Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3 (2): 25-35

Received: 25-02-2015
Accepted: 03-03-2015

Smaranika Sahu
Department of Biotechnology, Utkal University, Bhubaneswar, 751004, Orissa, India.

Jagneshwar Dandapat
Department of Biotechnology, Utkal University, Bhubaneswar, 751004, Orissa, India.

Nakulananda Mohanty
Department of Zoology, North Orissa University, Baripada, 757003, Orissa, India

Correspondence:
Jagneshwar Dandapat
Department of Biotechnology, Utkal University, Bhubaneswar, 751004, Orissa, India.

Foliar supplementation of zinc modulates growth and antioxidant defense system of tasar silkworm *Antheraea mylitta*

Smaranika Sahu, Jagneshwar Dandapat, Nakulananda Mohanty

Abstract
The present study was aimed to evaluate the growth and antioxidant defense protection of the silkworm (*Antheraea mylitta*) larvae (II[nd] instar), when fed on *Terminalia arjuna* leaves supplemented with a trace element, Zinc, for 10 days. Experimental animals, comprising control (group I; supplemented with distilled water) and the experimental groups (groups II, III and IV) were supplemented with Zinc, sprayed on leaves in doses of 68, 136, 272 mg/kg larval body weight, respectively. Antioxidant defense capacity in the whole body tissue of *A. mylitta* was found to be modulated with higher activity of Superoxide dismutase and Glutathione S- transferase in the experimental groups. Same trend was observed for total glutathione, reduced glutathione and ascorbic acid content. Results suggest that Zinc plays an important role in augmenting the growth and antioxidant protection of the larvae of *A. mylitta*, which may improve the larval fitness, quality and quantity of silk production.

Keywords: *Antheraea mylitta*, Foliar spray of Zinc, Oxidative stress indices, Reactive Oxygen Species

1. Introduction
Tropical tasar silkworm, *Antheraea mylitta* (Lepidoptera: Saturniidae) is a commercially important insect and a valuable component of Asian non-mulberry silk industry. Silkworms, like other insects, require various trace elements as micronutrients for normal growth and development [1]. Calcium, iron, magnesium, manganese, phosphorus and zinc are some of the trace elements reported for normal growth and development of mulberry silkworm [2]. However, the role of trace elements, particularly Zinc, needs to be studied in detail because it plays a vital role in the synthesis of proteins, lipids and carbohydrates and also reduces the duration of larval and pupal stages [3]. These studies provide information about the practical application of Zinc for quantitative and qualitative improvement of silk production. Tasar silkworm, *A. mylitta*, primarily feeds on the leaves of the host plants, *Terminalia arjuna*, *Terminalia tomentosa* and *Shorea robusta*. The larval stages are the only feeding stage in this silkworm. So, intake of balanced diet is essential for silk production. By supplementing the diet with vitamins and trace elements, various physiological functions can be modulated effectively for the improvement of silk production. In recent years, attempts have been made to improve the quality and quantity of silk through the enrichment of the leaves with micronutrients (Selenium, zinc etc.) in the silkworm, *Bombyx mori* [4, 5, 6], but no such report is available on tasar silkworm *A mylitta*. Moreover, most of the studies on *B. mori* related to Zinc are focused on growth performance and nothing is known about its antioxidant functions.
Zinc is an important trace element which plays important role in the regulation of cellular processes, antioxidant response and protection against apoptosis [7]. Aerobic organisms generate reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxides (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) because of oxidative metabolism. Oxidative stress is a condition relating to overproduction of reactive oxygen species (ROS). To attenuate the negative effect of ROS, organisms including insects possess antioxidant defense system that utilizes enzymatic and nonenzymatic antioxidants [8, 9]. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), Glutathione S- transferase (GST), Glutathione Peroxidase (GPX) and the nonenzymatic antioxidants include Vitamin E, C, A and Glutathione.
The production of silk depends on larval growth, nutrition and health status. Though few studies have been carried out on the effect of minerals on the growth, development and economic parameters of silk worm *B. mori* [4, 5, 6] no such reports are available on *A. mylitta*.

Zinc is involved in both the generation of ROS and defense against reactive oxygen species [10]. So the novel purpose of our study is to find out the effect of Zinc on antioxidant defense protection and its impact on the growth performance of the larvae of the tasar silkworm *A. mylitta*, through foliar spray on the host plant, *T. arjuna*.

2. Materials and Methods

2.1 Chemicals

Thiobarbituric acid (TBA), bovine serum albumin (BSA), sephadex G-25, 5, 5' dithiobis 2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. USA. Zinc Sulphate, Reduced glutathione, ascorbic acid, horse radish peroxidase, hydrogen peroxide, sodium dodecyl sulphate (SDS), 1- Chloro- 2, 4- dinitrobenzene (CDNB) were obtained from SISCO Research Laboratory, India. All other chemicals used were of analytical grade.

2.2 Test Insects

Larvae (II^{nd} instar) of trivoltine strains of tasar silk worm (*A. mylitta*) were collected from the identical host plants (*T. arjuna)* of the same age group maintained in the State Government sericulture field located at Baripada (21°56/6N, 86°43/17E), Orissa, India and were brought to the laboratory.

2.3 Experimental conditions and tissue preparation

Larvae were conditioned to the laboratory condition for one day. Following the conditioning period, larvae were weighed and randomly divided in to four groups (group I, II, III and IV). Larvae of Group II, III and IV were supplemented with the leaves of *T. arjuna* sprayed with the solution of Zn in the form of salt ($ZnSO_4$) in the increasing doses i.e. 68, 136, 272 mgZn/kg larvae body weight containing 20 larvae in each group. Group I larvae were supplemented with the *T. arjuna* leaves sprayed with distilled water and was served as control group. The doses were decided as per the report of Bentea *et al.* [4] in *B. mori.*

The feeding experiment was conducted for a period of 10 days. After the experiment the body weight of all groups of larvae was recorded and they were chilled at -20°C for 10 min and processed for homogenization. Whole body (WB) was homogenized (20%) in ice cold 50 mM phosphate buffer, pH 7.4 containing 0.1 mM EDTA and a pinch of PMSF using glass teflon mechanical homogenizer. Homogenates were centrifuged at 10, 000 x g for 20 min at 4°C and the post mitochondrial supernatant was collected. Endogenous H_2O_2 content and CAT activity were measured in the samples immediately after centrifugation. The remaining supernatant was kept at -80 °C for further analyses. All measurements were made in triplicate.

2.4 Zinc accumulation studies

For the analysis of Zinc in the whole body tissue of the silkworm (control and Zn treated) and in leaves of *T. arjuna*, samples were oven dried and digested in a mixture of two acids i.e. HNO_3: Hcl in the ratio 3:1 (v/v). Zinc levels in the digested samples were determined after suitable dilutions by atomic absorption spectrophotometer (AAS) (Perkin Elmer AA-400 model) using a specific cathode lamp of Zinc. Zn determinations in all samples were carried out in duplicate in pulled samples.

2.5 Measurement of Hydrogen peroxide (H_2O_2)

The hydrogen peroxide content in post mitochondrial fraction of WB homogenates was determined spectrophotometrically using horse radish peroxidase according to the method of Pick & Keisari, [11]. In this assay pure H_2O_2 was taken as standard and it was linear in the range of 20-80 nmol. In each tube 1.7 ml of phosphate buffer (50 mM, pH 7.4), 0.1 ml of phenol red solution and 50 µl of horse radish peroxidase (50 units) were taken and incubated for 5 min at room temperature. 0.1 ml sample was added to it, followed by immediate addition of 50 µl of NaOH (1 N) to stop the reaction. H_2O_2 content was expressed as nmoles H_2O_2/mg protein.

