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The Activity of Midgut Enzymes in the Fifth Instar Larvae of Silk Worm, Bombyx Mori (L) Fed with mulberry leaves treated with Water Solution of Eurhodin

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ABSTRACT

Eurhodin is well known as vital dye and used for addition to some growth media for bacterial and cell cultures. The present Attempt is concerned with the analysis of effect of Eurhodin treatment on the magnitude of velocity of midgut enzymes (protease and amylase) in the homogenate of mid gut tissue of fifth instar larvae of silkworm, Bombyx mori (L). Aqueous solutions of Eurhodin of different strengths (25 ppm; 50 ppm; 75 ppm & 100 ppm) was utilized to treat the leaves of mulberry. 400 ml of aqueous solution of Eurhodin powder was used to treat 100 grams of fresh mulberry leaves for feeding the group of hundred larvae for each time. Treated mulberry leaves were fed to the fifth instar larvae. The feeding the larvae with treated leaves was carried for first four days. Four feedings was the schedule for each day. For each feeding hundred grams of leaves were used. The larvae fed with untreated & water treated leaves were also maintained. The bioassay of mid gut enzymes was carried out on fifth day through the use of mid gut homogenate. The velocity of biochemical reaction catalyzed by mid gut protease and midgut amylase in larvae fed with untreated mulberry leaves was found measured 03.057 (±0.365) units and 6.539 (±0.918) units respectively. The midgut protease activity in larvae fed with mulberry leaves treated with various concentrations (25.0 ppm; 50.0 ppm; 75.0 ppm & 100.0 ppm) of the aqueous solution of Eurhodin powder was found measured 3.793 (±0.696); 5.115 (±1.109); 5.276 (±1.789) and 6.817 (±2.013) units respectively. There was 24.075 to 122.99 percent increase in the mid gut protease activity through Eurhodin treatment. The midgut amyase activity in experimental group larvae in attempt was found measured 6.869 (±1.217); 07.549 (±2.329); 07.719 (±3.313) and 08.539 (±3.789) units respectively. There was 05.046 to 30.585 percent amylase in the mid gut protease activity through Eurhodin treatment. The Eurhodin contents serve to improve the digestibility & exert the influence of efficient metabolism in the fifth instar larvae of silkworm, Bombyx mori (L). The Eurhodin powder treatment may gear overall biochemical constituency of silkworm larvae, through the significant improvement in the velocity of mid gut enzyme catalyzed biochemical reactions.

Keywords: Bombyx mori (L), Midgut Protease, Midgut Amylase, Eurhodin.

I. INTRODUCTION

Insect life cycle is generally complex involving several stages of the larval and pupal development. Adults are generally quite different from the larval forms. When the larvae undergo considerable change to become adults it is called metamorphosis. Insects show various types of metamorphosis as described below. There is close interlinking between the plant metabolites and life of insect herbivores. The metamorphosis is a biological process by which an animal physically develops after birth or hatching,

