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RESEARCH ARTICLE

EFFICIENCY OF ROOT EXTRACT OF *WITHANIA SOMNIFERA* GROWN IN BLACK SOIL IN ENHANCING FERTILITY OF MALE *DROSOPHILA MELANOGASTER.*

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ABSTRACT

Male infertility is a worldwide problem. Oxidative stress can damage the membrane of sperm, affecting motility and morphology. The alternative and complementary medicine for oxidative stress and infertility problems is antioxidant rich herbal medicine. Medicinal plants are considered to be the best source for antioxidant compounds, as plants produce significant amount of antioxidants to prevent the oxidative stress. One of the well-known herb extensively used in Indian Ayurveda for the treatment of male infertility is *Withania somnifera* (WS) commonly known as Ashwagandha, that has been studied for its potential effects on various aspects of health. The present study evaluates the antioxidant potential of ethanolic root extracts of *Withania somnifera* grown in black soil and red soil through *in vitro* analysis. A comparative phytochemical analysis has been carried out in *W. somnifera* grown in red and black soil. The bioenergetics molecules estimation and oxidative stress resistance ability has been done in extract fed group of *Drosophila melanogaster*. WS extract grown in black soil fed *D. melanogaster* increases the total antioxidants by 27%, increases 2.41 folds of resistance ability against oxidative stress with the increase of bioenergetics molecules in the flies. Further it increases testis size and sperm count in male *D. melanogaster*. This is due to high degree of antioxidants and natural polyphenols found in *Withania somnifera* grown in black soil compared to WS grown in red soil.

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INTRODUCTION

Infertility is a worldwide problem, male infertility is usually caused by problems that affect either sperm production or sperm transport, and 30%-40% of infertility is because of sperm abnormality (1). About two-thirds of infertile men have a problem with formation of sperm in the testes, either low numbers of sperm or non-functional. Oxidative stress has been identified as one of the many mediators of male infertility by causing sperm dysfunction (2). Oxidative stress can damage the membrane of sperm, affecting motility, morphology. Though various synthetic pharmaceutical drugs are available, many people are tend to use alternative and complementary herbal medicine. Medicinal plants are considered to be the best source for antioxidant compounds (3), as plants produce significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they considered as potential source of new compounds with antioxidant activity (4). Many herbal drugs are known to have a beneficial effect on male reproductive health, viz., *Allium cepa, Allium sativum, Tribulus terrestris, Cannabis sativa, Eurycoma longifolia, Capsicum frutescens,* and *Zingiber officinale* (5-8).

Medicinal plants are used as extracts, decoctions, semi-purified compounds to treat male infertility problems, dysfunctioning of the libido and sperm disorders. Extracts of *Panax ginseng, Panax quinquefolius* and *Lepidium meyenii* have shown positive effects on sexual desire; while extracts of *Andrographis paniculata* and *Acanthopanax senticosus* (9-12) improved sperm parameters. Several studies have been conducted to determine role of plant-derived antioxidants in improving sperm quality, such as the polyphenol of *Vitis vinifera* extract and crocin of *Crocus sativus* extract (13, 14).

One of the well-known herb extensively used in Indian Ayurveda in the treatment of male infertility for more than 3000 years is *Withania somnifera*, commonly known as A shwagandha or the 'Indian ginseng' (6-7). It is a small evergreen shrub with long tuberous roots that belong to the family *Solanaceae* (15). This plant can be found in tropical and subtropical areas, ranging from South Africa, Middle East India and China (16). It has small, greenish-yellow flowers and smooth, round fruits with numerous seeds, grown as a medicinal crop. Due to rich medicinal properties, the different parts of this plant are used as remedy in traditional medicine of different countries (17). It is widely used for the treatment of erectile dysfunction, oligozoospermia, reproductive endocrinological problems and other male reproductive health

problems. Since antiquity, and to this day, the root of *Withania somnifera* is used as an adaptogen, diuretic, sedative, antioxidant, and aphrodisiac (18). This plant is known to cure impotency and increase sex appeal and fertility when used solitarily or in combination with other medications (19). Further, it has been shown that Ashwagandha helps to balance oxidative stress pathways and improves sperm count and motility (20). Many phytochemicals have been extracted so far from this plant with possessing different pharmacologic and biological properties (21). Several phytochemical studies have been conducted to determine the chemical constituents of the various parts of *Withania somnifera* (22). The principal bioactive compounds of *Withania somnifera* are withanolides, which are triterpene. More than 40 different withanolides, 12 alkaloids and several sitoindosides have been isolated (23). The major biochemical constituents of *Withania somnifera* are withaferin-A, withanolide-D and withanone (24, 25).

