Ascorbic acid dualism in *Musca domestica* larvae

ABSTRACT

Aims: To examine the antioxidant and pro-oxidant properties of ascorbic acid in *Musca domestica* larvae.

Study Design: The pro-oxidant characteristics were evaluated by exposing the larvae to different doses of ascorbic acid. To validate its oxidant scavenging properties, the effects on antioxidant enzyme profiles were examined in both normal and D- galactose-induced stress conditions in *M. domestica* larvae.

Place and Duration of Study: Department of Zoology, Savitribai Phule Pune University, Pune between June 2009 and July 2010.

Methodology: The *M. domestica* larvae were subjected to different doses of AA for accessing the pro-oxidant nature. This study also investigated the antioxidant properties of AA in the D- gal induced oxidative stress condition by assessing the parameters as biochemical assays (Superoxide dismutase, Catalase, Glutathione S transferase, Lipid peroxidation and Protein carbonylation) and larval survival post treatment with AA and D- galactose.

Results: A significant correlation existed between larval death and AA concentration, with highest survival at 20 mM and 100% mortality at 100 mM, attributed to AA's pro-oxidant behavior. At the concentration of 10 mM, AA significantly reduced the oxidative stress induced by D- gal ($p < 0.05$) and also maintained the percent pupal survival and percent eclosion.

Conclusion: The transitional behavior of AA in *M. domestica* larvae is concentration-dependent, with reduced concentrations improving survival rates and higher concentrations resulting in increased mortality, underscoring the necessity for meticulous dose evaluation before administration in biological systems.

Keywords: Ascorbic acid; D- galactose; oxidative stress; Antioxidant; Pro-oxidant; *Musca domestica* larvae.

1. INTRODUCTION

Ascorbic acid (AA), also known as vitamin C, is a water-soluble vitamin that has gained immense popularity owing to its antioxidant potential [1]. Its discovery as an effective antiscorbutic agent was a breakthrough [2]. In 1928, Hungarian biochemist Albert Szent-Györgyi first isolated and described AA and got a Nobel Prize in 1937. The chemical structure of ascorbic acid is characterized by a six-carbon lactone ring, with two hydroxyl groups and a carbonyl group. This unique structure allows ascorbic acid to readily donate electrons, making it a potent reducing agent [3]. Under physiological conditions, AA takes two forms: L-ascorbic acid and L-dehydroascorbic acid. L-ascorbic acid is the primary dietary form with strong free radical scavenging activity. Animalia is widely known for its ability to produce AA, but not all organisms maintain this ability. ~~The organisms~~ Mammals such as guinea pigs, humans, a few other primates, and numerous other organisms fail to generate AA due to the absence of L-gulonolactone oxidase, an enzyme essential for the production of 2-keto-L-gulonolactone, which is later transformed to AA. These organisms thus depend on the dietary intake of AA [4].

AA is an essential nutrient that plays a crucial role in various physiological processes [5, 6]. ~~The o~~Extensive literature has given deeper insights into various aspects of AA, including its activity in biological systems. This information has provided opportunities to explore its potential against a wide range of diseases. Szent-Györgyi, shortly after his groundbreaking studies on AA, had speculated on its usefulness in treating infections (REFERENCE). Later on, a large number of studies asserted its efficiency in the prevention and treatment of atherosclerosis, AIDS, cataracts, the common cold, and cancer. Moreover, numerous studies have also highlighted the significance of AA consumption and its robust correlation with improvements in the immune system, wound healing, allergies, mental health issues, and numerous other stressors [7]. This vast pool of information also has studies that have refuted these findings, at least in part, as the claim that specific medication or therapy can treat a broad range of illnesses is usually met with skepticism. However, the fact that free radicals are involved in the etiology of the majority of these diseases may provide a satisfactory explanation for their promising role in their treatment [8-10].

Under typical physiological conditions, ~~circumstances~~, the cell continuously produces free radicals, the main byproducts of oxygen metabolism. Free radicals are essential for redox signaling in numerous cellular pathways, but when in excess, they severely hamper the biomolecules, inciting several cellular abnormalities [9]. Thus, these free radicals act as a double-edged sword requiring meticulous control. The cells efficiently establish this control by maintaining equilibrium in the production and elimination of these free radicals. An integrated network of enzymatic antioxidant systems within the cell maintains this balance [9]. Moreover, dietary antioxidants and vitamins such as AA provide additional assistance in keeping check on the uncontrolled generation of free radicals or their reactivity at biological sites [11]. AA contributes to the first line of antioxidant defense and prevents biomolecules from oxidative damage. AA is a strong reducing agent and a scavenger of free radicals. It donates a hydrogen atom, which pairs with the unpaired electron or free radical. The unreactive ascorbyl radical that is produced in this reaction is potentially harmless [12]. Since AA is a water-soluble molecule, it can function both inside and outside of cells, neutralizing and preventing damage from free radicals. AA has shown powerful scavenger activity against nitrogen and oxygen species, including singlet oxygen, hydrogen peroxide, the hydroxyl radical, and superoxide radical

ions, thus effectively shielding cellular constituents from free radical-induced damage [12]. Apart from being an efficient antioxidant, AA has shown several other beneficial properties. It acts as an important co-substrate for a wide range of enzymatic reactions, such as post-translational hydroxylation of proline and lysine in collagen and other connective tissue proteins, which allows proper production and maintenance of collagen, a structural protein found in many tissues [13-16]. It is also involved in the synthesis of carnitine, which is essential for transporting fatty acids to the mitochondria for energy production, as well as in the conversion of the dopamine to norepinephrine [17]. AA, owing to its ability to interact with various transcription factors, controls the expression of certain genes, particularly the collagen genes. Additionally, it facilitates the intestinal absorption of iron and functions as a cellular antioxidant, both independently and in conjunction with the antioxidant activity of vitamin E [17]. AA is also one of the important contributory factors for the proper functioning of the immune system, aiding in the development and maintenance of a healthy immune response [18,19].

