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Antioxidant effects of resveratrol against hydrogen peroxide induced oxidative stress in *Tribolium castaneum* (Herbst, 1797)

Sasikala Kalichamy^{1*} and Gunasekaran Chinnappan^{1*}

Abstract

Background Oxidative stress results from an imbalance between reactive oxygen species (ROS) and antioxidant. In the present scenario, knowingly and unknowingly, exposure to physical (UV radiation), chemical pollutants and toxicants triggers the excess production of ROS leading to an imbalance in the antioxidants, resulting in deleterious effects on biological systems. Plant-derived polyphenols, including resveratrol, possess potent antioxidant properties and may mitigate the impact of ROS, thereby protecting cells from oxidative damage. In this study, oxidative stress was induced in 4th instar larvae of *Tribolium castaneum* using a sub-lethal acute toxicity ($LC_{20} = 155 \mu\text{l}$) of hydrogen peroxide (H_2O_2) for 24 h. Following exposure, the larvae were topically post-treated with resveratrol (1 mM) for 24 h. The protective effects of resveratrol against H_2O_2 -induced oxidative stress were evaluated through the assessment of enzymatic (SOD, CAT, GPx and GST) and non-enzymatic (GSH and Vitamin C) antioxidant levels in the organism.

Results The total protein content and the activities of first-line antioxidant enzymes (SOD, CAT and GPx) were significantly reduced in H_2O_2 -exposed larvae compared with the control group. Post-treatment with resveratrol (1 mM) significantly increased total protein level and antioxidant enzyme activities relative to H_2O_2 -exposed larvae at 48 h. Levels of second-line antioxidant defenses, including GSH, GST and vitamin C, were increased in H_2O_2 -exposed larvae compared with the control group. Resveratrol treatment significantly elevated GST activity and GSH levels compared with the H_2O_2 -exposed group. In contrast, vitamin C levels showed a slight decrease in the resveratrol-treated larvae at 48 h, however, this was reduction was not statistically significant.

Conclusion The present findings reveal that resveratrol reactivates the first-line defense antioxidant system (SOD, CAT and GPx), which was declined in the H_2O_2 -exposed larvae of *T. castaneum* at 48 h. Second-line antioxidants (GST, GSH and vitamin C) were elevated in H_2O_2 -exposed larvae and further enhanced following resveratrol treatment, suggesting antioxidant effects of resveratrol attenuate the ROS, and prevent deleterious effects in the biological system. Further studies are required at the molecular gene expression level to elucidate the underlying mechanisms of resveratrol in the model organism.

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Keywords Hydrogen peroxide, Reactive oxygen species, Oxidative stress, Antioxidant, Resveratrol, Emergent model, *T. castaneum*

Background

Exposure to environmental pollutants, chemical toxicants and UV radiation induces oxidative stress in living organisms, disrupts the homeostasis of the biological system and accelerates the aging process (Peoples et al., 2019). The excessive production of reactive oxygen species (ROS) affects biomolecules such as lipids, proteins and DNA (Liguori et al., 2018), leading to several chronic pathological conditions, including diabetes, cardiovascular disease, cancer, neurological disorders and promoting aging (Liguori et al., 2018; Gulcin, 2020; Halliwell, 2020).

Antioxidant enzymes including Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are place crucial role in maintaining cellular redox homeostasis by detoxifying ROS (Ighodaro & Akinloye, 2018). Glutathione S-transferase (GST) promotes detoxification by conjugating of reduced glutathione (GSH) to reactive electrophilic compounds, while GSH and Vitamin C act as water soluble antioxidants that scavenges ROS and inhibit free radical chain reactions (Ighodaro & Akinloye, 2018; Phaniendra et al., 2015).

Hydrogen peroxide (H_2O_2) is widely used in industry and households (e.g., disinfectants, textiles, and bleaching) yet it can cause environmental contamination and occupational exposure risks (Agency for Toxic Substances and Disease Registry, 2026). Exposure may lead to respiratory, skin, and eye irritation, with severe toxicity at high concentrations. It also generates ROS, disrupting cellular function. Consequently, H_2O_2 is widely used as an oxidative stress inducer in model organisms to study oxidative stress responses (Subedi et al., 2017; Saputra et al., 2024). In the present scenario, knowingly or unknowingly, exposure to physical and chemical pollutants promotes excessive production of ROS, disrupting the antioxidant defense system and resulting in deleterious effects on biological system.

Polyphenols are naturally occurring bioactive compounds found in fruits, vegetables, legumes, and cereals. They are synthesized through the shikimic acid and phenylpropanoid pathways and contribute to plant defense against environmental stress conditions (Zhao, 2015). Polyphenols are classified into phenolic alcohols, phenolic acids, stilbenoids, flavonoids and lignans. These compounds act as antioxidants by scavenging free radicals and preventing the adverse effects of ROS in living organisms (Gan et al., 2018; Scalbert et al., 2005).