2.6 Activities of Antioxidant Enzymes

2.6.1 Assay of Xanthine Oxidase (XO) activity

Assay of xanthine oxidase was carried out according to the method of Roussos [12]. The assay mixture, in final volume of 3.0 ml, consisted of 0.30 ml Tris-HCl buffer, 50 mM, pH 7.4; 0.30 ml CuSO4, 10 mM; 0.05 ml Xanthine , 2.58 mM per ml. in 0.05 M glycine buffer, pH 7.4; 0.1 ml. of whole body homogenate and water to make up the volume. Change in absorbance was recorded at 290 nm at 15 seconds interval for two minutes. One unit of enzyme activity is defined as change in absorbance at 290 nm in 1 minute by 1 ml enzyme preparation (20-80µg protein).

2.6.2 Assay of Superoxide dismutase (SOD) activity

For the measurement of total SOD activity, 0.4 ml of post mitochondrial supernatant containing approximately 10-15 mg of protein was passed through a 2 ml column of sephadex G-25 and the elute was used for the activity assay according to the method of Das *et al.* [13] as described earlier[14]. In brief, superoxide radicals generated by photoreduction of riboflavin were allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with Naphthylamine to produce a red azo compound having absorption maxima at 543 nm. SOD scavenges superoxide radicals; therefore, nitrite formation in the reaction is inversely proportional to the amount of SOD present in the sample. One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. SOD activity was expressed as units/mg protein.

2.6.3 Assay of Catalase (CAT) activity

The post mitochondrial supernatant was used directly for assay of catalase activity following the decrease in absorbency of H_2O_2 at 240 nm [15] for a period of 2 min at 15 seconds interval. Calculation was done based on the decrease in absorbance of H_2O_2 at the end of 2 min by taking the extinction coefficient of H_2O_2 as 43.6 M^{-1} cm^{-1} at the end of 2 min . Activity was expressed as nkat/mg protein. One katal is defined as the amount of enzyme that transforms one mole of substrate per second.

2.6.4 Assay of Glutathione S-transferase (GST) activity

The column passed samples were used for the assay of glutathione S-transferase activity by the method of Habig & Jakoby [16]. In brief, 2.6 ml of 50 mM potassium phosphate buffer pH 6.5, 0.2 ml of 90 mM GSH and 0.1 ml of sample were added to make the reaction mixture and the reaction was initiated by adding 0.1 ml of 30 mM CDNB to it. Absorbance was recorded at 340 nm at 1 min interval till 6 min. Specific Activity of this specific enzyme was calculated by taking the extinction coefficient of CDNB i.e. 9.61 $mM^{-1}cm^{-1}$ and is expressed as nmol CDNB conjugate formed/min/mg protein.

2.7 Estimation of Protein content
Protein content of the samples for assay of oxidative stress indices and antioxidant enzymes was estimated according to the method of Bradford [17] using bovine serum albumin as standard.

2.8 Non-Enzymatic Antioxidants
2.8.1 Estimation of Glutathione (GSH) content
The supernatant from the whole-body homogenate were precipitated in ice cold 5% trichloroacetic acid containing 0.01N HCl and centrifuged at 1000xg for 15 min. Total glutathione content was measured in the supernatant of these samples by enzymatic recycling procedure and GSSG was measured after masking GSH with 2-vinylpyridine [18, 19]. GSH content was obtained from the difference between these two values. Only GSH plus twice the level of GSSG were denoted as total GSH equivalents. Oxidative stress index (OSI) was calculated as the ratio between GSSG and total GSH (OSI=100×2×GSSG/total GSH) as described earlier [20].

2.8.2 Estimation of Ascorbic acid (ASA) content
Whole-body samples were incubated with 5% (w/v) TCA in ice and centrifuged at 1, 000 x g for 10 min. The supernatant was taken for the estimation of ASA using Folin phenol reagent taking ascorbic acid as standard by the method of Jagota & Dani [21] and is expressed as µg/g tissue wet wt.

2.9 Statistical Analysis
Results are presented as means ± standard error of means (S.E.M.) for n=6. Difference among the means were analyzed by one way analysis of variance (ANOVA), through on line (Vassarstats) followed by Turkey's HSD test. Differences were considered statistically significant when $p < 0.05$.

3. Results
3.1 Changes in Body Weight (Fig. 1)
Changes in the body weight of the larvae fed with different concentrations of Zinc is shown in the figure 1. Body weight of Zn supplemented larvae increased progressively throughout the study. The increase in body weight was also in a dose dependent manner. The gain of body weight of Group II larvae was 57 % compare to earlier larvae. Similarly Group III (136mg/kg) and Group IV (272mg/ kg) larvae showed an increase in body weight 82% and 142% respectively. Control larvae (Group I) showed an increase in body weight of 15%.

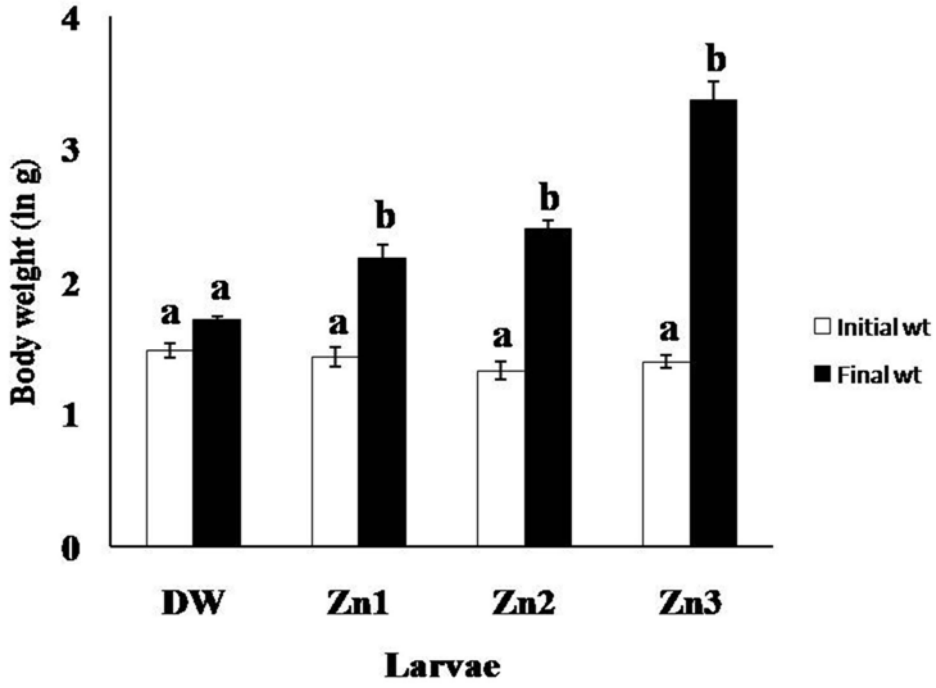

Fig 1: Effect of foliar supplementation of zinc on body weight gain of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=10). Bars sharing superscripts of different letters differ significantly.

3.2 Accumulation of Zinc
The accumulation of Zinc was analyzed in control, Zn treated larvae and also in the leaves of the host plant, *T. arjuna* and the Zinc content has been depicted in the following table.

