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EFFECT OF WITHANIA SOMNIFERA ON STARVATION RESISTANCE IN DROSOPHILA MELANOGASTER.

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Abstract: Most of the animals are facing the problem of periodical starvation, thus, expected to evolve adaptations in enhancing the starvation resistance. The capacity of an individual to survive prolonged period of starvation is depends on its diet and nutritional status. Many herbal and medicinal plants are known to have adaptogenic activity. Induction of such energy rich plants can increase many adaptive features in an organism. In the present study we have evaluated the adaptogenic property of *Withania somnifera* through *in vivo* studies by taking *Drosophila* as a model organism. There is an association between stress resistance and longevity in *D. melanogaster.* Starvation resistance and longevity were measured in methanolic extracts of *W.somnifera* supplemented flies of *D.melanogaster* through larval feeding. The result reveals that the extract supplemented flies increases the starvation resistance and extended the life span. Further through biochemical analysis the result supports that extract fed flies had more carbohydrate with high contents of glycogen that increases the resistance ability.

Keywords: *W.somnifera*, *D.melanogaster*, Starvation resistance, Longevity, Biochemical analysis.

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INTRODUCTION

Stress is an, any environmental factor that reduces the fitness of an organism. Under natural selection organisms have evolved with many specific adaptations to resist stress [1,2]. One of the major causes of stress is shortage of nutrition and certainly an animal experiences the periodical starvation, thus, expected to evolve adaptations in enhancing the starvation resistance. The capacity of an individual to survive prolonged period of starvation is depends on its diet and nutritional status. The amount and quality of nutrients intake by organisms have also a strong impact on life – history traits [1,3].Many herbal and medicinal plants are known to have adaptogenic activity. Induction of such energy rich plants can increase many adaptive features in an organism. One such medicinal plant having high adaptogenic with high antioxidant property is *Withania somnifera* (L.)Dunal. It is commonly known as Ashwagandha, belongs to the family *Solanaceae*. In Indian Ayurveda *W.somnifera* is used for various health remedy like arthritis, inflammations, conjunctivitis and tuberculosis [4,5]. The antioxidant potential from the root extracts of Ashwagandha, has been already studied through in-vitro assays [6]

The molecular mechanisms of physiological response to nutritional stress seem broadly conserved throughout the animal kingdom [7,8]. The best model to understand the physiology of starvation resistance is a Dipteran insect *Drosophila,* because of genetical homology with human beings [9]. The starvation resistance studies were analyzed in different artificial selection lines of *D.melanogaster* [10,11]. The stress resistance studies have also been reported in medicinal extract fed flies of *D.melanogaster* [12].

The basic mechanism that enhance to survival under starvation stress is the storage of higher energy reserves, especially in the form of lipids.There is an association between stress resistance and longevity with the storage levels of body lipids in *D. melanogaster* [13,14]. Hence, in the present study we have evaluated the adaptogenic property of *Withania somnifera* through invivo studies by taking *Drosophila* as a model organism. The goal of this study is to determine the relationship between starvation stress resistance, longevity and lipid as well as other biomolecular proportions in extract fed flies.

Materials and methods:

Preparation of extract:

The roots of *W.somnifera* were collected from in and around Mysore city, Karnataka, India. Roots washed and then dried under shade at room temperature. The powder form of *W. somnifera* was subjected to methanol solvent extractions using soxhlet apparatus. The extraction was carried out for 12-16 hours. The crude extract thus obtained was transferred to

flash evaporator for complete evaporation. The dried extract thus obtained was used for all the experiments. The concentrations of extracts were fixed based on LC_{50} value. All the experiments were carried out using two concentrations viz., 1mg/ml and 10 mg/ml.

***Drosophila* culture:**

Oregon-K strain of *D.melanogaster* were obtained from Drosophila Stock Centre, Department of studies in Zoology, University of Mysore, Mysore. The stocks were maintained at 22±1^{0}C with 60-70% relative humidity under 12hrs light and 12 hrs dark cycles in 30 ml of culture bottles containing wheat cream agar media seeded with yeast granules. All the experiments were carried out in the flies by supplementing with *W. somnifera* extract (10µl /3ml media) through larval feeding. Only yeast treated flies were considered as control group.