Plant-based natural compounds are an important source of drug discovery in pharmacological studies (Paul & Ma, 2011; Veerasham, 2012). Resveratrol (3,5,4'-trihydroxy -trans-stilbene) is a polyphenolic, antioxidant

compound first isolated by Takaoka from *Veratrum grandiflorum* root in 1939 (Takaoka, 1940). It is present in grape skin, mulberries, peanuts and red grape juice (Burns et al., 2002; Meng et al., 2021). The formation of resveratrol in plants is influenced by climatic conditions and the plant's resistance (Jeandet et al., 2002; Langcake & Pryce, 1977). It exhibits antioxidant, anti-inflammatory, antidiabetic, anticancer activities in experimental models (Halliwell & Gutteridge, 2015; Wong & Fiscus, 2015; Yang et al., 2022). Topical application of resveratrol induces antioxidant activity in human epidermal tissues by activating SIRT1 pathways and promoting tissue repair in case of inflammation and injuries (Pastore et al., 2012; Alonso et al., 2017; Christovam et al., 2019). However, translation of these findings into clinical practice remains controversial. It is often regarded as a "double-edged sword" exhibiting both beneficial and potentially adverse or context-dependent effects in the disease, dose and treatment duration (Salehi et al., 2018). Therefore, additional in-vitro and in-vivo studies are required to elucidate its underlying mechanisms in the biological system and to confirm its safety in pharmaceutical applications (Novelle et al., 2015; Liu et al., 2025; Wu et al., 2026).

Animal model organisms are frequently used in biomedical research. However, there are many limitations to using vertebrate models (such as mice, rats and primates) and there is an urgent need for alternative animal models for biomedical research to ensure the reduction, refinement and replacement (3Rs) of vertebrates (Wilson-Sanders, 2011). Over the last decade, scientific research has focused on insects, the largest and most widely distributed group in the animal kingdom. The red flour beetle, *Tribolium castaneum* (*T. castaneum*) (Herbst, 1797), (Order: Coleoptera), whose genome was sequenced (Tribolium Genome Sequencing Consortium, 2008), has emerged as an alternative animal model due to high fecundity, short generation gap, and being less expensive to rear and maintain (Knorr et al., 2013). It has been widely used in biomedical, toxicological studies and food gene interactions (Adamski et al., 2019; Pajaro et al., 2019; Bingsohn et al., 2016; Grunwald et al., 2013). The stress response pathways of *T. castaneum* make it a valuable model organism for investigating stress associated mechanisms and the underlying molecular pathways involved in adaptation (Freitak et al., 2012; Scharf et al., 2015; Schroder et al., 2008).

The present study investigated the antioxidant effects of resveratrol in fourth-instar larvae of *T. castaneum* following 24h exposure to H_2O_2 and subsequent topical post-treatment with resveratrol. This was achieved by the

evaluating of enzymatic antioxidants (SOD, CAT, GPx, and GST) and levels of non-enzymatic antioxidants (GSH and vitamin C) involved in the oxidative stress response induced by H_2O_2 .

Materials and methods

Insect rearing

The culture of *T. castaneum* adult beetles (Herbst) was procured from the Department of Entomology, Tamil Nadu Agricultural University, Coimbatore. The stock culture was prepared using 250 adult beetles, which were sub-cultured in 500 g of uninfested whole wheat flour mixed with 5% brewer's yeast under laboratory conditions. The cultures were maintained in a plastic jars covered with muslin cloth to ensure ventilation, prevent the escape of the insects and avoid the entry of other insects. The cultures were maintained under controlled conditions in an insect growth chamber (Model P-lab Make) at $29 \pm 2^\circ C$ with $75 \pm 5\%$ relative humidity (RH). The 4th instar larvae of *T. castaneum* (1.8 ± 2 mg, 18 ± 2 days old) were used for further experiments (Knorr et al., 2009).

Assessment of 24 h lethal concentration of H_2O_2

Fumigant exposure with H_2O_2 against fourth-instar larvae of *T. castaneum* and assessment of lethal concentration (LC_{20} & LC_{50}) according to method of Chaubey (2011) with slight modifications. Whatman Filter papers (diameter 7.0 cm) were impregnated with 50, 100, 150,

200 and 250 μ l concentration of hydrogen peroxide (30%) (Product No. 1.93407.0521, Emplura $^{\circ}$ grade) and placed in sterile glass Petri dish (100 mm diameter, 17 mm height). The solvent was allowed to evaporate for 30 seconds before larvae (ten) were placed in the Petri dish and the lid was sealed with parafilm. Non-treated H_2O_2 was used as a control. Three replicates were prepared for each treatment and control. The number of dead larvae was counted at 24 h after exposure. Mortality was determined based on the absence of movement and unresponsiveness to touch with a paintbrush. The percentage of mortality was calculated using Abbot's formula (Abbott, 1925). The lethal concentration of H_2O_2 was determined by Probit analysis using SPSS (Version 20.00).

$$\text{Mortality } P(\%) = \frac{T - C}{100 - C} \times 100$$

Where p = corrected mortality (%), T = percentage mortality of treated, and C = percentage mortality of control.