Despite the immense popularity and importance of AA, one cannot ignore its darker side. There exists a duality in the action of AA. In addition to its well-known antioxidant properties, AA can also act as a pro-oxidant depending on the environment and conditions in which the molecule is active [13, 20]. Few in vitro studies on AA have shown its pro-oxidant effects under specific conditions such as the presence of transition metals, excessive oxygen, and various other pathological conditions [21]. For example, chelation of Fe2+ by ascorbate results in the formation of an active catalyst for the production of reactive oxygen species. AA effectively aids in the conversion of Fe^{3+} to Fe^{2+}, which subsequently reacts with oxygen or hydrogen peroxide, resulting in the formation of superoxide anions and hydroxyl radicals [21]. Many studies have explained the significance of pro-oxidant property in the treatment of cancer [22, 23]. However, the literature available on its pro-oxidant nature is limited. The current study was designed to comprehend the transient pro-oxidant and antioxidant activity of AA in vivo in the larvae of the housefly, *M. domestica*. *M. domestica* –was selected for the investigation due to its wide availability and ease of rearing. Also, both humans and insects are incapable of producing AA and have to depend on dietary intake; the obtained results may be extrapolated to ~~can be easily used to correlate with~~ humans due to such similarity. The pro-oxidant properties of AA were examined by administering its different dosages to the larvae, whereas antioxidant potential was investigated by evaluating its protective action against D- galactose (D- gal)-induced oxidative stress. Though D- gal is a reducing sugar and a known physiological nutrient, its excessive intake may lead to increased production of reactive oxygen species hampering normal metabolism [24, 25]. D- gal has been widely used in investigating and understanding various aspects of stress and to evaluate the potential of various antioxidants in a range of animal models. Another noteworthy aspect of this study is its manipulation of the larval diet. Most of the research on evaluating the antioxidant properties of AA is pioneered mainly on manipulating adult diet; however, in this study, the effect of larval supplementation on adult eclosion and pupation was also examined.

2. MATERIAL AND METHODS

2.1 Fly stock rearing:

Nucleus culture of *M. domestica* was procured from the National Chemical Laboratory in Pune, India. Later the flies were acclimatized at 26 ±1°C with 70 ±1% relative humidity (RH) in the incubator (details….). The eggs were grown using controlled diet. Third generation of laboratory reared *M. domestica* larvae were used for further experimentation.

2.1.1 Diet:

The experimental diet was formulated by adding a mixture of 4 gm soya powder, 2gm rice bran and 1.5 gm milk powder in 15 ml of distilled water. The diet was supplemented with streptomycin and methylparaben to prevent microbial infection. Each experimental set comprised of 20 *M. domestica* larvae.

2.2 Pro-oxidant effect and selection of AA concentration:

Newly hatched first instar *M. domestica* larvae were reared on diets supplemented with different concentrations of AA viz. 20, 40, 60, 80, and 100 mM up to the third instar and larval survival was measured. Larval survival depicts the number of larvae survived till completion of third instar stage. Using the data of this experimentations further reduction in the AA concentration was done, where the larvae were exposed to 10, 15, and 20 mM concentrations. As the larval survival in all the three groups was significantly high further investigations were done based on biochemical analysis. Larvae reaching the third instar stage were used for biochemical assays such as LPO, PCC SOD and CAT. The obtained results were not only used to assess the *pro-oxidant* behavior but also for finalization of the AA dose for assessing its antioxidant nature. Based on the findings of these assays,10 mM concentration was selected for further investigations.

2.3 Antioxidant effect of Ascorbic acid against D- gal induced oxidative stress:

A concentration of 6 mg/ml of D- gal was selected for the investigation based on previously reported findings from our laboratory [26]. For this study, larvae (n = 20/replicate; 5 replicates per group) were exposed to four diet groups as, 1] Control, 2] 10 mM ascorbic acid (10 mM AA) 3] 6 mg/ml D- galactose, (D- gal) and 4] 10mM ascorbic acid + 6mg/ml D- gal (hereafter referred to as D- gal + 10 mM AA). Biochemical investigations were performed on third-instar larvae from each of the four treatment groups.

2.3.1 Gustatory assay:

The gustatory assay was performed to confirm that the results of the investigation are attributable to the experimental compound rather than variability in consumption, a gustatory assay was conducted. The food consumption of the house fly larvae was assessed by incorporating Sulforhodamine B, a visible dye, into their diets, followed by the measuring the gut redness [27]. Newly hatched first instar larvae were transferred on the four food sets and allowed to grow up to the second instar stage. At the late second instar stage, these larvae from each group were left unfed for 24 hours on tissue paper soaked with distilled water. After the starvation period, larvae were again relocated on their corresponding experimental food set blended with 2% sulforhodamine B. Larvae were fed for 3 hours on the dye-blended food. Later these larvae were placed on ice for immobilization and then dissected. The entire larval gut was dissected and homogenized in 0.1 M phosphate buffer, pH 7.2. The optical density of supernatant was measured at 540nm using a microplate reader.

2.3.2 Biochemical assays:

After growing the larvae on the diet supplemented with the treatment compound, third instar larvae from each of the four groups were immobilized on

ice and homogenized in respective assay buffers. For each assay 10 larvae were used. The obtained supernatant was subjected to centrifugation one more time at 12,000g for 10 minutes at 4°C. Supernatants were stored at -80°C until further use.

2.3.2.1 Superoxide dismutase (SOD) assay:

Larvae were homogenized in 50 mM sodium phosphate buffer (pH 7.0), and then centrifuged (12,000g, 10 min, 4°C). SOD activity was measured by formazon inhibition as described by Beauchamp and Fridovich (1971) with minor modification [28]. Absorbance was read at 560nm and enzyme activity was described in terms of units/mg of protein.

2.3.2.2 Glutathione-S-transferase (GST):

The GST activity of supernatants was assessed referring the method established by Habig et al. (1974), utilizing 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate [29]. The enzyme activity was quantified as nmol of CDNB conjugate produced per minute/mg of protein. A molar extinction coefficient of 9.6 mM-1 cm-1 was used.