Starvation resistance:

Starvation resistance assay was analyzed by following the standard method [15]. Newly emerged male and female flies were isolated from the synchronized extract supplemented culture bottles within 2hrs of eclosion. Then the flies were distributed in groups of 25 flies per vial of size 9 × 3 cm containing equal amounts of standard culture media with the extract. Then different aged flies were transferred to empty vials plugged with water soaked sponge. The vials were observed for every six hours once until all the flies reached mortality and calculated the resistance ability (hours) in all the analyzed groups. The experiments were carried out in10, 20 and 30 days aged unmated male and virgin female flies in different batches. 100 flies were maintained in control as well as extract fed groups.

Longevity:

Longevity studies were carried out in unmated flies of *D. melanogaster* based on standard protocol proposed [16]. Newly emerged male and female flies were isolated, separated from extract fed culture bottles using stereozoom under mild anesthetic conditions. Then 25 flies were transferred into vials of size 9×3 cm, containing equal quantities of culture media and supplemented with extract. The flies were transferred to fresh culture vials once in every three days without being etherized and survival rate was recorded in each vial, until all the flies reached the mortality. Longevity was measured in both the dose treatment groups and control groups. 100 flies were maintained in each control as well as in extract fed groups.

Biochemical analysis:

To know the association between starvation resistance and longevity with biomolecular components in the methanolic extract *W.somnifera* supplemented flies, few biochemical estimations were carried out. All the estimations were carried out in extract fed flies of

D.melanogaster at two different doses, only yeast treated flies were considered as control group. The estimations were carried out in whole body homogenate in three replicates in each group. All the biochemical estimations were analyzed in 20 days aged extract treated *D. melanogaster* flies of both sexes. All the chemicals used in the estimations were procured from SRL Pvt.Ltd, Mumbai.

Estimation of lipids:

Lipid estimation was carried out using the standard protocol [17].To estimate the lipid content in the extract supplemented flies, lipid sample was prepared from 1g wet weight of *D. melanogaster* flies and homogenized using solvent mixture, containing ethyl ether and ethanol in the ratio 3:1 in a pestle and mortar. Homogenized samples were centrifuged at 2000 rpm for 10 min. to get clear extract. To this, 0.05M KCl was added and shaken well to separate lipid layer from water layers, lipid layers was carefully transferred into a tube containing chloroform and copper reagent, mixed well for 2 minutes. The mixture was centrifuged at 2000 rpm for 10 minutes at 4°C, then upper aqueous layer was discarded and 0.1% of sodium diethyl dithiocarbamate solution was added to the lower chloroform layer, mixed well. Finally absorbance was read at 440 nm using colorimeter. The amount of lipid in the sample was calculated using Palmitic acid as standard.

Estimation of carbohydrate:

The total carbohydrate content was determined by following the standard method [18]. To carry out the estimation, flies were freezed and dried in a batch of 10 flies per vial for 36 hours at 70^0C. Then, measured the dry weight of flies in each batch followed by homogenization with distilled water. Further the samples were incubated in a water bath at 90^0C for 30 minutes, then, centrifuged the mixture at 5000 rpm and supernatant was mixed with anthrone reagent (2mg/ml). The sample was thoroughly mixed using vortex mixer and heated for 30 min, cooled at room temperature. The optical density of each sample was then measured at 620 nm using colorimeter. The total amount of glucose in each batch was estimated using a glucose standard graph. The fractional carbohydrate content was calculated by dividing the absolute carbohydrate content by the dry weight of the batch before extraction.

Estimation of Trehalose:

The quantity of trehalose in extract supplemented flies was estimated by following standard method [19]. For this, 10 flies were homogenized in 2% sodium sulfate solution using glass homogenizer and 1 ml of methanol was added to the homogenates, centrifuged at 3000 rpm. Then pellets were resuspended with distilled water twice. Further, 100 µl of the sample was mixed with 50µl of 1N HCL and heated at 90^0C for 7 minutes. Then 150µl of 1N NaOH was

added and heated the sample with anthrone reagent, sample was cooled and optical densities were measured at 555nm using the spectrophotometer. Trehalose concentration for each groups were calculated using a trehalose standard curve.

