Protective effects of Resveratrol Against Hydrogen Peroxide Induced Oxidative Stress in Model Organism *Tribolium castaneum* (Herbst, 1797)

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DOI: https://dx.doi.org/10.51244/IJRSI.2026.1305000041

Received: 29 April 2026; Accepted: 04 May 2026; Published: 25 May 2026

ABSTRACT

Oxidative stress is a biological condition characterized by an imbalance between free radicals and antioxidants in living organisms. It is triggered by various factors, including environmental pollution, toxins, radiation. Prolonged accumulation of reactive oxygen species (ROS) in the body leads to several chronic pathological conditions. Natural plant-derived polyphenolic compounds act as potent antioxidants by enhancing endogenous defense system and scavenging ROS, thereby reducing cellular damage. Resveratrol, a polyphenolic compound found in peanuts and berries, is known for its strong antioxidant properties. In the present study, the red flour beetle, *Tribolium castaneum*, was selected as an alternative model organism to evaluate the effects of resveratrol under oxidative stress conditions. DPPH scavenging activity, oxidative stress markers (lipid peroxidation and protein carbonyl) and cell viability were assessed in larvae oxidative stress induced by H_2O_2 for 24 h, followed by topical post treated with resveratrol (1mM). *In-vitro* DPPH assay showed lower IC_{50} for resveratrol (16.24 µg/ml) than ascorbic acid (25.96 µg/ml), indicating stronger antioxidant activity. *In-vivo,* DPPH scavenging activity significantly increased after resveratrol treatment (87.83%) compared to control (83.13%) and stressed larvae (81.37%) at 48 h ($p < 0.001$). There was a significant increase in lipid peroxidation and protein carbonyl levels in stress-induced larvae compared to the control and resveratrol treated larvae ($p < 0.001$). MTT assay showed reduced cell viability in H_2O_2-exposed larvae, which was restored by resveratrol. Further studies are needed to elucidate genetic molecular mechanisms of resveratrol in preclinical models, at different concentration to ensure safety and minimize potential adverse effects.

Keywords: Free radicals, Oxidative stress marker, Resveratrol, Cell viability, *Tribolium castaneum*

INTRODUCTION

Oxidative stress is an imbalance between excess production of oxidant and antioxidants during cellular metabolic process, which leads to damage the lipids, proteins, and DNA structure (Stadtman et al., 2004; Volodymyr, 2014). Generally, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) play a major role in contributing to oxidative stress. They are byproducts of cellular redox process driven by environmental pollutants, toxins, UV radiation, and lifestyle habits (smoking and alcohol consumption). ROS and RNS can be divided into two groups: free radicals such as superoxide (O_2^-•), hydroxyl radical (•OH), and Nitric oxide (NO•), and non-free radicals, such as hydrogen peroxide (H_2O_2) and nitrate (NO_3^-). Oxidation of membrane proteins and lipids produces byproducts such as protein carbonyls and malondialdehyde (MDA) which act as marker of oxidative stress (Ayala et al., 2014; Dalle et al., 2003). Excess level of oxidants is associated with several pathological conditions, including, cardiovascular diseases, neurological disorder, atherosclerosis, cancer, and aging (Pham-Huy et al., 2008; Forman and Zhang, 2021)

Plants are an important source of bioactive polyphenolic compounds, including flavonoids, anthocyanin, lignans, and stilbenes. These compounds consist antioxidant properties, and capable to scavenging the free radicals, and help prevent oxidative stress in the body (Zhao, 2015). Resveratrol (3,5,4′- trihydroxoy-trans-stilbene) is a secondary metabolite and a bioactive compound present in grapes, berries and peanuts. It belongs to the stilbenes

group and is a non-flavonoid phytoalexin. The trans-resveratrol form is more abundant than the cis form and less soluble in water (Veerasham, 2012; Akinwumi et al., 2018). Resveratrol activates sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, enhancing mitochondrial functional and promoting cell survival (Wu et al., 2024; Rogina et al., 2024). It is often used in the treatment of neurological and cardiovascular diseases, as well as cancer. Topical application of resveratrol has been shown to protect against UV-induced damage in SKH-mice and may help reduce edema, eczema, lesions, acne, and inflammation (Afaq., et al., 2003; Bozsányi et al., 2026; Szabolcs et al., 2006).

Alternative *in-vivo* models often used in biomedical research such as *Caenorhabditis elegans, Drosophila melanogaster*, and *Danio rerio* (Rollin, 2003; Seung et al., 2022). Insects are increasingly used as alternative models in preclinical research, and the red flour beetle, *Tribolium castaneum (T. castaneum)* is an alternative emergent animal model due to its short life cycle, high fecundity, ease of maintenance and less ethical concerns. Its genome has been fully sequenced and is more representative of higher animals than that of *Drosophila* (Schroder et al., 2008). It is an invaluable model for evaluating toxicological and pharmaceutical studies and as well as for screening transgenerational and epigenetic risk factors that are difficult to screen in humans (Campbell et al., 2022; Bingsohn et al., 2016).