Post-treatment with resveratrol

Acute oxidative stress (24 h) induced by a sub-lethal concentration (LC_{20} – 155 μ l) of H_2O_2 and the larvae were topically post-treated with resveratrol (2 μ L/larva) (>99% (HPLC, CAS-No: 501–36–0) permeabilization solution prepared with 1% Dimethyl Sulfoxide (DMSO) followed by the method of Murray et al. (2020) with slight

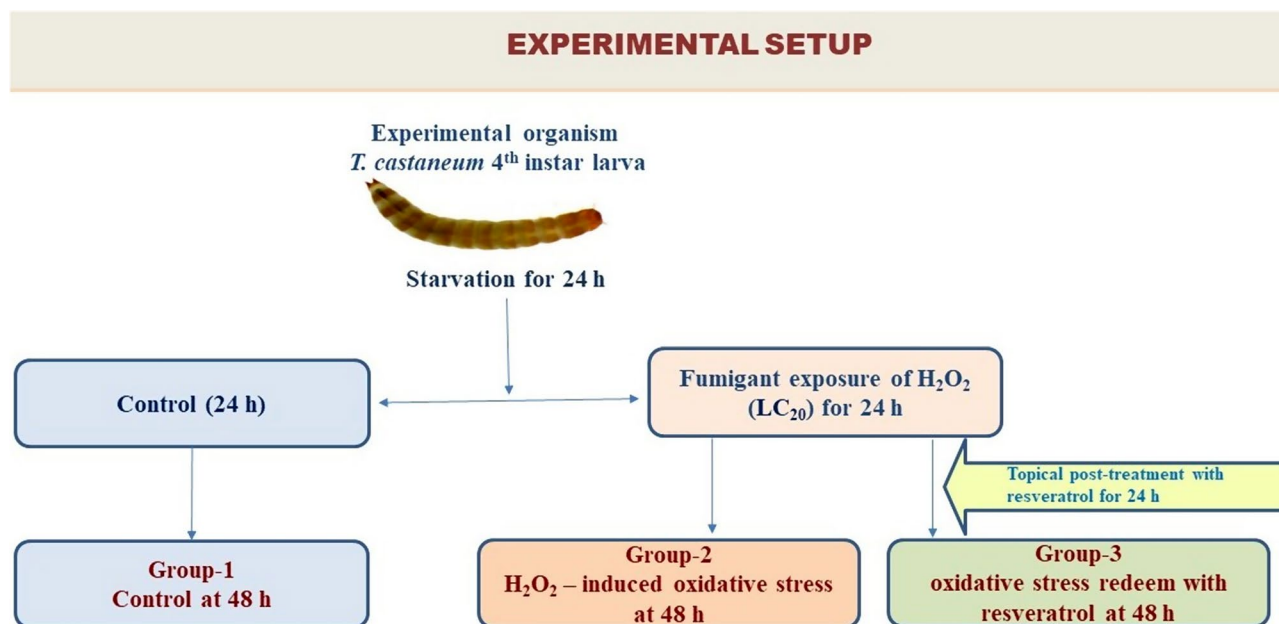


Fig. 1 The experimental groups are shown in Fig. 1. The larvae were starved for 24 h before the experiment. Group 1 served as the untreated control and was sampled at 48 h. Group 2 larvae were exposed to H_2O_2 at the LC_{20} concentration for 24 h to induce oxidative stress and were maintained until 48 h for sampling. Group 3 consisted of H_2O_2 -exposed larvae that received topical post-treatment with resveratrol. All groups were sampled at 48 h to evaluate the protective effect of resveratrol against H_2O_2 -induced oxidative stress

modifications. The dose level of resveratrol was determined by topical post-treatment with resveratrol against H_2O_2 -exposed larvae at different concentrations of resveratrol (0, 0.4, 0.8, 1 and 2 mM). The larvae exhibited abnormal wiggling movements when treated with 0.4 and 0.8 mM of resveratrol, similar to those observed in larvae exposed to H_2O_2 . However, at resveratrol concentration of 1 and 2 mM, the effects of H_2O_2 were reduced, and the larvae exhibited normal movement. Therefore, 1 mM resveratrol was used in further experiments to evaluate its antioxidant effects against H_2O_2 -induced oxidative stress in *T. castaneum* larvae. Larvae that were not exposed to either H_2O_2 or resveratrol were considered the control group (replicates $n=4$).

Determination of total protein level

The larvae were collected after 48 h and anesthetized by kept at 4 °C for 1–2 min and quickly the whole larvae ($n=10$) were homogenized in 50 mM Tris-HCl buffer, (pH 7.4) using mortar and pestle placed on an ice pack. The homogenate was then centrifuged at 10,000 g for 10 min at 4 °C, according to the method of Bamidele et al. (2013), with slight modifications. The supernatant was used to determine the total protein by Bradford's method (Bradford, 1976), with Bovine Serum Albumin (BSA) as the standard.

Determination of antioxidant capacity

Superoxide dismutase (SOD) activity was estimated according to the method of Marklund and Marklund (1974). The reaction mixture contained 0.5 ml of homogenate, 0.25 ml of cold absolute ethanol and 0.15 ml of chloroform. The components were mixed thoroughly, and the suspension was centrifuged for 15 min at 10,000 rpm. The assay mixture contained 2 mL of Tris-HCl buffer, 0.5 mL supernatant, 0.5 mL of pyrogallol, and distilled water to a final volume of 4.5 mL. The auto-oxidation rate of pyrogallol was determined by mixing 2 mL of Tris-HCl buffer, 0.5 mL of pyrogallol, and distilled water to a final volume of 4.5 mL. The blank contained 2.0 mL of Tris-HCl buffer and 2.5 mL of distilled water. The absorbance was measured spectrophotometrically at 420 nm at 1 min intervals for 3 min. The activity of SOD is defined as the amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50% and is expressed as $\mu\text{mol}/\text{min}/\text{ml}$.

The catalase activity was determined by the method of Sinha (1972). A total of 1 mL phosphate buffer (pH 7.0), 0.5 mL of H_2O_2 (30 mM), and 0.1 mL of supernatant were mixed. The reaction was stopped by adding 1 mL of dichromate acetic acid at 0, 30, and 60 seconds, and the mixture was then kept in a boiling water bath for 10–15 min. The rate of absorbance was measured at 590 nm, and the enzyme activity was expressed as $\mu\text{mol}/\text{min}/\text{ml}$.