Estimation of Glycogen:

The concentration of the Glycogen was performed using methods as described [10]. To quantify the glycogen in *W.somnifera* supplemented *D. melanogaster* flies, ten flies were homogenized in 200µl of 2% sodium sulfate solution. Further, 1ml of methanol was added to the homogenate and centrifuged for 1 minute at 2000 rpm. The obtained supernatant was decanted and evaporated to a volume of 200µl, and then anthrone reagent was added to the sample. The solutions were thoroughly mixed, heated to 90^0C, and after cooling the sample at room temperature, the optical densities were measured in a wavelength of 625nm using spectrophotometer. Glycogen estimations were carried out in both extract fed flies and control flies. The total amount of glycogen was calculated using glycogen standard graph.

Estimation of Protein:

Protein estimation was quantified by following Lowry's method [20]. For this estimation 20µl of the test sample was mixed with distilled water and Lowry's reagent, and then allowed to incubate at room temperature. Further, Folin-Ciocolteu's solution was added followed by incubation. The optical density was measured at 660 nm against blank after incubation using colorimeter. Then calculated the amount of protein in each group with the Bovine serum albumin (BSA) standard graph.

Statistical analysis;

The data obtained from all the experiments were subjected to statistical analysis using SPSS software (version 16.0).To know the level of significance among the analyzed groups in starvation resistance and longevity, data were subjected to one way ANOVA analysis, followed by DMRT. A probability of $P < 0.05$ was considered as significant. The male and female longevity of the analyzed groups were measured through survival curve analysis. Data from biochemical estimations were expressed as mean ± SE. To know the relationship between longevity and starvation resistance test, Pearson's correlation coefficient was applied.

Result and discussion:

The amount and quality of nutrients intake by organisms have a strong impact on life-history traits, fertility, longevity and stress resistance. The ability of animals to withstand prolonged periods of food deprivation is called 'starvation resistance'. It is an important key trait for the survival of insect ability to survive under starvation [1,21]. Starvation resistance is usually

quantified as the time until death under starved conditions. Various environmental factors causing change in the phenotypic expression of starvation resistance and its fitness, among them nutrient composition of foods are the most important factor affecting starvation resistance because the physiological capacity of animals to survive starvation depends on their nutritional status and dietary history [22,23]. In the present study starvation resistance and longevity were analyzed in *W. somnifera* extract supplemented flies of *D.melanogaster*. Virgin females, showed a general declining tendency of starvation resistance as females aged [14]. Hence, in the present study the resistance ability was measured in different aged extract supplemented unmated flies (10, 20 and 30 days) of both the sex in two dose treatment batches. The results of starvation resistance in the extract supplemented flies are compiled in Table.1. The resistance ability was found to be low at 10 days, reached maximum at 20 days, then declined at 30 days in both extract treated as well as control batches. Similar such observation was observed in both the sex. Under starvation, the mortality in control group was begins at 24th hrs. In 10 days aged male batch, while, it was found to be 30^{th} hrs in rest of the group analyzed. In 10 days treatment batch, female of dose-II shows more resistance ability under starvation with the mean resistance ability was 60.96±1.32hrs. Similarly, in 20 days and treatment batches, the maximum resistance ability was observed in dose- II of female batch in 20 days, where mortality was extended up to 96^{th}hr, with the mean resistance ability 74.04± 1.83hrs (Fig.1). However, in 30 days, female of Dose-II batch had the highest starvation resistance 62.34±1.55, where mortality extends up to 84hr. The data on statistical analysis reveals that both the dosage treatment batches showed significant differences with the control batches ($P<0.05$). Further, it shows that among the analyzed batches, females of 20 days treatment had highly significant differences with the F value is 62.62.