Resveratrol exhibits a double-edged sword, act as a pro-oxidant properties and toxic effects depending on the dosage level and route of administration (Salehi et al., 2018). Further need more *in-vivo* studies are required to better understand and mitigate side effects. Hydrogen peroxide is a toxic compound and produce ROS, leading to abnormal physiological functions and reduced cell viability (Subedi et al., 2017; Saputra et al., 2024). The current study evaluated oxidative stress marker (lipid peroxidation (LPO), protein carbonyl), DPPH (2,2-Diphenyl-picrylhydrazyl) free radical scavenging activity and cell viability in *T. castaneum*, oxidative stress induced by hydrogen peroxide-induced and followed by topical post-treatment with resveratrol.

MATERIAL & METHODS

Adult insects of *T. castaneum* were obtained from a stock culture and sub-cultured in un-infested whole wheat flour. They were maintained in the insect growth chamber at 29±1ºC and 70-85 % relative humidity. Healthy fourth instar larvae (weight 18±2 mg) were harvested, and randomly selected for the further bioassays.

Induction of oxidative stress and post treatment with resveratrol

Fourth instar larvae were transferred using a fine brush to Borosil glass Petri dish (100 mm diameter) and subjected to starvation for 24 h. Fumigant sub-lethal acute toxicity of hydrogen peroxide was determined by Chaubey et al., 2008 with slight modifications. Borosil@ filter paper (90 mm diameter) was impregnated with different concentration of hydrogen peroxide (50, 100, 150, 200, and 250 µl) and placed inside the glass Petri dish. The solvent was allowed to evaporate for a few seconds. Ten larvae were then placed in each Petri dish and sealed with parafilm. The sub-lethal concentration (LC_{20}- 155 µl) of hydrogen peroxide (H_2O_2) was determined for 24 h, while non-exposed with H_2O_2 larvae were used as the control group.

Post-treatment with resveratrol was performed following oxidative stress induction. After 24 h of H_2O_2 exposure, larvae were topical post treatment with 2 µL of resveratrol (>99 % HPLC, CAS No. 501-63-0) (prepared in 1% DMSO) against H_2O_2 exposed larvae according to Murray *el al*., 2020 with slight modifications. Doses of 0.5, 1, and 2 mM were tested against H_2O_2 exposed larvae. The H_2O_2- exposed larvae showed sluggish movement. The 1 mM dose of resveratrol showed effective recovery of stressed larvae, therefore, 1mM was selected for subsequent assays. Meanwhile, both the H_2O_2 exposed larval group (without resveratrol treatment) and the control group were maintained (n=3).

DPPH assay from larval homogenate

The method followed by according to the method of Karakus et al., 2026 with slight modifications. The larvae ere anesthetized by kept at 4ºC for few minutes and homogenized with 70% ethanol using mortar and pestle. The homogenized samples were centrifuged at 10,000 rpm for 10-15 min. An aliquot of 0.5 ml of supernatant mixed with 1 ml of ethanol and added with equal volume of 0.1 mM of DPPH (DPPH solution prepared in ethanol). The reaction mixture was incubated in the dark at room temperature for 20-30 min. DPPH solution in

ethanol was used as the control, and ethanol alone served as the blank. The absorbance rate was measured at 517 nm against the blank.

$$\text{Free radical scavenging activity (\%)} = \frac{\text{OD control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

DPPH-in-vitro Assay

The method followed by according to the method of Brand et al., 1995. Different concertation of resveratrol 5, 10, 20, 40 and 80 µg/ml were prepared and made up to 1ml using ethanol. Ascorbic acid was prepared and used as the standard in the same way. To each sample, 1 ml of DPPH (0.1mM) solution was added. A blank solution was prepared containing the same amount of ethanol and DPPH. The mixture was incubated at room temperature for 30 min, and absorbance was measured at 517 nm.

$$\text{Free radical scavenging activity (\%)} = \frac{A - B}{A} \times 100$$

Where A is absorbance rate of blank, B is the absorbance of the sample.

Lipid peroxidation (LPO)

The method was determined by Devasagayam and Tarachand, 1987. Larvae were collected and washed with PBS to remove debris, the larvae were kept on ice for few min and homogenized with tris HCL buffer (tris hydrochloric acid, 7.4 pH). The homogenate was centrifuged 10,000 rpm for 4 ºC. 0.2 ml of supernatant mixed with 0.5 ml of tris-HCl buffer (0.15M, pH 7.4) and 0.15 ml 10 mM dipotassium phosphate (KH_2PO_4). Added 0.4 ml distilled water in the mixture and incubated at 37 ºC for 20 min with constant shaking. The reaction was stopped by the addition of 1 ml 10% TCA (Trichloroacetic acid). 0.75 ml of TBA (thiobarbituric acid) (0.67%) was added and kept in boiling water bath for 30 min. The tubes were centrifuged and supernatant was collected. The absorbance was measured at 532 nm. The content of malonodialdehyde expressed as nanomoles per mg of protein, and the formula is given below.