The Enzymatic GP_x activity was determined by using method of Rotruck et al. (1973). A total of 0.1 ml sodium phosphate buffer (pH 7.0), 0.1 ml 200 mM reduced glutathione (GSH), 0.1 ml H_2O_2 (0.042%) and 0.1 ml of a 1:10 diluted aliquot of the homogenate was taken, and made up to 2.0 ml with distilled water. The tubes were incubated at 37 °C for 3 minutes. The reaction was terminated by adding 0.5 ml of 10% Trichloroacetic acid (TCA), followed by centrifugation at 10,000 rpm for 10 min. From this, 0.5 mL of the supernatant was taken and mixed with 2 mL of disodium hydrogen phosphate solution (0.3 M) and 0.5 mL of the DTNB (5,5' Dithiobis - 2-nitro benzoic acid) reagent. The color developed was read at 412 nm against a blank reagent containing only buffer and the DTNB reagent. Suitable aliquots of the standard were also treated similarly, and enzyme activity was expressed as units per mg of protein.

The activity GST was determined using the method of Habig et al. (1974). Ten larvae were collected and washed with ice-cold PBS to remove debris. The samples were homogenized in 1 ml of ice-cold 0.1 M PBS and centrifuged at 10,000 RPM for 15 min 4 °C. The supernatant was collected and used as the enzyme source. The reaction mixture contained 1.7 ml 0.1 M PBS buffer (pH 6.5), 0.1 ml of 10 mM reduced glutathione, and 0.1 ml of enzyme extract. The reaction was initiated by adding 10 mM, 1-chloro-2,4-dinitrobenzene (CDNB) prepared in ethanol. The increase in absorbance was measured at 340 nm for every 1 min intervals for 3–5 min using a V-730 JASCO UV-Visible spectrophotometer. A blank contained all reagents except the enzyme extract. The GST enzyme activity was calculated using the extinction coefficient of the GSH-CDNB conjugate ($9.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as $\mu\text{mol}/\text{min}/\text{ml}$.

GSH was assayed using Ellman's method (Ellman, 1959). 1 mL of tissue homogenate was mixed with 1 mL of ice cold TCA (10%), and the volume was made up to 3 mL with distilled water. The mixture was then centrifuged at 5,000 rpm for 5 min. From this 1 mL of supernatant was taken, and 4 mL of PBS solution and 0.5 mL of DTNB were added. The rate of absorbance was read at 412 nm. A series of standard (glutathione reduced-Himedia-TC134-5 G), treated similarly, was also run to determine the glutathione content, which was expressed as $\mu\text{mol}/\text{ml}$.

Estimation of Vitamin C according to Roe and Kuether (1943) method. A total of 1 ml of homogenate mixed with 1 ml 10% TCA and centrifuged at 10,000 rpm for 20 min. 1 ml of supernatant was added with 0.2 ml of DTC reagent and mixed well. The reaction mixture was incubated at 37 °C water bath for 3 hours. To this 0.75 ml of 65% cold sulfuric acid (H_2SO_4) was added and incubated for room temperature for 30 min. the red color developed was read at 520 nm.

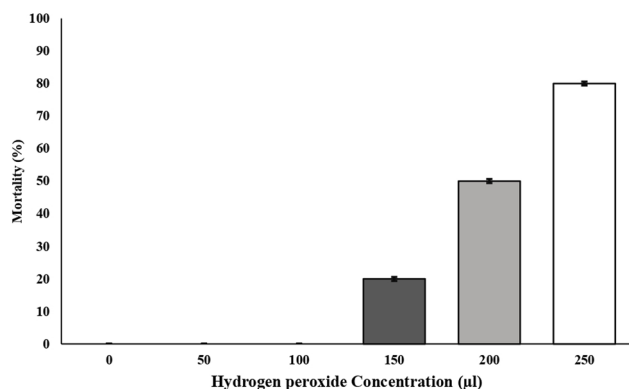


Fig. 2 Percentage of mortality rate of *T. castaneum* 4th instar larvae exposed against H_2O_2 (30%) at different concentrations (0, 50, 100, 150, 200 and 250 µl) at 24 h ($n=3$)

Table 1 Lethal toxicity of H_2O_2 against 4th instar larvae of *T. castaneum* for 24 h

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

χ^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)

Statistical analysis

Lethal concentration values (LC₂₀ and LC₅₀), their 95% confidence intervals, and the Chi-square goodness-of-fit statistics were estimated using Probit analysis in IBM SPSS Statistics version 20.0. Results are expressed as mean $\pm$ SD, and a p value of <0.05 was considered statistical significance, ($n=3$).

Data were analyzed using parametric statistical methods with IBM SPSS Statistics version (20.0). Group comparisons were performed using one-way analysis variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post hoc multiple comparison test. Results are expressed as mean $\pm$ SD, and a p value of <0.05 was considered statistical significance, ($n=4$).