Table 1: Zinc content (mg/g tissue wet wt) in the host plant leaves and in the whole body tissue (Group I-IV) of the larvae

Zn content in the Leaves of *T. arjuna* (mg/g tissue wet wt)	Zn content in the Group I Larvae (mg/g tissue wet wt)	Zn content in the Group II Larvae (mg/g tissue wet wt)	Zn content in the Group III Larvae (mg/g tissue wet wt)	Zn content in the Group IV Larvae (mg/g tissue wet wt)
0.040 -0.043	0.019-0.020	0.104-0.108	0.112-0.116	0.160-0.169

3.3 Hydrogen peroxide content (Fig. 2)
Figure 2 indicates the effect of supplementation of Zinc on the H_2O_2 content of the whole body tissue in the larvae of *A. mylitta*. H_2O_2 content was significantly increased in all Zn treated groups as compared to control. However, no significant change was observed among the treated groups.

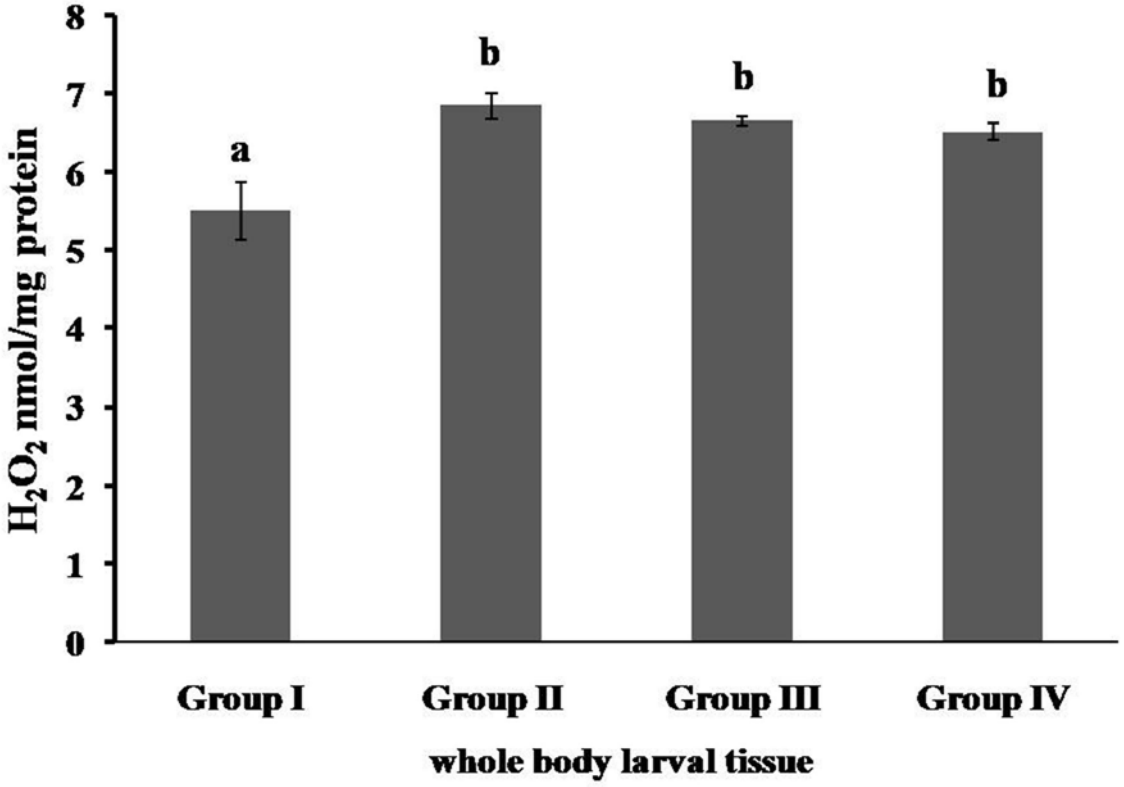

Fig 2: Effect of foliar supplementation of zinc on H_2O_2 content (nmol/mg protein) in whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

3.4 Changes in Oxidative stress indices (Fig. 3, 4)

Foliar supplementation of Zinc showed a significant reduction in the oxidative stress indices in all the Zn treated groups as compared to control. The GSH:GSSG ratio was significanly increased in the whole body tissue of *A. mylitta* larvae after the adminstration of Zinc through foliar spray.

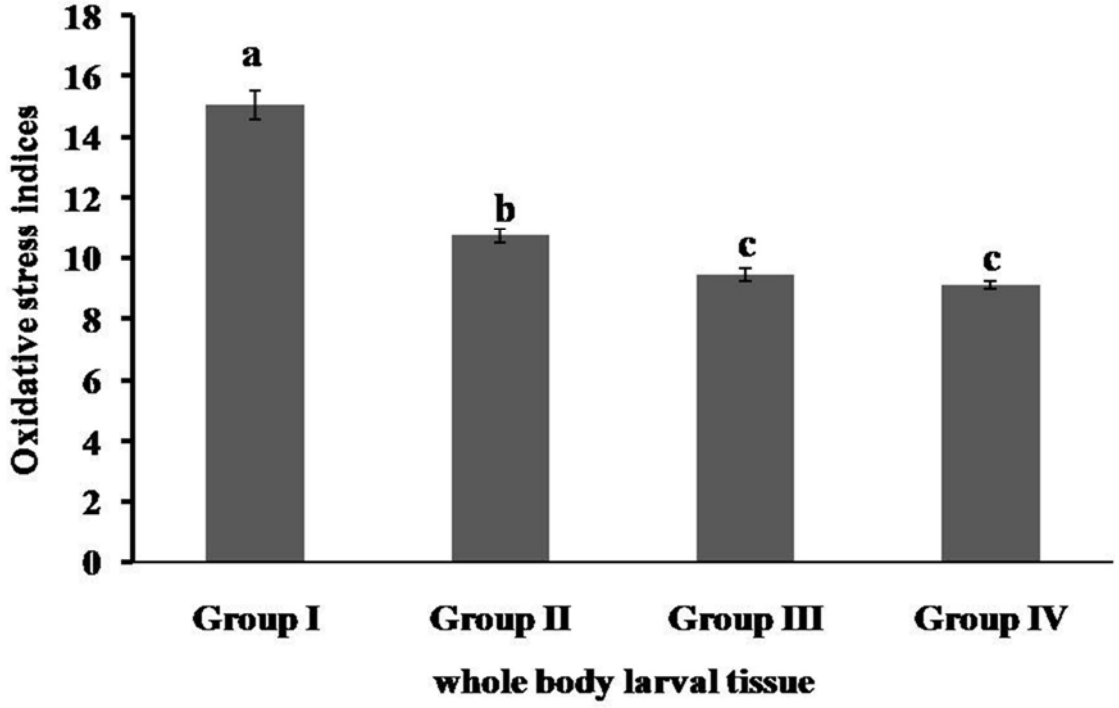

Fig 3: Effect of foliar supplementation of zinc on oxidative stress indicies in the whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