The starvation resistance ability study has been proved in the artificial selection lines of *D.melanogaster*. [10,11] The starvation resistance ability study also been analyzed in different diet fed *D. ananassae* and shown that the resistance ability was increased in carbohydrate rich diet fed flies [24]. The starvation tolerance study was analyzed in different ayurveda rasayana fed flies of *D.melanogaster,* tolerance was improved in Amalakki rasayana fed flies [25]. Further, it has been reported that phytoecdysteroids of fenugreek extract increases the resistance under stress [12]. The starvation resistance analysis has been studied in *Phyllanthus debilis* extract supplemented *D. melanogaster* and they opined that extract supplemented flies showed increased starvation resistance [26]. Similar observation is exists in the present study, among the extract supplemented batches, dose-II treatment groups (10mg/ml) showed maximum resistance ability, male increased by 46.14%, female increased by 43.48% of resistance than control flies. Current analysis supports the observation reported by others.

Increase stress resistance has often been linked with life span extension. The evolutionary relationship between starvation and longevity has been confirmed in some selection experiments. There is an evidence for a positive relationship between stress resistance and longevity in *D. melanogaster.* Mating shortens the lifespan of female flies and that effect may carry nutritional and metabolic costs [27,28]. Adult diet manipulation has been the principle means of longevity extension in fruit flies [29,30]. Hence, in present study longevity was carried out in unmated male and virgin female flies to know the association with starvation resistance by supplementing methanolic extract of *W. somnifera* through adult feeding. The results of longevity in extract supplemented flies are given in Table.2. The longevity was carried out in both the sex from two dose treatment batches. The maximum fly survived in control male was 83 days, while female was 86 days with mean lifespan of 65.08 and 61.32 days respectively. In male treatment batch, the maximum fly survived in extract treated male flies from dose I and dose II treatments were 95 and 108 respectively, the mean life span in dose-I group was 83.46 days and the dose-II group was 88.86 days. In female treatment batch, the maximum fly survived until 102 days with the mean life span 84.23 days in dose-I group and 112 days, with the mean life span 90.97 days in dose-II group. The statistical analysis shows that all the groups showed highly significant differences with each other with the F value 107.56 in male group and 118.40 in female group. Further, the data reveals that the extract supplemented groups in both male and female group shows significant increase mean life span when compared to control groups. Among the dose treatment batches, dose-II of female batch has highest lifespan. The life span extension study was made in the fruit flies, supplemented with various medicinal plant extracts. The high antioxidant rich plants *Curcuma longa, Emblica officinalis* and *Rhodiola rosea,* increases the life span significantly in *D. melanogaster* [31,32]. Further, it has been reported that a bioactive polyphenolic compound curcumin of *Curcuma longa* and flavonoids and polyphenols of Cocoa increases the life span of *D. melanogaster* [33,34]. The longevity study has been analyzed in *Asparagus racemosus* extract fed flies of *D. melanogaster* and reported that antioxidants of the plant increases the longevity by 41% than control flies [35]. Further, longevity study has been made in two species of *Phyllanathus* and reported that *P.amarus* increases the lifespan by 75% and *P.debilis* increases by 66.66% than control flies of *D.melanogaster* [26]. These reports clearly indicate that plants have significant role in life span extension. Based on the obtained result, the present study revealed that the longevity was increased in both the dose treatment groups of *W.somnifera* extract fed flies when compared to control group. The male batch showed an increase of longevity by 28.24% in dose-I fed groups and 36.53% in dose II treatment. The longevity in female group was increased by 37.47% and 48.35% in dose I and dose II treatment respectively. The survival curve of longevity are compiled in Fig.2. A sex specific lifespan extension has been reported in *Cynomorium songaricum* extract treated *D. melanogaster* batches [36]. Acai pulp increases the lifespan by 22% in female flies [37]. The life span of females fed with *Asparagus racemosus* was increased

by 41.17% [35]. In the present study, similar such observation is found in *W. somnifera* extract supplemented flies, where all the females showed increased longevity than males. This result is in line with the findings of earlier reports [26,35,37]. The antioxidant activity of *W. somnifera* has been reported through many *invitro* studies [38,39]. Hence antioxidants present in *W. somnifera* increase the life span in *D.melanogaster*.

