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Efficacy of *Withania somnifera* against oxidative stress in male *Drosophila melanogaster*.

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ABSTRACT

Oxidative Stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to repair the resulting damage that leads to deleterious effects in living organisms. Stress has been reported to be a causative factor for male infertility. *Withania somnifera* has been documented in Ayurveda for its stress-combating properties. However, limited scientific literature is available on aspect of *W. somnifera* grown in different soil at different regions. We undertook the present study to understand the role of stress in male infertility, and to test the ability of *W. somnifera* grown in black soil and red soil to combat stress and treat male infertility. Plants containing high amount of antioxidants that protect the body cells against stress. Antioxidants have the ability to scavenge and neutralize the generated free radicals. In the present study we evaluated the antioxidant potential of ethanolic root extracts of *Withania somnifera* grown in black soil (BWS) and *Withania somnifera* grown in red soil (RWS) in *Drosophila melanogaster* under oxidative stress state. To know about the effect on male fertility, sperm count and accessory gland protein estimation were carried out in extract fed flies. The efficacy of *Withania somnifera* in scavenging reactive oxygen species was measured through biochemical analysis. The total sperm count and ACp amount was more in BWS and in OS induced BWS fed flies. Further, it reveals that BWS extract fed flies increases the SOD activity by 11.27%, CAT activity by 7.13%, GSH increases by 7.34% when compared to control group. The activity levels of antioxidant enzymes in Paraquat induced BWS extract fed batches showed significant increase in SOD (18.23%), CAT (11.29%) and GSH (14.17%) when compared to control flies with the induction of OS. Lipid peroxidation also decreased in BWS fed flies under stress (70.13%) and non-stress conditions (91.14%). This was due to high degree of antioxidants and natural polyphenols in *Withania somnifera* grown in black soil region.

Key Words: *Withania somnifera*, *Drosophila melanogaster*, Oxidative stress, Paraquat, sperm count, Antioxidant enzymes, LPO.

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Introduction:

Male infertility is an alarming global health issue, more than 40-50% of couples are affected by this problem. One of the major causes for male infertility is oxidative stress, which is an imbalance between production of reactive oxygen species (ROS) and antioxidant defense mechanisms in the body. The increased ROS level leads to oxidative stress that can impair the sperm viability and reduce sperm count (1). ROS can induce cell death pathways in sperm, leading to decreased sperm viability (2). Oxidative stress can also affect the sperm production in the testes, leading to reduced sperm count or oligozoospermia (3). Furthermore, oxidative stress can disrupt the antioxidant defense system in the seminal plasma, leading to decreased antioxidant capacity and increased susceptibility to oxidative damage (4). Oxidative stress can also lead to direct damage to the sperm plasma membrane, causing changes in its fluidity, integrity and impair sperm function leading to reduced fertility (5). Infertility is usually further connected with low levels of antioxidants in semen compared to fertile men. Sperm is particularly susceptible to oxidative stress resulting from the consumption of large amounts of polyunsaturated fatty acids, or from small amounts of intracellular antioxidative enzymes (6).

Supplementation of antioxidants is one of the method to reduce these harmful effects. Antioxidants play a vital role in improving sperm quality by increasing count, motility, viability and morphology. Antioxidants are biological compounds that are able to reduce the adverse impacts of ROS, acting as safeguards against oxidative stress. The super oxidase dismutase and catalase are the major antioxidants that attack against the oxidative stress by breaking down harmful ROS into harmless components (7). Glutathione is an antioxidant in the body functioning against oxidative stress. Hence both these antioxidant and antioxidant enzymes provide a powerful defense against oxidative stress, enhancing sperm health and male reproductive function. Seminal plasma contains an antioxidant mechanism that creates an ideal environment for sperm function and fertility (8). Fertility impairments are associated with decreased total antioxidant capacity, as well as decreased non-protein antioxidants. About 3% of the proteins contained in seminal plasma are enzymes. SOD, CAT, GPx, and GR have been found to exhibit relatively high activity in the fractions of ejaculate rich in sperm (9). SOD, and CAT tend to be lower in infertile men compared to fertile controls. CAT is an enzyme which plays an important role in the protection of sperm against ROS, it converts millions of hydrogen peroxide molecules into water and oxygen each second, and elevated CAT levels can be achieved by various antioxidant therapies leading to improved sperm parameters. Further it has been proved that the synergistic actions of both SOD and CAT has more beneficial effects in male infertility (10).