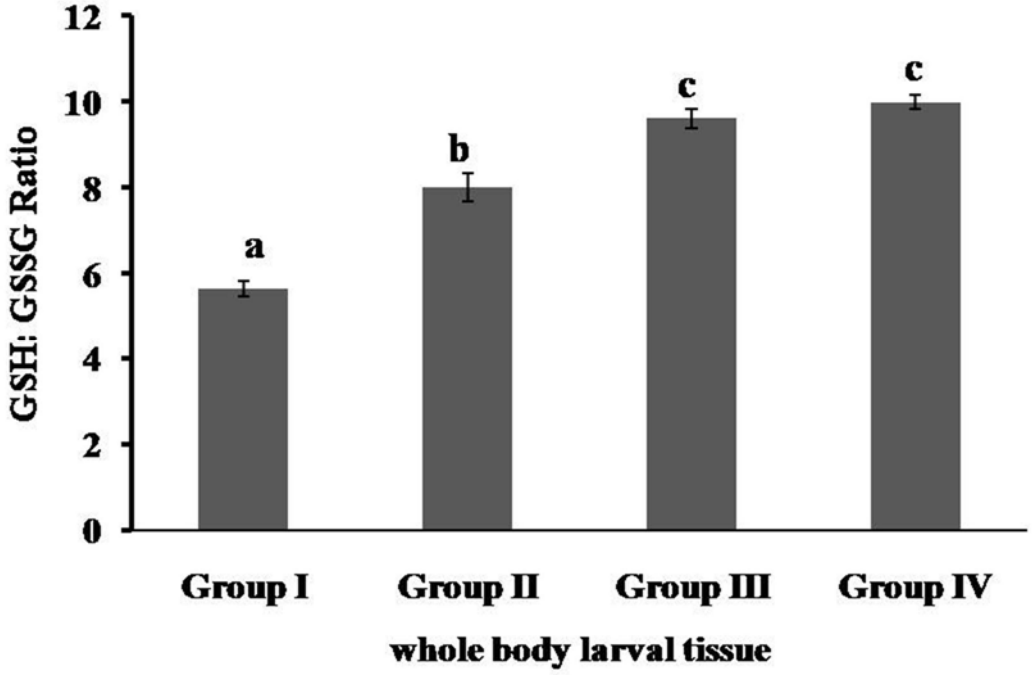

Fig 4: Effect of foliar supplementation of zinc on GSH: GSSG ratio in whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

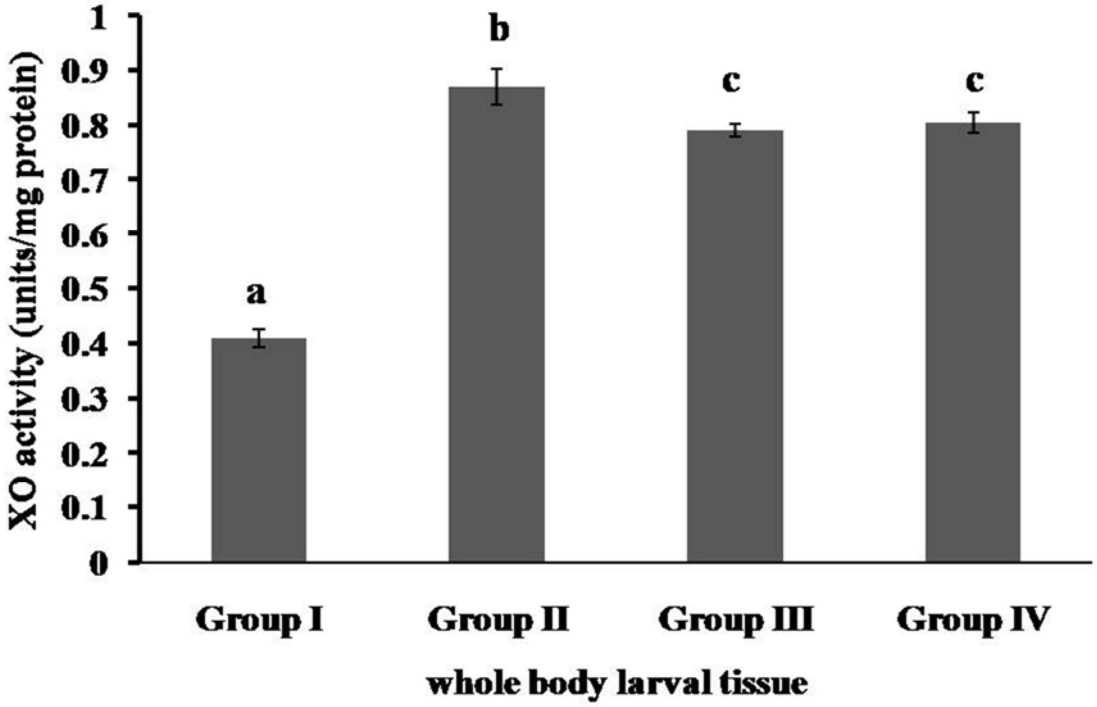

Fig 5: Effect of foliar supplementation of zinc on XO activity (units/mg protein) in whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

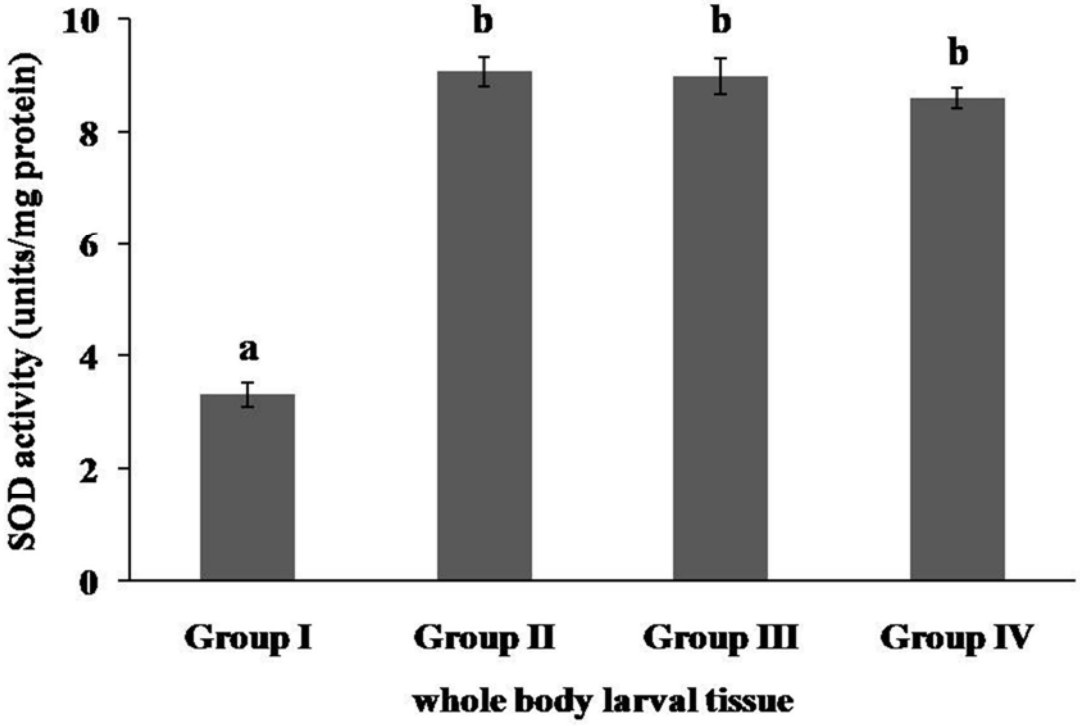

Fig 6: Effect of foliar supplementation of zinc on SOD activity (units/mg protein) in whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

