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Research Article

Withania somnifera promotes stress resistant activity in *Drosophila melanogaster*

Renuka Prasad B.P and Ashadevi J.S*

Genetics Research Laboratory, Department of Zoology, Yuvaraja's College, University of Mysore, Mysuru, India

ABSTRACT

Stress is a state of mental or emotional strain of an individual. In recent years nutritional antioxidants study have gain more attention in minimizing the stress like oxidative stress. The stress resistant ability in an organism can be increased by the supplementation of herbal resources. However, few plant extracts are known to have stress resistant ability and increases the tolerance capacity. Plants containing high antioxidant and other bioactive compounds promote tolerance capacity. An antioxidant rich plant has been proved to decreases the lipid peroxidation. Here, we investigated the potential protective effect of ethanolic extract of *Withania somnifera* (WS), against Paraquat toxicity on stress tolerance capacity using *Drosophila melanogaster*. Wild-type fruit flies of Oregon-K strain were fed with standard food media with 1mg/ml and 10 mg/ml of WS. The oxidative stress was induced by exposing the extract supplemented flies to Paraquat (20 mM). The stress tolerance capacity of flies was measured by subjecting to desiccation and oxidative stresses. Further, locomotor activity, lipid peroxidation were also studied along with the quantification of triglycerides, glycogen in WS fed flies under stress conditions. Our result reveals that PQ induced WS fed flies showed greater survivability, better locomotor ability when compared to PQ induced flies. WS fed flies increases about 73.55% of resistance ability under oxidative conditions and increased by 59.15% under desiccation than PQ induced flies. WS was more effective in protecting against Paraquat toxicity. The flies fed with high dose of WS (10mg/ml) showed greater improvement of the tolerance ability when subjected to desiccation and oxidative stresses. Further, the data on biochemical analysis reveals that lipid peroxidation activities were found to be significantly low and the triglyceride as well as glycogen quantities were found to be significantly high in WS fed flies compare to -ve control under both desiccation and oxidative stress conditions. Together, these findings suggest that WS promotes stress resistant ability by modulating metabolism and reducing oxidative damage.

Keywords: *Drosophila melanogaster*, *Withania somnifera*, Oxidative stress assay, Desiccation Assay, Negative Geotaxis, Lipid Peroxidation, Triglyceride.

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***Address for Correspondence:** Dr. Ashadevi J.S, Head of the Department, Department of Zoology, Yuvaraja's College, University of Mysore, Mysuru, India

INTRODUCTION

Stress is a state of mental or emotional strain of an individual. This is due to external environmental factors or internal alterations in the metabolism. The major one is oxidative stress, which is an imbalance between free radicals and antioxidants in the body. Oxidative stress occurs when the production of reactive oxygen is exceeds than the body's ability to detoxify the reactive intermediates. This imbalance leads to oxidative damage to biomolecules. Naturally body produces antioxidants that defend against free radicals. The antioxidants neutralize the free radicals, thereby rendering them harmless to other cells.

In recent years nutritional antioxidants study has gain more attention in minimizing the stress like oxidative stress. Perusal of literature demonstrated that phytochemicals of plants promote health and prevents stress ¹⁻⁴. Hence, stress resistant ability in an organism can

be increased by the supplementation of herbal resources that are good source of natural antioxidant. Food consumption acts as a major environmental factor that plays a pivotal role in extending the stress resistance of an organism. Several nutraceutical compounds have ability to tolerate the stress and increases the survivability in many organisms. *Withania somnifera*(WS) is also known as Ashwagandha, is a well-known herb in the Ayurvedic and indigenous medical system and long been known for its antioxidant properties ^{5,6}. Few active compounds of WS have been proved for possessing antioxidant effects that destroys free radicals ⁷. In Indian Ayurveda, Ashwagandha is used as an "adaptogen" to help the body cope with daily stress; however, there is no proper experimental evidence in support of stress resistant against stress molecules by *in vivo* analysis. Hence, in the present study it has been design to analyze the potential protective effect of ethanolic extract of *Withania somnifera* (WS), against Paraquat toxicity on stress tolerance capacity through *in-*

vivo using *Drosophila melanogaster*. This insect served as a unique and powerful model to study human genetic diseases and for screening synthetic and natural compounds ⁸. The stress tolerance capacity of flies was measured by subjecting to desiccation and oxidative stresses. Further, locomotor performances through negative geotaxis assay and lipid peroxidation were also investigated along with the quantification of triglycerides and glycogen in WS fed flies under stress conditions.