$$\text{Malonodialdehyde (nM per mg of protein)} = \frac{\text{A sample} - \text{A blank (at 532nm)}}{\varepsilon \times L \times \text{Vsample} \times \text{protein (mg)}} \times V_{\text{total sample}}$$

Where A is absorbance, ε – molar extinction coefficient ($1.56 \times 10^{-5} M^{-1}cm^{-1}$ (for MDA-TBA complex), Vtotal sample- Total volume of sample, L-path length, and Vsample -Volume of sample

Protein carbonyl

Protein carbonyl was estimated by according to Levine et al., 1990. A total 200 µl of sample was mixed with 800 µl of DNPH (2,4 dinitrophenyl hydrazine) and 800 µl of 2.5 N HCL. The reaction mixture was incubated at room temperature for 45 min in the dark and vortexed for every 15 min. after incubation 20% TCA was added and mixed well and kept on ice for 5 min. The tubes were centrifuged at 10,000 rpm at 4 ºC. The supernatant was discarded and the pellet was suspended in 1 ml of ethanol: ethyl acetate mixture. The sample were centrifuged at 10,000 rpm for 10 min at 4 ºC. The pellet was washed by the above step was repeated by 2-3 times. After final wash, the pellet was resuspended in 500 µl of 6 M guanidine hydrochloride and mixed well by vortexing. The tubes were centrifuged at 10,000 rpm for 10 min at 4 ºC. The absorbance was measured at 370 nm. The protein carbonyl level expressed as nanomole per milligram of protein and the formula is given below.

$$\text{Protein carbonyl (nM per mg of protein)} = \frac{\text{A sample} - \text{A blank (at 370nm)}}{\varepsilon \times L \times \text{Path length} \times \text{protein concenration(mg)}} \times 10\text{^}6$$

Where A is absorbance, ε- molar extinction coefficient = 22,000 $M^{-1}cm^{-1}$ (for DNPH derivatives)

MTT assay

Determination of cell viability by method of Catalani et al., 2023. Whole larval tissue was homogenized in PBS and centrifuged at 10,000 rpm for 10 min. The supernatant was collected. 1 ml of larval supernatant mixed with 100 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5mg/ml). The mixture was incubated at room temperature for 2-4 h. During incubation, purple formazan crystals formed. After incubation, the supernatant was carefully removed and the crystals were dissolved in 1ml of DMSO. The absorbance was measured at 570 nm using a spectrophotometer.

$$\text{Cell viability (\%)} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

Control: Non-treated larvae, Test: Treated larvae

Statistical analysis:

All the data were analyzed by one way ANOVA using SPSS/20 software. The values were expressed as mean ± standard deviation followed by Tukey's post hoc multiple comparison test. $p < 0.05$ was considered as statistical significance.

RESULTS

Table 1. shows that the acute lethal toxicity of hydrogen peroxide (30%) was evaluated using dose-response analysis, and the result indicates the LC_{20} value was 155.13 µl, with 95% confidence limit of 106.08 µl to 179.08 µl. The R^2 value of 0.955 demonstrates an excellent fit of the dose-response model, explaining nearly all variability in the data.

Table 1. Sub-lethal (LC_{20}) acute toxicity (24 h) of hydrogen peroxide against *T. casatneum* (4^{th} instar larval stage)

Compound	**LC_{50} (µl)**	**LC_{20} (µl)**	**χ^2 (df)**	***R^2***
	95% Confidence limit (LCL-UCL)	**95% Confidence limit** (LCL-UCL)		
Hydrogen Peroxide (30%)	196.97 (167.99 – 236.87)	155.13 (106.08- 179.082)	0.174 (3)	0.995

$\chi 2$ - chi-square test, R^2 - Coefficient of determination, df – Degree of Freedom, LCL- Lower confidence limit, UCL- Upper confidence limit. (No heterogeneity factor was used in calculation of confidence limits).

The results of the *in-vitro* free radical scavenging activity of resveratrol and ascorbic acid (standard) are shown in Figure 1 and 2, respectively. Resveratrol is a potent antioxidant and scavenges free radicals in a dose-dependent manner. The scavenging activity of resveratrol and ascorbic acid was tested at different concentrations such as 5, 10, 20, 40, and 80 µg/ml. Ascorbic acid showed scavenging activities of 30.41, 39.56, 44.74, 70.82, and 81.52 % respectively, while resveratrol showed 37.88%, 43.61%, 50.65%, 80.98%, and 92 %, respectively. These finding revealed that both resveratrol and ascorbic acid effectively scavenging free radicals in a dose-dependent manner. The IC_{50} values of resveratrol and ascorbic acid, which were 16.24 µg/ml and 25.96 µg/ml, respectively, indicating that resveratrol exhibits higher free radical quenching activity at a lower concentration compared to ascorbic acid.