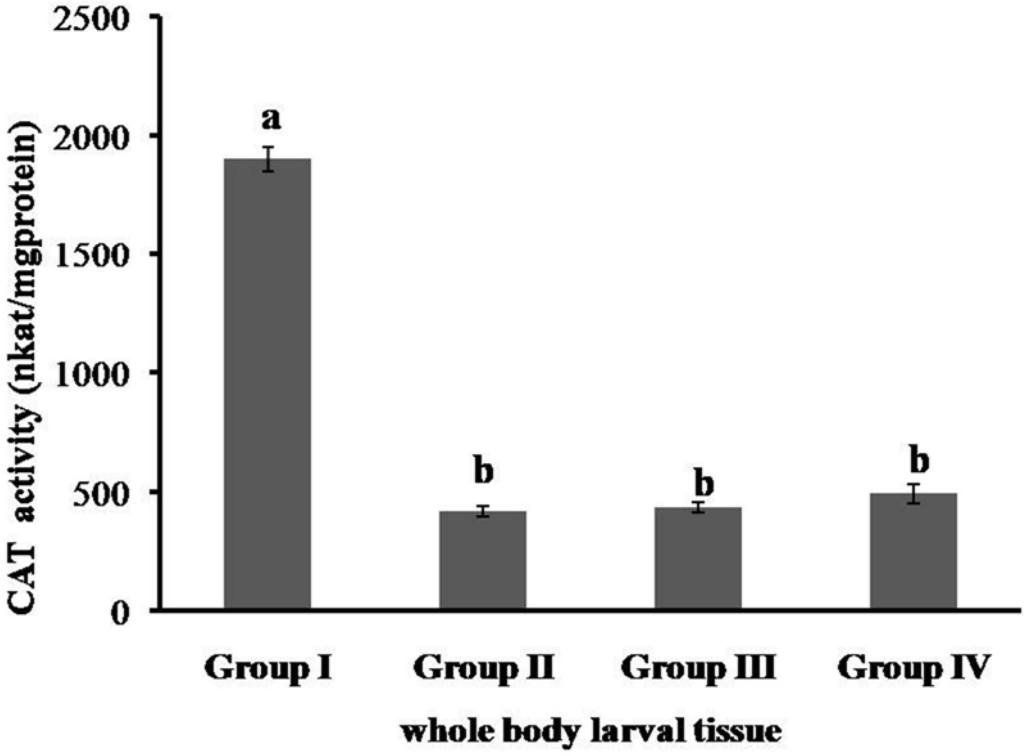

Fig 7: Effect of foliar supplementation of zinc on Catalase activity (nkat/mg protein) in whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

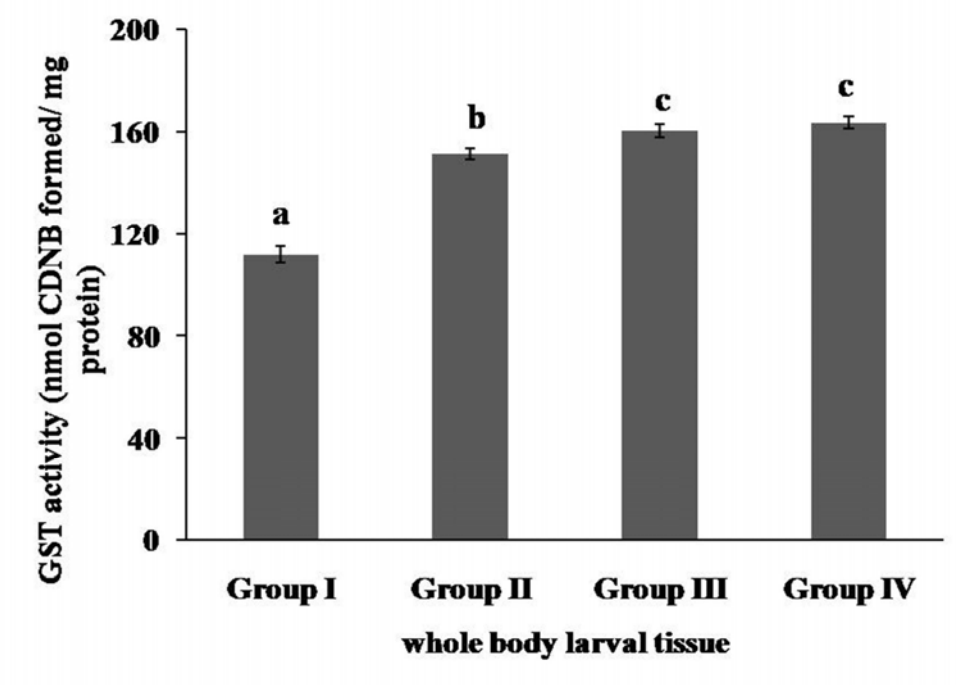

Fig 8: Effect of foliar supplementation of zinc on GST activity (nmol CDNB formed/mg protein) in whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

3.5 Changes in Activities of Antioxidant Enzymes (Fig. 5, 6, 7 & 8)

Supplementation of Zinc through foliar spray resulted in a significant increase of xanthine oxidase activity in whole body homogenate of the larvae of *A. mylitta* (Fig.5). Total SOD activity in the whole body tissue (Fig.6) was increased significantly in all the three Zn treated groups. Highest SOD activity was observed in the larvae of group III. After Zinc supplementation, catalase activity (Fig.7) was decreased significantly in the whole body homogenate. Foliar supplementations of Zinc increased the activity of Glutathione S-transferase significantly in the whole body homogenate of treated groups.

3.6 Changes in Non-enzymatic antioxidants (Fig. 9, 10, 11 & 12)

Supplementation of Zinc through foliar spray elevated the total glutathione and reduced glutathione content in comparision to control. A clear dose dependent and significant elevation of both total glutathione and reduced glutathione was observed in the whole body tissue of *A. mylitta*. Oxidised glutathione (GSSG) content was not significantly affected in response to Zinc supplementation. Ascorbic acid (ASA) content was increased significantly in whole body of *A. mylitta* in group III and IV, but was unaltered in group II as compared to group I (control).

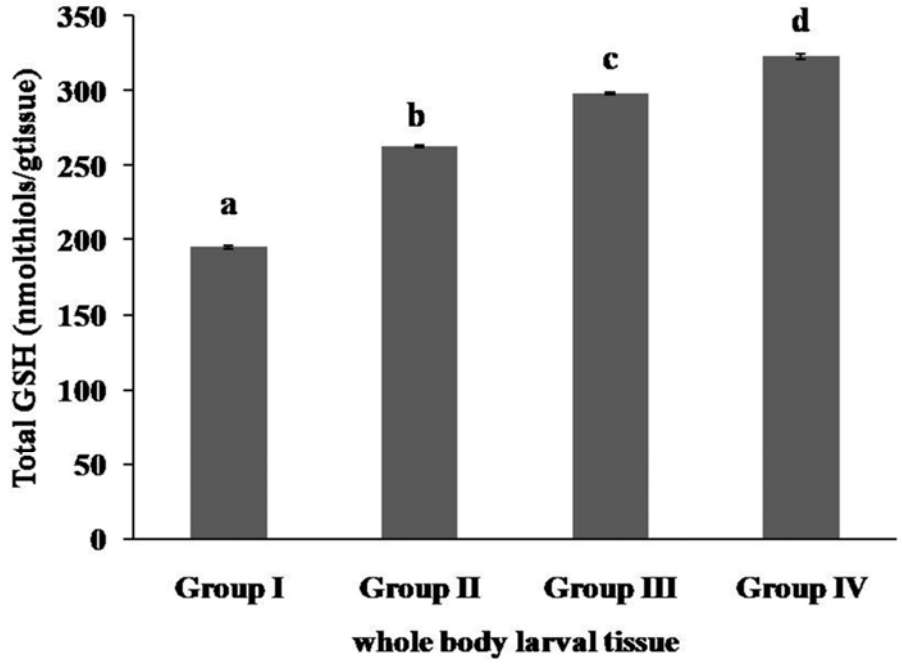

Fig 9: Effect of foliar supplementation of zinc on Total GSH content (nmol thiols/g tissue wet wt) in the whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