MATERIALS AND METHODS

Chemicals: Paraquat (1,1'-dimethyl-4-4'-bipyridinium dichloride), Bovine serum albumin (BSA) were procured from Himedia, Mumbai, India. 1,1,3,3-tetramethoxypropane purchased from Sisco Research laboratory, Pvt. Ltd., Mumbai. Triglycerides and Glycogen were procured from Sigma Aldrich. All other chemicals and reagents used for the analysis were procured from reputed companies.

Extract preparation:

WS plant was collected from natural habitat in Mysore, Karnataka, India. The extract was prepared by using the root of the plant. The root powder was subjected to Soxhlet unit to get ethanolic extraction. The collected dried extracts were stored and used for the analyses. All the experiments were carried out using two different concentrations of the WS (dose-I: 1 mg/ml and dose-II: 10 mg/ml).

Drosophila culture:

Wild-type *D. melanogaster* of strain Oregon-K flies were obtained from National *Drosophila* Stock Center, Department of Zoology, University of Mysore, Mysore, Karnataka, India. Flies were maintained on the standard wheat cream agar medium seeded with dry yeast granules at $22 \pm 1^{\circ}\text{C}$ 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 ⁹ and immediately after emergence, male and female flies were sorted under light anesthesia. Then, flies were maintained in WS supplemented culture media and aged them for 10, 20 and 30 days for different stress and biochemical analysis.

Oxidative stress assay

To test susceptibility to acute oxidative stress, Paraquat is used as a stress inducing molecule ¹⁰. The oxidative test was carried out as per the standard procedure ¹¹. 3-days-old males were exposed to 20 mM PQ. Ten flies were transferred to empty vials and maintained for 2 hours before the test was set up. Flies were transferred to a vial of size 9 x 3 cm, contained Whatmann filters wetted with 20mM PQ in 5% sucrose solution. The control flies were placed into vials containing Whatmann filters wetted with only 5% sucrose. The flies were monitored for every 2hrs. The rate of survival was recorded for every 2 hrs., once until all the flies reached mortality in all the vials. The experiments were carried out in 10 such vials.

Desiccation Assay

Desiccation assay was carried out as per the standard protocol ¹². To carry out this test, male flies were isolated under light anesthetic condition and maintained in standard culture vials for aging. Then flies were introduced into a desiccation chamber which contains 1.0g of CaCl_2 , maintained constant relative humidity (5%) and constant temperature ($24 \pm 1^{\circ}\text{C}$). Further, vials were sealed with two layers of parafilm to prevent the entry of external moisture. The total number of flies reached the mortality was

recorded for every 2 hrs and data was collected until all the flies were dead. The experiment was repeated thrice.

Treatment for subjecting to oxidative stresses and desiccation;

To subject the oxidative stress, different aged WS fed flies were exposed to 20mMPQ with 5% sucrose solution in a empty vial for 24hrs. on every alternate days for 8 days. Flies were further maintained in normal wheat cream media fed with WS and used for further analysis. To subject the desiccation stress, different aged WS fed flies were treated with 20mM PQ and introduced the flies into a desiccation chamber which contains CaCl_2 with constant relative humidity and temperature; flies were exposed for 2 hrs. Then, flies were further maintained in normal wheat cream media fed with WS and used for further analysis. After the subjection of oxidative stress and desiccation, the flies were used for climbing assay (Negative Geotaxis), Lipid peroxidation assay, triglycerides and glycogen estimation. All the parameter were carried out in WS supplemented flies in dose-I (1mg/ml) concentration with 20mM PQ and dose-II (10mg/ml) concentration with 20mM PQ. Untreated wild flies of *D. melanogaster* without WS supplemented flies were considered as +ve control, while, 20mM PQ treated flies were considered as -ve control. All the studies were carried out in 10days, 20 days and 30 days aged flies.

