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Archana Ashok Sharbidre
Department of Zoology, Savitribai Phule Pune University, Pune, 411007, Maharashtra State, India.

Mohini B. Bamane
Department of Zoology, Savitribai Phule Pune University, Pune, 411007, Maharashtra State, India.

Prajakta D. Dhage
Department of Zoology, Savitribai Phule Pune University, Pune, 411007, Maharashtra State, India.

D. L. Bharmal
Department of Zoology, SPK College, Sawantwadi, Maharashtra State, India.

*These authors contributed equally to the work*

Correspondence:
Archana Ashok Sharbidre
Department of Zoology, Savitribai Phule Pune University, Pune, 411007, Maharashtra State, India.

Prevention of D-galactose induced oxidative stress in gut compartments of *Musca domestica* larvae by Melatonin.

Archana Ashok Sharbidre #, Mohini B. Bamane #, Prajakta D. Dhage #, D. L. Bharmal

Abstract
The effect of melatonin on the oxidative stress produced by the dietary uptake of D-galactose (6mg/ml) was studied in the gut compartments of *Musca domestica* larvae. The larvae were simultaneously treated with melatonin (25 µg/ml). The effects of D-galactose were evaluated as elevated levels in the quantity of Superoxide Dismutase, Glutathione Transferase, Catalase activities and Lipid peroxidation in different gut compartments of four groups of *M. domestica* L. larvae ($P < 0.05$). All these changes were prevented by addition of melatonin in the diet. These findings indicate (i) D-galactose induced oxidative stress in the gut compartments, characterized by a high level of lipid peroxidation, and of antioxidative enzymes activities, and (ii) melatonin prevents the deleterious effects induced by D-galactose. In conclusion, the results illustrate the ability of melatonin to amend the oxidative stress response to D-galactose by protecting gut membranes.

Keywords: D-galactose; *Musca domestica;* antioxidant enzymes; melatonin.

1. Introduction
Melatonin (N-acetyl-5-methoxy-tryptamine) is a chief secretory product of the pineal gland synthesized from tryptophan [1]. Melatonin has been shown to reduce the accumulation of the major products of lipid per oxidation [2]. The antioxidant capacity of melatonin is through the cascade reaction when scavenging free radicals [3]. Quite the reverse with the classic antioxidants such as vitamins C and E, it is estimated that during this cascade reaction, one melatonin molecule could scavenge up to 10 free radicals, whereas one glutathione that detoxifies one radical per molecule [4]. Numerous studies have confirmed that melatonin regulates certain antioxidant enzymes like superoxide dismutase and glutathione peroxidase [5] or acting as a scavenger of Reactive Oxygen Substances, such as peroxyl radical [6], nitric oxide [7], hydrogen peroxide [8], and singlet oxygen [9]. Since no pro-oxidant function and toxicity has been reported for melatonin, it is concluded that this hormone has the characteristics of a very good radical scavenger.
D-galactose is a naturally-occurring reducing sugar in the body, which gets completely metabolized at normal concentrations. In contrast, at higher concentrations it is converted to aldose, hydrogen peroxide, and galactose oxidase; producing superoxide anions and oxygen-derived free radicals which damage the function of macromolecules and cells [10]. Oversupply of D-galactose could contribute to generation of ROS through oxidative metabolism of D-galactose as well as through glycation end products [11]. ROS are the normal by-products of oxygen metabolism mainly found in mitochondria have the potential to damage the DNA, proteins and lipids by oxidizing them leading to cellular malfunctions. In order to overcome this excess production of ROS; dietary dose of the antioxidants proves as a great defense system. Both enzymatic and non enzymatic anti-oxidants exist in the intracellular and extracellular environments to quench ROS. The enzymatic anti-oxidants include superoxide dismutase (SOD) [12], catalase (CAT) [13] and glutathione peroxidases (GPx) [14].
Musca domestica L. 1758 is an excellent model system to investigate longevity promoting properties of neutraceutical extracts and phytoconstituents [15]. This aging model may be useful in identifying effective antioxidants and their mechanisms in slowing aging and age-related degenerative diseases [16]. The effect on melatonin supplementation in larva of *M. domestica* is not yet studied so far. So this is the first attempt to study such effect.