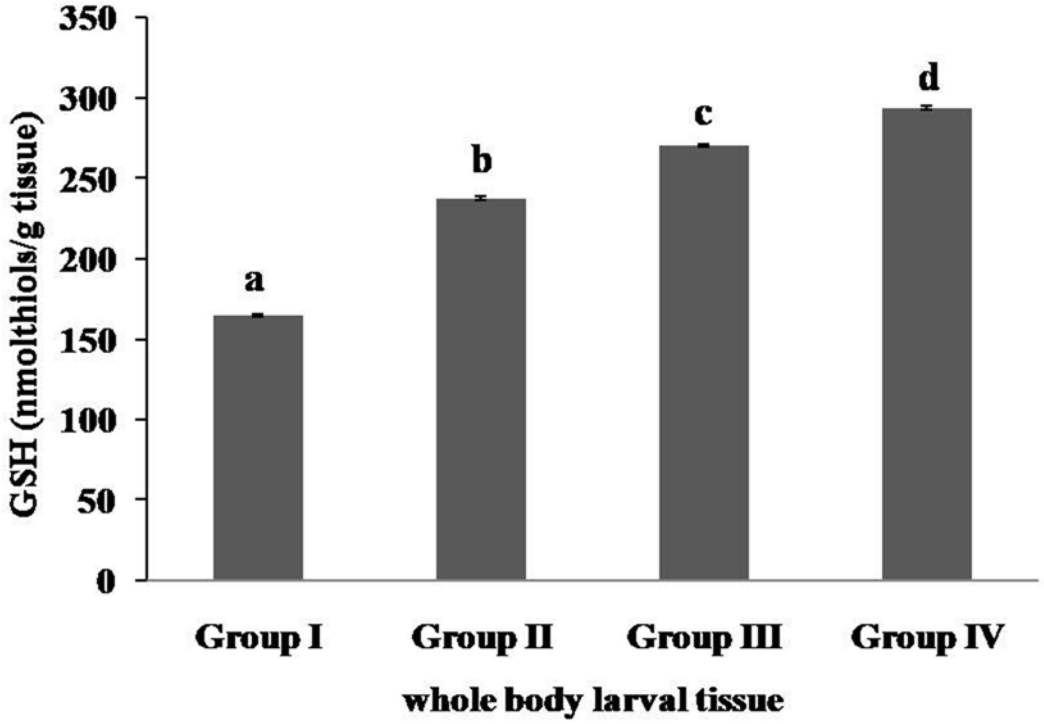

Fig 10: Effect of foliar supplementation of zinc on GSH content (nmol thiols/g tissue wet wt) in the whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

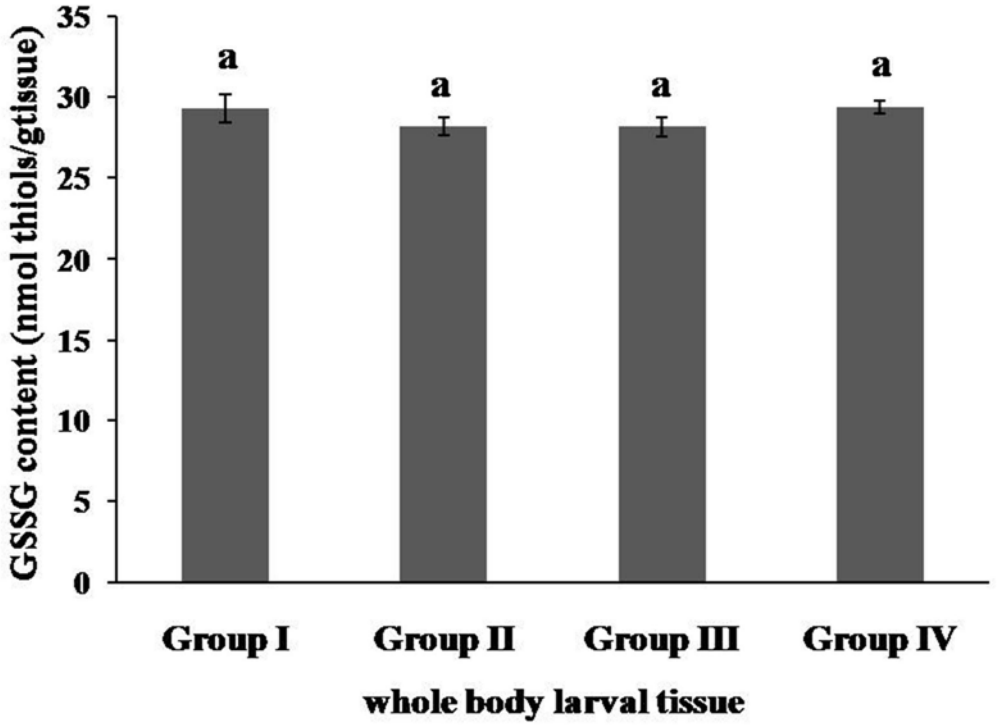

Fig 11: Effect of foliar supplementation of zinc on GSSG content (nmol thiols/g tissue wet wt) in the whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

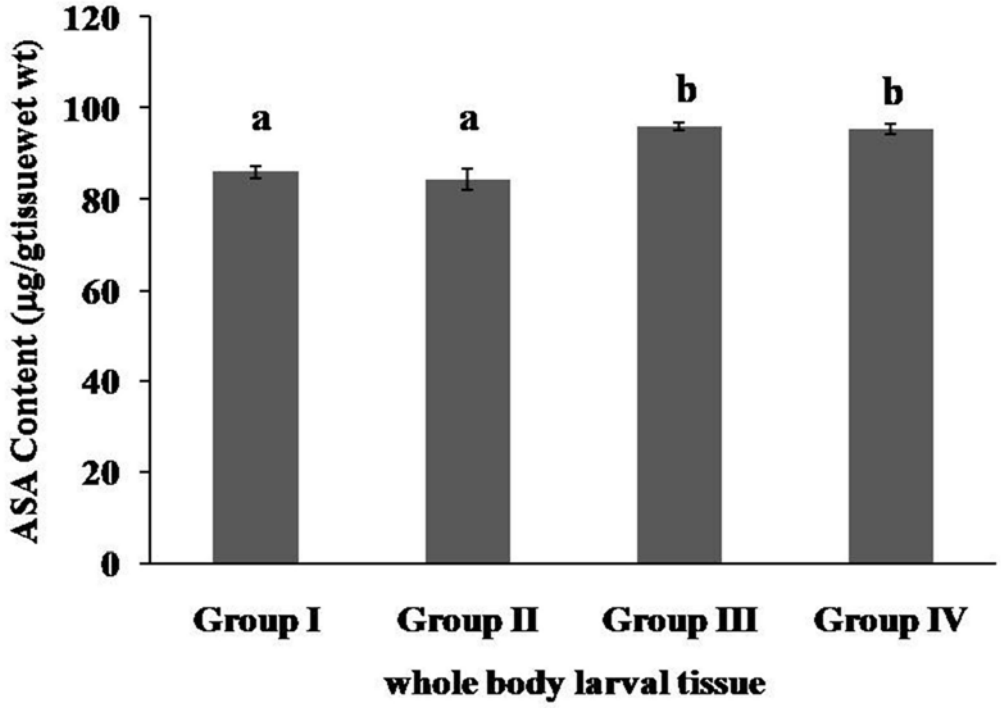

Fig 12: Effect of foliar supplementation of different doses of zinc on ASA content (µg tissue wet wt) in the whole body tissue of A. mylitta larvae. Data are expressed as mean± standard error of mean (n=6). Bars sharing superscripts of different letters differ significantly.