Negative Geotaxis

To analyze the effect of WS on locomotion pattern in PQ induced flies, -ve geotaxis assay was carried out based on the standard protocol ¹³. Flies were transferred with light anesthetic condition in to a labeled vertical glass column tube (15 cm length and 1.5 cm in diameter). After 30 min of recovery from regular media, flies were gently tapped to the bottom of the tube, and the number of flies that climbed up to 6 cm mark of the column (i.e., the top) in 5 sec as well as those that remained below the mark was counted separately. Vertical climbing ability of flies in each batches were assessed. The procedure was repeated five times per group at 1 min intervals.

Lipid Peroxidation

Lipid Peroxidation assay was measured by employing thiobarbituric acid (TBA) by following the standard procedure ¹⁴. Assay was carried out in different aged WS supplemented flies under stressed conditions. 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. MDA was measured per mg of protein.

Protein estimation

Protein estimation was quantified by following Lowry's method ¹⁵. To quantifying the proteins, test sample was mixed with Lowry's reagent and allowed to incubate at room temperature for 15 min. then Folin-Ciocolteu's solution was added, mixed and again incubated at room temperature for 30 min. Optical density (OD) was measured at 660 nm using colorimeter and calculated the amount of protein by making use of BSA standard graph.

Estimation of Triglyceride

Triglyceride content was determined by using standard protocol ¹⁶ with slight modifications. 20 flies were homogenized in 100 μl PBS, 0.5% Tween 20, and immediately incubated at 70°C for 5 min. Heat-treated

homogenate was centrifuged, and supernatant was incubated with Triglyceride reagent for 30 min at 37°C. Samples were then incubated with Glycerol reagent for 5 min at 37°C, and the quantity was estimated at a wavelength of 525 nm using spectrophotometer.

Estimation of Glycogen

Glycogen levels were determined as described by using standard protocol¹⁶ with some modifications. In concise, there were 20 flies collected, homogenized in ice cold extraction buffer which contains HEPES(4-2-hydroxyethyl-1-piperazineethanesulfonic acid), Sodium azide, PMSF(phenylmethane sulfonyl fluoride) and EDTA of pH 7.0), centrifuged at 1700rpm for 3min at 4°C and the supernatant was aliquoted. Incubated the mixture with glucose reagent and optical density was measured at 540nm using spectrophotometer.

Statistical analysis

Stress tolerant survival probability analyses were performed between different treatment batches and control. All the Statistical analysis was performed using SPSS 19. For biochemical analyses, activity of LPO and the quantity of protein, glycogen, triglycerides were expressed as mean \pm SE. The level of significance was measured by one-way and multivariate ANOVA followed by tukey's test, with $p<0.05$ being statistically significant.

RESULTS

Oxidative resistance test

Oxidative resistance test was carried using Paraquat (PQ) as oxidative stress molecule. For this test, WS supplemented flies were exposed to PQ. The result obtained from the oxidative resistance test was represented in Fig. 1A. The highest mean survival ability in control flies (non-PQ induced) was observed on 20 days (43.04hrs), while PQ induced flies to wild flies were died on 22 hrs. itself. However, the mean survivability of WS fed flies with PQ treated flies was found to be 49.81hrs. in 20 days aged flies, but highest increase of survivability was noticed in dose-II at 30 days (73.55%). Statistical analysis reveals that both the dose treatment batches showed significant differences with the -ve control batches ($F=401.69$, $p=0.001$)

Desiccation test (DS Test)

DS test was performed in PQ induced flies as well as WS supplemented flies induced by PQ. The result obtained from the desiccation resistance test was shown in Fig. 1B. The mean desiccation resistance in control flies (without PQ induced) was more at 20 days flies (46.24). The highest mean survivability under desiccation in PQ induced flies at 20 days was 25.2hrs. While it was found to be 51.86 hrs in WS prefed PQ induced flies. The percentage of survivability was significantly more in WS prefed Dose-II at 10 days flies. ($F=396.29$, $p=0.001$). There was a significant increase in the survivability under desiccation status of the flies due to WS supplementation.