2.3.2.3 Catalase (CAT) assay:

Larvae were homogenized in 66 mM phosphate buffer (pH 7.0), centrifuged (8000g, 5 min at 4°C) and supernatants were obtained. Following Abei's (1984) method CAT activity was measured and expressed as mmol H2O2 reduced per minute (= 1 EU)/mg of protein using extinction coefficient 39.4 mM -1cm-1 [30].

2.3.2.4 Ascorbate Peroxidase (APOX):

Supernatants containing 0.5 mM ascorbic acid were combined with 0.3% H2O2 [31], and the reduction in absorbance at 290 nm was recorded for 10 minutes. Boiled samples were evaluated concurrently. Enzyme activity was quantified as mmol of ascorbate oxidized per minute (=1EU)/mg of protein using 2.8 mM 1cm1 as molar extinction coefficient.

2.3.2.5 Lipid Peroxidation (LPO):

The lipid peroxidation (LPO) was assessed using the thiobarbituric acid reactive substances (TBARS) assay [32]. Results are presented in nmoles of malondialdehyde (MDA)/ gm of wet tissue. LPO was determined as nanomoles of TBARS produced per gram of tissue, utilizing a molar extinction coefficient of 1.56 x 10-5 M-1cm-1.

2.3.2.5 Protein Carbonylation assay (PCC):

Reznick and Packer (1994) method was used to quantify protein carbonyl content [33]. The concentration of carbonyls was measured at 370 nm and expressed as nmol/mg protein, using a molar extinction value of 22 $mM^{-1}cm^{-1}$. Using Bovine Serum Albumin (BSA) standard curve protein concentration in the guanidine solutions was assessed by measuring absorbance at 280 nm.

2.3.2.6 Determination of protein concentration:

Protein concentrations in all test samples were quantified using Bradford reagent and measuring the OD at 595 nm [34]. Using BSA as standard protein concentration was measured.

2.4 Effect of AA and D- gal on pupal viability and percent eclosion studies:

To investigate the impact of AA and D- gal supplementation on the pupation and eclosion of *M. domestica* , larvae were grown in each of the four treatment groups (n=20, 5 replications). The larvae were allowed to pupate, and the number of pupae developed was documented in terms of percentage pupation. The quantity of flies that emerged from the pupa was recorded, and percentage eclosion was examined.

2.5 Statistical Analysis:

All experiments were repeated in 5 replicates. Results analysis was based on the significance of differences between control and exposed larvae by one-way analysis of variance (ANOVA), followed by Tukey's test (means comparison) performed by applying a statistical software, SPSS -2011. Significance was set at $p < 0.05$. All data values were presented as mean ± standard error (SE). Means sharing the same letter represent no statistical significance, $p< 0.05$.

3. RESULTS AND DISCUSSION

3.1 Pro-oxidant nature of AA:

The present study contributes to understanding the bimodal behavior of AA in *M. domestica* larvae *in vivo*. It demonstrated that AA exhibited fatal effects in the larvae at higher concentrations while the lower concentration mitigated the -induced oxidative stress. This investigation revealed a strong correlation between larval death and an increase in the AA concentration, with the highest survival at 20 mM and 100% mortality at 100 mM concentration (Figure 1).

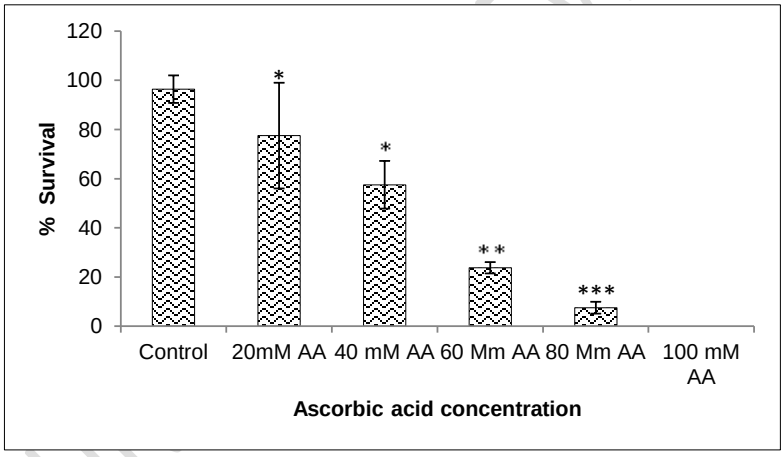


Figure 1. Standardization of Ascorbic acid dose.
Values are shown as means ± SD (n=20).
Significance is based on $p<0.05$ compared with Control group values.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Increased lethality observed at the concentrations of 60 mM, 80 mM, and 100 mM can be attributed to the pro-oxidant behavior of AA. Previous studies in vitro have documented the similar dual behavior of AA *[35-37].* A survey of bovine hemoglobin revealed both antioxidant and pro-oxidant properties of AA owing to its radical scavenging property and reaction with oxygenated hemoglobin, causing an increase in the H_2O_2 and choleglobin production [38]. In the U-937 cells, it exhibits a transition from a pro-oxidative to antioxidant behavior [39]. In