4. Discussion

Oxidative stress is an imbalance between the production of Reactive Oxygen Species (ROS) and the cell's ability to neutralize ROS. Like all other organisms insects have also a regulatory mechanism for balancing prooxidants and antioxidants and have a well-developed antioxidant defense system to prevent the damages from oxidative stress [8]. Minerals are the major constituents of the silkworm diets and the production parameters of raw silk depend on larval nutrition and health status [4]. Many studies have been carried out on the effect of minerals to improve the quantity and quality of silk [22-25]. We observed that the body weight of zinc treated larvae increased progressively throughout the study in comparison to control and the rate of increase was dose dependent. The maximum body weight was observed in the larvae which feed on 272 of Zn/kg body weight of larvae through foliar spray (142%). Ashfaq *et al.* [26] reported that Zn increases the weight of larvae and serigene gland in mulberry silkworm. Etabery *et al.* 2003; Islam *et al.* 2004 [24, 27] confirmed that the mineral supplementation (N, P and K) of mulberry leaves enhances the final body weight of the silkworm larvae, *B. mori*. The findings of the present study indicate that the higher level of hydrogen peroxide in the Zinc treated groups and group II shows the highest H_2O_2 content. The elevation of H_2O_2 content may be related to the increase in SOD activity in Zn treated larvae. Virgli [28] reported that in the presence of Zinc, superoxide dismutase is able to reduce superoxide radical to hydrogen peroxide. This may the reason for the enhancement of H_2O_2. Cong *et al.* [29] reported that SOD activity was more sensitive to Zn. Zinc is an essential component of Copper/zinc SOD. Therefore, supplementation of zinc may stabilize the copper zinc SOD and enhances SOD enzyme activity [30, 31]. Xanthine oxidase (XO), is a rate-limiting enzyme of purine metabolism. It catalyzes the oxidation of hypoxanthine and xanthine to produce superoxide anion or H_2O_2. In insects, catalase and ascorbic peroxidase are responsible for the elimination of H_2O_2 [32, 33]. H_2O_2 was higher in all Zn treated groups than the control, and same trend was observed for Xanthine oxidase activity. Catalase activity shows the reverse trend i.e. catalase activity was less in Zinc treated groups. This may be due to higher concentration of H_2O_2 in Zn treated group. Higher XO and lower CAT activity may also be the reasons for enhancement of H_2O_2. XO catalyzes the production of H_2O_2 and Catalase is responsible for the elimination of H_2O_2 [34]. Our result corroborates with the finding of Cong, *et al.* [29] who found that Zn treated mollusks, *Crassostrea gigas* also exhibited lower catalase activity in both, gills and hepatopancreas.

Glutathione is the major low molecular weight thiol compound in cells and exists in both reduced thiol (GSH) and disulphide–oxidized (GSSG) forms and it is one of the central component of antioxidant defense system. The oxidation of GSH is catalyzed by GST which is an important enzyme in protecting cells against oxidative injury [35]. Total glutathione is defined as GSH + 2GSSG, because 1 mol GSSG can be reduced to 2 mol of GSH [36]. In our experiment increased GST activity, total GSH and GSH content was observed in the Zinc treated group which is in agreement with earlier reports of Saliu and Bawa-Allah [37] and Faramobi *et al.* [38]. They also found similar result i.e. increased activity of GST and GSH content in Zn treated African cat fish, *Clarias gariepinus.* The observed augmentation of reduced glutathione contents and GST activity following zinc treatment may be explained by Zinc's property of inducing metallothionein as a free radical scavenger [39]. GSH: GSSG ratio is a dynamic indicator of oxidative stress with a high ratio indicating reducing condition, while a low ratio indicates pro-oxidative conditions [40]. In our experiment control has lower level of GSH/GSSG ratio indicating pro-oxidative condition and Zinc treated groups exhibited reducing condition with higher GSH-GSSG ratio. GSH can be synthesized by insect cells; ascorbic acid must be obtained from the diet. Ascorbic acid (ASA) protects critical macromolecules from oxidative damage and it can scavenge ROS directly with and without enzyme catalysts [41].

The reducing functions of GSH and ASA are to some extent complementary [42]. It was also reported by Meister [42] that GSH deficiency should lead to deficiency in ascorbic acid level, which may be correlated in our study where both GSH and ASA contents were higher in zinc treated groups. Tupe *et al.* [43] reported that ascorbic acid may have protective role in oxidative stress with reference to Zinc uptake. This may be the reason of enhancement of ascorbic acid content in our study with the supplementation of zinc.

Taken together, the findings of the investigation suggest that, supplementation of Zinc through foliar spray lead to an improvement of the body weight of the larvae and attenuate oxidative stress indices by modulating antioxidant protection in the larvae.

5. Acknowledgements

The work was generously funded by DST, Govt. of Odisha, India. Authors are thankful to Head, Department of Biotechnology, Utkal University, Bhubaneswar, India for the provision of laboratory facilities which enabled us to complete the work. We thank Mr. Mihir Kumar Gajendra Mohapatra, Technical Inspector, Directorate of textile and Handloom, sericulture section, State Silk Board (Baripada, Odisha) for providing the larvae. Financial assistance from Department of Biotechnology, Govt. of India and DST-PURSE grant (DST-India) to the P.G. Department of Biotechnology, Utkal University, is gratefully acknowledged.

