



## **The contents of Total Protein 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 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 levels of protein in the 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. Total protein bioassay was carried out on fifth day through the use of silk glands, fat bodies and haemolymph. The total protein content of silk glands of untreated group of larvae was found measured 22.819 units. Eurhodin was found resulted into increase in the salivary gland proteins (total) from 21 to 113 percent. The total protein content of fat bodies of untreated group of larvae was found measured 19.934. Eurhodin treatment was found resulted into increase in the fat body proteins (total) from 34 to 89 percent. The total protein content of haemolymph of untreated group of larvae was found measured 14.564 units. Eurhodin treatment was found resulted into increase in the haemolymph proteins (total) from 22 to 115 percent. Efficient use of Eurhodin may open a new avenue for "Roseus Silk Protein".

**Keywords:** *Bombyx mori* (L), total proteins, Eurhodin

### **1. Introduction**

There is close interlinking between the life of insect herbivores and plant metabolites in plants. 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) [3] 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) [20], 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 its 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) [11, 12, 13]. 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) [19] & minerals (Subramanyam Reddy, 1992) [24]. 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) [23]. 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) [8] 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) [27] 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)<sup>[26]</sup> kho-go ( Desai, *et al*, 2011)<sup>[5]</sup> 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)<sup>[36]</sup>. 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 counterstain 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)<sup>[36]</sup> 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)<sup>[36]</sup>, 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 dyeing 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, Shardashai Pawar Mahila Mahavidyalaya, Shardashai 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).

## 2. Material & Methods

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; Protein Bioassay and Statistical analysis.

### 2.1 Silkworm Rearing

The egg cards or disease free layings (DFL) of bivoltine, 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)<sup>[17]</sup> & explained in earlier attempts by Khyade (2004)<sup>[16]</sup>; Vitthalrao & Kulkarni (2011)<sup>[26]</sup>; Desai, *et al*, (2011)<sup>[5]</sup> 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)<sup>[15]</sup> 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.

## 2.2 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.

## 2.3 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.

## 2.4 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.

## 2.5 Bioassay of Total Proteins from the Salivary Glands; Fat bodies and Haemolymph

The bioassay of total proteins from salivary glands; fat

bodies and haemolymph was carried out on fifth day of fifth instar. Twenty larvae from each group were selected randomly. Ten larvae were utilized for total protein estimation from silk glands. And remaining ten larvae were used for haemolymph total protein estimation. The chloroform soaked cotton pads were used for the provision of anaesthesia to the fifth instar larvae of silkworm, *Bombyx mori* (L). Weight of individual larva was recorded. Individual larva was dissected open from dorsal side. Both the silk glands from individual larva were separated. The larval dissection was carried in chilled saline (0.9 percent sodium chloride solution). The fifth instar larvae are dissected for silk glands and fat bodies in chilled saline (0.9 percent NaCl). Similarly, the fat bodies from each dissected larva were separated. The tissues were blotted separately and weighed accurately on electronic balance. Both the tissues were washed separately in ice cold saline. There after, each tissue was blotted; blotted & weighed accurately on electronic balance. Each tissue was then processed for fragmentation followed by homogenization in chilled in chilled distilled water. Clean & sterilized mortar & pestle were used for tissue homogenization. Each tissue assay sample was processed for keeping at 37°C for twenty four hours in the solution of sodium hydroxide of normal (1.0 N) strength.

Bioassay of total proteins from silk glands and fat bodies was carried in triplicate (for assay sample three test tubes were taken). 1 ml assay sample was transferred to each test tube. Addition of 5.0 ml Lower's —C solution was made in each of the test tube mixed well and kept for 15 min to allow the formation of copper protein complex. A blank was also prepared simultaneously. After 15 min, 0.5 ml Folin's phenol reagent was added to each tube and mixed well. Then they were allowed to develop colour for 30 min at room temperature. After it, the optical density was recorded at 660 nm on spectrophotometer. The results were replicated three times. The protein concentration of assay sample was calculated by referring the optical density obtained for sample and by using standard graph and expressed in the unit as  $\mu\text{g}$  proteins per mg tissue.

Through pricking the prolegs with sterilized needle, the haemolymph from fifth instar larvae was collected in separate small vials pre-coated with phenyl thiourea (phenyl thiourea prevent melanization of content). Volume of haemolymph was measured. Each vial was weighed accurately. Weight of empty vial was subtracted to get the weight of haemolymph (mg/ml). It was stored at  $-20^{\circ}\text{C}$  and used for bioassay of total proteins. For estimation of total proteins, in a clean centrifuge tube 0.1 ml of haemolymph was taken, to this 1ml of 10% TCA solution was added and centrifuged for 10 min at 3000 rpm. The content was mixed with 2ml of 0.1 N NaOH and processed for estimation of proteins by Lowry's method as described earlier.

## 2.6 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](https://en.wikipedia.org/wiki/Student%27s_t-test)) and explained by Norman & Bailly (1955).