KG1A myeloid leukemia cells, AA supplementation up to 250 µM resulted in the intracellular antioxidant behavior, whereas its higher concentrations exhibited pro-oxidant effects [20]. Similarly, in the yeast *Saccharomyces cerevisiae*, AA effectively protected cells against chromium (VI)-induced oxidative damage but with a concentration-dependent pro-oxidant activity [40]. In an Alzheimer's disease model, a 6-month treatment of AA decreased the oxidative damage and Aβ oligomer formation, soluble Aβ42 to Aβ40 ratio, and restored the synaptophysin and phosphorylation of Tau at Ser39 [41]. However, higher concentrations reversed its beneficial effects, indicating a pro-oxidant nature [41]. The afore-mentioned studies are in coherence with our results, where 80 mM and 100 mM doses exhibited maximum larval mortality. The direct correlation between the rise in larval mortality and increasing concentration of AA may be ascribed to extracellular auto-oxidation and H_2O_2 production. AA exhibits its pro-oxidant nature via the Fenton reaction [36]. Under aerobic conditions, AA tends to react with molecular oxygen, generating superoxide radicals, resulting in its dismutation to H_2O_2 [20]. Upon entering the cell, H_2O_2 reacts with reduced metal ions, leading to the production of potentially harmful OH radicals [20]. One of the studies has also suggested that the pro-oxidant activity associated with redox cycling occurs when the ratio of antioxidant to metal ion is relatively low [42]. At higher doses, AA causes DNA aberration by increasing the production of H_2O_2 [43]. It causes severe effects on the survival and outgrowth of neuritis by causing glycation of the cellular proteins [44]. In dietary supplementation studies, it exhibited divergent results, significantly improving the average life span in mice but not in guinea pigs [45-47]. AA plays a substantial role in the generation of ROS, primarily via the mitochondrial respiratory chain and specific enzymes like NADPH oxidases (NOXs) [48-51]. NOXs also generate O^{-2} anion or H_2O_2, which can be prevented by ascorbate, provided the levels of NOX are high [50-51]. AA effectively reduced the levels of NOX in the microvascular endothelial cells, thus reducing the chances of sepsis [50-51]. However, it activated NOX in embryonic stem cells, causing cardiomyogenesis [52]. These investigations confirm that AA can generate ROS in numerous ways, and the dosages need to be strictly regulated in order to derive benefits from its antioxidant properties and avoid its pro-oxidant behavior.

3.2 Antioxidant nature of AA:

Amongst all the concentrations tested in this study, the larvae treated with the 20 mM AA dose showed the highest survival rate of 80%. But this value prompted speculation of a 20 mM dose still being potentially toxic to the larvae, albeit with lesser severity; hence, further investigation was done by progressively reducing the concentration to 10 mM, 15 mM, and 20 mM, achieving nearly 100% survival. We assume that the doses below 20 mM concentration allow survival in *M. domestica* larvae. Additional validation of observed results was done by analyzing the levels of PCC, LPO, and SOD. In comparison to the control and 10 mM AA dose, a substantial increase in PCC and LPO activity was

seen at 15 mM and 20 mM concentrations of AA ($p < 0.05$ and $p < 0.01$, respectively), as shown in Figure. 2. However, there was no significant difference between the control and 10 mM AA-treated groups. Consequent to these results, 10 mM AA was used for further investigation.

Co-supplementation of 10 mM AA with D- gal treatment significantly reduced the stress caused by D- gal. D- gal is a monosaccharide commonly found in dairy products, fruits, and vegetables. It elicits oxidative stress through multiple interconnected pathways [24, 25, 53]. The auto-oxidation of D- gal, catalyzed by minute quantities of free transition metals like iron and copper, can produce reactive oxygen species (ROS) *in vivo*. In numerous studies concerning aging, D- gal was used to induce oxidative stress. In *D. melanogaster* and *M. domestica* it has been reported to expedite the aging process by enhancing oxidative stress [54].

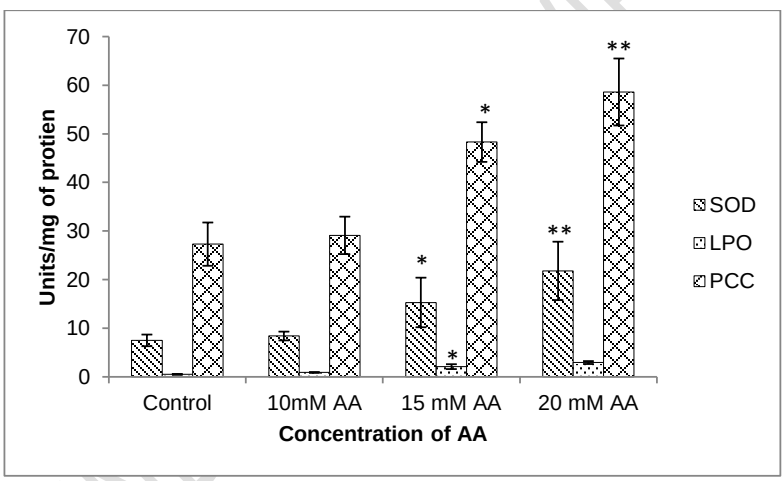


Figure 2. Standardization of Ascorbic acid concentration by antioxidant assays

Superoxide dismutase (SOD) activity is expressed as Units/mg Protein
Lipid Peroxidation (LPO) levels are expressed as nmol MDA formed/mg tissue
Protein carbonylation (PC) values are expressed as nmol /mg protein
Values are shown as means ± SD (n=10).
Significance is based on $p<0.05$ compared with Control group values.
* $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

On performing a gustatory assay with sulforhodamine B, it was observed that there was no significant alteration in the color index among the larvae of all four treatment groups (one-way analysis of variance, $p > 0.05$) (Figure 3). The gustatory assay thus validated that the results of this study are attributable to the treatment substance (AA and D- gal) rather than modifications in eating behavior.

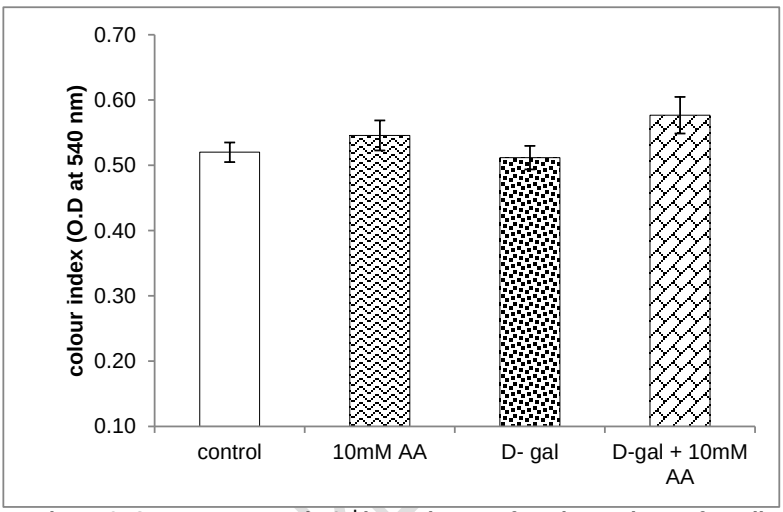


Figure 3. Gustatory assay in 3rd instar larvae of *M. domestica* on four diet groups:

Values are shown as means ± SE (n=10).

