and fifth day recorded values were 2.212 ± 0.008 and 2.45 ± 0.01 mg/gm weight of the tissue of ovary, respectively (Figure 3).

Adult: The first day of the adult emergence recorded a value of 2.824 ± 0.008 mg/gm weight of the tissue. Later on from the second day of the adult period (28th day of the life cycle) onwards the protein content in the ovaries recorded a considerable decrease from 2.64 ± 0.01 mg/gm weight of the tissue to 0.308 ± 0.013 mg/gm weight of the tissue of the ovary on the fourth day of the adult life i.e., at the end of its life cycle (Figure 3).

2. Protein content in treated insects

2.1. Haemolymph proteins

Larval stages: The protein content in the haemolymph of the fifth and sixth instar Andrographolide treated resultant larval stages of *Tribolium confusum* showed a prominent decrease when compared to the haemolymph proteins of the control larvae.

The haemolymph protein content on the first day of the fifth instar (15 day old) larvae showed 0.97 ± 0.01 mg/ml which is less when compared to the haemolymph protein of the control larvae. The second and third days of the fifth instar also exhibited a low protein content of 1.118 ± 0.008 mg/ml and 1.158 ± 0.008 mg/ml respectively when compared to the haemolymph protein content of the control larvae.

The first day of the sixth instar (18 day old) larvae recorded a value of 1.196 ± 0.005 mg/ml and on the final day showed a definite decrease in protein content when compared to the controls exhibiting 1.268 ± 0.008 mg of protein/ml of haemolymph (Figure 1).

Pupa: The fifth and sixth instar treated resultant pupae showed a decrease in protein content when compared to the control**.** The first day recorded a protein content of 1.066 ± 0.005 mg of protein/ml which further decreased exhibiting lower levels of 0.982 ± 0.004 mg/ml, 0.742 ± 0.008 mg/ml, 0.686 ± 0.005 mg/ml and 0.624 ± 0.005 mg of protein /ml of haemolymph on the second, third, fourth and fifth days respectively (Figure 1).

Adult stage: The haemolymph protein content in the treated resultant adults showed a decrease of 0.566 ± 0.005 mg/ml**,** 0.34 ± 0.007 mg/ml, 0.274 ± 0.013 mg/ml and 0.21 ± 0.007 mg of protein/ml on first, second ,third and fourth days respectively (Figure 1).

Larval pupal intermediate: The protein content in the haemolymph of the larval pupal intermediate was 0.96 ± 0.01 mg of protein/ml of haemolymph.

Pupal adult intermediate: The protein content in the pupal adult intermediate recorded a protein level of 0.39 ± 0.015 mg/ml of haemolymph.

Abnormal adult: The protein content in the abnormal adult recorded a value of 0.28 ± 0.015 mg/ml of haemolymph**.**

2.2. Fat body proteins

Larval stages: There was a drastic decline in the protein content in the fat body of the Andrographolide treated resultant insects compared to the fat body protein content of the control. The first, second and third days of the fifth instar larvae recorded a protein content of 0.974 ± 0.013, 0.996 ± 0.005 and 1.034 ± 0.005 mg/gm weight of the tissue, respectively. The protein content further changed from 1.074 ± 0.005 mg/gm weight of the tissue on the first day of the sixth instar to 1.1 ± 0.007 mg/gm weight of the tissue on the second day of the sixth instar. The third and fourth days recorded a value of 1.118 ± 0.004 and 1.146 ± 0.005 mg/gm weight of the tissue respectively. These values are significantly lower when compared to that of control (Figure 2).

Pupa: The first day of the pupal period exhibited a protein content value of 1.192 ± 0.021 mg of protein/gm weight of the tissue in the fat body. It declined to 1.066 ± 0.005 and 0.822 ± 0.008 mg/gm weight of the tissue on the second and third days respectively. The protein content further decreased from 0.736 ± 0.008 mg of protein/gm weight of the tissue on the fourth day to 0.662 ± 0.008 mg of protein/gm weight of the tissue on the fifth day (Figure 2).