involving a conspicuous and relatively abrupt change in the animal's body structure through cell growth and differentiation. Some insects, fishes, amphibians, mollusks, crustaceans, cnidarians, echinoderms, and tunicates undergo metamorphosis, which is often accompanied by a change of nutrition source or behavior. Animals that go through metamorphosis are called metamorphoses. The metamorphosis in insects is said to be in the orchestrate progression. The insect metamorphosis is closely interlinked with plant metabolites. According to Bowers, et al (1966) the chemical constituents of plants (Roots; Stems; Leaves and Fruits) could have been the factors of growth & metamorphosis for insects. The plant eating insects are able to avoid poor quality food. That is to say, the insects are able to select food from variety available for them. The larvae of silkworm, Bombyx mori (L) are monophagous. They are feeding exclusively on the leaves of mulberry Morus alba (L). For the purpose of getting qualitative silk cocoons, it is essential to fortify either the quality of food (mulberry leaves) appetite of larval instars of silkworm, Bombyx mori (L). According to Murugan and George (1992), the factors responsible for influencing the growth, development & subsequent physiology of body of silkworm larvae include: quality of nutrition, that is to say the biochemical status of nutrients in the food (Leaves of mulberry, Morus alba L); quantity of hormones (hormonal level) in the body & the conditions of climate (environmental conditions). Each and every element in body of larva is primarily derived from it's source of food material. The leaves of mulberry, Morus alba (L) are exclusive source of nutrients for the life of larval instars of silkworm, Bombyx mori (L). The leaves of mulberry, Morus alba (L) are containing the nutrients and many stimulants for the life of larval instars of silkworm, Bombyx mori (L) (Ito, 1960,1961; Nayar & Fraenkel, 1962; Ito, et al, 1964; Ito & Hyashiya, 1965). The quality of the nutrition (leaves of mulberry, Morus alba L.) serves a lot to accelerate the growth, metamorphosis in larval instars of silkworm, Bombyx mori (L). The entire credit of life of silkworm, Bombyx mori (L) goes to the nutrients in the leaves of mulberry, Morus alba (L). Therefore, the leaves of mulberry, Morus alba (L) forms the physiological foundation for sericulture. The leaves of mulberry are the mulberry, Morus alba (L). The leaves of mulberry, Morus alba (L) biochemically constituted with proteins, lipids, carbohydrates (Murali, 1992) & minerals (Subramanyam Reddy, 1992). The biochemical profile of the leaves of mulberry, Morus alba (L.) exert influence on the corresponding diversity of larval mid-gut enzymes capable of hydrolyzing the biocompounds in the body of larval instars of silkworm, Bombyx mori (L). The proteins; lipids; carbohydrates (glycogen) are stored in the body tissues of larval instars of silkworm, Bombyx mori (L) especially, the fat bodies.

There is variation in the food consumption in phytophagous insects. This may be for varied biochemical processes, ultimately for successful adaptations (Slansky, 1982). It has been suggested that, there is a functional difference between the activity of digestion by the digestive fluid in mid gut & tissue of mid gut. It has been reported by Horie, et al (1963) that, molecular proteins are hydrolyzed into peptides by digestive fluid content & into aminoacids with peptidases in the mid gut tissue. Likewise, the polysaccharides, are digested in the insect gut lumen by digestive fluid & disaccharides and/or trisaccharides get hydrolysed into their constituent monasaccharide sugars mainly in the gut tissue (Horie, 1967). Yamafugi and Yonezawa (1935) reported the analogy of insect lipase, the lipid digesting enzyme of the insect mid gut with pancreatic lipase of vertebrates. The attempts towards production of the qualitative silk through the improvement in the efficiency of consumption & utilization of food by larval instars of silkworm, Bombyx mori (L) include: improvement in the quality of mulberry leaves & supplementation of nutrient biocompounds like soya protein; potassium iodide, copper sulphate, other mineral salts, herbal products (or drugs) like digoxin

(Vitthalrao & Kulkarni, 2011) kho-go (Desai, et al, 2011) and stevia inulin (Shubhangi Pawar, et al, 2017). Quality of mulberry leaves get reflected into the quality of the cocoons spun by fifth instar larvae of silkworm, Bombyx mori (L). There are reports on Use of soya protein; potassium iodide, copper sulphate, mineral salts, herbal products for improvement of the quality of leaves of mulberry, Morus alba. Herbal products are well known for the acceleration of metabolism in the body of larval instars of silkworm, Bombyx mori (L).

Neutral red (toluylene red, Basic Red 5, or C.I. 50040) is a eurhodin dye used for staining in histology. It stains lysosomes red (Winckler, 1974). It is used as a general stain in histology, as a counterstain in combination with other dyes, and for many staining methods. Together with Janus Green B, it is used to stain embryonal tissues and supravital staining of blood. Can be used for staining Golgi apparatus in cells and Nissl granules in neurons. In microbiology, it is used in the MacConkey agar to differentiate bacteria for lactose fermentation. Neutral red can be used as a vital stain. Live cells incorporate neutral red into their lysosomes. As cells begin to die, their ability to incorporate neutral red diminishes. Thus, loss of neutral red uptake corresponds to loss of cell viability. It is also used to stain cell cultures for plate titration of viruses. Neutral red is added to some growth media for bacterial and cell cultures. It usually is available as a chloride salt (Repetto, et al, 2008).Neutral red acts as a pH indicator, changing from red to yellow between pH 6.8 and 8.0.