Another major factor that affects the physiology of sperm is formation of MDA under oxidative stress condition, generated during lipoperoxidation. MDA level can provide information about the extent of oxidative stress, increased seminal MDA concentrations, decreases the seminal plasma total antioxidant capacity (11). The elevated CAT levels also decrease lipid peroxidation.

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An anti-oxidative rich diet, improve sperm quality as well as lessen oxidative damage (12). Hence, antioxidants based therapies for male fertility are seems to be more relevant. Perusal of above literature suggested that antioxidant rich plants promote male reproductive health against the oxidative stress. One such good source of natural antioxidant documented in Indian Ayurveda and Unani systems of medicine is *Withania somnifera*, belongs to the family *Solanaceae*. *Drosophila melanogaster* served as a unique and powerful model to study oxidative stress and to evaluate synthetic as well as natural compounds. In the present study a comparative study has been made in *Withania somnifera* root extract grown in red and black soil to know the efficacy on male fertility in *Drosophila melanogaster* through sperm count and biochemical analysis under stressed condition.

MATERIALS AND METHODS

Chemicals

Paraquat (1,1′-dimethyl-4-4′-bipyridynium dichloride), EDTA, Hydrogen peroxide, Nicotinamide adenine dinucleotide phosphate (NADP), Trichloroacetic acid (TCA), Reduced glutathione, 5, 5'– dithiol-bi's (2-nitrobenzoic acid) (DTNB), Bovine serum albumin (BSA) were procured from Sigma Aldrich, India. All other chemicals and reagents used for the analysis were procured from reputed companies.

Preparation of plant extracts

The roots of *Withania somnifera* plant grown in red and black soil were collected in Mysore and Gadag districts of Karnataka, India. Collected roots were washed separately then dried in the laboratory at room temperature and powdered using an electric blender. The obtained root powder was subjected to Soxhlet unit to get ethanolic extraction. The collected extracts grown in red soil from Mysore region was named as RWS, while collected extracts grown in black soil from Gadag named as BWS, further extracts were stored in separate vials and used for the analysis. All the experiments were carried out using two different concentrations of the RWS and BWS (Dose-I: 2mg/ml and Dose-II: 10mg/ml).

Culturing of flies

Wild-type *D. melanogaster* of strain Oregon-K flies were obtained from National *Drosophila* Stock Center, Department of Zoology, University of Mysore, Mysuru, Karnataka, India. The flies were cultured in a standard wheat cream agar media seeded with dry yeast granules and maintained at 22 ± 1º C with relative humidity of 60 - 70%. Flies were multiplied by subculture and synchronized eggs were collected from the Delcour technique as per the standard procedure and immediately after emergence, male flies were sorted under light anaesthesia. Then, only male flies were considered and supplemented with BWS and RWS extracts and 10 days aged flies were used for all the analysis.For all the experiments synchronized flies were used from isofemale line stocks.

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Oxidative stress assay

Induction of oxidative stress by Paraquat (PQ): To know the efficacy of BWS and RWS extracts on total count of sperms, Acp quantity and antioxidant enzymes as well as LPO, flies were subjected to oxidative stress molecule. Paraquat dichloride (PQ) has been employed as OS molecule by following the method of Hosamani and Muralidhara, (2013). 10 days aged BWS and RWS extracts fed flies were used for OS induction. The flies were starved in empty vials of size 9 x 3cm for 2hrs. Then flies were exposed to 20mM PQ in 5% sucrose solution through a soaked filter paper for 2hrs.duration. Survival was determined after 24 hrs. and survived flies were used for homogenization for biochemical assays. 50 flies were maintained in each batch (10 flies/vial) and each assay was repeated thrice.

Sperm count Analysis

The sperm count of the flies has been carried out from the testes of different batches of 20 males. Experiments were carried out in four different batches namely BWS/RWS fed batch without PQ, BWS/RWS fed batch with PQ, without PQ and extract were considered as control batch, control flies induced with PQ. Testes from the 20 days age group male flies were dissected in PBS using fine micro needle under stereomicroscope and were transferred into 50 µl of saline to release the sperm. After 15minutes 10-15µl diluted sperm sample were transferred to Haemocytometers (chamber depth 0.1mm) to count the number of sperm. Placed the counting chamber on the microscope stage and observed the sperms using the 40X objective. Spermatozoa in 5 of the large squares on each side of the counting chamber were counted. The haemocytometer was 0.1 mm depth and the 25 large squares represent an area of 1 square mm.