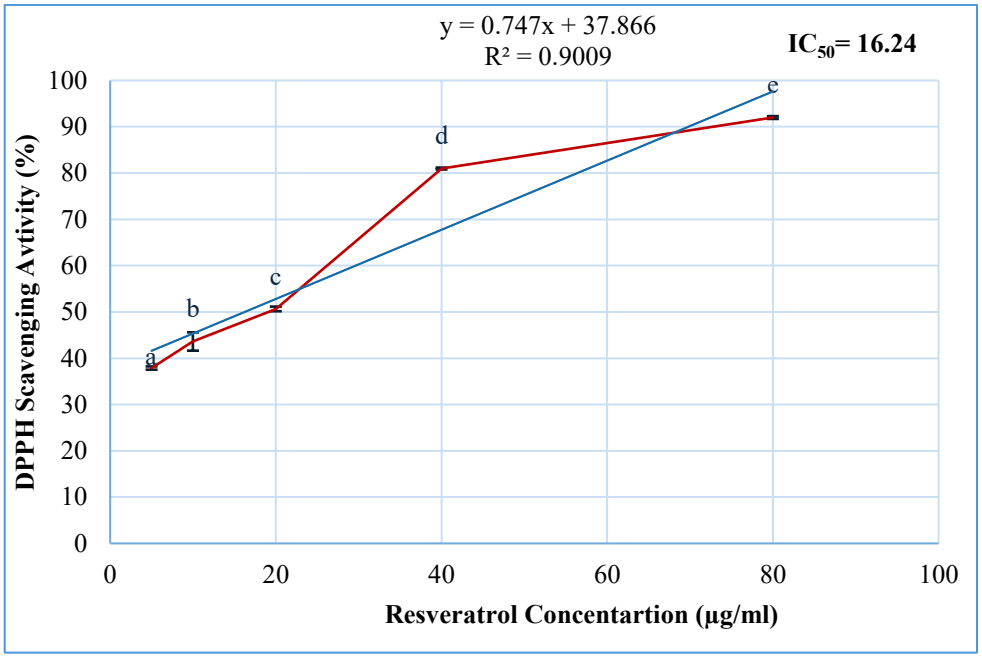

Figure 1. DPPH free radical scavenging activity of resveratrol at different concentration 5 10, 20, 40, and 80 µg/ml. Significant differences were observed at $p < 0.05$ based on Tukey's test. 50% inhibitory concentration (IC_{50}) of resveratrol was 16.96 µg/ml (R^2=0.9009). Different letters (a-e) above the bars indicate significant differences at $p < 0.05$ based on Tukey's test. Data are presented as mean ± SD (n=3).

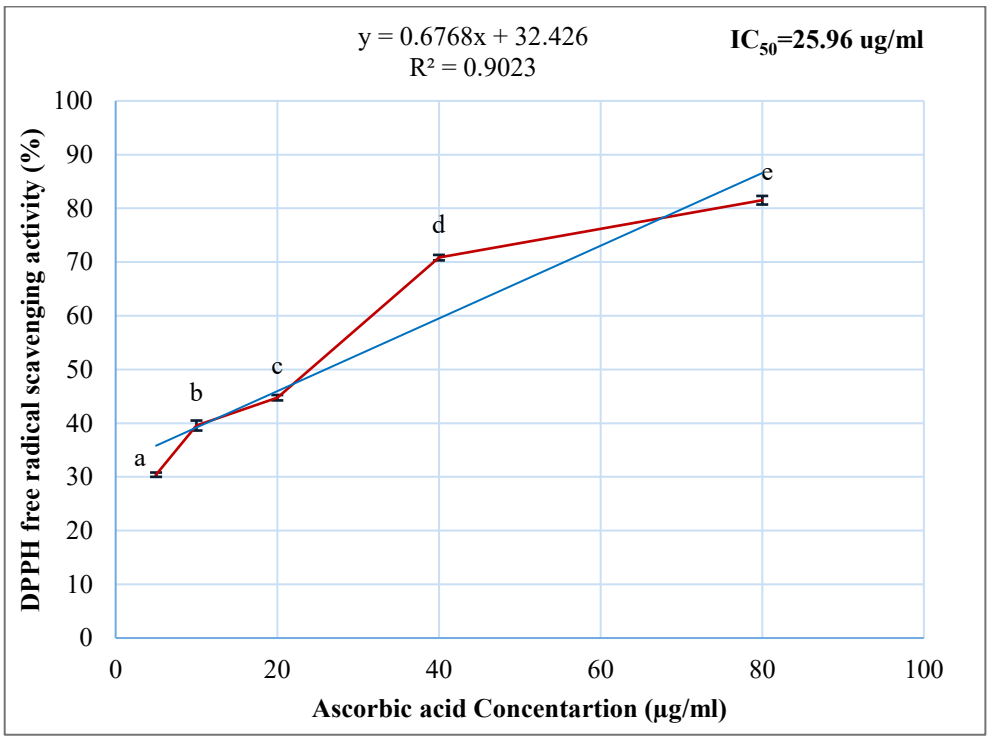

Figure 2. DPPH free radical scavenging activity of ascorbic acid at different concentration 5, 10, 20, 40, and 80 µg/ml. significant differences were observed at $p < 0.05$ based on Tukey's test. 50% inhibitory concentration (IC_{50}) of ascorbic acid was 25.96 µg/ml (R^2=0.9023). Different letters (a-e) above the bars indicate significant differences at $p < 0.05$ based on Tukey's test. Data are presented as mean ± SD (n=3).