Compartmentalization of digestive enzymes and nutrient absorption in insect gut has been reviewed by Terra *et al.*, 1996 [17], also a specific and apparently regulated spatial distribution of oxidative radicals and their scavenging enzymes in *Spodoptera littoralis* have been reported by Krishnan and Sehnal [18] who suggested that the insects exploit oxidative stress in the process of digestion but manipulate the stress spatially to minimize oxidative threat to the gut tissue. But till date there is no such report on larval *M. domestica* gut oxidative stress. The present study was carried out to test the hypothesis that the oxidative stress elicited by the ingestion of D-galactose is associated with antioxidant defense and that the stress and defense are allocated to different gut compartments. In present investigation the housefly *Musca domestica* larvae were used to study the effect of D-galactose- induced oxidative stress and the antioxidant properties of melatonin.

2. Materials and methods

2.1 Chemicals

Ascorbic acid, D-galactose, sulforhodamine B and methyl paraben was procured from sigma Chemical Co., St. Louis, USA.. All other chemicals used were of analytical grade.

2.2 Fly stock rearing

Nucleus culture of houseflies was procured from National Chemical Laboratory, Pune and then acclimatized in laboratory at 26±1 ^{0}C with 70 ±1% RH.

2.3 Standardization of the doses

Prior standardizations of melatonin and concentration of D-galactose were done.

2.4 Diet

The experimental diet was formulated by earlier done work in the laboratory (unpublished data). The control diet was prepared using 9 gm soya food (4 gm soya powder+ 2 gm rice bran +1.5 gm milk powder) in 15 ml of distilled water. In order to avoid microbial infection diets were supplemented with Streptomycin and methyl paraben.

2.5 Preparation of experimental sets

Administration of dose to *M. domestica* larvae

Form the mother culture, the eggs were collected and divided into four groups. Each group contained 50 eggs and was reared during their postembryonic development, from emergence until third instar stage on above mentioned diet. Group I insects were reared during their postembryonic development, from emergence until third instar stage, on control media. In addition to the control media, Group II larvae were reared 6 mg/ml D-galactose, Group III larvae were reared on 25 µg/ml melatonin and Group IV larvae were reared on control diet added with 6mg/ml D-galactose + 25µg/ml melatonin.

2.6 Gustatory assay

Eggs were grown on the artificial food dissolved in the respective concentration up to late second instar stage. Later these larvae were starved for 24 hours and transferred on respective food mixed with 2% streptorhodamine B; where they were allowed to feed for 3 hours. The entire gut region of these flies was dissected and homogenized in the phosphate buffer. O.D was taken at 540 nm. Streptorhodamine B was used to detect the amount food taken in by the larvae. This assay was done in order to ensure that the results that are observed are not because of the altered feeding behavior but because of the added concentration of the respective doses.

2.7 Sample Collection and Photometry

After four days of treatment, third instar larvae from all four groups were collected, immobilized on ice and dissected in ice-cold 0.1M Phospahte buffer, pH 7.0. Foregut, midgut and hindgut were separately collected in assay buffers, homogenized and centrifuged at 4 °C for 15 min at 3000 rpm. Supernatant was again centrifuged at 12,000 g, 10 min for 4 °C. Supernatants were stored at -80 °C and used for the further analysis within a week.

2.8 Biochemical assays

2.8.1. Antioxidant enzymes

2.8.1.1 Superoxide dismutase (SOD)

SOD activity was measured at 560 nm and was calculated using inhibition percentage of formazon formation [12]. Absorbance was read at 560 nm and the enzyme activity was calculated in terms of Units/mg Protein.

2.8.1.2. Glutathione-S-transferase (GST)

GST activity was measured spectrophotometrically by the method of Habig *et al.* [19] using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The enzyme activity was calculated as nmol CDNB conjugate formed min/mg protein using a molar extinction coefficient of 9.6mM^{-1} cm^{-1}.

2.8.1.3. Catalase (CAT)

Supernatants (8000g, 5 min, 4 °C) of protein extracts in 66 mM phosphate buffer (pH 7.0) were taken for the assay with 3% hydrogen peroxide [20].