Concept of production of natural colored silk is not new. It has been handled by many more attempts of researches. Environmentally protected technology for colored silk has been introduced by the authorities of National Chemical Laboratory (NCL) of Pune and Central Sericultural Research and Training Institute (CSRTI) of Mysore (Nisal, et al. , 2013). Trivedy, et al (2016) reported the production of cocoons with remarkable color change persisting even after degumming. Selection of dye suitable for the life of silkworm and sustainable for silk industry is crucial. On this line of studies, the vital dyes may fulfill the necessities to establish the environmentally protective method of obtaining natural colored silk from the larval instars of silkworm, Bombyx mori (L).

The dye named eurhodin is appearing in the literature reviewed as a natural and vital dye. It is also recognized by the common name as neutral red. The labels such as toluylene red and basic red seems to belong to chemical nomenclature. Winckler (1974) reported eurhodin as histological staining. Lysosomes are the organelles stained with this eurhodin stain. This eurhodin stain is used in laboratories of biochemistry as a general stain in histology. It may also be used as a counter-stain in combination with other stains. It has also been reported to be used to stain embryonal tissues. It is used together with Janus Green B stain. In hematology, eurhodin is used as supravital stain. It can be also used for staining cell organelles like Golgi apparatus and Nissl granules. Eurhodin is well known for using in the MacConkey agar. The eurhodin, in MacConkey agar help to differentiate bacterial population for lactose fermentation. Repetto, et al (2008) reported eurhodin to be used in the study of viability of cells. Lysosomes are stained by eurhodin. That is to say the living cells use eurhodin to incorporate into their lysosomes. The cells that are loosing their life are loosing the ability of incorporation of eurhodin stain. Through such type of studies, one can analyze the pattern of loss of cell viability. Repetto, et al (2008), further reporting use of eurhodin stain in cell culture, especially, for plate titration of viral bodies. Use of eurhodin for addition in growth media for bacterial cultures and cell cull cultures is well recognized. Eurhodin is usually is available as a chloride salt. In chemistry laboratories, eurhodin is used as a pH indicator, changing from red to yellow between pH 6.8 and 8.0. Few reports on use of neutral red as food supplement are available (Natalia, et al, 2011; Anumol, et al, 2018). The attempt of Natalia, et al (2011) belong to Singapore Institute recognized as IMRE. This team established

green technology to get rid of traditional dying process necessary to obtain colored silk. This attempt claims that, a simple addition of fluorescent dye as the supplements of diet for feeding the silkworms results into colored silk. Consumption of fluorescent dye treated mulberry leaves leads into change the color of silkworms. Soon after maturation, such silkworms spin colored cocoon. The color of silk reeled from such cocoons is matching exactly to the dye used for treating the mulberry leaves. The recent attempt of Agricultural Development Trust, Baramati through Science Association, Shardabai Pawar Mahila Mahavidyalaya, Shardanagar Tal. Baramati Dist. Pune – 413115 (India) (Vitthalrao Bhimasha Khyade and Eric Richard Kandel, 2018) deals with attempt on use of vital dye to treat mulberry leaves before feeding. Through the integration of natural dye material directly into the silk deserve environmentally appreciations and exert friendly influence in the colored silk production. The aim present attempt is to screen correct dosage for treating the mulberry leaves with Eurhodin and analysis of total protein contents of silk glands; fat bodies and haemolymph from the fifth instar larvae of silkworm, Bombyx mori (L).

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polysaccharides, are digested in the insect gut lumen by digestive fluid & disaccharides and/or trisaccharides get hydrolysed into their constituent monasaccharide sugars mainly in the gut tissue (Horie, 1967). Yamafugi and Yonezawa (1935) reported the analogy of insect lipase, the lipid digesting enzyme of the insect mid gut with pancreatic lipase of vertebrates. The attempts towards production of the qualitative silk through the improvement in the efficiency of consumption & utilization of food by larval instars of silkworm, Bombyx mori (L) include: improvement in the quality of mulberry leaves & supplementation of nutrient biocompounds like soya protein; potassium iodide, copper sulphate, other mineral salts, herbal products (or drugs) like digoxin (Vitthalrao & Kulkarni, 2011) kho-go (Desai, et al, 2011) and stevia inulin (Shubhangi Pawar, et al, 2017). Quality of mulberry leaves get reflected into the quality of the cocoons spun by fifth instar larvae of silkworm, Bombyx mori (L). There are reports on Use of soya protein; potassium iodide, copper sulphate, mineral salts, herbal products for improvement of the quality of leaves of mulberry, Morus alba. Herbal products are well known for the acceleration of metabolism in the body of larval instars of silkworm, Bombyx mori (L).