The DPPH• free radical scavenging activity of *T. castaneum* fourth instar larvae was assessed under sub-lethal acute oxidative stress induced by H_2O_2 and compared with control larvae at 24 h. Furthermore, larvae were topically post-treated with resveratrol following H_2O_2 exposure, and the activity was evaluated at 48 h (Figure 3). The results demonstrated a reduction in free radical scavenging activity in oxidative-stress induced larvae at 24 h and 48 h, recording as 84.31% and 81.37% compared to the control group such as 85.6% and 83.13%

respectively. Meanwhile, the resveratrol-treated larvae exhibited a statistically significant enhancement in DPPH radical quenching activity (87.83%) compared to both H_2O_2-exposed ($p < 0.05$).

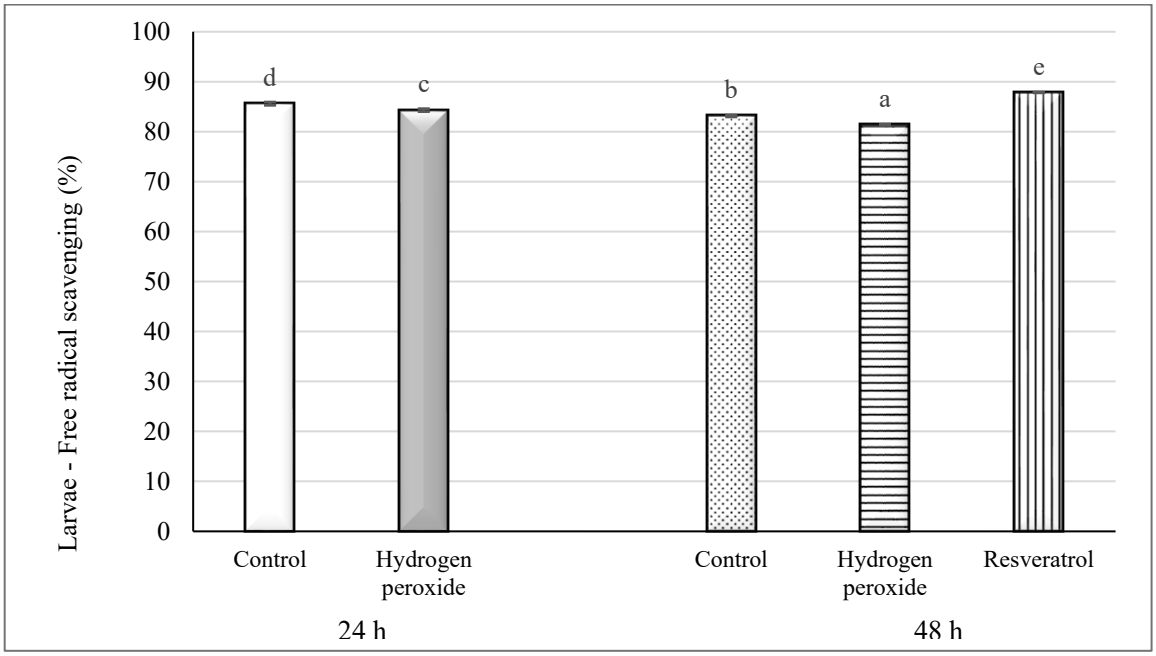

Figure 3. DPPH free radical scavenging activity in larval homogenates measured in control and oxidative stress state induced by H_2O_2 and post-treatment with resveratrol. Different letters (a-e) above the bars indicate significant differences at $p < 0.05$ based on Tukey's test. Data are presented as mean ± SD (n=3).

Figure 4. Shows that level of MDA significantly increased in H_2O_2- exposed larvae at 24 h and 48 h (15.27 and 37.22 nmol/mg of protein, respectively) compared to the control (4.55 and 12.08 nmol/mg of protein respectively). Post-treatment with resveratrol, the MDA level reduced to 13.49 nmol/mg of protein.