Significance is based on $p<0.05$ compared with Control group values.

3.2.1 Biochemical assays:

Superoxide dismutase (SOD) facilitates the dismutation of the O^{-2}, thus neutralizing it [55, 56]. In this study, the larval group treated with D- gal showed a three-fold rise in the SOD activity (Figure 4). However, a 10 mM AA dose improved the SOD activity in the D- gal co-treated group, demonstrating its role in scavenging O^{-2}. At higher concentrations, AA showed divergent behavior, decreasing the SOD activity and exhibiting pro-oxidant behavior. Low doses of AA may have averted the decline in SOD activity caused by D- gal-induced oxidative damage. AA has been shown to up regulate SOD activity in various cell types and animal models, while D- gal induces oxidative stress and mitochondrial dysfunction, leading to a reduction in SOD activity [57,58]. Ascorbate was reported to increase the activity of SOD activity *in vitro* in cultured cells and animal plasma; however, ascorbate supplementation orally did not affect the SOD activity in human plasma [59].

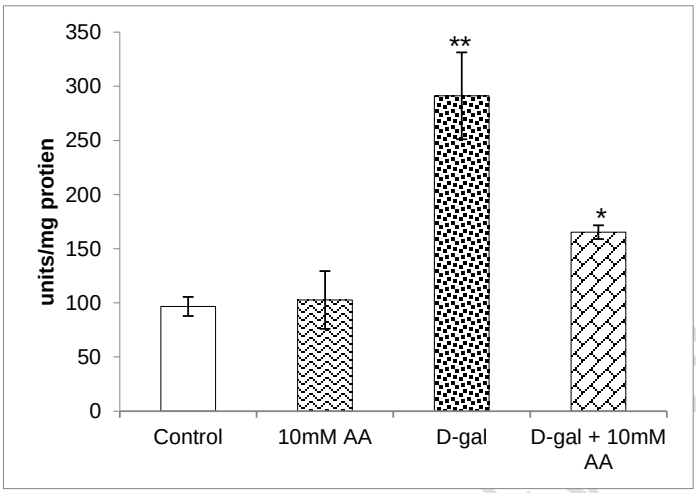


Figure 4. SOD activity (Units/mg Protein) in *M. domestica* larvae

Values are shown as means ± SD (n=10).

Significance is based on $p<0.05$ compared with Control group values.

* $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

In this study there was no significant difference in the GST activity between control and 10 mM AA-treated groups. The larvae treated with D- gal (three-fold) and 10 mM AA + D- gal (two-fold) showed a significant increase (Figure 5). The rise in GST activity seems to be a reaction to elevated oxidative radicals. Glutathione is an essential antioxidant. Studies have advocated the probable role of AA in augmenting the GST activity, thus enhancing the innate detoxification processes of the body [60].

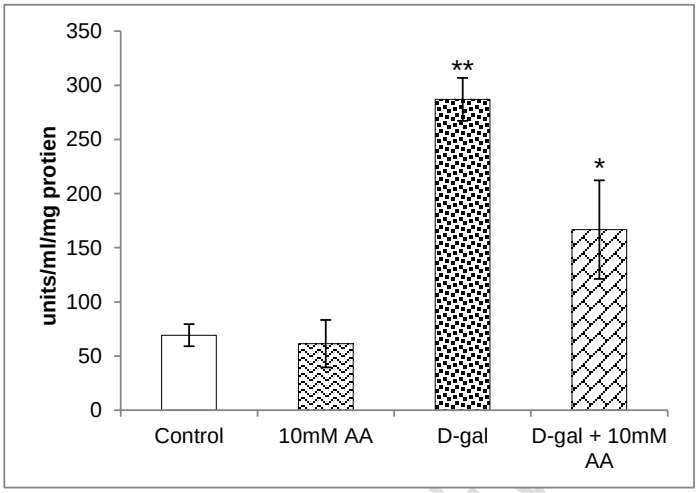
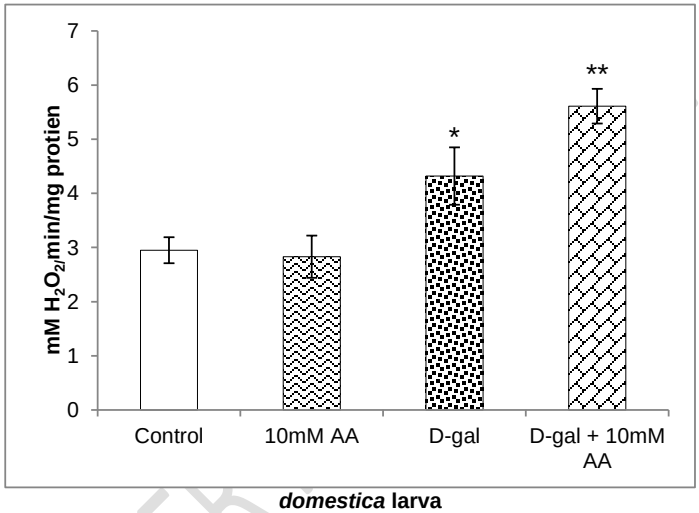


Figure 5. GST activity (Units/ml/mg Protein) in *M. domestica* larvae
Values are shown as means ± SD (n=10).
Significance is based on p<0.05 compared with Control group values.
* p<0.05, **= p<0.01, ***= p<0.001