Results

Assessment of 24 h lethal concentration of H_2O_2

Mortality rate of *T. castaneum* larvae at 24 h exposed to H_2O_2 (30%)

The percentage of mortality rate of *T. castaneum* larvae exposed to H_2O_2 (30%) for 24 h at different concentrations, such as 50, 100, 150, 200 and 250 µl, was shown in Fig. 2. The highest percentage (80%) of mortality rate was observed at 250 µl. 20 and 45% mortality were noted at 150 µl and 200 µl, respectively. There was no mortality observed at the lower concentrations (50 µl and 100 µl).

Lethal concentration of H_2O_2

The lethal (LC₅₀) and sub-lethal (LC₂₀) values of H_2O_2 (30%) against *T. castaneum* 4th instar larvae were estimated as 196.97 µl and 155.13 µl, respectively, using SPSS probit analysis (version 20) based on mortality rate at 24 h (Table 1).

Post-treatment with resveratrol

Morphological changes in fourth- instar larvae of *T. castaneum*

T. castaneum fourth-instar larvae exposed to H_2O_2 (LC₂₀: 155 µl) exhibited abnormal wriggling, sluggish movement and showed black spots on the thoracic appendages and abdominal segments (Fig. 3b), while subsequent post-treated with resveratrol attenuated the H_2O_2 induced abnormalities (Fig. 3c)

Total protein content

The total protein content was significantly reduced in H_2O_2 (LC₂₀-155 µl) exposed larvae compare to the control. The larvae post-treated with resveratrol (1 mM) were significantly increased compared with the H_2O_2 exposed larval group at 48 h ($p < 0.05$) (Fig. 4a).

Determination of antioxidant capacity

The enzymatic SOD activity was inhibited in the H_2O_2 -exposed larvae compared with the control group. SOD activity was significantly increased in larvae post-treated



Fig. 3 Morphological changes in fourth- instar larvae of *T. castaneum* at 48 h. (a) Control larva; (b) larva exposed to H_2O_2 at LC₂₀ (155 µl) for 24 h and showed black spots on the thoracic appendages and abdominal segments; (c) H_2O_2 exposed larva topically post-treatment with resveratrol (1 mM) for 24 h appeared similar to the control larva

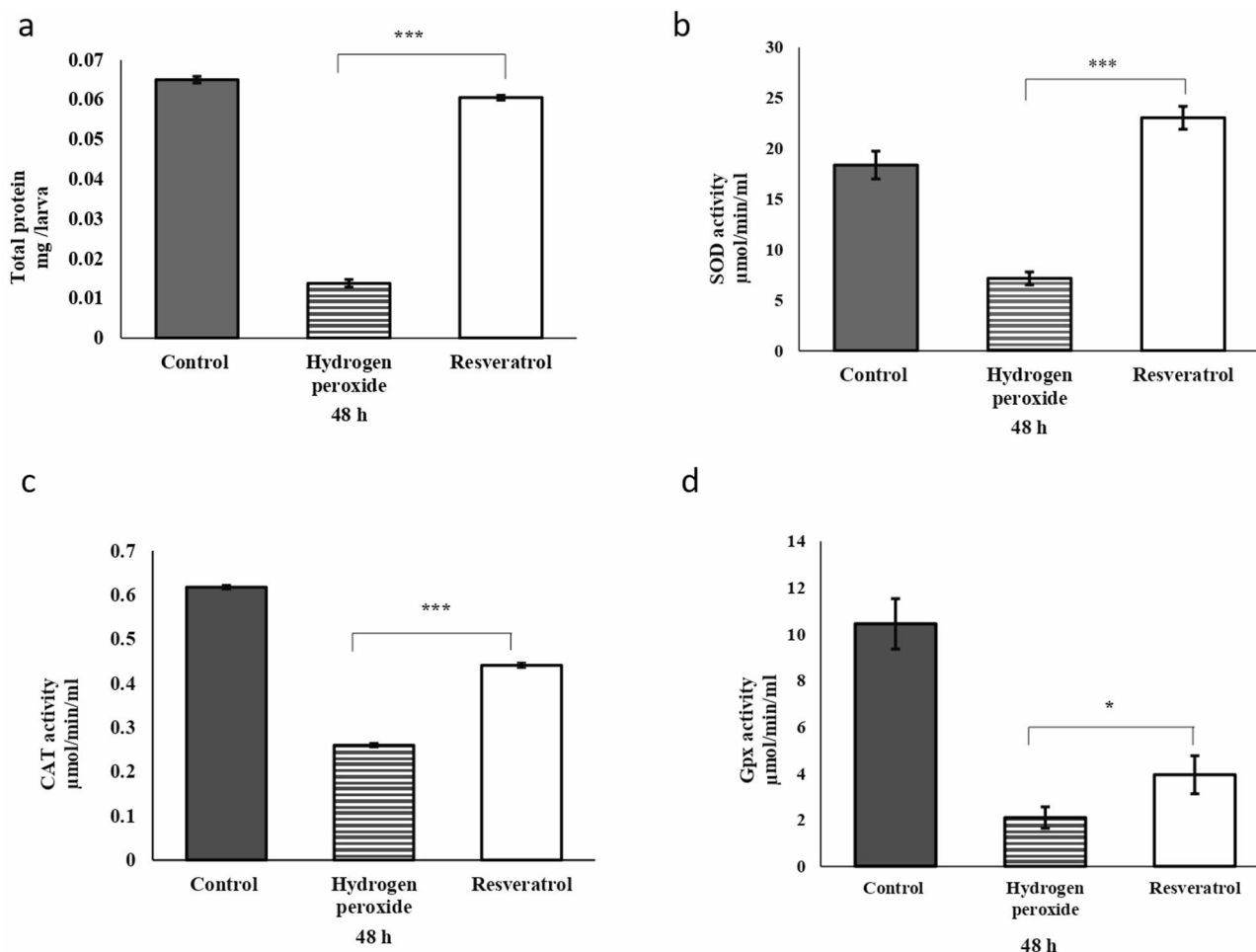


Fig. 4 Total protein level (a) and first-line enzymatic antioxidant activities: SOD (b), CAT (c), and GPx (d) In fourth-instar larvae of *T. castaneum* exposed with H_2O_2 (LC_{20}) and attenuated with 1 mM resveratrol at 48 hrs. Data are presented as mean $\pm$ SD, significant levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one way ANOVA followed by Tukey's post hoc multiple comparison test)