Starvation resistance can influence the longevity [40]. It has been reported that the enhanced longevity in selected lines of *D. melanogaster* are relatively more resistant to starvation stress [41,42]. Further, it has been shown that long lived *D. melanogaster* mutants are usually stress resistant [43]. Mutation of *chico* of *Drosophila* extends life-span by up to 48% and also increases starvation resistance in females [44]. Correlation analysis between starvation resistance and longevity was also been observed in flies supplemented with *Cynomorium songaricum* extract [45]. Further it has been reported that the increased resistance to starvation was positively correlated with lifespan extension [26,46]. In the present study, the maximum starvation resistance was observed in 20 days aged extract fed flies, hence, to study the association between starvation resistance and longevity, *W. somnifera* extract fed flies, Pearson correlation analysis has been made in 20 days aged flies. The result of correlation study revealed that there was a high degree of positive correlation exists between the longevity and starvation resistance in the extract treatment groups at $p<0.01$. Further, it was noticed that dose-I of extract fed flies of both the sex has highest 'r' value (0.983 in male and 0.982 in female). The present result of correlation analysis confirms the existence of positive correlation between lifespan extension and starvation resistance in *W.somnifera* extract supplemented flies. These results are in line with the observation reported by others [26, 46]

Biochemical analysis:

Starvation resistance ability is associated to energy storage molecules in the diet such as carbohydrates, proteins and lipids. Increased starvation resistance can have an indirect response on larval developmental time, and can cause higher adult lipid content [47]. In *Drosophila*, the level of body lipid storage has been considered as the most significant determinant of starvation resistance [48]. Hence, in the present study differential biomolecular abundances were measured in *W.somnifera* extract supplemented flies. The starvation resistance was significantly high in 20 days extract fed flies, hence to understand the association between starvation resistance and major energy yielding molecules, the biochemical estimations namely lipids, carbohydrates and proteins were made in 20 days extract fed flies of both the sex. The results of lipids, carbohydrates and proteins estimations are compiled in Fig. 3. It was observed that the quantities of all the three biomolecules were found to be high in *W.somnifera* fed flies when compared to control flies. Further, it was observed that the female group of Dose-II were had more quantity in all the analyzed

biochemical parameters. The highest quantity of lipid was observed in dose-II batch (16.13 µg/ml), which was found to be increased by 2.56 folds than control group. Similarly, the highest carbohydrate content in dose-II batch was 59.33 µg/fly, which increased by 1.18 folds than control group. Though, the highest quantity of protein was observed in Dose-II of female group (5.76 66 µg/fly), the overall protein quantities were found to be least among the analyzed biochemical estimation. Further, the result reveals that the quantity of carbohydrate was found to be high among analyzed biochemical estimations. The statistical analysis reveals that the quantity of all the three biochemical parameters in both the dose treatment groups of extract fed flies shows highly significant differences with the control flies.

Sugars are the major carbon sources for production of energy. Trehalose and glycogen are the major energy yielding carbohydrate. Trehalose is a nonreducing disaccharide, synthesized in the fat body that becomes the major haemolymph sugar in most of the insects including *Drosophila* [49]. Similarly, the glycogen is also as another main energy storage molecules, store the glucose in tissues. Since, the quantity of carbohydrate was found to be high among analyzed biochemical estimations, to detect the level of specific carbohydrate, the quantity of trehalose and glycogens were estimated in 20 days aged *W.somnifera* extract fed flies of *D.melanogaster.* The results of trehalose and glycogen estimations are compiled in Fig.4. It reveals that the quantity of trehalose and glycogen in control batch of both the sex were remains same, while irrespective of the dosage, both the quantities were more in female than male groups of extract fed batches. The maximum amount of trehalose was noticed in dose-II treatment of female batch (12.33µg/fly). Similar such observation was observed in glycogen estimation, the highest amount of glycogen (19.60µg/fly) was recorded in dose-II treatment of female batch. The statistical analysis reveals that in both male and female groups, the extract treated dose-I and dose-II batches showed significant differences with control groups. Further, the result reveals that in all the analyzed groups, the major part of the carbohydrates was exist in the form of trehalose and glycogen. In dose-II of both male and female groups, 20% of carbohydrate was found to be as trehalose, 30 to 33 % of carbohydrate as glycogen.