**3. Results & Discussion**

The results on the contents of total protein in the fifth instar larvae of bivoltine, crossbreed, silk worm, *Bombyx mori* (L) fed with mulberry *Morus alba* (L) (M-5: variety) leaves treated with water solution of Eurhodin powder are summarized in table 1 and Figure- 1.

The total protein contents of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27)] silkworm, *Bombyx mori* (L) recipients of untreated leaves of mulberry, *Morus alba* (L) (M-5: variety) (untreated control group) in present attempt were found measured 22.819 ( $\pm 0.443$ ); 19.934

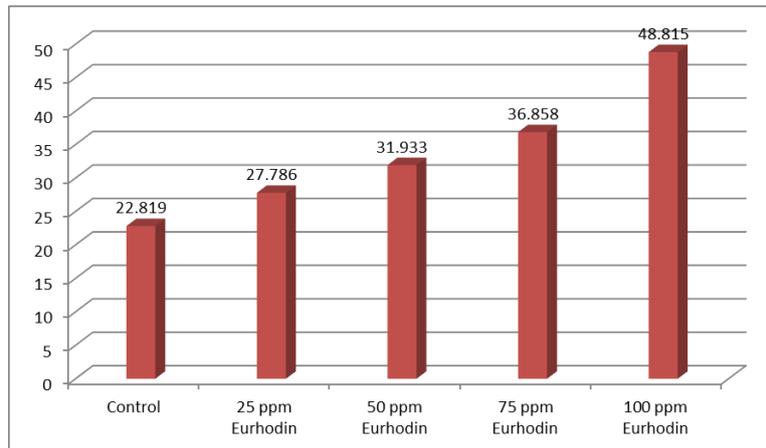
( $\pm 1.043$ ) and 14.564 ( $\pm 0.687$ ) units respectively.

The quantitative estimation of total protein of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27)] silkworm, *Bombyx mori* (L) recipients of leaves of mulberry, *Morus alba* (L) (M-5: variety), treated with aqueous solution of Eurhodin powder, with 25 ppm strength in present attempt was found measured 27.786 ( $\pm 0.646$ ); 26.875 ( $\pm 1.357$ ) and 17.898 ( $\pm 0.859$ ) units respectively. In comparison with the control group, there was 21.766; 34.819 and 22.892 percent increase in total proteins respectively in of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27)] silkworm, *Bombyx mori* (L) through treating leaves of mulberry, *Morus alba* (L) (M-5: variety) with 25 ppm aqueous solution of Eurhodin powder.

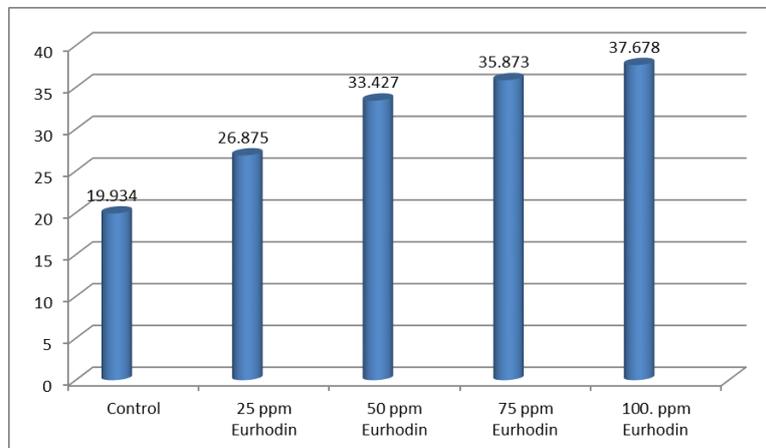
**Table 1:** Total Protein Contents proteins in Silk Glands, Fat Bodies and Haemolymph in the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: Bivoltine Cross Breed [(CSR6 x CSR26) x CSR2 x CSR27)] fed with the leaves of mulberry, *Morus alba* (L) (M-5: variety) treated with aqueous solution of Eurhodin powder.

Group Tissue	Untreated Control	25 ppm	50 ppm	75 ppm	100 ppm
Silk Glands	22.819 ( $\pm 0.443$ ) 00.000	27.786 ( $\pm 0.646$ ) 21.766	31.033 ( $\pm 0.758$ ) 35.996	36.858 ( $\pm 3.758$ ) 61.523	48.615 ( $\pm 6.638$ ) 113.04
Fat Bodies	19.934 ( $\pm 1.043$ ) 00.000	26.875 ( $\pm 1.357$ ) 34.819	33.427 ( $\pm 0.786$ ) 67.688	35.873 ( $\pm 0.838$ ) 79.958	37.678 ( $\pm 1.632$ ) 89.013
Haemolymph	14.564 ( $\pm 0.687$ ) 00.000	17.898 ( $\pm 0.859$ ) 22.892	19.078 ( $\pm 0.751$ ) 30.994	24.076 ( $\pm 0.786$ ) 65.311	31.381 ( $\pm 1.445$ ) 115.46