Negative Geotaxis

The negative geotaxis activity was performed in both oxidative and desiccation status under stress conditions with the extract supplementation. The results obtain from these experiments were shown in Fig 2A and B. The physical activity was highest in 10 days of control flies (92.81%) when compared among analyzed groups. There was a significant decline in the physical activity with PQ exposure in the flies in different age in oxidative stress. The

physical activity was increased in the analyzed groups at 10 days aged flies. The similar such result was observed under desiccation status. The highest geotaxis behavior was observed in Dose-II treatment batch at 10 days flies of both oxidative and desiccation status. However, there was a significant increase in the physical activity of the flies due to supplementation with WS ($F=364.69$, $p= 0.001$, Figure 5A) in Oxidative stress (Fig.2A) and Desiccation ($F=379.25$, $p= 0.001$, Fig. 5B). The flies reared on low to moderate supplementation of WS have significantly higher activity during later age. The result summarizes that stress decreases the physical activity of flies.

Lipid Peroxidation

The lipid peroxidation was measured by TBARS assay in different age grouped flies under stressed conditions. The data obtained of LPO performed in extract supplemented flies under both oxidative and desiccation status was represented in Fig. 3. The data on oxidative status revealed that MDA level was significantly more in batches where flies were treated with only PQ, where as LPO level was decreased in both the doses of WS extracts supplemented groups when compared to PQ treated group. The data shows that the extract treated flies, in all the age profiled groups showed significant reduction in MDA when compared to PQ treated without WS extract ($p<0.05$),. The highest MDA was noticed in 30 days aged PQ treated flies (0.40nmol MDA/ mg protein). The highest MDA reduction (0.21nmol MDA/ mg protein) was observed in 10 days Dose-II WS fed with PQ treated flies (Fig. 3A). Similar such results were noticed in desiccated WS fed flies when induced to PQ (Fig. 3B). The LPO activity was significantly decreased after the supplementation of WS. The LPO activity was least in Dose-II batch at 10days aged flies. In general, WS treated flies at different age intervals showed highest reduction in lipid peroxidation in both stressed conditions.

Estimation of Triglycerides

To know the effect of WS on triglyceride levels, quantification of triglycerides were made in WS supplemented flies under stress conditions. The result of the triglyceride estimation of both oxidative and desiccation status were expressed in Fig 4. The triglyceride quantity was found to be least in PQ treated (-ve control), while PQ treated in WS fed flies were found to be significantly high under oxidative status ($F_{2,8}=13.777$, $p=0.005$). The maximum quantity was obtained in WS dose-I pre-fed PQ treated 10 days aged flies (28.71 μ g/fly). However, there was significant increase of triglyceride levels in WS pre-fed flies when compare to control flies. Similar result was observed in batches under desiccation status. The quantity of triglycerides was significantly more in WS prefed 10 days aged flies both under oxidative and desiccation status.

Estimation of Glycogen

To know the effect of WS in the amount of glycogen, quantification of glycogens were made in WS supplemented flies under stress conditions. The result of the estimation of both oxidative and desiccation status were expressed in Fig 5. The triglyceride quantity was found to be least(11.38 μ g/fly) in PQ treated 30 days aged flies (-ve control), while PQ treated in WS pre fed flies were found to be significantly high under oxidative status (26.21 μ g/fly) at both 10 and 20 days aged flies. However, there was significant increase of glycogen quantity in WS pre-fed flies when compare to control flies. Similar result was observed in batches under desiccation status. The quantity

of glycogen was significantly more (26.21 $\mu\text{g}/\text{fly}$) in WS prefed 10 days aged flies both under oxidative and desiccation status. The estimated triglycerides and

glycogen were found to be high in WS prefed flies under both oxidative and desiccation status when compared to PQ treated (-ve control) ones.