2.8.2. Ascorbate Peroxidase (APOX)

Supernatants containing 0.5 mM ascorbic acid were mixed with 0.3 % H_2O_2 [21] and the decrease of absorbance at 290 nm was measured for 10 min with the photometer. Boiled samples were assayed in parallel. Enzyme activity was expressed as mM ascorbate oxidized per min (= 1 EU) per mg protein using a molar extinction coefficient 2.8 mM^{-1}cm^{-1}.

2.8.3. Lipid Peroxidation (LPO)

The Thiobarbituric acid reactive substances (TBARS) Assay method was used to evaluate the peroxidation of lipids (LPO) [22]. LPO was calculated as nanomoles of thiobarbituric acid reactive substances (TBARS) formed per gram of tissue using a molar extinction coefficient of 1.56 10^{-5}M^{-1}cm^{-1}.

2.8.4. Acid Phosphatase (ACP)

The acid phosphatase activity was estimated using the Linhardt and Walter [23] method with some modifications. ACP activity was calculated in terms of units formed per mg of tissue using a molar extinction coefficient of p-nitrophenol 18.8 µM^{-1}cm^{-1}.

2.8.5. Protein carbonyl content in *M. domestica* larvae

Protein carbonyls in the gut tissues were determined according to Reznick and Packer [24] method. Protein carbonyl values were corrected for the interfering substances by subtractingthe OD_{370}/mg protein measured without DNPH.

2.9. Determination of concentration of protein

Protein concentrations in all test samples were determined using Bradford reagent and measuring the OD at 595 nm using

a UV–Visible spectrophotometer [25]. The amount of protein was quantified using bovine serum albumin as the standard.

3. Statistical Analysis

All the values were expressed as mean ± standard error (SE). The experiments were repeated on three different occasions in triplicate. Results were analyzed for significance of differences between control and exposed by one-way analysis of variance (ANOVA), followed by Tukey's test (means comparison) using a statistical software, SPSS -2011. Significance of results was set at $p<0.05$.

4. Results and Discussion

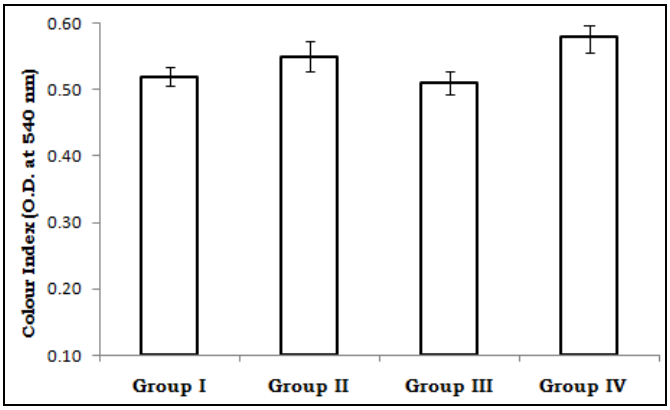

Fig 1: Gustatory assay in third instar larvae of *M. domestica* on four diet groups.

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
Significance is based on $p < 0.05$, compared with Control group values.

Fig. 1 shows that the all the four groups of larvae did not show significant difference in their feeding assay. Thus the results obtained from the following experiments are not due to the alteration in the feeding behaviour of the larvae, it was due to the presence of the treatment compound itself.

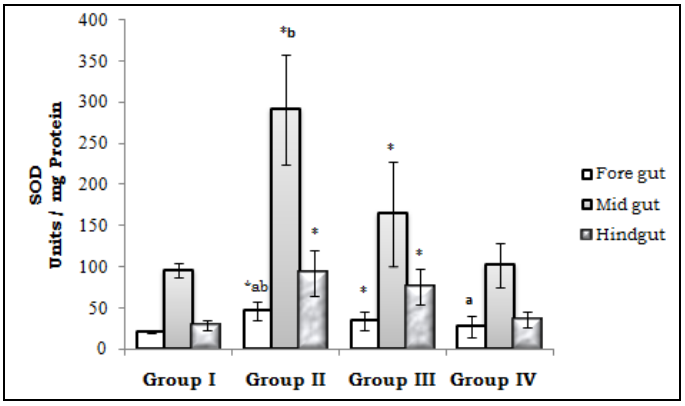

Fig 2: Superoxide dismutase activity *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
SOD values are expressed as Units/mg of protein
Significance is based on $p < 0.05$, $*p < 0.05$, compared with control group values.
Different letters (a and b) indicate significant difference between respective gut compartments ($p < 0.05$).