with resveratrol compared to H_2O_2 -exposed larvae at 48 h ($p < 0.05$) (Fig. 4b).

Catalase enzyme activity decreased in the H_2O_2 -exposed larval group compared with the control. However, CAT activity was significantly elevated in the resveratrol-treated larvae compared to the H_2O_2 -exposed larvae at 48 h ($p < 0.05$) (Fig. 4c).

The GPx activity also reduced in H_2O_2 -exposed larvae compared with the control. Topical post-treatment with resveratrol reactivated GPx activity and resulting in a significant increase compared with the H_2O_2 exposed larval group at 48 h ($p < 0.05$) (Fig. 4d).

GST activity was slightly increased in the *T. castaneum* larvae exposed to H_2O_2 compared to the control, although this change was not statistically significant. In contrast the resveratrol treated resulted in a significant elevation in GST activity compared to the both control and H_2O_2 -exposed larvae ($p < 0.05$) (Fig. 5b).

The second-line antioxidant GSH level was higher in the H_2O_2 -exposed larvae than in the control. After

post-treatment with the resveratrol, the GSH level was significantly elevated compared to the H_2O_2 -exposed larval group at 48 h ($p < 0.05$) (Fig. 5b).

The non-enzymatic antioxidant Vitamin C increased in H_2O_2 exposed larvae compared to the control. In resveratrol (1 mM) treated larvae, vitamin C levels were slightly reduced compared to the H_2O_2 exposed larval groups at 48 h, however, the difference was not statistically ($p < 0.05$) (Fig. 5c).

Discussion

Preclinical in-vitro and in-vivo screening tests of pharmaceutical drugs ensure safety assessment and prevent or reduce side effects of pharmaceutical products. The present study investigated the antioxidants effect of resveratrol in the alternative emergent model *T. castaneum*.

Fourth-instar larvae of *T. castaneum* larvae were fumigant-exposed to H_2O_2 (30%) for 24 h at different concentrations (50, 100, 150, 200 and 250 μl). Mortality rate of 80, 45 and 20% were observed at 250 μl , 200 μl , and