Biochemical analysis

(i) Quantitative estimation of accessory gland protein (Acp)

Sample preparation; Accessory glands of unmated males were separately dissected using PBS solution with the help of entomological needles, then the glands were fixed in 95% ethanol. Further, outer membrane was removed from the fixed glands using micro needles, only secretions of the accessory gland was considered for the analysis, hence the secretion were washed in a mixture of methanol and chloroform (1:1) and dried at 37° C in incubator for 15 min. About 100 µL of sample buffer (0.625 M tris-HCL pH 6.8, 1% βmercaptoethanol, 1% SDS, and 10% glycerol) was added to each sample to dissolve the secretions. Ten pairs of accessory glands from different groups were used for quantitative estimation of accessory gland protein.

Estimation of Accessory protein: The estimation of accessory gland protein was carried out by Bradford method as per the standard procedure (13). 50µl of Acp samples collected from BWS and RWS fed groups was mixed with 5 mL of Bradford reagent (100 mg of Coomassie Brilliant Blue G-250 in 50 mL of 95% ethanol. Then, add 100 mL of 85% phosphoric acid and 850 mL of distilled water).Then solution was allowed to stand for 5 min to develop blue color. The optical density of the solution was measured using a spectrophotometer at

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absorbance of 595 nm. The quantity of accessory gland protein in the sample was calculated using bovine serum albumin (BSA) as the standard. 25 trials were taken individually for BWS and RWS fed groups.

(ii) Antioxidant enzyme assay: All the antioxidant enzyme (SOD, CAT, GSH) activities, were measured in BWS and RWS extract fed flies in two different doses (Dose-I and Dose-II). All the experiments were carried out in 10 days age grouped flies under stressed and non-stressed condition. The oxidative stress molecule (PQ) induced flies with extract treatment were considered as stressed group. The wild flies without exposed PQ treatments were considered as control group.

(a) Superoxide dismutase (SOD): SOD activity was measured by using NBT method as per the standard procedure (14). About 3ml reaction mixture was prepared by adding 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 0.1 mM EDTA, 75 µM NBT and 50 µL of enzyme extract. All the experimental tubes were exposed to 400 W bulbs for 15 min. and at 560 nm absorbance was read. The inhibition of the reaction between riboflavin and NBT in the presence of methionine was considered as one unit of SOD activity and it was expressed in units/mg of protein

(b) Catalase (CAT): CAT activity was measured based on the quantity of the H_2O_2 substrate remaining after the action of CAT present in the enzyme extract (15). To measure this, 0.4 ml of enzyme extract was mixed with 2.6 ml of phosphate buffer along with 30% of H_2O_2. The activity was measured by determining the decomposition of H_2O_2 at 240 nm. CAT activity was calculated by using the millimolar extinction coefficient of 43.6 and expressed in terms of µm/min/mg of protein.

(iii) Glutathione (GSH)

The activity of GSH was measured by using DTNB method as per the standard procedure (16). For this flies were homogenized in 10% TCA (ice-cold) and 10 mM EDTA (1:1). Homogenate was centrifuged at 5000 rpm. Further, enzyme reaction mixture was prepared by adding 200 µl of supernatant, 0.2M Tris-buffer (pH8.0) and 50 µl of DTNB. The reaction mixture was incubated for 10 min. at room temperature to get yellow coloured complex. The absorbance was read at 412 nm and the activity was expressed as µg/mg protein.

(iv) Lipid Peroxidation Assay

Lipid Peroxidation assay was measured by employing thiobarbituric acid (TBA) as per the procedure (17). Assay was carried out in different aged fly groups. The reaction mixture consists of 0.2 µl of homogenate sample, 0.2 µl of SDS (8.1 % w/v), 1.5 ml of acetic acid (pH 3.5, 20%), 1.5 ml of TBA (0.8% w/v) and reaction mixture was made up to 4 ml with distilled water, mixed well. The mixture was incubated in a water bath at 90° C for 60 min. and then cooled in ice-bath. After cooling, samples were mixed with 3 ml of n-butanol and centrifuged at 5000 rpm for 10 min. Further, supernatant was carefully transformed into another test tube and the absorbance was measured at 532 nm. Lipid peroxidation was quantified as malondialdehyde (MDA) equivalents using 1,1,3,3-tetramethoxypropane as the standard (molar extinction coefficient value is 15600 M^{-1} cm^{-1}). Blank solution was prepared by mixing all the reagents except sample homogenate.