Adult: The first day of the adult emergence recorded a protein value of 0.508 ± 0.017 mg/gm weight of the tissue**.** The second, third and fourth day adults recorded value of 0.398 ± 0.010, 0.27 ± 0.01 and 0.194 ± 0.005 mg of protein /gm weight of the fat body tissue respectively revealing a drastic decrease in the protein content (Figure 2).

Larval pupal intermediate: The fat body protein content in larval pupal intermediate was 1.1 ± 0.14 mg/gm weight of the tissue.

Pupal adult intermediate: The fat body protein content in the pupal adult intermediate was 0.51 ± 0.01 mg/gm weight of the tissue.

2.3 . Ovarian proteins

Abnormal adult: The fat body protein content in the abnormal adult was 0.35 ± 0.02 mg/gm weight of the tissue.

Larval stages: The first day of the sixth instar recorded ovarian protein content of 0.354 ± 0.008 mg/gm weight of the tissue. It gradually increased to 0.394 ± 0.005, 0.45 ± 0.012, and 0.546 ± 0.008 mg/gm weight of the tissue on the second, third and fourth days of the sixth instar respectively (Figure 3).

Pupa: The ovarian protein content in the treated resultant pupae recorded a value of 0.618 ± 0.008 mg/gm weight of the tissue on the first day of the pupal period. It steadily increased from 0.692 ± 0.008 mg/gm weight of the tissue on the second day to 0.768 ± 0.010 mg/gm weight of the tissue on the third day. The fourth and fifth days recorded protein values of 0.826 ± 0.008 and 0.89 ± 0.01 mg/gm weight of the tissue respectively (Figure 3).

Adult: The protein content in the ovaries on the first day of Andrographolide treated resultant adults of *Tribolium confusum* recorded a value of 0.936 ± 0.013 mg/gm weight of the tissue. On the second day it decreased to 0.61 ± 0.01 mg/gm weight of the tissue. The third and fourth days recorded a value of 0.406 ± 0.011 and 0.188 ± 0.008 mg/gm weight of the tissue respectively (Figure 3).

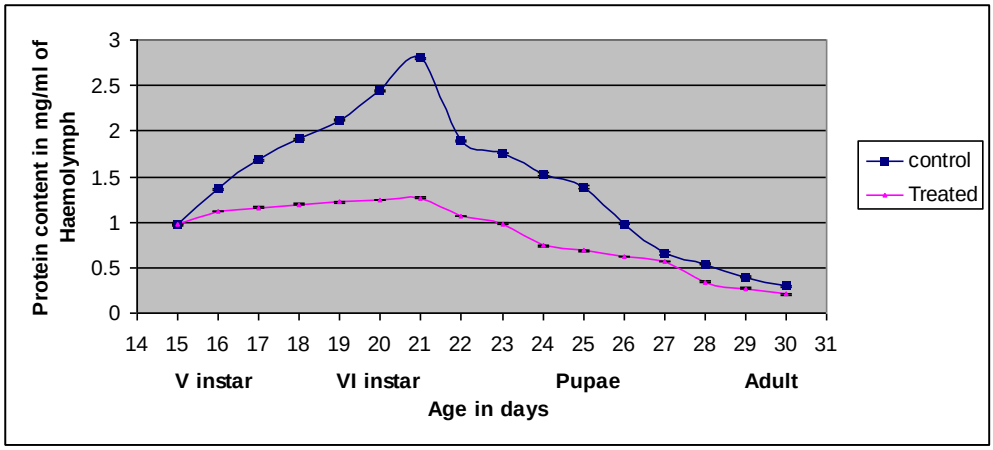

Figure 1: Quantitative changes in the protein content of the Haemolymph of *Tribolium confusum* in control and Andrographolide treated resultant insects during different stages of the life cycle