Sericultural practices are basically related to the nutrition and physiology of digestion in silkworm. Moreover, nutrition and physiology of digestion in silkworm are the most fundamental and important challenges in the sericulture. Significant sericulture may occur if and only if a species of silkworm can be grown quickly and economically. Distinguishing feature of larval instars of silkworm is digestion of albumin, fat and carbohydrates except cellulose (Kellner, et al , 1887). The nutrient composition of the meal get reflect on ability of secretion of digestive enzymes in larval instars of silkworm. The leaves of mulberry, Morus alba (L) should be supplemented with various nutrients. This may help for silkworm feeding to promote silk quality and quantity (Mahmood et al , 2002) . Studies carried out by Mahmood et al (2002) was reported significant consumption of food material followed by gain in the larval weight through feeding “Farm yard manure and ammonia solution” treated mulberry leaves. There is relation among factors like the nutritional status of mulberry leaves and silkworm growth, silk yield and disease resistance Ravikumar (1988). According to Sengupta, et al (1972), nutrients like essential sugars, amino acids, proteins and vitamins are obligatory for normal growth of larval instars of silkworm. Javed and Gondal (2002) have reported higher growth and lower mortality of silkworm larvae fed with nitrogen and ascorbic acid supplemented mulberry leaves. Kanekatsu (1972; 1978); Eguchi and Iwamoto (1976); Abraham (1992) and Sumida, et al (1994) studied on midgut digestive enzymes of larval instars of silkworm, Bombyx mori (L). Kanekatsu, et al (1989) reported rationalization of some of midgut enzymes in larval instars of silkworm, Bombyx mori (L).

Vital stains separate active mycorrhizal structures from structures with no enzymatic activity. Nitroblue tetrazolium (NBT) is a commonly used vital stain that is coupled to the activity of succinate dehydrogenase, an enzyme that is involved in the Kreb's cycle. Active mycorrhizal structures (those that contain active succinate dehydrogenase) stain purple. As mentioned above, counterstaining with a non-vital stain such as acid-fuchsin allows the detection of both active and inactive mycorrhizae. Fluorescein diacetate (FDA) is hydrolysed by esterases in mycorrhizal cells, and causes those cells to become fluorescent. Active mycorrhizae glow green, while dead mycorrhizal structures are not stained. Unfortunately, FDA also stains the cell walls of plant roots, and the fluorescence of these cell walls interferes with the observer's ability to measure mycorrhizal infection. FDA can only be used accurately with root sections. A more specific vital stain, alkaline phosphatase (ALP) stains only fungal structures. ALP-stained mycorrhizae are violet-

black. There is conflicting evidence about ALP's usefulness as a marker of mycorrhizal structures involved in phosphorus uptake. Tisserant et al. (1993 in Varma 1998) found that ALP activity in intraradical hyphae increased prior to a growth response of the host plant. However, Larsen et al. (1996 in Varma 1998) found that mycorrhizae that had been treated with a fungicide showed reduced phosphorus transport, but no reduced ALP activity. Regardless, ALP is a more specific stain for mycorrhizae than FDA and has been used with root segments as well as root sections (Gianinazzi and Gianinazzi-Pearson, 1992).

On this much background, the study has been planned to analyze the effect of feeding the leaves of mulberry, Morus alba (L) aqueous solution of Eurhodin powder on the velocity of biochemical reactions catalyzed by midgut protease and midgut amylase in the fifth instar larvae of silkworm, Bombyx mori (L) (Race: Bivoltine Double Hybrid).

II. METHODS AND MATERIAL

The attempt was divided into the steps like: Silkworm Rearing; Eurhodin solution Preparation; Grouping the Fifth Instar Larvae; Treating the mulberry leaves and feeding the larvae; Bioassay of mid gut enzymes (protease and amylase) and Statistical analysis.