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(v) Protein estimation

Protein estimation was quantified by following Lowry's method (18). In a test tube 20 µl of the sample and 980 µl of distilled water were added. To this 5 ml of Lowry's reagent was added and allowed to incubate at room temperature for 15 minutes. Then 0.5 ml of Folin-Ciocolteu was added, mixed well and again incubated at room temperature for 30 minutes. Optical density was measured at 660 nm using colorimeter and calculated the amount of protein by making use of BSA as standard graph.

Statistical analysis

All the Statistical analysis was performed using SPSS 19. The data of all the biochemical activities of were expressed as mean ± SE. The level of significance was measured by one-way and multivariate ANOVA followed by tukey's test, with $p<0.05$ is statistically significant.

Result and Discussion

Antioxidants are used in worldwide for the treatment of male infertility. Oxidative stress is considered as a major causative factor for male infertility. Sperm are especially sensitive to oxidative stress. Several studies have shown that certain plant-derived antioxidants would improve sperm quality, polyphenol-rich grape *Vitis vinifera* extract and crocin of *Crocus sativus* extract (19, 20). Polyphenols of Grape seed extracts, are able to neutralize ROS and increase motility and viability of bovine spermatozoa (21). *W. somnifera* is reported to have several beneficial effects on male fertility, but its mode of action based on reports from human and animal studies has not yet been properly documented. Hence in the present study comparative account has been made on sperm analysis under stress in *Drosophila melanogaster* using *W. somnifera* extract grown in two different soil conditions. To know the efficacy of the extracts on endogenous antioxidant system few antioxidant enzyme activity has been measured in extract fed flies.

Sperm count: The sperm count of the flies has been carried out from the testes of 20 males. Analyses were carried out in BWS fed batch and RWS fed batch under stressed and non-stressed conditions. The result of sperm count analysis has been illustrated in Fig.1. The total number of sperm count in the control group was 2460, while it was reduced after PQ induction (2070). The sperm count was increased in both BWS and RWS extract fed groups and even after the PQ induction. The highest percentage of increased sperm count was seen in dose-II of BWS fed group flies. It was increased by 21.58% when compared to control group, while it was increased by 11.35%, when exposed with PQ when compared to control with OS.

In *Drosophila*, the accessory glands are simple sacs consisting of a single layer of secretory cells around a central lumen. The accessory gland is composed of two types of binucleated epithelial cells: a main cell and a secondary cell. In *Drosophila* males, the accessory gland is responsive to nutrient-dependent regulation of fertility. The male accessory glands synthesize and secrete a complex mixture of proteins, carbohydrates, lipids, and amino acids (22). Accessory glands produce the structural proteins needed for spermatophore formation, it

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secretes accessory gland proteins (Acps) into the seminal fluid, which are essential for male fertility (23, 24). The amount of Acp differs between species and between strains of the same species. Nutrition is a significant factor and has great impact on the reproduction, survival, and health of an organism. Quantity of nutrients has a major influence on the size of the gland and the amount of Acps (25). In the present study, to know the efficacy of WS on accessory gland secretion, Acp has been estimated in extract fed flies. The result of Acp estimation was given in the Table 1. The amount of Acp in the control groups was 0.071 µg/pair of glands, while amount is decreased by 6.34% in PQ induction batch. In contrast to this the amount of Acp was increased in extract batches. The greater amount was observed in Dose-II of RWS (0.485 µg/pair of glands), in Dose-II of BWS (0.70 µg/pair of glands). The careful observation of Table 1 shows that RWS extract fed flies increases by 6.81 % and BWS extract fed flies increases by 9.86 %. However, in batches where extract fed flies induced with PQ, the quantity was more when compared to batch without extract. Dose-II of BWS possesses highest quantity (0.56 µg/pair of glands) than rest of the analyzed groups. It was increased by 8.42%.