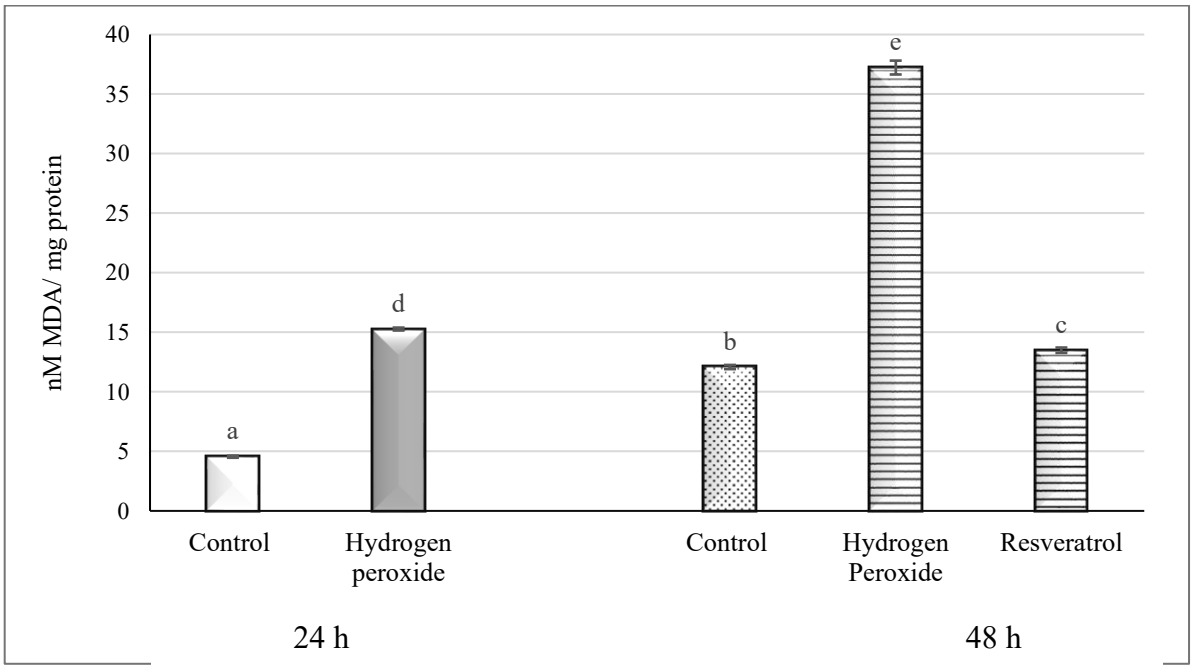

Figure 4. The level of LPO in larval homogenates measured in control and oxidative stress state induced by H_2O_2 at 24 and 48 h, including post-treatment with resveratrol. Different letters (a-e) above the bars indicate significant differences at $p < 0.05$ based on Tukey's test. Data are presented as mean ± SD (n=3).

Figure 5. Shows that protein carbonyl levels significantly increased in H2O2- exposed larvae at 24 h and 48 h (20.91 and 29.64 nmol/mg of protein, respectively) compared to the control (5.67 and 14.41 nmol/mg of protein respectively). Post-treatment with resveratrol declined level to 13.1 nmol/mg of protein.