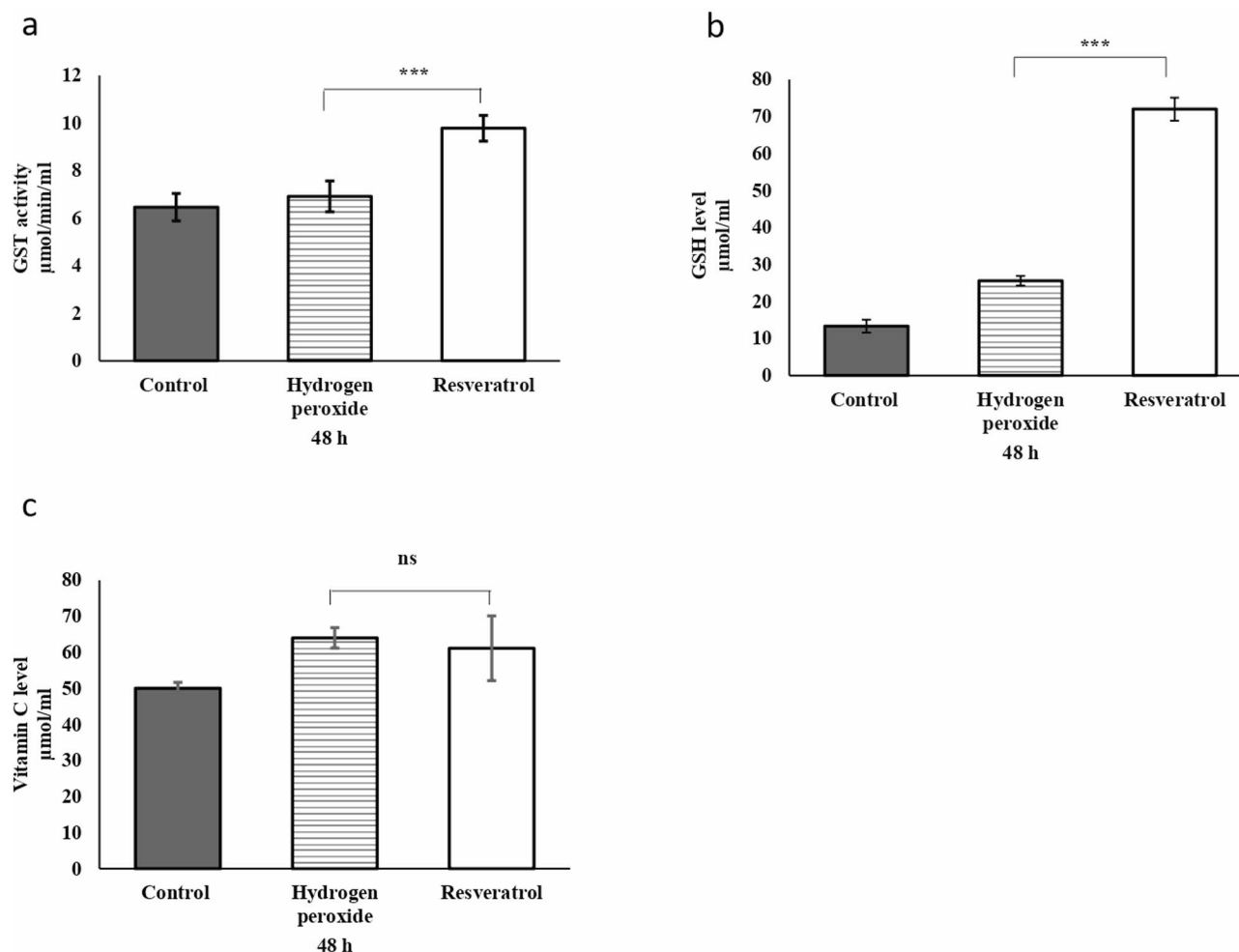


Fig. 5 Second-line enzymatic antioxidant activities: GST (a), GSH (b), and vitamin C (c) in 4th instar larvae of *T. castaneum* exposed with H_2O_2 (LC_{20}) and attenuated with 1 mM resveratrol at 48 hrs. Data are presented as mean $\pm$ SD. Significant levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns – non significant (one way ANOVA followed by Tukey's post hoc multiple comparison test)

150 μ l, respectively. There was no mortality observed at the lower concentrations of 50 and 100 μ l. H_2O_2 induce dose-dependent mortality in *T. castaneum* larvae (Fig. 2). Based the mortality rate, the lethal concentration (LC_{20} , LC_{50}) of H_2O_2 were determined such as 155.13 μ l and 196.97 μ l respectively (Table 1).

The sublethal concentration of pesticides are used in some toxicological tests to study the deleterious effects in the population without causing immediate death, evaluate fitness parameters of the organism and molecular mechanism in the biological system (Blair et al., 2015). The exposure to hydrogen peroxide induce ROS and led to oxidative cellular damage and apoptosis due to imbalance with antioxidants (Zhou et al., 2016). In this study, exposure of *T. castaneum* 4th instar larvae to a sublethal concentration of H_2O_2 (LC_{20} -155 μ l) resulted in abnormal wiggling, sluggish movement, and the appearance of black spots on the appendages and the abdominal segments, which may be attributed to oxidative damage of

tissues. In the post-treatment with resveratrol (1 mM) the black spots were not observed and, the larvae were exhibited normal wiggling behavior with slightly reduced sluggishness indicating that resveratrol mitigated adverse effects of induced by H_2O_2 (Fig. 3).

Oxidative stress reduces functional protein levels by degradation of proteins, repressing protein synthesis (Vogel et al., 2011). The total protein level was reduced in H_2O_2 -exposed larvae compared with the control group. However, the resveratrol-treated group, showed the higher protein level than the H_2O_2 -exposed larvae, indicating that oxidative stress caused by ROS may damage proteins, whereas resveratrol prevents protein oxidation (Fig. 4a).

The antioxidant enzymes SOD, CAT, and GPx are the first line of defense against ROS. GST enzyme together with the non-enzymatic antioxidant GSH (cofactor) and Vitamin C are involved in the second line of defense

mechanism against free radicals and prevent cell damage (Jomova et al., 2024; Halliwell & Gutteridge, 2015).

SOD is a very important group of metalloenzymes which are present in all aerobic living organisms (Flynn & Melov, 2013; Zelko et al., 2002). SOD converts the highly toxic superoxide radical anions ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and thus it prevents accumulation of oxygen free radicals which is primary protection against ROS (Genestra, 2007). The H_2O_2 cleaved into water molecule (H_2O) and oxygen (O_2) by catalase enzyme. The excess production of ROS disturbs the endogenous antioxidant defense system (Jomova et al., 2024; Halliwell & Gutteridge, 2015). In our study the activities of SOD and CAT significantly reduced in H_2O_2 exposed larvae than control at 48 h (Fig. 4b & c). This may be due to H_2O_2 exposure may promote ROS generation through Fenton reactions, resulting formation of hydroxyl radicals that contribute to oxidative stress and leading to deleterious effects in the organism. H_2O_2 readily diffuses across biological membranes and contributes to oxidative stress when its intracellular concentration exceeds the antioxidant capacity of the cell. In the presence of transition metal ions, H_2O_2 generates highly reactive hydroxyl radicals ($\bullet OH$) through the Fenton reaction, resulting reduced SOD and CAT activity efficiency (Halliwell & Gutteridge, 2015; Sies, 2017). Topical post-treatment with resveratrol against H_2O_2 larval group increased SOD and catalase activities, indicating that resveratrol improves the first-line defense mechanism in the organism under stress conditions (Fig. 4b and c). Similar results found in drosophila flies exposed by 1-methyl-4 phenyl-1, 2, 3, 6 tetra-hydropyridine, where resveratrol treatment, improved oxidative stress (Abolaji et al., 2018).