The endogenous antioxidant system can scavenge the excess amount of ROS. The major antioxidant enzymes serving as primary endogenous antioxidants to deactivate the ROS. Superoxide dismutase (SOD), catalase, and glutathione reduced (GSH) activity has been assayed in clinically diagnosed infertile males .Catalase enzyme activity was significantly decreased while SOD and GSH were substantially increased in infertile men in comparison to non-infertile.(26). The result of antioxidant enzyme activity has been shown in Fig.2. The data reveals that the SOD activity in the control groups was 20.27 µg/ml of protein, it was reduced after induction of PQ (18.87 unit/mg of protein). The analyzed both the extract fed flies of both the dosage group showed highly significant increased SOD activity when compared to the control flies. Similarly the activity was high in the PQ induced BWS and RWS grouped flies. The result of CAT and GSH also shows increased activity in both extract fed group flies. The overall result reveals that dose-II of BWS extract fed group flies increases the SOD activity by 11.27%, increases CAT by 7.13% and GSH by 7.34% when compared to control group. In stress induced dose-II of BWS extract batches SOD increases by 18.23%, CAT increases by 11.29% and GSH increases 14.17% when compared to control flies with the induction of OS.

Sperm membranes are mostly constituted by poly-unsaturated fatty acid and susceptiblity to oxidative stress was assessed by evaluating LPO (27). LPO levels can be detected by measuring lipid oxidation end products as MDA (28). Malondialdehyde (MDA) is the final product of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals leads to overproduction of MDA, this level is commonly known as a marker of oxidative stress. The activity of LPO in WS extract fed flies were measured through Thiobarbituric Acid Reactive Substances (TBARS) Assay. The result of LPO activity has been shown in Table 2.

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The activity of LPO in control group was 2.54 unit/mg of protein, it was increased by 81.88% after immediate PQ induction. The data reveals that LPO activity was decreased in WS extract fed groups. Further, it noticed that LPO activity was decreased as dosage increases. The maximum reduction of LPO was observed in BWS fed group (0.21 unit/mg of protein), which was reduced by 91.14% than control group. In the OS induced batch again the maximum reduction of LPO was noticed in BWS with PQ fed group (1.34 unit/mg of protein), which was reduced by 70.13% when compared to PQ induced control group.

The antioxidant molecules may alter spermatozoa maturation, interfering with physiological sperm function. The therapeutic supplementation of antioxidants on semen quality has beneficial effects in infertile men under oxidative stress condition (29). SOD and GSH levels were found to positively correlate with sperm count and motility, while enhanced MDA levels were associated with altered sperm morphology (30). In the present analysis BWS extract fed flies exhibits superior result than RWS extract fed flies. High dose of BWS fed batch flies has more sperm count with high accessory gland protein, their antioxidant enzyme and glutathione levels were also found to be high with the reduction of LPO activity. This was due to rich antioxidant property of *Withania somnifera* grown in black soil. Based on the observation, the present analysis revealed that WS grown in black soil has greater influence in increasing SOD, CAT and GSH with reduced LPO under oxidative stress state, there by increases the sperm count and Acp quantity than WS grown in red soil.

Our study supports the work of Shiva *et al.*, (31), where SOD and CAT are positively associated whereas LPO are negatively associated with elevated sperm count.

In conclusion, the studies summarizes that the supplementation of BWS increases sperm count and amount of accessory gland protein in *D. melanogaster* under oxidative condition. Further it increases antioxidant enzyme activities and reduces the Lipid peroxidation under stress and non-stress conditions. This is due to high degree of antioxidants and natural polyphenols in *Withania somnifera* grown in black soil of Gadag region.