The larvae exposed to D-galactose alone showed a significant increment of SOD activity. There is significant three-fold increment in mid gut of D-galactose treated group compared with control group. SOD activity in foregut and midgut of D-galactose+ melatonin were also increased by two-fold. But there was no significant difference in the SOD levels in all three gut tissues of control and melatonin treated groups.

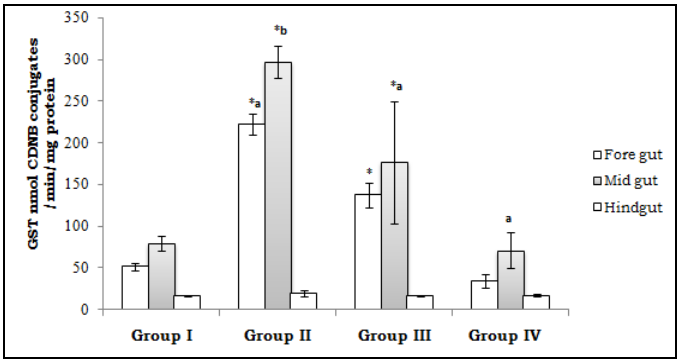

Fig 3: GST activity in *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
GST levels are expressed as nmol CDNB conjugates /min/mg protein.
Significance is based on $p < 0.05$, $*p < 0.05$, compared with control group values.
Different letters (a and b) indicate significant difference between respective gut compartments ($p < 0.05$).

Effects of D-galactose and melatonin treatment on GST activity in 3rd instar larvae of *M. domestica* are depicted in Fig. 3. It was observed that in all gut compartments there is significant three-fold increment in the GST activities of the D-galactose treated group. The foregut and midgut compartments of the Group IV shows two-fold increment as compared with control group and there was not much difference in the SOD activity in all three guts of the control and melatonin treated larvae.

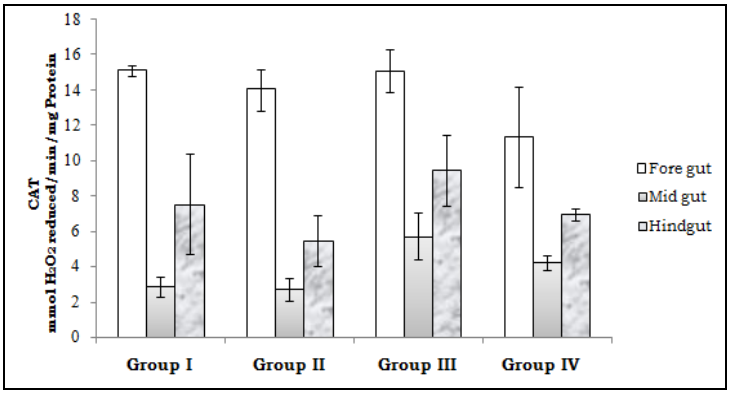

Fig 4: CAT activity in *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
CAT levels are expressed as mmol H_2O_2 consumed/min/mg protein.
Significance is based on $p < 0.05$, $*p < 0.05$, compared with control group values.

A slight decrease was observed in CAT activity in Group II compared with control (Group I) in all gut samples but these values are not found statistically different (Fig 4). In midgut and hindgut tissues, CAT activity values were seen to rise in Group III than other groups, but those are not significant.

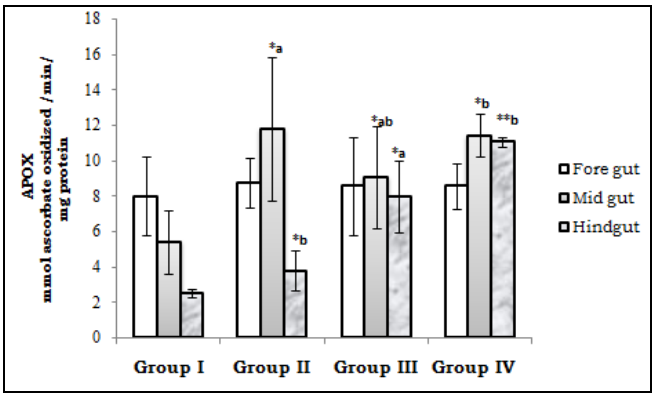

Fig 5: Ascorbate Peroxidase (APOX) activity in *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
APOX levels are expressed as mmol of ascorbate oxidized /min/mg protein tissue.
Significance is based on p < 0.05, *p < 0.05, **p < 0.001, compared with control group values.