M. domestica larvae treated with D- gal revealed a two-fold increase in CAT activity; however, this activity was significantly higher in 10 mM AA + D- gal co-treated groups (Figure 6.). Interestingly, AA has been reported to have a direct impact on CAT regulation. It effectively enhances the CAT activity, thus improving antioxidant defense [61-65]. The precise mechanisms of AA that modulate this antioxidant enzyme remain inadequately understood; however, multiple hypotheses have been suggested. One of the mechanisms involved is the ability of AA to maintain the prosthetic metal ions of CAT in their reduced state, which is a vital dynamic for maintaining enzymatic activity [65]. Moreover, AA has been reported to enhance the production of CAT, possibly via the activation of transcription factors or signaling pathways that govern its gene expression [65]. Prolonged D- gal administration in mice has led to a substantial reduction in CAT activity across multiple tissues, including the brain, liver, and kidneys [66,67]. In another study, D- gal-induced oxidative stress in rat brains was proportional to the reduction in expression and activity of SOD and CAT [68,69]. However, the reason for our contrasting results, where the levels of CAT were high in both D- gal and 10 mM AA + D- gal co-treated groups, remains to be understood. AA has shown differential action on the CAT activity depending on the cell type. In rapidly proliferating mammalian cells, especially cancer cells, higher concentrations of AA have shown a reduction in CAT activity and an increase in H_2O_2 levels, allowing the enzyme to perform its signaling function [70,

71]. However, non-neoplastic cells like keratinocytes significantly enhance CAT activity, increasing their antioxidant potential due to their peripheral location in the skin [50]. Following UV-induced oxidative stress, AA has been shown to affect CAT activity in different skin cells in various ways. In UV-irradiated keratinocytes, AA stimulates CAT activity and protects cells against H_2O_2 overexpression, while this effect is not observed in UV-irradiated skin fibroblasts [50].

Figure 6. CAT activity (in mmol H_2O_2 reduced/min/mg Protein) in *M. domestica* larva



Values are shown as means ± SD (n=10).
Significance is based on p<0.05 compared with Control group values.
* p<0.05, **= p<0.01, ***= p<0.001

In insect cells, the elimination of H_2O_2 is assessed based on CAT and/or APOX activity [72]. APOX enzymes utilize ascorbate as a substrate for the detoxification of peroxides, including H_2O_2 [73]. Consequently, they facilitate the electron transfer process from ascorbate to peroxide, producing dehydroascorbate and water [73]. The D- gal treatment significantly increased APOX activity three-fold, while the D- gal + 10 mM AA group showed a two-fold increase, with no significant difference observed between the control and 10 mM Asc groups (Figure 7). APOX activation in D- gal-treated larval tissue suggests its function in the elimination of intracellular H_2O_2 residues that CAT is unable to remove.

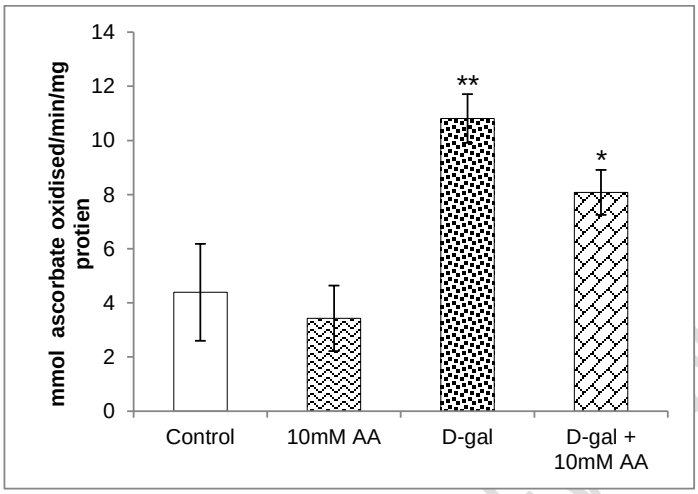


Figure 7. APOX activity (mmol ascorbate oxidized /min/mg Protein) in *M. domestica* larvae

Values are shown as means ± SD (n=10).

Significance is based on p<0.05 compared with Control group values.

* p<0.05, **= p<0.01, ***= p<0.001

MDA levels, a biomarker for lipid peroxidation, were dramatically elevated (two-fold) in D- gal-treated *M. domestica* larvae and marginally (but not significantly) decreased in a group co-treated with ascorbic acid (Figure 8). The remaining groups exhibited no notable differences in their MDA levels. The lipids in the bilayer and non-membrane-forming lipids frequently experience oxidative reactions, resulting in the production of lipid peroxidation byproducts such as reactive aldehydes, e.g., 4-hydroxynonenal (4-HNE) and MDA, along with cyclization derivatives [74]. Lipid peroxidation impairs the membrane fluidity and integrity, thus severely affecting cell physiology and pathology [75]. AA is essential for inhibiting lipid peroxidation and effectively protects the cells from increased levels of 4-HNE and MDA during stress conditions, such as UV-induced oxidative stress in skin cells, the toxic effects of xenobiotics in liver cells, and oxidative stress-associated myonecrosis [59]. Reduced lipid peroxidation levels in the AA co-treated group compared to the D- gal alone treated group are indicative of its antioxidant effects.

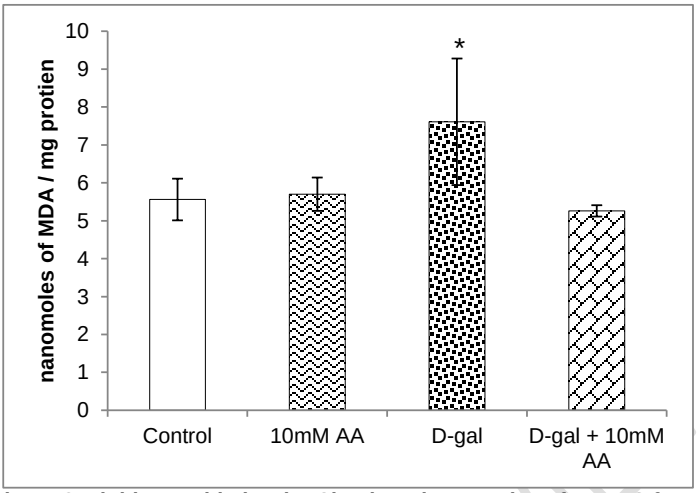


Figure 8. Lipid Peroxidation (LPO) values (nanomoles of TBARS formed /mg Protein) in *M. domestica* larvae

Values are shown as means ± SD (n=10).