GPx is a family of antioxidant enzymes that has an important role in various biological functions which regulation depends on selenium availability (Pei et al., 2023; Duntas, 2012). GPx enzyme scavenges reactive species and reduces lipid peroxides and hydroperoxides in the cell membrane and cytoplasm (Yang et al., 2003; Rocha et al., 2015). Li et al. (2022) stated that GPx knockout mice exhibited extremely elevated level of acute oxidative stress, resulting in deleterious effects on the animals. The present results revealed that the GPx activity was decreased in H_2O_2 -exposed *T. castaneum* larvae at 48 h compared with the control, suggesting that oxidative stress induced imbalance state of antioxidants and free radical production (Fig. 4d). The observed reduction in GPx activity in H_2O_2 exposed larvae, also indicates impairment of the glutathione-dependent antioxidant defense system. GPx detoxifies H_2O_2 and lipid hydroperoxides by utilizing reduced glutathione (GSH) as an electron donor. Under prolonged oxidative stress, GPx may become inactivated due to excessive peroxide generation,

leading to decreased enzyme activity (Brigelius-Flohé & Maiorino, 2013). GPx activity restored in resveratrol-treated larvae following H_2O_2 exposure at 48 h indicating that resveratrol attenuated H_2O_2 -induced oxidative stress through its antioxidant properties (Fig. 4d).

Glutathione S-transferase (GSTs) are phase II metabolic detoxification enzymes that play an important antioxidant role in removal of xenobiotic by conjugating reduced glutathione (GSH), acting as a second-line defense in the antioxidant system (Hattem et al., 2014; Ighodaro & Akinloye, 2018; Mhlana, 2018). Song et al. (2021) reported that GST antioxidant activity was significantly higher in the resveratrol-treated silkworm larvae compared to untreated larvae. The present findings indicate a slight increase in glutathione S-esterase activity in H_2O_2 exposed larvae compared to the control group. Although GPx activity was reduced following H_2O_2 exposure, the levels of GSH and GST activity were increased (Figs. 4d, 5a and b). Excessive H_2O_2 generation may have caused oxidative modification and inhibition of GPx, reducing its ability to detoxify peroxide despite availability of GSH. Meanwhile, increased GST activity suggests enhanced utilization of GSH for the conjugation and removal of oxidative stress-derived electrophilic metabolites (Lu, 2013; Hayes et al., 2005). Thus, the increase in GSH and GST activity together with decreased GPx activity may reflect compensatory GST mediated detoxification in H_2O_2 -oxidative stress induced larvae at 48 h. However, post-treatment with resveratrol resulted in significant elevation of GPx, GST enzyme activity and GSH levels in larvae at 48 h, which indicates that resveratrol polyphenolic compound act as an antioxidant stimulator in the organism.

Vitamin C also known as ascorbic acid which is micro-nutrient has most important role in various biological processes of the organisms such as induce host immune response against infectious diseases, act as a cofactor in enzyme oxidation process, and promotes collagen synthesis which is essential in structural connective tissue development both in vertebrates and insects. Vitamin C synthesis in insect by activity of L-gulonolactone oxidase enzyme. It scavenges free radicals and at excess level it acts as a pro-oxidative factor (Hou et al., 2021; Kazmierczak et al., 2020). It is a non-enzymatic antioxidant involved in the second line antioxidant defense mechanism against free radicals. Vitamin C level increased during stress conditions to prevent oxidative cell damage (Padayatty et al., 2007; Marik, 2020). In this study, the antioxidant vitamin C was significantly increased in H_2O_2 exposed *T. castaneum* larvae than control at 48 h. The post treated with resveratrol slightly decreased than H_2O_2 exposed organisms, which elucidated that Vitamin C acts against ROS, meanwhile, balances in the host antioxidant system (Fig. 5c).

Conclusion

The present study revealed that fumigant exposure of H_2O_2 , induced oxidative stress in *T. castaneum* larvae, leading to inhibition of the first-line enzymatic antioxidant defenses such as SOD, catalase, and GPx due to excess level of ROS. In response, second-line enzymatic antioxidant GST and non-enzymatic antioxidants, including GSH and vitamin C level, increased as a compensatory mechanism to counteract free radicals. Resveratrol restored both first and second-line antioxidant defense systems in H_2O_2 -exposed organisms, thereby mitigating oxidative stress. A limitation of this study is the use of single concentration resveratrol (1mM) to evaluate its antioxidant effects. Resveratrol may act as both an antioxidant and a pro-oxidant depending on its dosage level. Further studies are required to investigate its molecular mechanism of resveratrol and developmental effects in model organisms at different doses for better understanding and ensure safety of its therapeutic applications.

Abbreviations

UV	Ultraviolet
ROS	Reactive oxygen species
LC	Lethal concentration
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
°C	Degree Celcius
RH	Relative humidity
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
TCA	Trichloro acetic acid
DTNB	5,5' Dithiobis – 2-nitro benzoic acid
DMSO	Dimethyl Sulfoxide
Rpm	Round per minute
Nm	Nanometer
DTC	Dinitrophenyl hydrazine – Thio urea – Copper sulfate
ANOVA	Analysis of Variance

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Author contributions

The first author S.K. designed the study, conduct experiments, statistical analysis of data and drafted the original manuscript and G.C. reviewed the manuscript. All the authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

We grant consent for the publication of our work. We acknowledge that this consent extends to the initial publication and any subsequent editions or reprints.

Competing interests

The authors declare no competing interests.

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