Due to rich therapeutic value, in recent days there is a high demand in cultivation of WS. The commercial value of ashwagandha roots depends on their physical quality and morphology. Medicinal values are mainly depends on high-yielding improved variety based on the agro climatic conditions of a particular area. To identify the finest variety, comparative and systematic scientific evaluation of high-yielding ashwagandha phytochemistry and pharmacological properties are required. The organic sources of nutrients have a beneficial impact on soil properties and produce quality bioactive compounds (26). Organic manures have several advantages like they supply plant nutrients, including micronutrients, and improve soil biological properties (27). Fertilizer, pesticides and chemicals has greater negative impact for yields and quality of WS. Hence, organic cultivation technology can produce the quality medicinal herb by avoiding excessive use of synthetic fertilizers and chemicals. Organic nutrient sources like vermicompost can be utilized as plant growth media and soil conditioner which supply plant nutrients slowly but steadily throughout the plant growth period (28).

Based on the comprehensive detailed information on ethnobotanical knowledge in Karnataka, Gadag is the place where highest WS is cultivating (29&30). Black soil or heavy soils are suitable for cultivation. Gadag is the district located in central part of Karnataka state and lies between 75° 16 to 76° 03" E longitude and 14° 56 to 15° 53" N latitude. Vegetation is hilly, deciduous, semi evergreen, scrubby types of forest with average rainfall of 500 to 650 mm. The minimum temperature recorded during winter is 18°C and highest recorded in summer is 42.5°C, the type of soil present is black. Though Ashwagandha grow well in both red soil and black soil conditions, in the preparation of Ayurvedic formulation preferably WS grown in black soil is considered. Since, the root of WS is being used in the treatment of male fertility, an attempt has been made to evaluate the comparative studies of phytochemical constituents of WS extract collected from Mysore region grown in red soil and collected from Gadag region grown in black soil through *invitro* analysis and further morphometric parameters of testis, sperm count and oxidative stress study has made with the quantification of bioenergetics molecules through *invivo* analysis using *Drosophila melanogaster.*

MATERIALS AND METHODS

Chemicals: All the chemicals used are of analytical grade. Propionic acid, Acetic acid, Sucrose, Ethanol, Agar-agar, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Chloroform, Ethyl acetate, Methanol, 5'–dithio-bi's 2-nitrobenzoic acid (DTNB), Quercitin, Phosphate buffer, Potassium ferricyanide, Trichloroacetic acid, Ferric chloride, Gallic acid, Aluminium Chloride, Pottasium acetate, Tannic acid, Sulphuric acid were obtained from SRL ltd, Mumbai, India. Paraquat dichloride (PQ) was obtained from Sigma Aldrich, USA.

Preparation of plant extract: The roots of *W. somnifera* were collected from in and around Mysuru and Gadag. Collected roots were cleaned and then dried under shade at room temperature and powdered mechanically using electric blender. The powder form of *W. somnifera* was subjected to ethanol solvent extractions using the soxhlet apparatus. The crude extract thus obtained was transferred to a flash evaporator for complete evaporation. The dried extract thus obtained was used for all the experiments. The extract of WS grown in red soil, collected from Mysore is abbreviated as RWS and WS grown in black soil, collected from Gadag is abbreviated BWS. The concentrations of extracts were fixed based on the LC_{50} value. All the experiments were carried out using two concentrations viz., 2mg/ml and 10 mg/ml.