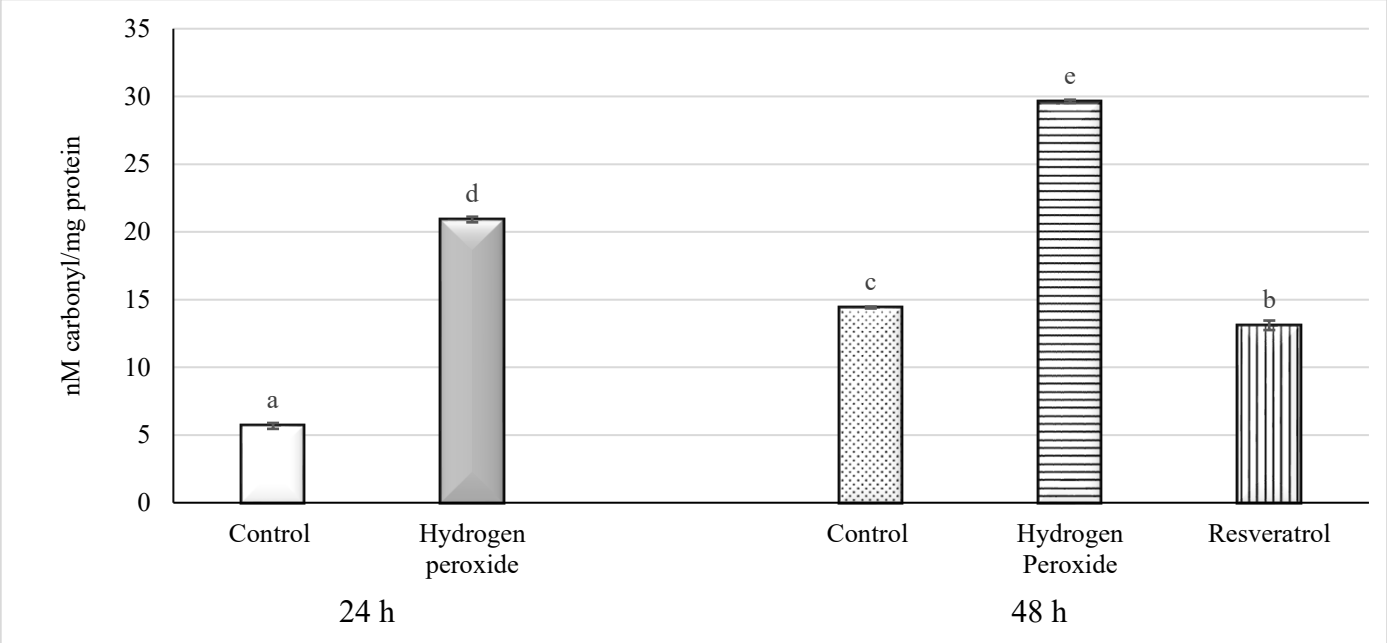

Figure 5. Protein carbonyl content in larval homogenates measured in control and oxidative stress state induced by H_2O_2 at 24 and 48 h, including post-treatment with resveratrol. Different letters (a-e) above the bars indicate significant differences at $p < 0.05$ based on Tukey's test. Data are presented as mean ± SD (n=3).

Figure 6. Shows cell viability assessed by the MTT assay, with the control larval group denoted as 100%. The cell viability of the H_2O_2- exposed larval group was significantly reduced at 24 and 48 h compared to the control, reaching 22.75% and 29.08% respectively. However, topical post-treatment with resveratrol rejuvenated the cells and increasing viability to 51.7% compared to the oxidative stress-exposed larvae at 48 h.

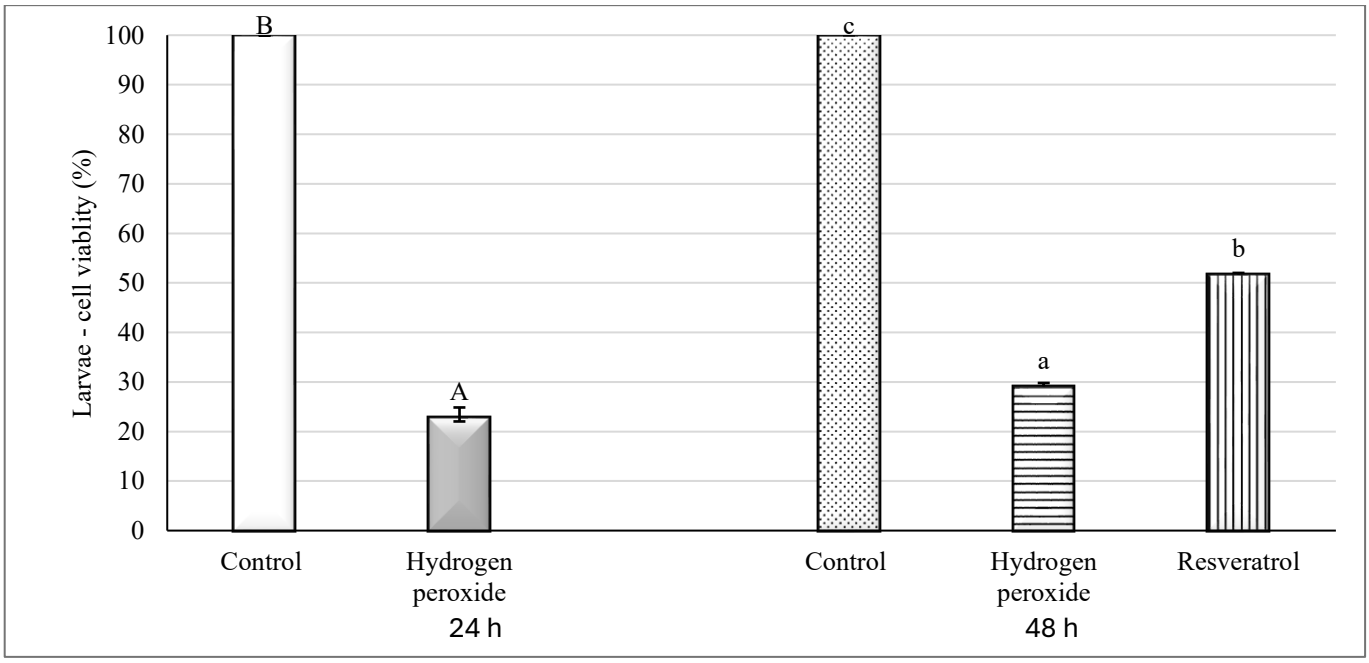

Figure 6. Cell viability (%) determined by MTT assay in larval homogenates under control conditions and acute oxidative stress induced by H_2O_2 at 24 h. Letters A and B denoted significant differences between groups at 24 h. Cell viability (%) in Control, H_2O_2 exposed group as well as following post-

treatment with resveratrol at 48 h were assessed. Different letters (a, b, and c) above the bars indicate significant differences at $p < 0.05$ based on Tukey's test. Data are presented as mean ± SD (n=3).