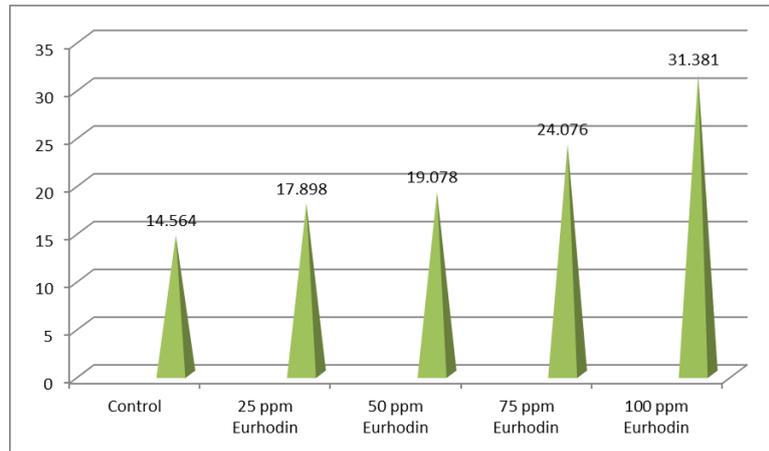
- Each figure is the mean & three replications. \*
  - Figure in parenthesis with  $\pm$  sign is the standard deviation. \*\*
  - Figure below parenthesis is percent change. \*\*\*
- : P<0.05  
: P<0.01  
: P<0.001



**Fig 1:** Contents of proteins in Silk Glands of 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:** Contents of proteins in Fat bodies of 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 3:** Contents of proteins in Haemolymph of 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 total proteins of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) recipients of leaves of mulberry, *Morus alba* (L) (M-5: variety), treated with aqueous solution of Eurhodin powder, with 50 ppm strength in present attempt were found measured 31.033 ( $\pm 0.758$ ); 33.427 ( $\pm 0.786$ ) and 19.078 ( $\pm 0.751$ ) units respectively. In comparison with the control group, there was 35.996; 67.688 and 30.994 percent increase in total proteins respectively in of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) through treating leaves of mulberry, *Morus alba* (L) (M-5: variety) with 50 ppm aqueous solution of Eurhodin powder.

Treating leaves of mulberry, *Morus alba* (L) (M-5: variety), with aqueous solution of Eurhodin, with 75 ppm strength and feeding them to the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) for continuous four days was found reflected into quantity of total proteins in silk glands; fat bodies and haemolymph, which measured 36.858 units (with Standard Deviation  $\pm 3.758$ ) (61.523 percent increase); 35.873 (with Standard Deviation  $\pm 0.838$ ) (79.958 percent increase) and 24.076 (with Standard Deviation  $\pm 0.786$ ) (65.311 percent increase) respectively. In comparison with the control group, there was 61.523; 79.958 and 65.311 percent increase in total proteins respectively in of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) through treating leaves of mulberry, *Morus alba* (L) (M-5: variety) with 75 ppm aqueous solution of Eurhodin powder.

The quantitative estimation of total protein of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) recipients of leaves of mulberry, *Morus alba* (L) (M-5: variety), treated with aqueous solution of Eurhodin powder, with 100 ppm strength in present attempt was found measured 48.615 units (with Standard Deviation ( $\pm 6.638$ )) (113.04 percent increase); 37.678 units (with Standard Deviation ( $\pm 1.632$ )) (89.013 percent increase) and 31.381 units (with Standard Deviation  $\pm 1.445$ ) (115.46 percent increase) respectively. In comparison with the control group, there was 113.04; 89.013 and 115.46 percent increase in total proteins

respectively in of silk glands; fat bodies and haemolymph of the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) through treating leaves of mulberry, *Morus alba* (L) (M-5: variety) with 100 ppm aqueous solution of Eurhodin powder.

Change in the strength of aqueous solution of Eurhodin powder from 25 ppm to 100 ppm for treating the leaves of mulberry, *Morus alba* (L) (M-5: variety) and feeding the fifth instar larvae of bivoltine race [(CSR6 x CSR26) x CSR2 x CSR27] silkworm, *Bombyx mori* (L) was observed to exert considerable change amount of the total protein of silk glands; fat bodies and haemolymph. Significant increase in the levels of total proteins of silk glands; fat bodies and haemolymph of the fifth instar larvae of silkworm, *Bombyx mori* (L) fed with mulberry leaves treated with various concentrations of aqueous solution of Eurhodin powder, eco-friendly formulation may be explained away as due to enhanced break down of contents of mulberry leaves. The Eurhodin may improve appetite & digestion.

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) [34, 36]. 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) [38-39]. 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)<sup>[36]</sup>.

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)<sup>[1]</sup>, 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.

#### 4. Acknowledgement

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