The abundance of lipids energy storage molecules are correlated with starvation resistance [50,51]. Starvation-resistant flies may carry greater lipid reserves at eclosion itself [47]. Further they concluded that the changes in lipid metabolism underlying starvation resistance already occur during larval stage and starvation-resistant lines continue to increase their lipid reserves during early adulthood. The relationship between starvation resistance, body lipid content and lifespan in *D. simulans* population has been reported a negative relationship, suggesting a fitness cost to increasing lipid reserves [52]. The starvation resistance study has been made in *D. ananassae,* which were fed with different nutrient rich food media and reported that higher starvation resistance for flies developed on carbohydrate-rich diet compared to those

developed on protein-rich diet [53]. Females stored higher level of body lipids and glycogen contents, utilized both of these energy resources under starvation stress, whereas the starved males metabolized only body lipids as a source of energy [54]. In the current study, proves that *W.somnifera* extract fed flies have more carbohydrate than lipids, thereby which increases the resistance under starvation. The glycogen is becomes a major carbohydrate in serving bulk energy reserves for developing resistance under starvation. The obtained observation was similar as reported earlier [53,54].

Conclusion: In conclusion, the studies reveal that *W.somnifera* is high energy reserving and antioxidant rich plant, the supplementation of this increases the starvation resistance and longevity in *D.melanogaster*. The high amount of carbohydrate (glycogen) in extract fed flies responsible for developing increase resistance under starvation. Hence it proves the adaptogenic and antiageing property of *W.somnifera* in *D.melanogaster*

Table.1. Result of Starvation resistance in *D.melanogaster* supplemented with *W.somnifera.*

Groups	Starvation resistance in days (expressed in hours)					
	10days		20 days		30 days	
	Male	Female	Male	Female	Male	Female
Control	44.22±1.01[(a)]	49.62±1.06(a)	45.90±0.95(a)	51.60±1.02(a)	40.20±1.01(a)	48.96±1.09(a)
Dose-I	51.00±1.04(b)	56.64±1.17(b)	53.88±1.10(b)	60.12±1.31(b)	48.48±1.03(b)	55.50±1.25(b)
Dose-II	55.80±1.19(c)	60.96±1.32(c)	67.08±1.46(c)	74.04±1.83(c)	52.86±1.19(c)	62.34±1.55(c)
F value	28.69	23.02	80.03	62.62	34.95	25.86

Df= (2,297) Note: The strains with the same letter in the parenthesis are not significantly different at 5% level according to DMRT.

Table.2. Result of Longevity of *D.melanogaster* supplemented with *W.somnifera.*

Groups	Male				Female			
	Minimum (days)	Maximum (days)	Mean ± SE (days)	% of increase	Minimum (days)	Maximum (days)	Mean± SE (days)	% of increase
Control	42	83	65.08±1.23(a)	-	41	86	61.32±1.54(a)	-
Dose-I	60	95	83.46±0.90(b)	28.24%	60	102	84.23±1.16(b)	37.47%
Dose-II	60	108	88.86±1.40(c)	36.53%	60	112	90.97±1.53(c)	48.35%
F value	107.56						118.40	

Df= (2,297) Note: The strains with the same letter in the parenthesis are not significantly different at 5% level according to DMRT.