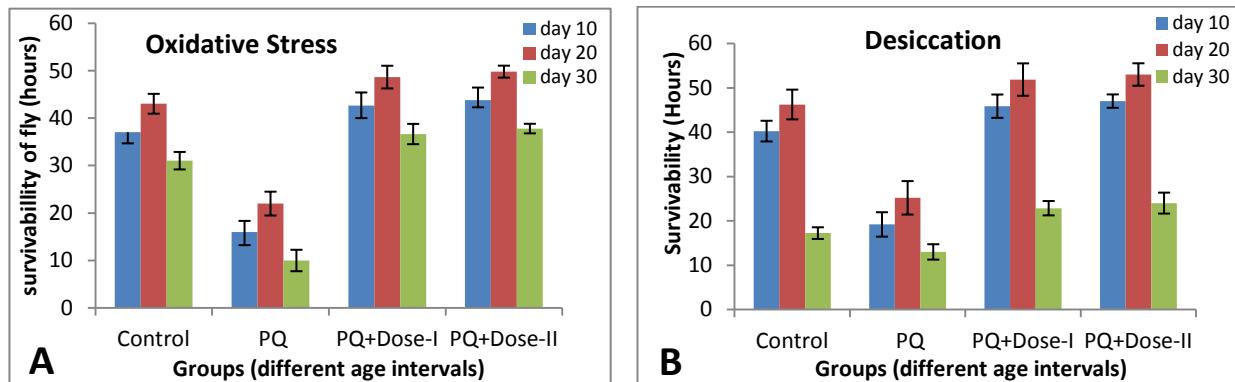


Figure 1: Effect of *Withania somnifera* extract against paraquat on survivability in *D. melanogaster*

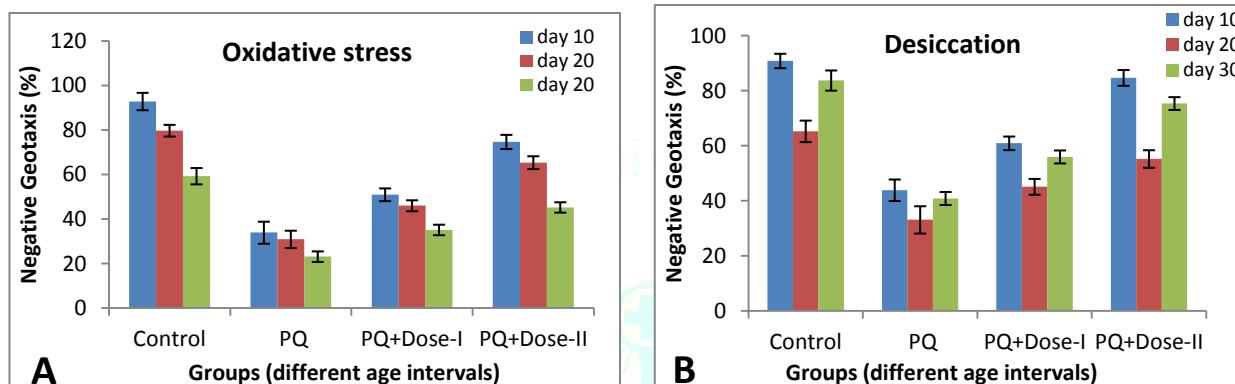


Figure 2: Effect of *Withania somnifera* extract against paraquat on locomotory behavior in *D. melanogaster* by negative geotaxis test.