***Drosophila melanogaster* culture:** Oregon-K strain of *Drosophila melanogaster* were obtained from *Drosophila* Stock Centre, Department of studies in Zoology, University of Mysore, Mysuru. The stocks were maintained at 22 ± 1^0C with 60-70% relative humidity in containing wheat cream agar media seeded with yeast granules in 30ml culture bottles. All the experiments were carried out in the flies by supplementing *W. somnifera* extract of two geographical regions through larval feeding.

Phytochemical analysis of *Withania somnifera*: The ethanolic root extract of RWS and BWS was subjected to *in-vitro* phytochemical analysis to quantify the antioxidant activity by superoxide radical scavenging, total antioxidant estimation (DPPH radical scavenging activity), reducing power assay. Screening tests were performed for the identification of phytochemical constituents such as, phenolics, flavonoids, tannin and saponin compounds, antioxidants such as total contents, reducing power activity. All the *in vitro* studies of RWS and BWS were made of a concentration 5mg/ml and the quantities were measured using UV Spectrophotometer (ELICO Ltd. India).

Total antioxidant estimation: The total antioxidant activity was measured by DPPH radical scavenging method followed (31) with minor modifications. 1mg of extract was used for the estimation and mixed with 2ml of DPPH solution. Ethanol was used as a control and Quercetin was used as standard (1mg/ml). The mixture was shaken vigorously and kept in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm. The scavenging activity of each extract on DPPH radical was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{(1-Absorbance of sample) x 100}}{\text{Absorbance of control}}$$

Determination of Phenolics: The total phenolic content in the ethanolic extract of RWS and BWS was determined by Folin-Ciocalteu reagent method (32). 0.5 ml of plant extract was mixed with 2.5 ml of Folin-Ciocalteu reagent and the mixture was incubated for 15 minutes at 45° C after adding 2 ml of 7.5% sodium carbonate. Then the absorbance was measured at 765 nm with Gallic acid as standard.

Determination of total flavonoids: Determination of flavonoids in RWS and BWS was made as per the procedure of (33). 1 ml of plant extract was mixed with 3 ml of ethanol, 0.2 ml of aluminium chloride and 0.2 ml of 1M potassium acetate and 5.6 ml of distilled water. The mixture was incubated for 30 minutes at room temperature. Quercetin was used as standard and the optical density was measured at 415 nm.

Determination of Tannin: The estimation of tannin in RWS and BWS was made as per the standard procedure (34). For this estimation, 0.5 ml of extract was mixed with 0.25 ml of 1N Folin–Ciocalteu reagent and 1.25 ml of 20% sodium carbonate. The mixture was incubated at room temperature for about 40 minutes. Tannic acid was used as standard and the absorbance was measured at 725 nm against blank.

Invivo analysis: Newly emerged virgin male flies were segregated and isolated from the culture bottles under stereozome in mild aesthetic conditions in an interval of time fixed. They were released into individual vials containing equal quantities of wheat cream agar medium with BWS and RWS extracts separately. Flies in each culture vial were transferred to fresh vials in every three days. All the estimations were carried out in whole body homogenate of 10 days RWS and BWS extract fed flies with two different doses, only yeast fed flies were considered as control group.

Estimation of Total Carbohydrates: The total carbohydrate content was determined by following the standard method (35). To carry out the estimation, flies were freezed and dried for 36 hours. Then the dry weight of flies measured in each batch followed by homogenization with distilled water. Further the samples were incubated in a water bath 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. Then each sample optical density was measured at 620 nm using colorimeter. The total amount of glucose in each batch was estimated using a glucose standard graph. The carbohydrate content was calculated by dividing the absolute carbohydrate content by the dry weight of the batch before extraction.

Estimation of Total Proteins: Protein estimation was quantified by Lowry's method (36). 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.

Estimation of Total Triglycerides: Triglyceride content was determined by using standard protocol (37) with slight modifications. Flies were homogenized in 100 µl PBS, 0.5% Tween 20, and immediately incubated at 70°C for 5 minutes. 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 using spectrophotometer at 525 nm.