(A). Silkworm Rearing:

The loose egg mass or disease free layings (DFL) of biivoltne, crossbreed race: [(CSR6 x CSR26)] x [CSR2 x CSR27)] of silkworm, Bombyx mori (L) were procured through the sericulture unit of Agriculture Development Trust, Malegaon. Black boxing was followed for incubation. The early age larvae (First and Second instared larvae) (Chawki) and late age larvae (Third; Fourth and Fifth instared larvae) were reared in the laboratory of "Dr. APIS" through the methods prescribed by Krishnaswami, et al (1978) & explained in earlier attempts by Khyade (2004); Vitthalrao & Kulkarni (2011); Desai, et al, (2011) Shubhangi Pawar, et al (2017); Ramprakash Verma, et al (2018); Pranita Rajendra Vare, et al (2018); Manisha Mahendra Nalwade, et al (2018); Seema K. Dongare, et al (2018) and the others. The larvae were fed with fresh and appropriate quality leaves of mulberry, Morus alba (L) procured from sericulture unit at Malegaon Sheti Farm of Agricultural Development Trust Baramati, Shardanagar, (Malegaon Khurd). The schedule of feeding prescribed by Sharad G. Jagtap (2014) was followed for both early age larvae (First and Second instared larvae) (Chawki) and late age larvae (Third; Fourth and Fifth instared larvae). The fifth instared larvae were preferred for the analysis of effect of treating the mulberry leaves with aqueous solution of Eurhodin on total protein contents.

(B). Eurhodin Solution Preparation: The neutral red (toluylene red, Basic Red 5, or C.I. 50040) is well recognized as "Eurhodin dye". It is used for staining in histology. It stains lysosomes red (Winckler, 1974). The quantity about 0.02 weight percent of eurhodin, the neutral red dye is reported for "No Harmful Effects on Silkworm" (Anumol, et al , 2018). For the present attempt, 0.01 weight percent of was selected. This eurhodin, the neutral red dye was procured from Nice Chemicals Pvt. Ltd (PB No: 2217, Manimala Road, Edappally, Kochi, Kerala, 682024, India) through local dealer. Four different concentrations of Eurhodin solution were prepared, which include: 25 ppm; 50 ppm; 75 ppm and 100 ppm.

(C). Grouping the Fifth Instar Larvae: Soon after the fourth moult, the the fifth instared larvae were divided into six groups, each with hundred individuals. The groups include: Untreated Control; Water treated Control and four treated groups. The four treated groups include: 25 ppm; 50 ppm; 75 ppm and 100 ppm. 400 ml of aqueous solution of Eurhodin powder was used to treat 100 grams of fresh mulberry leaves. The treatment was carried out for half an hour before feeding. The treated mulberry leaves were drained off completely & then fed to the fifth instar larvae of silkworm, Bombyx mori (L) in respective groups. Feeding treated mulberry was carried out for the first four days of fifth instars.

(D).Treating the mulberry leaves and feeding the larvae:

Mulberry leaf treatment was carried half an hour before each feeding. 400 ml of aqueous solution of Eurhodin powder was used to treat 100 grams of fresh mulberry leaves for feeding the group of hundred larvae for each time. Fresh leaves of mulberry, Morus alba (L) were weighed. The known volume of solution of each strength was taken in separate glass jar. Known quantity of mulberry leaves was kept immersed separately in aqueous solution of each strength. The treatment was carried out for half an hour before feeding. The treated mulberry leaves were drained off completely & then fed to the fifth instar larvae of silkworm, Bombyx mori (L) in respective groups. Four feedings were followed (5.00 a.m.; 11.00 a.m. ; 5.00 p.m. ; 11.00 p.m.). One hundred grams leaves of mulberry, Morus alba (L) were used for feeding the group of hundred larvae for each time. The feeding treated mulberry was carried out for the first four days of fifth instars. The larvae fed with untreated mulberry leaves and water treated mulberry leaves were also maintained.