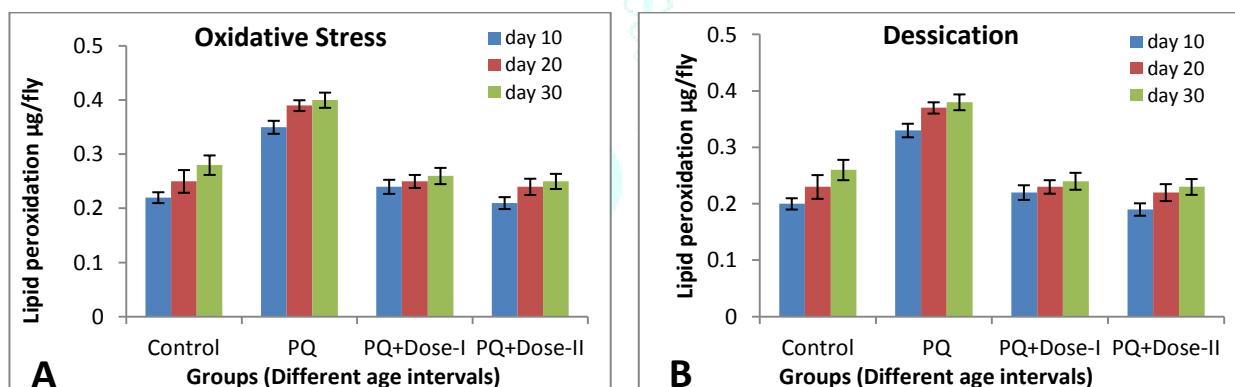


Figure 3: Effect of *Withania somnifera* extract on Lipid peroxidation level against paraquat in *D. melanogaster* by TBARS assay

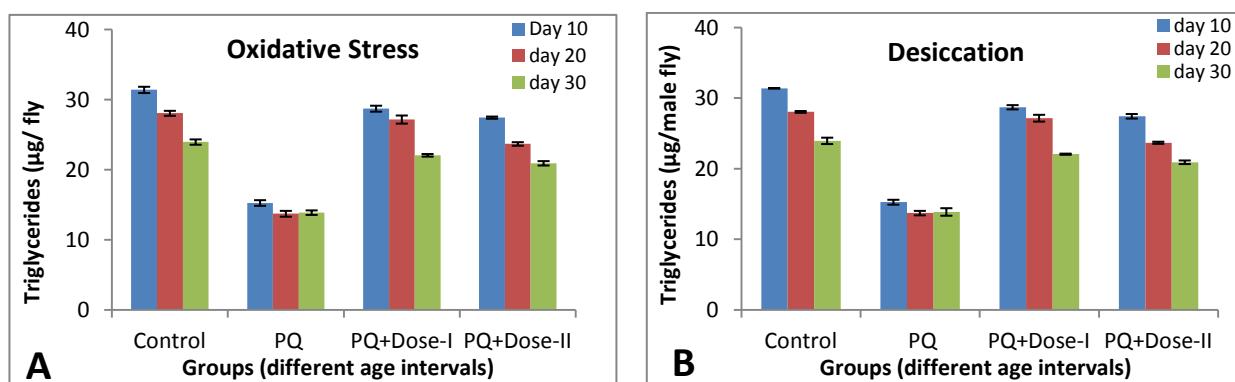


Figure 4: Effect of *Withania somnifera* extract on quantity of Triglycerides against paraquat in *D. melanogaster*