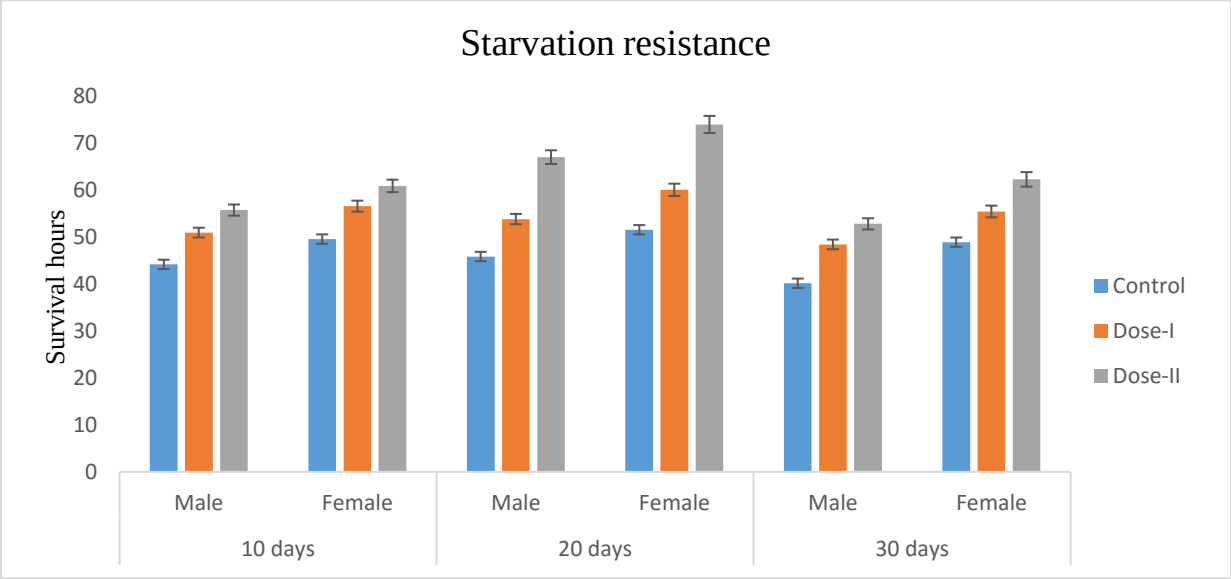
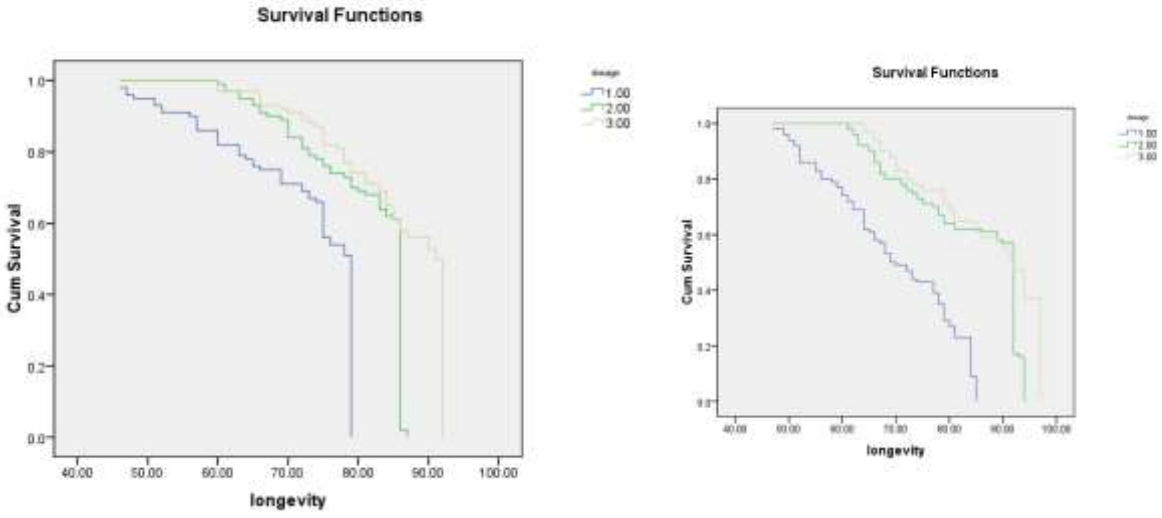


Figure1. Result of Starvation resistance in *D.melanogaster* supplemented with *W.somnifera.*



a) Male b) Female

Figure 2: Survival curve of *D.melanogaster* flies supplemented with *W.somnifera*