(E). Bioassay of midgut Soluble Proteins; midgut protease and amylase:

The bioassay of midgut Soluble Proteins; midgut protease and amylase was carried out on fifth day of fifth instar. Twenty larvae from each group were selected randomly. Weight of individual larva was recorded. They were anaesthetized with chloroform soaked cotton pads. Individual larva was dissected open from dorsal side. The entire alimentary canal was separated from individual larva. The alimentary canal was flushed with ice cold saline so as to remove the debris of mulberry leaf & washed with ice cold saline. The alimentary canal was blotted & weighed accurately on electronic balance. The mid gut tissue was fragmented & then homogenized in chilled saline. Homogenate was centrifuged at 400C for 15 min. at 10000 rpm. The supernatant was equalized to the volume, aliquots of which contain 10 mg per ml & used as assay sample. Half the volume of assay sample was utilized for bioassay of soluble proteins & another half for mid gut enzymes (protease & amylase).

Bioassay of soluble proteins was carried out through the methods of Lowery, et al, (1951). For each assay sample (of each group), bioassay was carried in the triplicate set. One ml of assay sample was added in each test tube. The blank test tube was also prepared simultaneously, in which the assay sample was replaced with distilled water. Addition of 5 ml Lowery's "C" solution was made in each test tube, mixed well & kept for 15 minutes for the purpose to form the copper-protein complex. After fifteen minutes; 0.5 ml Folin's phenol reagent was added in each test tube & mixed well. The content in each test tube was allowed to develop colour. Then the optical density of content of each test tube was recorded at 660 nm on spectrophotometer. The concentration of soluble proteins of each assay sample was calculated through the reference of optical density assay sample & standard proteins (BSA) (the plot of optical density against concentration of BSA).

The activity of mid gut protease was carried out according to the method of Brik, et al, (1962) with modifications suggested by Isshaya, et al, (1971) & outlined by Chougale (1992) & Khyade (2004). The mid gut protease activity was determined in triplicate set along with the blank. The mixture of incubation consisted of substrate (one ml of ten percent casein solution) ; source of enzyme (0.5 ml assay sample) & 0.5 ml of 0.2M Trisbuffer (pH= 8.4). For the blank, assay sample was replaced by distilled water. The incubation was carried out in water bath at 300C for 20 minutes with constant shaking. Addition of 6 ml of 2 percent trichloroacitic acid was made. The content was centrifuged at 8000 rpm for 15 minutes. The supernatant was used to read the optical density at 280 nm on spectrophotometer. Amount of tyrosine liberated from the casein due to action of mid gut protease was calculated through the use of optical density readings for assay sample; tyrosine (from

standard graph) & predetermined soluble protein contents of each assay sample. The activity of mid gut protease was expressed in terms of specific activity: microgram tyrosine liberated per mg protein per minute.

The activity of mid gut amylase was determined according to the methods of Bernfeld (1955); explained by Ishaaya & Swirski (1970), with modifications suggested by Gaikwad (1998) & outlined by Khyade (2004) & Desai, et al, (2011). For the purpose to determine the activity of mid gut amylase, 20 larvae were selected randomly & processed for assay sample preparation as described for soluble proteins. Mid gut amylase was determined in triplicate set along with blank. The incubation mixture consisted of one ml of one percent starch solution (as substrate), phosphate buffer (pH=9.2) & 0.5 ml of assay sample. For the blank, assay sample was replaced by distilled water. The process of incubation was carried out in water bath at 300C for 20 minutes. After incubation the termination of activity of enzyme was made by addition of 2 ml DNSA & 2 ml distilled water. The contents were heated in boiling water bath exactly for five minutes, cooled immediately & the optical density of content was read at 540 nm on spectrophotometer.

For the purpose to calculate the mid gut amylase activity; the optical density readings for each assay sample; standard solution of maltase (from graph) and soluble proteins were utilized. The enzyme activity was expressed in specific activity: micrograms of maltose liberated per mg protein per minute.

(F). Statistical analysis:

Consistency in the results is qualitative parameter in research studies. Therefore, the whole experimentation in the present study was repeated for thrice. The data of all the three attempts was collected and subjected for statistical analysis. The statistical parameters for analysis considered in the study include mean, standard deviation, percent change & significance through student t – test introduced by William Sealy Gosset (a chemist working for the Guinness brewery in Dublin, Ireland. "Student" was his pen name) (https://en.wikipedia.org/wiki/Student%27s_t-test) and explained by Norman & Baily (1955).

Table – 1: The Activity of Mid Gut Protease and Mid Gut Amylase in the Fifth Instar Larvae of Silkworm, Bombyx mori (L) (Race: Bivoltine Cross Breed [(CSR6 x CSR26) x CSR2 x CSR27)] Fed With the Leaves of Mulberry, Morus alba (L) (M-5: variety) Treated With Aqueous Solution of Eurhodin Powder.