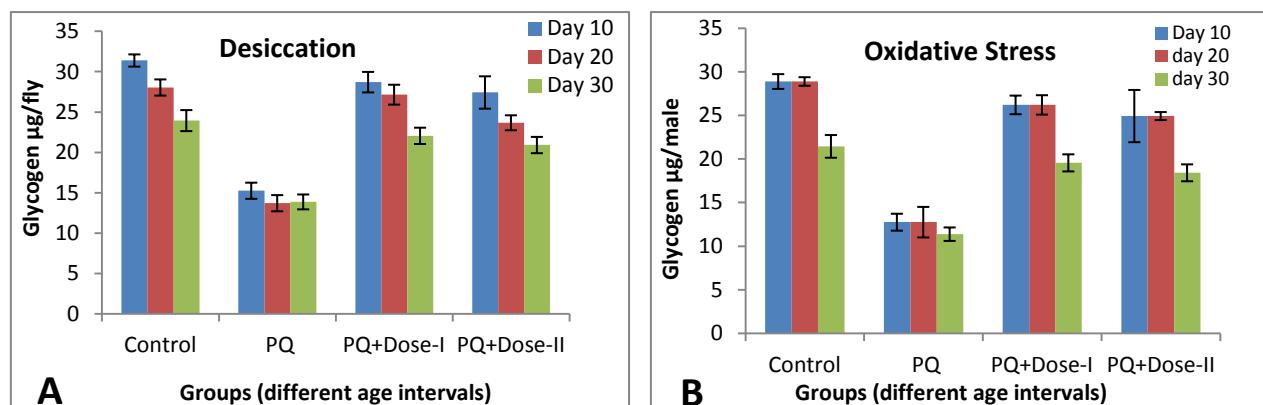


Figure 5: Effect of *Withania somnifera* extract on quantity of glycogen against paraquat in *D. melanogaster*

DISCUSSION

Paraquat, is a quaternary ammonium herbicide widely used in agriculture. It undergoes reduction, and generates a stable paraquat radical during in-vivo administration. Paraquat reacts with oxygen to generate superoxide anion like reactive oxygen species (ROS) ¹⁷. Paraquat can cause lipid peroxidation of ROS, protein carbonylation etc. The fruit fly *Drosophila melanogaster* is extensively used as an alternative model organism in biomedical research for many toxicity analyses. Various plant extracts and their bioactive compounds have ability to reduce the paraquat toxicity and minimize the oxidative stress. The consumption of high antioxidant compound reduces oxidative stress, ^{18,19}. It has been reported that the pre-fed blueberry extracts in *D. melanogaster* causes the reduction of paraquat-induced mortality ²⁰. Pomegranate juice and resveratrol decreases the paraquat-induced oxidative stress ²¹. Plant extracts contains many polyphenols of phytochemical family, having health promoting benefits ²². In the present study *Drosophila* was selected as a model to evaluate the stress tolerance capacity of WS against paraquat toxicity. The stress tolerance capacity of flies was measured by subjecting to desiccation and oxidative stresses in WS supplemented flies. WS has a potent antioxidant and possess various nutraceutical compounds, their biomedical properties are already been proved ²³.

In this study, oxidative and desiccation stress resistance test were carried out by inducing PQ. Resistant ability was increased in both the doses of WS under stress (PQ induced) conditions. Among the extract treated groups, both the dosage supplemented flies showed significant increase in survivability. 20 days WS fed flies showed higher survivability which increased by 2 folds over PQ treated -ve groups both under oxidative and desiccation stress conditions. Surprisingly resistance ability was increased by 2.4 times more in WS fed flies with PQ treated batches. The percentage of survivability was more in WS flies even with the control flies. In conclusion, result indicated that PQ induction affects the survival of the flies due to the oxidative stress that minimized by WS extract, thus the antioxidants of WS increased the percentage of survivability.

Locomotor activity of an organism was analyzed by negative geotaxis behavior. It is an ability to move against gravity and climb, is suggested to indicate the level of physical fitness of test animals ²⁴. PQ induced flies showed locomotors deficits, while WS fed flies with PQ has showed better locomotor activity under stress condition when compared to PQ induced flies. -ve geotaxis behavior in Dose-II of WS fed flies showed 54.62% more activity under

both oxidative and desiccation stress than PQ induced flies. The oxidative stress caused by various types of stress molecules like paraquat, H₂O₂, methotrexate and acrylamide could be minimized by antioxidants^{25,26}. Studies have shown that *Anacardium microcarpum* reduces the oxidative stress caused by paraquat in *D. melanogaster*²⁰. The result of present study is in line with Muller and his team ²⁰. In conclusion, PQ induction causes oxidative stress that could be reduces by WS extract.