Different letters (a and b) indicate significant difference between respective gut compartments (p < 0.05).
There was no significant difference in APOX activity in foregut tissues of all groups (fig 5). In midgut, a general increase was observed in all groups. The highest activity was found in Group IV (p<0.001) whereas Group II and Group III values were also statistically different compared with Group I (p<0.05). In hindgut all treated groups showed significant increase in APOX values than Group I (p<0.05).

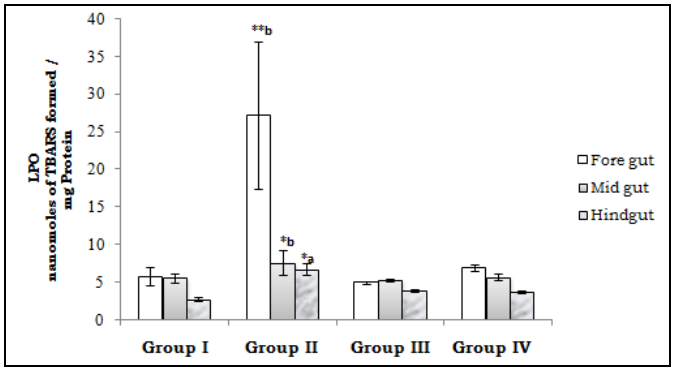

Fig 6: LPO (nmol TBARS /mg tissue) in in *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
Significance is based on p < 0.05, *p < 0.05, compared with control group values.
LPO levels are expressed as nmol TBARS formed/mg tissue.

Different letters (a and b) indicate significant difference between respective gut compartments (p < 0.05).
The data on TBARS pertaining to lipid peroxidation of *M. domestica* larvae are presented in Fig. 6. The results show a significant increase in all three gut sample lipid peroxidation values in D-galactose treated larvae (Fig-6). About 5-fold rise in foregut (p <0.001), 1.5-fold increase in midgut (p <0.05) and 3-fold increment in the hindgut tissues (p <0.01), indicating the increased oxidative stress due to D-galactose

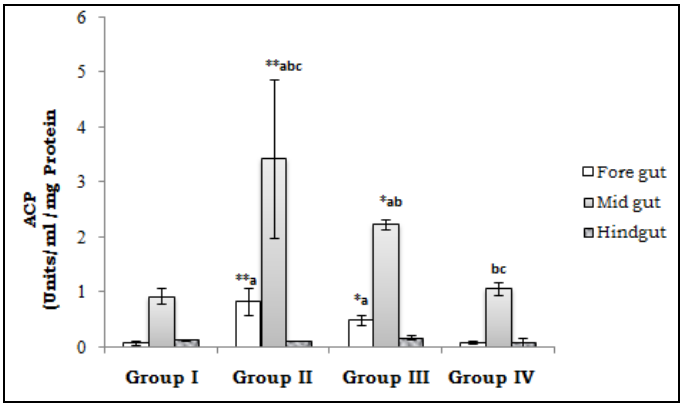

Fig 7: Acid Phosphatase (ACP) activity in *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
ACP values are expressed as Units/mg of protein.
Significance is based on p < 0.05, *p < 0.05, **p < 0.001, compared with control group values.
Different letters (a, b and c) indicate significant difference between respective gut compartments (p < 0.05).

The results show a significant increase in foregut ACP activity (Fig. 7), Group II showed 10-fold rise in D-galactose treated larvae (p <0.01), while Group III was found with 6-fold more activity and about 5-fold rise in foregut (p <0.05). In midgut the Galactose treated Group II showed four times more activity (p <0.01) and that of Group III showed 3-fold rise (p <0.05). There was no significant difference in hindgut tissue.