Induction of Oxidative stress (OS test): To know the oxidative resistance ability of BWS and RWS extracts of *Withania somnifera*, flies were subjected to oxidative stress molecule. Paraquat dichloride (PQ) has been employed as OS molecule by following the standard method (38). To determine the resistance ability in under stress condition, 10 days age grouped BWS and RWS extracts fed 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. The control flies were placed into vials containing Whatmann filters wetted with only 5% sucrose. For this experiment, 50 flies (10 flies/vial) were maintained in each batch. For every 6hrs the survival rate is recorded. Mortality rates were observed in both control and extract treated with OS induction till all fly reaches mortality.

Morphometric analysis of Testis: Testes of 20 unmated male flies cultured in BWS extract and PQ induced BWS extract were dissected in phosphate buffered saline PBS (pH 7.2) fixed in 4% paraformaldehyde in PBS for 20min and rinsed. The testes are stained by acetoorcein for 20 minutes then 45% acetic acid is added and sealed. The size of the testes i.e., length and width were measured by micrometry. The 20 testes samples were measured in both from BWS and BWS with PQ induced flies.

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 fed batch without PQ, BWS fed batch with PQ, without OS 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.

Statistical analysis: Data from all the estimations were expressed as mean ± SE. The data obtained from all the experiments were subjected to statistical analysis using SPSS software (version 20.0).To know the level of significance among the analyzed groups in data were subjected to one way ANOVA, followed by DMRT. A probability of $P < 0.05$ was considered as significant.

RESULTS

In vitro studies for phytochemical analysis; the phytochemical screening of the root extract of RWS and BWS were carried out to detect the amount of major phytochemicals.

Table 1. Phytochemical estimation of *Withania somnifera* grown in black soil and red soil

Phytochemical Analysis	Quantitative analysis in Ethanolic extracts	
	RWS µg/ml	BWS µg/ml
Total antioxidants	0.033±0.03	0.042±0.01
Phenolics	163.33±0.12	181±1.52
Flavonoids	123.33±1.88	139.23±0.04
Tannins	0.41±0.03	0.50±0.01

Table 2. Sperm count analysis in different group of male flies under stress and non-stress conditions

Groups	Sperm Count
Control	2460±0.55
Control + OS	2070±0.27
BWS Dose - I	2883±0.09
BWS Dose - II	2991±0.64
BWS Dose - I+OS	2106±0.81
BWS Dose - II+OS	2305±0.11

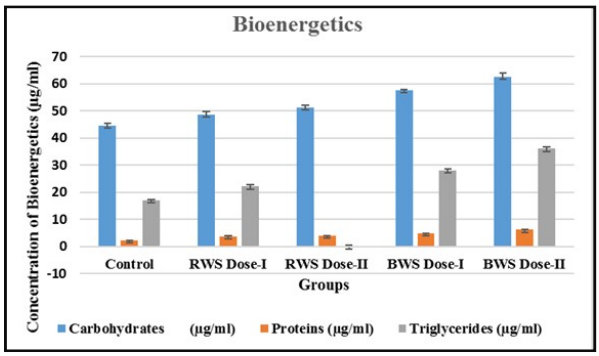

Fig. 1. Estimation of Carbohydrates, Proteins and Triglycerides in ethanol extract of *W. somnifera* treated *D. melanogaster* male flies

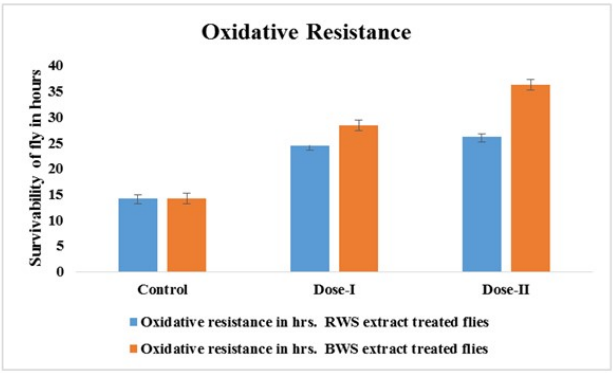

Fig. 2. Result of Oxidative resistance in ethanol extract of *W. somnifera* treated male flies of *D. melanogaster*