Lipid peroxidation analysis is one of the key biochemical parameter to detect the oxidative stress toxicity. This assay was made based on reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). MDA is a byproduct of lipid peroxidation, by quantifying MDA amount of lipid peroxidation can be measured. Researchers observed that supplementation of green tea catechins and broccoli extract reduces total body LPO level. Oxidative stress resistance abilities and LPO were analyzed in *Lycium chinese* extract fed *D. melanogaster* ²⁷. Perusal of literature evidences showed that lipid peroxidation decreases the level of MDA in pre-fed various extract flies induced by Paraquat^{28,29,30}. In the present study LPO was studied by TBARS assay in WS supplemented flies under oxidative and desiccation stressed conditions. WS supplemented flies showed decreases in MDA level significantly compared to both stress control and normal control. Further the result indicated that the WS treated flies higher dose at 10 days showed greater reduction of MDA, it was decreased by 40% than stressed condition, while in desiccated stress condition, WS fed flies in higher dose at 30 days showed highest reduction in lipid peroxidation, that reduced by 65.21% than stress condition. Here, WS supplementation plays a pivotal role in reducing lipid peroxidation. Present result shows similar observation was made on supplementing different plant extracts in *D. melanogaster* by many researchers^{31,32}. It has proved that *Lycium chinese* extract scavenges the free radicals *in vivo* by up-regulating the antioxidant enzymes and thus inhibits lipid peroxidation in flies ³³. These studies clearly demonstrated that up-regulation of endogenous antioxidants levels inhibits lipid peroxidation that in turn increases stress tolerance ability of the organisms. In the present analysis antioxidants of WS were responsible for reduction of lipid peroxidation in *D. melanogaster*. Therefore, by down regulating MDA, WS increases stress tolerance ability, locomotor behavior in WS supplemented *D. melanogaster*

Sugars are the key carbon sources for production of energy. Stress resistance ability is associated to energy storage molecules in the diet including carbohydrates and lipids.

Triglycerides and glycogen are two predominant energy storage molecules in an animal and both were decreased steadily when exposed to stress^{34,35,36}.

In the present analysis, triglycerides and glycogen estimations were made in WS fed flies after subjection of oxidative stress and desiccation by induction of PQ. The result confirms that the quantities of both triglycerides and glycogens were found to high in both the dosage groups of WS fed flies under oxidative as well as desiccation stress conditions. WS fed flies increases 1.8 folds of triglycerides with PQ treated flies under oxidative as well as desiccation stress conditions. The result of glycogen in oxidative conditions reveals that the quantity was 2 fold increases in 20 days of Dose-I WS fed flies, while in other age group it increases by 1.5 to 1.9 folds when compared to PA treated flies without extract. However, in desiccated batches, both the dosage fed groups increases by 2 fold of glycogen in 10 and 20 days aged flies, when compare with PQ treated flies without extract. The improvement of both triglycerides and glycogen is due to WS supplementation. Though flies were stressed by PQ, the quantities of these energy yielding molecules acquired more, because of WS, even under oxidative and desiccation stress conditions. It has been proved that the antioxidants and bioactive compounds of WS are much greater³⁶. This increases the major energy

yielding molecules such as triglycerides and glycogen; thereby it increases the stress tolerance ability. Our results suggest that, WS administration in the diet can cause metabolic changes and stress resistant activity in *Drosophila*. Hence it proves the adaptogenic and antiaging property of *W. somnifera* in *D. melanogaster*. This study is a first kind of report showing stress, desiccation and oxidative stress resistant effect of *W. somnifera* in *D. melanogaster*.

CONCLUSION

The potential protective effect of *Withania somnifera* on stress tolerance capacity has been analyzed against paraquat toxicity using *Drosophila melanogaster*. PQ induced WS fed flies showed greater survivability in oxidative stress as well as desiccation condition when compared to PQ induced flies. The stress tolerance capacity of flies was measured by subjecting to desiccation and oxidative stresses, PQ induced WS fed flies showed better locomotor ability, low LPO activity with high triglycerides and glycogen contents. PQ induction causes oxidative stress that could be reduced by WS extract. Increase of these two major energy yielding molecules responsible for the increase of stress tolerance ability.

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