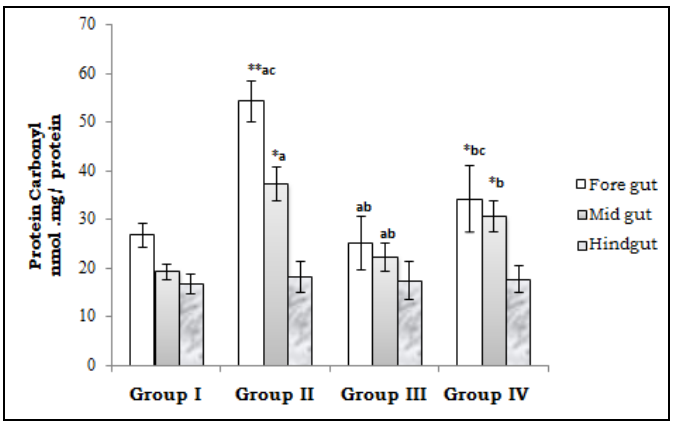

Fig 8: Protein carbonyl content in *M. domestica* larvae:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
Significance is based on p < 0.05, *p < 0.05, **p < 0.001, compared with control group values.
Protein carbonyl values are expressed as nanomols/mg of protein.
Different letters (a, b and c) indicate significant difference between respective gut compartments (p < 0.05).

As shown in Fig 8, the foregut of *M. domestica* suffered greater damage marked by protein carbonylation. In comparison to the controls, the titer of carbonyl groups in proteins of the foregut of *M. domestica* larvae was enhanced significantly ($P < 0.001$) after D-galactose feeding.

In midgut also significant rise in protein carbonyl content was found in Group II ($p < 0.05$). In group IV, both foregut and midgut tissue showed significantly higher level of protein carbonylation ($p < 0.05$). In all four groups concentrations of carbonyl content proved insignificant.

Our data demonstrate a distinct compartmentalization in the distribution of antioxidant enzymes in the digestive tract of *M. domestica* larvae. The spatial distribution patterns are retained and differences among the gut compartments are enhanced upon D-galactose addition to the diet. Diet supplementation with D-galactose, increased the level of antioxidant enzyme activities, presumably in response to the oxidative stress. The ingestion of D-galactose elevated significantly the activities of all studied enzymes in the tissue. In our study we did not find significantly different CAT activity. But we found increased GST activity. The enhancement of the GST activity appears to be an innate foregut response to the rise of oxidative radicals. The insect GST is effective in targeting the hydro-peroxides but not the hydrogen peroxide [26], suggesting that it may prevent lipid peroxidation in the foregut tissues without interfering with high H_2O_2 concentration in the foregut lumen. APOX stimulation with galactose in the midgut tissue suggests a role in the scavenging of intracellular H_2O_2 residues that are not eliminated by the catalase [27].

The gut antioxidant enzymes provide protection against oxidative radicals rendering the generation of new radicals less likely. Gut compartmentalization with respect to the generation of oxidative radicals and the production of antioxidant enzymes appears to be an important component of the gut function. The insects seem to possess an efficient strategy for the deployment of oxidative radicals in the initial digestion steps without endangering the midgut where final digestion and nutrient absorption occur [18].

Lysosomal acid phosphatise is transported as a trans-membrane protein to dense lysosomes, the pathway of lysosomal ACP to lysosomes include the passage through the plasma membrane [28]. Injured lysosomes release hydrolytic enzymes in cytoplasm leading to auto degradation of cellular proteins; damage to endoplasmic reticulum interferes with protein synthesis and intracellular transport of vital compounds [29]. Lipid peroxidation in membrane lipids plays an important role in cell physiology and pathology, there are number of membrane bound enzymes and their activity is altered by lipid peroxidation. Decreased Lipid peroxidation values in melatonin co-treated group than the D-galactose treated group showing their antioxidant properties indicate lysosomal clearance, which is the indication of healthy status of the cell. As the oxidative radicals are responsible for formation of the carbonyl groups in the side chains of certain amino acids, the carbonyl content in proteins is commonly employed as a marker of oxidative protein damage [30-31]. In our experiment, D-Galactose treated group showed high level of protein carbonylation which was found to be decreased upon Melatonin treatment.

5. Conclusion

The present study thus concludes that, D-Galactose induces oxidative stress in the gut compartments of *Musca domestica* larvae. This stress can be reduced by Melatonin. Thus, Melatonin acts as an efficient free radical scavenger by protecting gut membranes and also tries to prevent D-galactose induced oxidative stress.

6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

7. Acknowledgments

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