6. References

1. Dadd RH. Nutrition: organisms. In: Comprehensive Insect Physiol. Pergamon Press, Oxford 1985, 313-390.
2. Ito H. Nutrition of the silkworm, *Bombyx mori* L. Proceedings of Japan Academic Sci 1967; 43:57-61.
3. Bhattacharya A, Kaliwal BB. Biochemical content of the Fat body and Hemolymph of silkworm (*Bombyx mori*) larvae fed with mulberry leaves fortified with mineral magnesium chloride ($MgCl_2$). The Phillippine Agr Sci. 2005; 88:359-362.
4. Benţea M, Şara A, Mărghitaş LA, Dezmirean D, Gabor E, Vlaic B, Creţa C. Effect of selenium and zinc supplementation on production parameters and DNA of the mulberry silkworm (*Bombyx mori* l.), baneasa white variety. J. of Agricul and Biol Sci 2012; 7:871-876.
5. Kavita S, Prasad SS, Yellamma K. Determination of minimum effective dose of Zinc chloride for modulation of metabolism and silk production in the silkworm, *Bombyx mori*. The Bioscan 2011; 6:147-152.
6. Mihal B, Marghitas AL, Sara A, Dezmirean D, Gabor E, Vlaic B, *et al*. The effect of Selenium and Zinc supplementation on Growth and Reproduction of the Mulberry Silkworm (*Bombyx mori* L.) Pro Environ 2011; 4:302-307.
7. Kafel A, Rozpędek K, Szulińsk E, Zawisza-Raszka A, Migula P. The effects of cadmium or zinc multigenerational exposure on metal tolerance of *Spodoptera exigua* (Lepidoptera: Noctuidae) Environ Sci Pollut Res 2014; 21:4705-4715.
8. Ahmad S. Biochemical defense of prooxidant plant allelochemicals by herbivorous insects. Biochem Systema and Ecol 1992; 20:269-296.
9. Felton GW, Summers CB. Antioxidant systems in insects. Arch of Insect Biochem Physiol 1995; 29:187–197.
10. Palmiter RD. Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proc Natl Acad Sci USA 1994; 91:1219-1223.
11. Pick E, Keisari Y. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages. Induction by multiple nonphagocytic stimuli. Cell Immunol 1981; 59:301-318.
12. Roussos GG. Xanthine oxidase from bovine small intestine. In: Methods in Enzymology; (Grossman L, Moldave K, editors). Academic Press, New York 1967; 12:5-16.
13. Das K, Samanta L, Chainy GBN. A modified spectrophotometric assay of superoxide dismutase using nitrite formation of superoxide radicals. Indian J of Biochem and Biophys 2000; 37:201-204.
14. Dandapat J, Chainy GBN, Rao KJ. Lipid peroxidation and antioxidant defence status during larval development and metamorphosis of giant prawn, *Macrobrachium rosenbergii***.** Comp. Biochem. Physiol. C 2003; 135:221-233.
15. Aebi H. Catalase. In: Methods in Enzymatic Analysis. (Bergmayer HU Ed). Academic Press, New York 1974, 673-678.
16. Habig WH, Jakoby WB. Glutathione S-transferases (rat and human). Method in Enzymol 1981; 77:218-231.
17. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.
18. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione; applications to mammalian blood and other tissue. Anal Biochem 1969; 27:502–522.
19. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem 1980; 106:207–212.
20. Olsvik PA, Kristensen T, Waagbo R, Rosseland BO, Tollefsen KE, Baeverfjord G *et al*. MRNA expression of antioxidant enzymes (SOD, CAT and GSH-Px) and lipid peroxidative stress in liver of Atlantic salmon (Salmo salar) exposed to hyperoxic water during smoltification. Comp. Biochem. Physiol. Part C 2005; 141:314-323.
21. Jagota SK, Dani HM. A new colorimetric technique for the estimation of vitamin C using folin phenol reagent. Anal Biochem 1982; 127:178-182.
22. Bhattacharya A, Kaliwal BB. Influence on mineral potassium permanganate on the economic parameters of the silkworm, *Bombyx mori* L. Proceeding of National Conference on Tropical Sericulture for Global Competitiveness, 2003, 91.
23. Etebari K, Fazilati M. Effect of feeding on mulberry's supplementary leaves with N, P and K in some biological and biochemical characteristics of silkworm. J of Sci Technol Agric and Natur Resour. 2003; 7:233-244.
24. Etebari K, Ebadi R, Matindoost L. Effect of feeding mulberys enriched leaves with ascorbic acid some biological, biochemical and economical charecterstics of silkworm *Bombyx mori* L. J Indust Entomol 2004; 7:81-87.
25. Khan MA, Akram W, Ashfaq M, Khan HAA, Kim YK, Lee JJ. Effects of optimum doses of nitrogen, phosphorus, potassium and calcium on silkworm. *Bombyx mori* L. growth and yield. Entomol Res 2010; 40:285-289.
26. Ashfaq M, Afzal W, Asif Hanif M. Effect of Zn (II) deposition in soil on mulberry-silk worm food chain. African J. of Biotech 2010; 9:1665-1672.
27. Islam R, Abdul A, Dipak P, Shaheen S, Nilufa B, Islam R. Effect of salt, nickel chloride supplementation on the

growth of silkworm *Bombyx mori* L. J Biol Sci 2004; 4:170-172.
28. Virgili F, Canali R, Figus E, Vignolini F, Nobili P, Mengheri E. Intestinal damage induced by zinc deficiency is associated with enhanced CuZn superoxide dismutase activity in rats: Effect of dexamethasone on thyroxine treatment, Free Radic Biol Med, 1999; 26:1194-1201.
29. Cong M, Wu H, Liu X, Zhao J, Wang X, Lv J, *et al.* Effects of heavy metals on the expression of a zinc-inducible metallothionein-III gene and antioxidant enzyme activities in *Crassostrea gigas* Ecotoxicol DOI 10.1007/s10646-012-0926-z, 2012
30. Kajihara J, Enomoto M, Nishijma K, Yabuuchi M, Katoh K. Comparision of properties between human recombinant aand placental copper-zinc SOD. J Biochem 1988; 104:851-854.
31. Jing MY, Sun JY, Zi NT, Sun W, Qian LC, Weng XY. Effects of zinc on hepatic antioxidant systems and the mRNA expression levels assayed by cDNA microarrays in rats. Ann Nutr Metab 2007; 51:345-351.
32. Bolter CJ, Chefurka W. The effect of phosphine treatment on superoxide dismutase, catalase, and peroxidase in the granary weevil, Sitophilus granaries. Pestic Biochem Physiol 1990; 36:52–60.
33. Yamamoto K, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. Catalase from the silkworm, *Bombyx mori* Gene sequence, distribution, and overexpression. Insect Biochem Mol Biol 2005; 35:277–283.
34. Zhao LC, Shi LG. Metabolism of hydrogen peroxide between diapuse and nondiapuse eggs of the silkworm, *Bombyx mori* during chilling at 5 °C. Arch of Inscet Biochem and Physiol 2010; 74:127–134.
35. Forman HJ, Zhang H, Rinna A. Gluthathione: overview of its protective roles, measurement and biosynthesis. Mol asp of med 2009; 30:1-12.
36. Meng G, Yao J, Wang Li, Zhao L. Variation in glutathione status associated with induction and initiation of diapauses in eggs of the bivoltine strain of the silkworm, *Bombyx mori*. Physiol Entomol 2011; 36:173-179.
37. Saliu JK, Bawa-Allah KA. Toxicological Effects of Lead and Zinc on the Antioxidant Enzyme Activities of Post Juvenile *Clarias gariepinus.* Resear and Environ 2012; 2:21-26.
38. Faramobi EO, Adewole OA, Ajimoko YR. Effect of Butachlor on Antioxidant Enzyme Status and Li-pid Peroxidation in Fresh Water African Catfish, (*Clarias gariepinus).* Intern J of Environ Resear and Publ Heal 2007; 5:423-427
39. Seagrave J, Tobey RA, Milderbrans CE. Zinc effects on glutathione metabolism. Relationship to zinc induced protection from alkylating agents, Biochem pharmacol 1983; 32:3017-3021
40. Asensi M, Sastre J, Pallado FV, Lloret A, Lehner M, Asuncion JG, *et al*. Ratio of reduced to oxidized glutathione as indicator of oxidative stress status and DNA damage. Methods Enzymol 1999; 299:267-276.
41. Krishnan N, Kodrik D, Kludkiewicz B, Sehnal F. Glutathione-ascorbic acid redox cycle and thiredoxin reductase activity in the digestive tract of *Leptinotarsa decemlineata* (say). Insect Biochem Mol Biol 2009; 39:180-188.
42. Meister A. Minireview: glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 1994; 269:9397-9400.
43. Tupe RS, Chiplonka SA, Aqte VV. Changes in zinc uptake in response to ascorbic acid and folic acid in rat liver slices under normal and oxidative stress conditions. Biofactors 2007; 30:27-34.