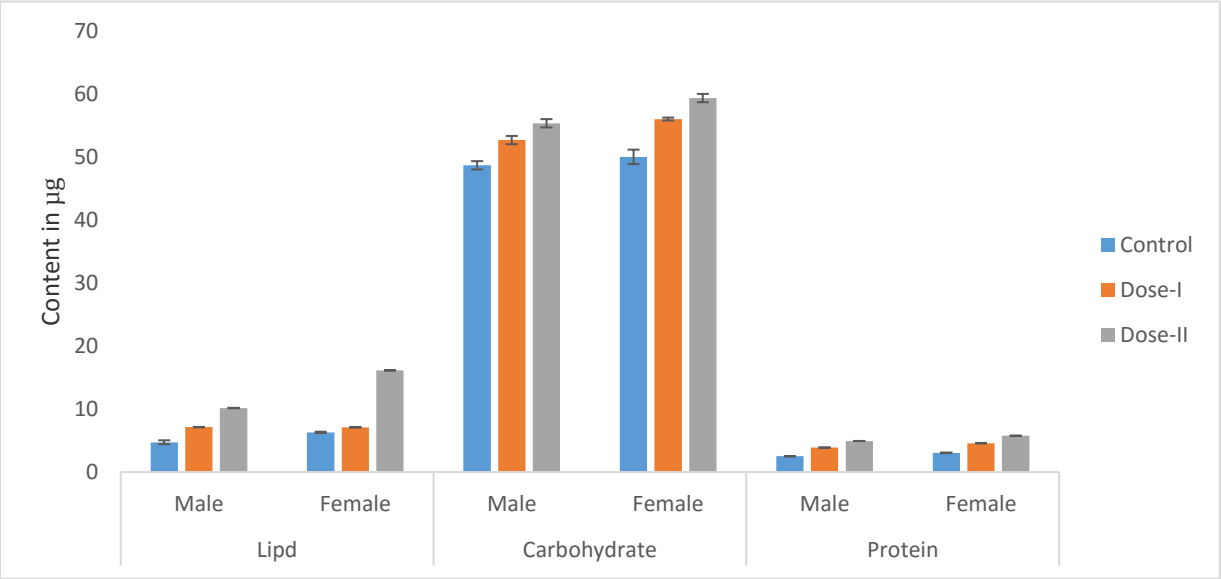


Figure.3. Result of quantification of biomolecules in *W. somnifera* extract supplemented flies of *D. melanogaster*.

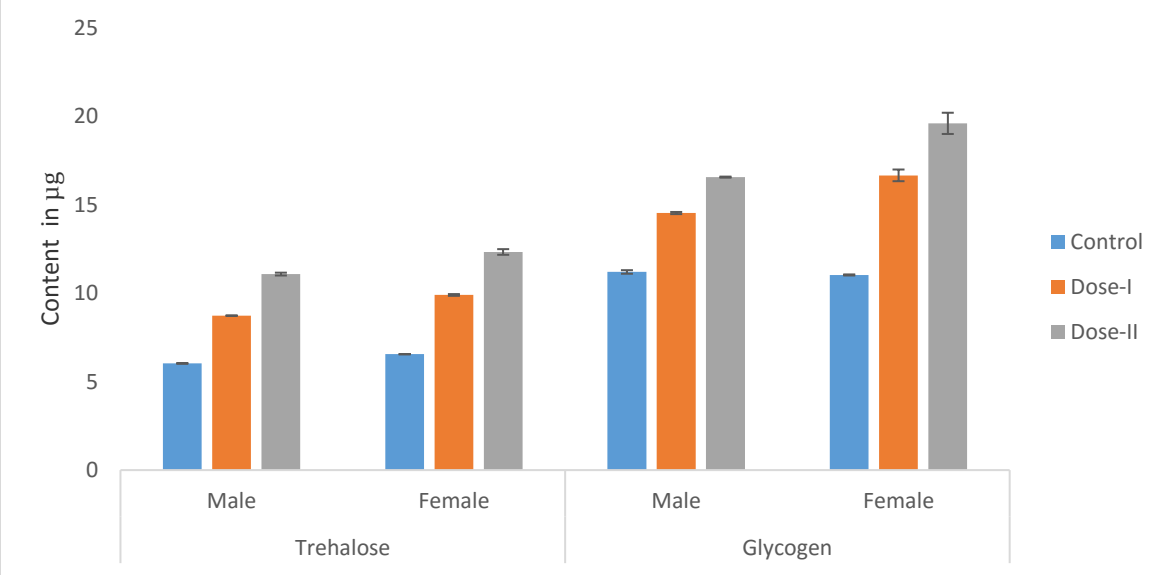


Figure.4. Result of quantification of Trehalose and Glycogen in *W. somnifera* extract supplemented flies of *D. melanogaster* .

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