Significance is based on p<0.05 compared with Control group values.

* p<0.05, **= p<0.01, ***= p<0.001

PCC is an irreversible post-translational modification that arises owing to the interaction between ROS and the side chains of specific amino acids, such as lysine, arginine, proline, and threonine [76]. Carbonylated proteins may form cytotoxic aggregates that are associated with Parkinson's disease, Alzheimer's disease, and cancer [76, 77]. Protein carbonyls are the markers of protein oxidation [78]. In this study, PCC levels of the control and 10 mM AA-treated groups showed no significant difference, but a three-fold rise in PCC levels was observed in the D- gal group, whereas a two-fold rise was observed in the D- gal + 10 mM AA co-treated groups. (Figure 9). The elevation of PCC values in D-gal-treated groups may stem from the detrimental impact of oxidative stress induced by D- gal on proteins [79]. ROS can interact with various amino acids, resulting in the formation of oxidatively modified proteins with impaired function [76]. Methionine/cysteine oxidation, carbonyl group formation, and cross-linking are commonly found in ROS-induced modifications [76]. Most of these modifications are irreversible, and the proteolysis of these malformed proteins becomes a tenuous task due to damage in the proteasome functioning under oxidative stress. Ascorbate may eliminate various proteasome inhibitors and promote the removal of oxidatively damaged proteins [80, 81]. However, this activity is strictly concentration-dependent [80, 81].

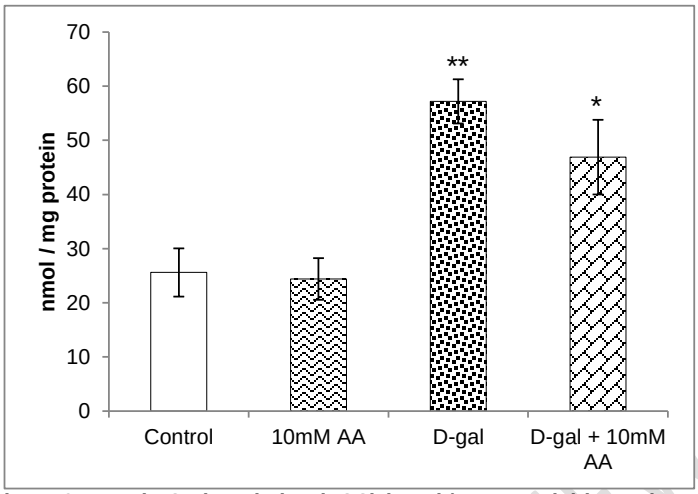


Figure 9. Protein Carbonylation (PCC) (nmol / mg protein) in *M. domestica* larvae

Values are shown as means ± SD (n=10).
Significance is based on p<0.05 compared with Control group values.
* p<0.05, **= p<0.01, ***= p<0.001.

3.3 Effect of ascorbic acid and D- gal on pupal viability and percent eclosion studies:

Our results indicated no significant variation in survival rates of the larvae across four treatment groups. However, there were variations in pupation percentages [Figure 10]. The larval group treated with D- gal showed a significant reduction (9.38%) in pupation rate compared to the control group. The D- gal + 10 mM AA co-treated group had a lower percentage (6.81%) but was not significantly different from the control group. The group treated with 10 mM AA had a larger pupation percentage (70.27%). The percentage of eclosion was used to determine the survival of the pupa. In comparison to the control, the observations indicated a significant decrease in the eclosion percentage of pupae treated with D- gal (7.27%) relative to those treated with D- gal + 10 mM AA (5%). Although the eclosion percentage in the group treated with 10 mM AA was greater (5.23%), it was not significantly different from that of the control group (Figure 11). Our results showing higher pupation percentage in AA treated group are in coherence with the outcomes of Jumbo-Lucioni et al. (2013), where similar outcomes in D. melanogaster were observed [82]. The reduction in adult eclosion percentage of the D- gal treated group is indicative of its adverse effect on pupal survival while larval survival remained unaffected. While the pupation rate of the Control and D- gal + 10 mM AA co-treated groups did not differ, ascorbic acid was observed to boost the percent pupation rate of *M. domestica*. Thus our observations suggest that larval supplementation affected the rate of eclosion.

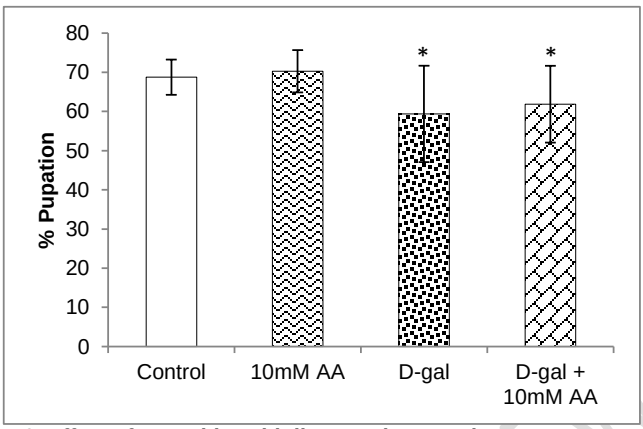


Figure 10. Effect of Ascorbic acid diet supplementation on percentage (%) pupation in *M. domestica* larvae
(The bars represent mean of live pupae ± SE. *p < 0.05 vs. control group.)

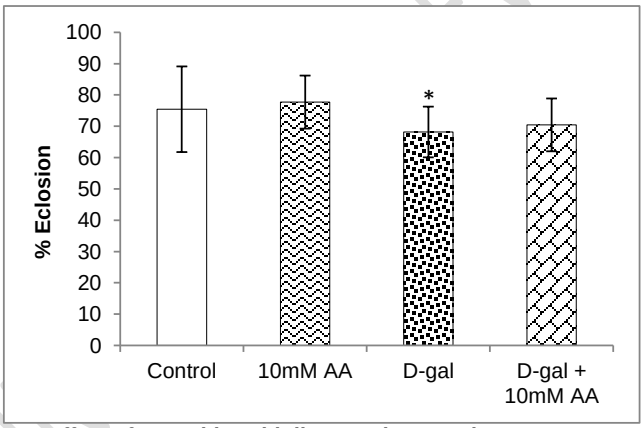


Figure 11. Effect of Ascorbic acid diet supplementation on percentage (%) eclosion in *M. domestica* larvae (The bars represent mean of adult eclosion form pupae ± SE. *p < 0.05 vs. control group.)