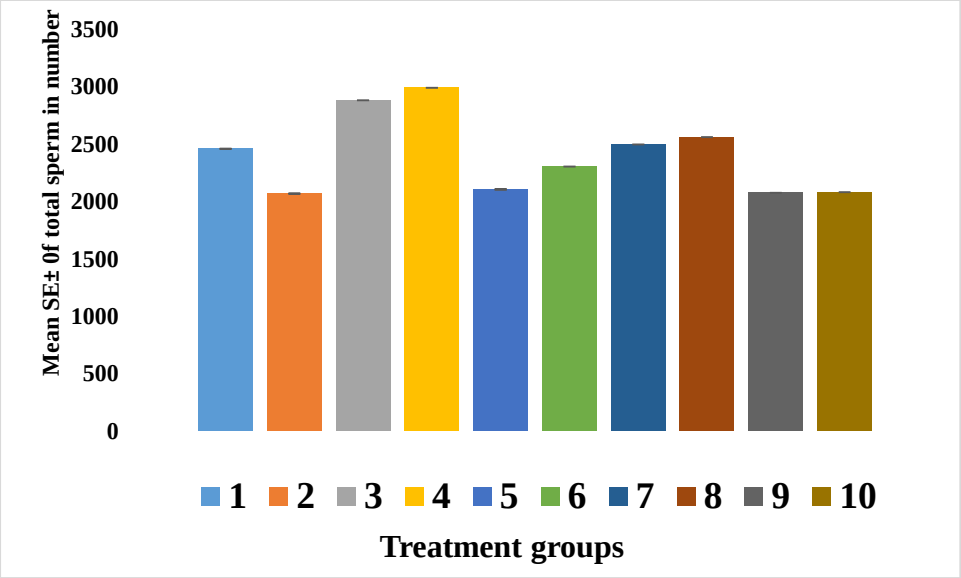

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Figure 1: Result of sperm count in WS extract fed groups of *Drosophila melanogaster* under stressed and non-stressed condition. 1-Control, 2-Control+PQ, 3-BWS Dose-I, 4-BWS Dose-II, 5-BWS Dose-I+OS, 6-BWS Dose-II+OS, 7-RWS Dose -I, 8-RWS Dose-II, 9-RWS Dose-I+OS, 10-RWS Dose-II+OS

Acps (µg/pair of glands)			
Control	0.071±0.12		
Control + OS	0.066±0.05		
BWS Dose - I	0.058±0.08	**RWS Dose -I**	0.36±0.08
BWS Dose - II	0.7±0.05	**RWS Dose -II**	0.48±0.09
BWS Dose - I+OS	0.54±0.050	**RWS Dose - I +OS**	0.53±0.06
BWS Dose - II+OS	0.56±0.07	**RWS Dose - II +OS**	0.54±0.07

Table 1: Result of Accessory gland protein estimation (Acps) in WS extract fed groups of *Drosophila melanogaster* under stressed and non-stressed condition

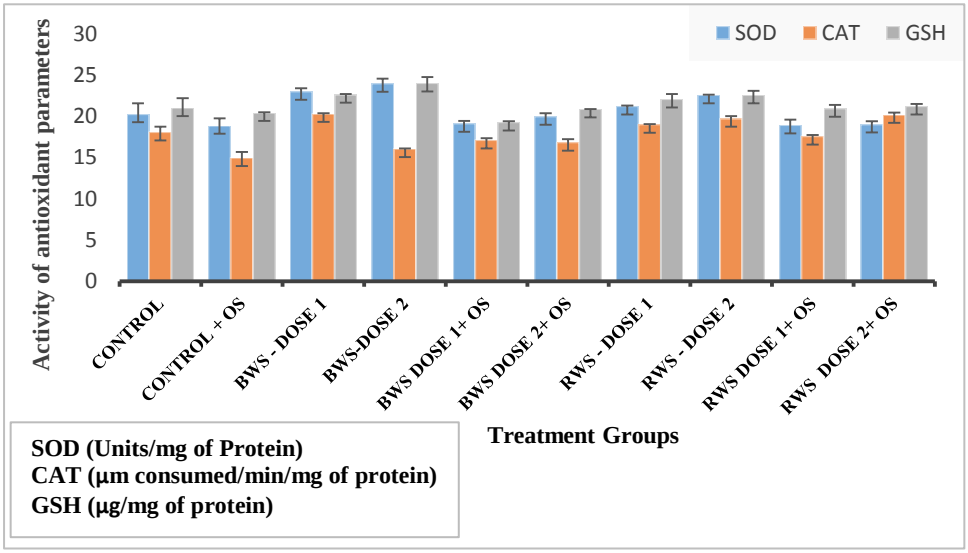

Figure 2: Result of antioxidant enzymes estimation in WS extract fed group of *Drosophila melanogaster* under stressed and non-stressed status.

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LPO in different groups (nmole MDA/mg of protein)			
Control	2.54±0.12		
Control + OS	4.62±0.08		
BWS - Dose 1	0.23±0.07	**RWS - Dose 1**	0.28±0.05
BWS – Dose 2	0.21±0.06	**RWS - Dose 2**	0.26±0.05
BWS 1 + OS	1.38±0.08	**RWS 1 + OS**	1.18±0.05
BWS 2+ OS	1.34±0.05	**RWS 2 + OS**	1.16±0.05

Table 2: Result of Lipid peroxidation (LPO) in WS extract fed group of *Drosophila melanogaster* under stressed and non-stressed status.

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