DISCUSSION

Oxidative stress is an endogenous physiological state in biological system; however, prolonged exposure of environmental physical and chemical toxicants leads to negative impact on the organism. An imbalance between the pro-oxidants and antioxidants leads to several acute and chronic pathological conditions. Topical application of natural extract and antioxidant serums neutralize ROS before and after exposure with blue light in human skin (Coat et al., 2021).

Topical application of resveratrol has been reducing hyperpigmentation in guinea pig exposed to UV radiation (Lee et al., 2014). The present study investigated the DPPH free radical quenching activity of resveratrol and its ameliorative effects against H_2O_2 induced oxidative stress in *T. castaneum* fourth instar larvae.

Hermanto et al., 2024), stated that resveratrol can reduce ROS levels, with an inhibitory concentration (IC_{50}) is 32.10 µM, indicating very strong free radical scavenging activity. Resveratrol's phenolic hydroxyl group (at the 3, 5, and 4' positions) donate hydrogen atoms to neutralize radicals and inhibit DPPH. The present findings also revealed that resveratrol exhibited *in-vitro* DPPH• free radical scavenging activity, effectively quenching DPPH with a low IC_{50} value (16.24 µg/ml) than ascorbic acid (25.96 µg/ml).

H_2O_2 induces oxidative stress by increasing ROS in *Drosophila melanogaster*. The exposure of H_2O_2 stimulates excess production of ROS and impairs cellular function (Subedi et al., 2017). Our findings demonstrated that a reduction in DPPH free radical scavenging activity in whole larval homogenates of (fourth instar) *T. castaneum* after 24 h exposure to an acute sub-lethal concentration H_2O_2. Subsequent topical post-treatment with resveratrol significantly enhanced DPPH• radical scavenging activity indicating that resveratrol mitigates oxidative stress and helps restore the antioxidant defense system in the model organism.

Lipid are essential macromolecules for maintain the integrity of cell membranes in the organisms. The chemicals and toxicants exposure leads to membrane damage through free radical generation, lipid peroxidation, and protein oxidation (Danieli et al., 2023; Elsaesser and Howard 2012). Malondialdehyde MDA, a main byproduct of LPO. These compounds have cytotoxic properties contributes to the acute and chronic pathological conditions (Aguilar et al., 2020; Tsikas, 2017; Dalle-Donne et al., 2006). The present finding revealed that MDA level was significantly increased in H_2O_2 exposed larvae at 24 h and 48 h compared to the control, which can be evidenced by the increase in the LPO in acute oxidative stressed larvae. Post treatment with resveratrol reduces the MDA and rejuvenates oxidative stress induced by H_2O_2.

Proteins carbonyl is oxidized form of proteins by the free radicals. It is the most commonly used marker for oxidative stress. (Dalle-Donne et al., 2003). In our study, the Protein carbonyl was significantly increased in the H_2O_2 exposed larval groups indicating the protein damage due to oxidative stress, while resveratrol treated group were significantly decreased the protein carbonyl level suggesting the polyphenolic compound prevent protein damage. Pandey et al., 2009 was also observed same protective effects of resveratrol on formation of protein carbonyls.

We evaluated cell viability in stress-induced larvae, following topical-post treatment with resveratrol (1mM). Huang et al., 2016 stated that resveratrol mitigates the cytotoxicity induced by radioactive agent ioxitalamate in HK-2 human renal proximal tubule cell. The present finding of MTT assay revealed that the cell viability significantly decreased in H_2O_2 exposed larvae at 24 h and 48 h compared to the control and post treatment with resveratrol, indicates the polyphenolic compound mitigates the cytotoxic effect induced by H_2O_2.

CONCLUSION

The findings of this study revealed that *T. castaneum* serves as a promising alternative model organism to vertebrate for preliminary pharmaceutical and toxicological investigations. H_2O_2-induced oxidative stress significantly increased the oxidative stress marker, reduced cell viability and DPPH free radical scavenging

activity in *T. castaneum* larvae. However, post-treatment with resveratrol significantly improved cell survival and reduced protein carbonyl and lipid peroxidation indicating its protective and restorative effects against oxidative stress. The present study has certain limitations, as only a single concentration of resveratrol (1mM) was evaluated in the model organism under oxidative stress. Further investigations are required to explore pro-oxidant and antioxidant properties of resveratrol and its dose-dependent effects and underlying genetic and molecular mechanisms in biological to better understand its pharmacological applications

ACKNOWLEDGEMENT

We gratefully acknowledge the ANRF (DST-SERB), Govt of India for providing grant for this study (SERB sanction order no: EEQ/2021/001049). We thank to the Department of Zoology, Bharathiar University, Coimbatore, Tamil Nadu, India for providing essential facilities needed for this study.

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