In this investigation, D- gal ingestion markedly enhanced the activities of all the assessed antioxidant enzymes. Similar findings were observed in the gut compartments of *M. domestica* [26]. However many of the studies have reported a reduction in the levels of SOD, CAT, and GST after administration of D- gal [69, 83]. The increase in the levels of these antioxidant enzymes in D- gal treated larvae would be mainly to curb the levels of increased oxidative radicals, thus protecting biomolecules from damage. Another reason may be because the larvae were exposed to the sub-lethal doses of D- gal and thus may have

developed a hormetic response. We also speculate that the results of the larvae treated with AA doses did not differ from that of the control dose due to the absence of oxidative stress or the cells utilizing the antioxidant capacity of AA instead of triggering the antioxidant network. Several studies advocate the positive effects of AA administration/supplementation on the cells or organisms challenged with a stressor. In a colchicine-induced rat model of Alzheimer's disease, AA administration resulted in recovery of memory impairments and prevented neurodegeneration and neuroinflammation owing to its antioxidant potential [84]. Nevertheless, its higher doses exhibited neurotoxic effects owing to its pro-oxidant nature [84]. The neuroprotective effects of AA and Curcumin were studied in the lead acetate-induced neurotoxicity in rats [85]. Both the antioxidants efficiently antagonized the toxicity via reducing tumor necrosis factor-α (TNF- α), interleukin-6, caspase-3, and MDA levels, while upregulating the C/EBP homologous protein and mammalian target of rapamycin kinase, thereby showing antioxidant and antiapoptotic properties [85]. Dietary supplementation of AA has been hypothesized to decelerate the progression of Parkinson's disease via antioxidant mechanisms; however, no significant associations have been found [86]. The higher AA supplementations improved the expression of anabolic, myogenic, and oxidative metabolism genes and also antioxidant enzyme activity [86]. In albino rats challenged with azithromycin and etoricoxib, a notable elevation in serum glutathione (GSH) and glutathione peroxidase (GPx), alongside a reduction in MDA, was noted post-AA treatment [87]. Furthermore, AA also repaired the liver histomorphological impairment in the liver caused by azithromycin and etoricoxib [87]. In the salivary glands of diabetic rats, it enhanced the activity of the antioxidant system without lipid peroxidation, it also led to decreased levels of O^{-2} [88]. In the parotid glands of albino rats exposed to silver nanoparticles, AA administration ameliorated the toxicity and degeneration [89]. In one of the studies, addition of antioxidants such as Uric acid and AA to stored red blood cells helped to maintain the quality parameters, protected them from oxidative defects, and enhanced the antioxidant power [90]. Various studies in chicken have also proved the efficacy of antioxidant properties of AA either alone or in combination with other antioxidants in various aspects of its development as well as good hatchability [91-93]. AA is also reported to stimulate the production of low molecular weight antioxidants, such as glutathione (GSH), thioredoxin (Trx), coenzyme Q, α-tocopherol, and retinol [59]. AA owing to its antioxidant properties is reported to lower oxidative DNA modification and single-strand breaks, thus reducing the DNA mutations [94]. It elevates GSH levels without influencing the activity of other associated enzymes. In UV-irradiated skin cells, AA led to down-regulation of GSH-Px and GSSG-R with a simultaneous increase in the GSH level [59].

Our observations in alignment with vast literature validated the efficient antioxidant nature of AA. This vital nutrient has proved of utmost benefit in the treatment of many ailments owing mostly to its antioxidant and protective nature.

However, scientists have developed ways to draw benefits from its pro-oxidant properties too. The pro-oxidant properties of AA have been potentially used in the treatment of cancer. Many studies have advocated its anti-cancer properties [22, 23, 95, 96]. The different ways in which AA affects cancer cells to become a potent anticancer agent have been well reviewed [22, 23, 95, 96]. In animals, ascorbate donates an electron to copper and iron metals, producing O^{-2}, an H_2O_2-like ROS. However, a higher concentration of AA is required for this reaction. Ascorbate reacts with transition metal ions, reducing them to ferric and cupric ions, and oxidizing them into ascorbate free radicals. Ascorbate degrades Fe3+/Cu2+ to ferrous/cuprous ions oxidizing into ascorbate free radicals. These ions react with oxygen to generate O^{-2}, which disintegrates into H_2O_2. During the Fenton reaction, H_2O_2 produces hydroxyl peroxide radical ($HO\cdot$), which hampers the cancer cells [23]. Tumor tissues contain proteins in interstitial fluid and extracellular fluid, which contain catalytic metals like iron and copper [97, 98]. Tumor vessels have greater permeability, amplifying metal ion conversion and enhancing AA transformation [97]. Elevated copper levels in cancer make cancer cells vulnerable to ROS-generated selective cytotoxicity [99]. The action of AA has been proven to be selective towards cancer cells and normal cells [23]. The probable reason for its selective nature may arise from already high levels of endogenous ROS and low levels of antioxidants in cancer cells as compared to normal cells [23]. In cancerous cells, relatively lower levels of catalase, glutathione peroxidase, and peroxiredoxins may result in reduced efficacy of the free radical removal [100].

4. CONCLUSION

This work provided significant insights into this transitional behavior of AA in *M. domestica* larvae under normal and oxidative stress conditions emphasizing that its effects are highly concentration-dependent. While lower concentrations of AA can alleviate oxidative stress and enhance survival rates in larvae, higher concentrations lead to increased mortality due to pro-oxidant nature. These findings underscore the importance of carefully considering AA dosage in biological systems to harness its beneficial properties while mitigating its potentially harmful effects.

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