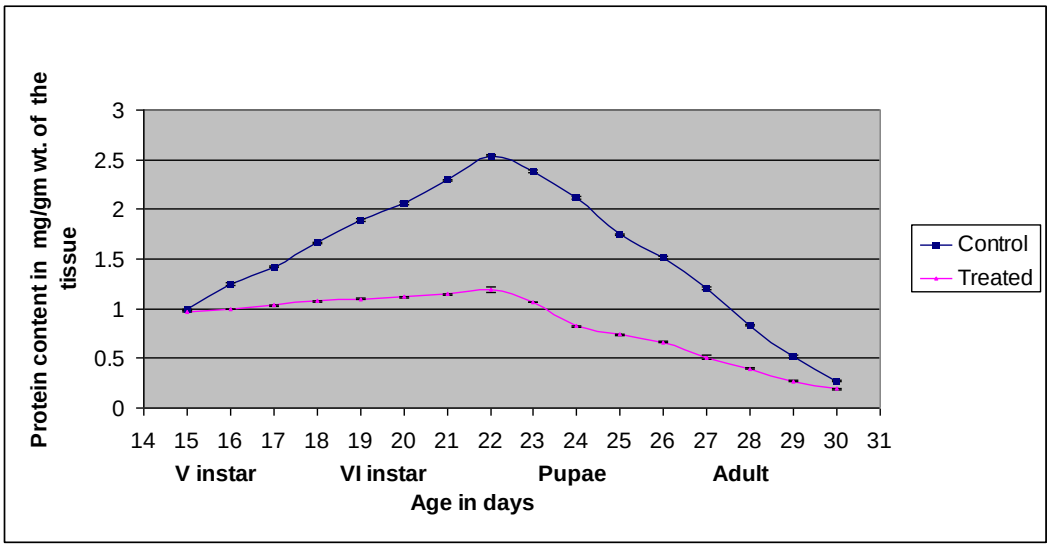

Figure 2: Quantitative changes in the protein content of the Fat body of *Tribolium confusum* in control and Andrographolide treated resultant insects during different stages of the life cycle.

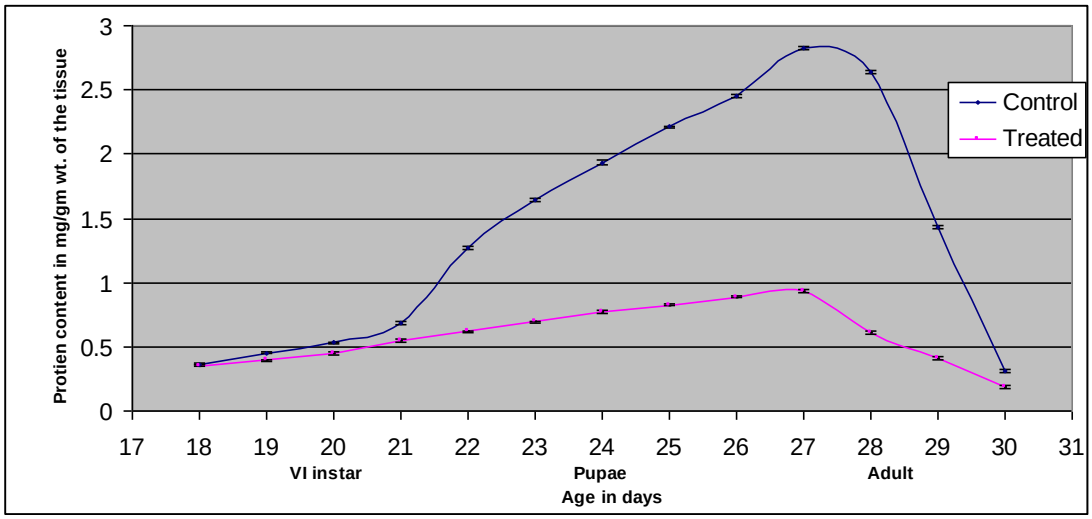

Figure 2: Quantitative changes in the protein content of the Ovary of *Tribolium confusum* in control and Andrographolide treated resultant insects during different stages of the life cycle.

DISCUSSION

The results of this study suggest pronounced changes in the concentration of the total soluble protein in the haemolymph, fat body and ovary during the development of Andrographolide treated larvae of T*ribolium confusum* when compared to the controls.

In holometabolous insects a high proportion of protein is synthesized during the larval development and used later for the synthesis of adult organs. From a morphogenetic point of view studies on haemolymph proteins have a special interest because they provide us with an adequate background to judge the synthetic activity associated with the differentiation process in developing organism. The changes in the protein content of the haemolymph are mainly caused by protein synthesis, incorporation into the fat body cells, histolysis of these cells and histogenesis of the imaginal organs.