Mid Gut Enzymes Group	Protease	Amylase
Untreated Control	03.057 (±0.365) 00.000	6.539 (±0.918) 00.000
25 ppm	3.793* (±0.696) 24.075	6.869* (±1.217) 05.046
50 ppm	5.115** (±1.109) 67.320	07.549** (±2.329) 15.445
75 ppm	5.276*** (±1.789) 72.587	07.719*** (±3.313) 18.045
100 ppm	6.817*** (±2.013) 122.99	08.539*** (±3.789) 30.585

- Each figure is the mean & three replications.
- Figure in parenthesis with + sign is the standard deviation.
- Figure below parenthesis is percent change.

* : P<0.05

** : P<0.01

*** : P<0.001

III. RESULTS AND DISCUSSION

The results on the effect of feeding the leaves of mulberry, Morus alba (L) aqueous solution of Eurhodin powder on the velocity of biochemical reactions catalyzed by midgut protease and midgut amylase in the fifth instar larvae of silkworm, Bombyx mori (L) are summarized in table 1 & presented in Fig. 1 and 2. Treating the mulberry leaves with various concentrations of aqueous solution of Eurhodin powder & feeding them to the fifth instar larvae of silkworm, Bombyx mori (L) for four days was found variously reflected in the levels of activity of enzymes (protease & amylase) in the mid gut tissue homogenate.

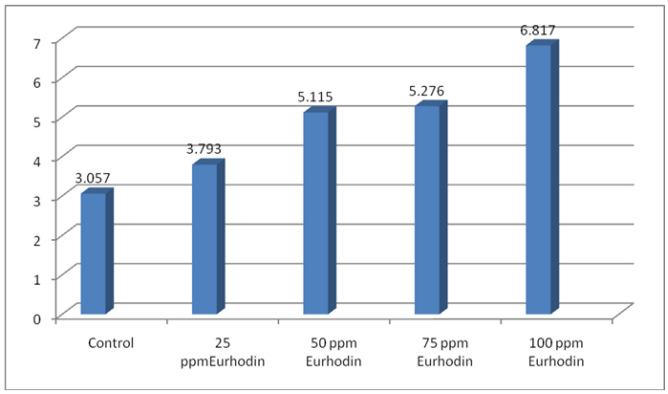

Fig – 1: The Activity of Mid Gut Protease in the Fifth Instar Larvae of Silkworm, Bombyx mori (L) (Race: Bivoltine Cross Breed [(CSR6 x CSR26) x CSR2 x CSR27)] Fed With the Leaves of Mulberry, Morus alba (L) (M-5: variety) Treated With Aqueous Solution of Eurhodin.

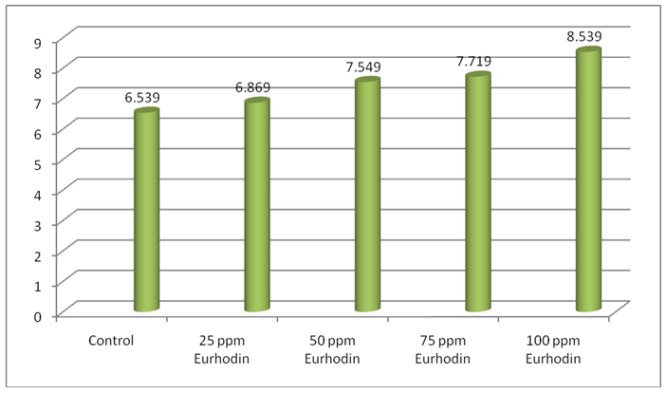

Fig – 2: The Activity of Mid Gut Amylase in the Fifth Instar Larvae of Silkworm, Bombyx mori (L) (Race: Bivoltine Cross Breed [(CSR6 x CSR26) x CSR2 x CSR27)] Fed With the Leaves of Mulberry, Morus alba (L) (M-5: variety) Treated With Aqueous Solution of Eurhodin.

The velocity of biochemical reaction catalyzed by mid gut protease and midgut amylase in larvae fed with untreated mulberry leaves was found measured 03. 057 (±0.365) units and 6.539 (±0.918) units respectively.

The midgut protease activity in larvae fed with mulberry leaves treated with 25.0 ppm; 50.0 ppm; 75.0 ppm & 100.0 ppm (four different concentrations) of the aqueous solution of Eurhodin powder was found measured 3.793 (±0.696); 5.115 (±1.109); 5.276 (±1.789); and 6.817 (±2.013) units respectively.

The midgut amyase activity in larvae fed with mulberry leaves treated with 25.0 ppm; 50.0 ppm; 75.0 ppm & 100.0 ppm (four different concentrations) of the aqueous solution of Eurhodin powder was found measured 6.869 (±1.217); 07.549 (±2.329); 07.719 (±3.313) and 08.539 (±3.789) units respectively. Percent increase in the midgut protease activity through Eurhodin treatment in present attempt was ranging from 24.075 to 122.99 (table – 1). Percent increase in the midgut amylase activity through Syzigium treatment in present attempt was ranging from 05.046 to 30.585 (table – 1).

Significant improvement in the activities of midgut protease and amylase in the larval instars of silkworm, Bombyx mori (L) (Race: Bivoltine Cross Breed [(CSR6 x CSR26) x CSR2 x CSR27)] fed with the leaves of mulberry, Morus alba (L) (M-5: variety) treated with aqueous solution of Eurhodin powder may be explained away as due to enhanced break down of contents of mulberry leaves through respective enzymes in the mid gut.

The uptake of neutral red depends on the cell's capacity to maintain pH gradients, through the production of ATP. At physiological pH, the dye presents a net charge close to zero, enabling it to penetrate the membranes of the cell. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and is retained inside the lysosomes. The method is cheaper, presents less interference, and is more sensitive than other cytotoxicity tests (tetrazolium salts, enzyme leakage, or protein content) (Borenfreund et al., 1988; Repetto et al., 2008). The neutral red assay is more sensitive and requires less equipments than the estimation of cell death by enzyme leakage using lactate dehydrogenase. It also compares favorably to estimation of total cell number by assaying protein content. The neutral red uptake assay is simpler, detecting only viable cells; however, once initiated it must be completed immediately, as it is not possible to freeze the cells, as is done for the determination of total protein assay. Nevertheless, this assay is compatible with the determination of total protein content because it is possible to perform both the total protein content and the neutral red assays on the same culture, that is, neutral red estimates can be obtained and then the protein determination can be carried out (Arranz and Festing, 1990; Vichai and Kirtikara, 2006). In common to other cell culture procedures, there are certain limitations due to the character of the compounds to be tested: substances that are volatile, unstable or explosive in water, or with low solubility, present problems (Repetto et al., 2008).

According to Sen (1988), there is enhanced synthesis of poly (A) RNA in phytophagous insects through exogenous compounds. It may be possible for of Eurhodin to accelerate the rate of synthesis of total proteins in the tissues like silk glands; fat bodies and haemolymph in fifth instar larvae of silkworm Bombyx mori (L). According to Applebaum (1985), continuous feeding in insects get reflect into improvement in the rate of metabolism through the enhanced activities of the enzymes. Individual dosage of Eurhodin may be responsible for improved contents of total proteins of silk glands; fat bodies and haemolymph should screened out. Eurhodin should be utilized efficiently for significant improvement in the total silk proteins in the larval instars of silkworm, Bombyx mori (L) for commercial silk fiber.

For the conclusive remarks on the role of vital in digestion, present attempt is taking support of "Neutral Red Stainable Cytoplasmic Inclusions in the food vacuoles of Paramoecium caudatum (L)" reported by Prowazek (l897). The granules stainable with neutral red have long been associated with digestive enzymes. Prowazek (l897) termed these neutral red stainable inclusions "Fermentrâger," Nirenstein (1905) stated that in P. caudatum the neutral red inclusions entered the food vacuoles during the acid phase of digestion. His work was supported by that of Rees (1922). Fortner (1926) and Muller (1932) reported the penetration of food vacuoles by neutral red granules in P. caudatum. Eurhodin, the vital stain may have had capabilities to make the real form of enzyme more efficient to utilize available system and there by significant increase in the velocity of enzyme catalyzed biochemical reactions.

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V. REFERENCES

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