The protein concentration of the haemolymph of control *Tribolium confusum* increased gradually during larval development and reaches its highest value in the last instar larvae when larval transformation was nearing the completion but declines during the pupal and adult stages of development. In case of treated resultants the trend was similar but the protein content was very low when compared to the controls. This indicates that the Andrographolide induced the hormonal imbalances which influenced the protein synthesis for the further development of the insect. Similar results were observed by Raja *et al.* (1986) and Anitha *et al*. (2000) in *Chilo partellus.*

The increase in total soluble protein of haemolymph which occurs during the last instar has been correlated with a high rate of protein synthesis by the fat body and the rapid uptake of this protein into the haemolymph (Martin *et al*., 1971). This large build-up was essential because the energy required during metamorphosis must be derived from endogenous sources (Pant and Agarwal, 1965). In the latest development the decline in haemolymph protein was suggested to be due to the increased retention of the newly synthesized protein in the fat body and the selective uptake of haemolymph proteins by the fat body (Kinnear *et al*., 1971).

Though the haemolymph protein content was highest during the final day of each instar it was lower in the treated when compared to the control *Tribolium confusm*. In several species of insects the developmental pattern of the synthesis of haemolymph proteins is attributed to the influence of hormones (Tojo *et al*. 1985; Janarthan *et al*. 1999).

Epidermal reprogramming and synthesis of new cuticular proteins at metamorphic moults are regulated by the moulting hormone (Anderson *et al.*, 1995; Brisca and Sahayaraj, 2009). The Andrographolide treatment might have depleted the critical ecdysteroid level in *Tribolium confusum* as observed in azadirachtin and plumbagin treated *Helicoverpa armigera* leading to defective metamorphic moult (Josephraj kumar *et al*., 1999; Brisca and Sahayaraj, 2009).

The protein concentration in the fat body exhibited a steady increase during larval development and thereafter the increase is markedly accelerated during the early pupal period. The accelerated increase in protein content of the fat body during the early pupal period was suggested to be due to the change of the role of the fat body from synthetic organ to storage organ (Wang *et al.*, 2010).

Locke and Collins (1968) suggested that proteins are sequestered from the haemolymph by the fat body during the last larval instars and are stored as intracellular multivascular bodies. The sequestered proteins in the fat body during pupal phase are used as metabolic fuel and building blocks for adult development (Hauverland, 1996, Burmester, 1999). In case of treated resultants there was a drastic decline in the protein content of the fat body when compared to the controls. This clearly indicates that Andrographolide influenced the synthesis and sequestration of proteins by the fat body cells. The findings of Khatter and Abuldahab (2010) are in conformity with our results.

The protein content in the ovaries of control *Tribolium confusum* gradually increased from the sixth instar to the adult until oviposition. This is correlated to vitellogenesis and to the possibility that the proteins synthesized in the fat body are utilized by the growing oocytes, confirming the results of Prabhu and Nair, 1971; Raja *et al*., 1988). In case of treated resultants there was a significant decrease in the protein content of the ovaries when compared to the controls.

Different studies confirmed that biosynthesis and uptake of vitellogenin are under hormonal control (Hagedorn, 1985; Kunkel and Nordin, 1985). In insects vitellogenin is synthesized in the fat body and transported into developing acts after being released into the haemolymph (Pan *et al*., 1969). Treatment with Andrographolide might have inhibited protein sequestration by the ovaries and oocytes which led to the reduced fecundity.

The decline in protein concentration of various tissues at different stages of the treated resultants of *Tribolium confusum* was apparently due to the action of Andrographolide. This was expressed by the production of morphologically abnormal treated resultants such as larval-pupal intermediates, pupal-adult intermediates and abnormal adults. Similar effects were observed in *Helicoverpa armigera* (Hub.) by the treatment of *Lantana camara* (L.) leaf extract (Prasad and Purohit, 2009).

CONCLUSION

This study clearly enhances the fact that Andrographolide inhibits protein synthesis which is a major biochemical process underlying morphogenesis, yielding a weapon for the control of the stored grain pest *Tribolium confusum*.

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