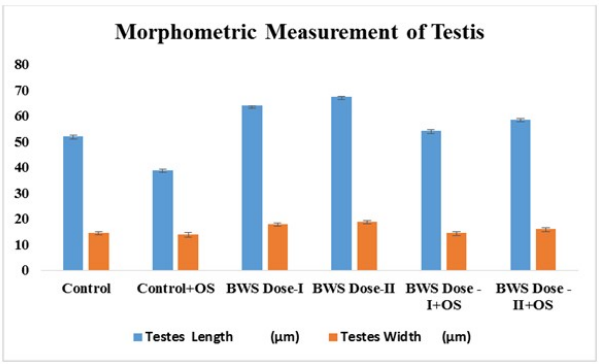

Fig. 3. Result of Morphometric measurement of testes in ethanol extract of *W. somnifera* treated *D. melanogaster* male flies

The data on phytochemical analysis (Table 1) reveals that the total antioxidants were found to be 0.042µg/ml in BWS extract, 0.033µg/ml in RWS extract. The major neutraceuticals present in both the groups were identified as phenolic, flavonoids and tannins. The amount of phenolics, flavonoids and tannin in BWS extract were 181µg/ml, 139.23µg/ml and 0.5 µg/ml respectively. The amount of phenolic in RWS extract was 163.33µg/ml and flavonoids was 123.33µg/ml. The result reveals that all the analyzed phytochemicals are more in BWS extract than RWS extract.

Invivo analysis

Quantification of Bio-energetic molecules: Three major bioenergetics molecules has estimated in BWS and RWS extract fed 10 days old flies of *D. melanogaster.* The estimation was carried out in different dose treatment batches. The amount of all the bioenergetics molecules were found to high in both extract fed flies group when compared to control group. The results of estimations were compiled in Fig.1. The highest carbohydrate contents was noticed in the flies fed with Dose-II of BWS extract 62.66 (µg/ml), least content was observed in Dose-I of RWS extract fed flies. Similarly the amount of proteins and triglycerides were high in Dose-II of BWS extract fed flies. The estimated highest protein was 6.28 (µg/ml) and triglycerides was 36.12 (µg/ml)

OS test: Oxidative resistance test was carried using Paraquat (PQ) as oxidative stress molecule. For this test, both BWS and RWS extracts supplemented 10 days aged flies were exposed to PQ. The survival rate was recorded for every 6 hrs. The result obtained from the oxidative resistance test was represented in Fig. 2. Mortality rates of both extract treated flies showed significantly more compared to control batch. The mean mortality rate in control group was 14 hrs. While dose-I and dose-II of BWS fed grouped flies mortality was reached on 28hrs and 36hrs respectively. However in dose-I and dose-II of RWS extract fed group flies it was 24hrs and 26hrs. The highest mean mortality was noticed in Dose-II of BWS extract fed flies. The data on statistical analysis reveals that both the dosage treatment batches showed significant differences with the control batches. Among the extract supplemented batches, dose-II BWS treatment groups (10mg/ml) showed maximum result than RWS and control groups.

Morphometric analysis of Testis: The size of the testes i.e., length and width were measured using micrometry, expressed as µm. The length and width of control fly was 63µm and 17.66 µm respectively, it was slightly reduced in the flies where BWS fed groups after OS induction, but significantly increased in the BWS fed flies, Dose-II of BWS extract fed flies shows the testis length of 67.33 µm with width of 18.96 µm. However, in the batch of BWS extract fed flies with OS induction, the size of the testis was increased when compared to Control group with PQ induction.

Sperm Count Analysis; The data on sperm count analysis of the BWS extract fed flies is compiled in Table 2. The total sperm count in control group was 2460, it was decreased after PQ induction (2070). The count was increased in the batch of BWS fed grouped flies. However, the count was more in does-II fed groups than dose-I groups. Similar such results were obtained in the batch where BWS extract fed flies exposed to PQ.

The total count in dose-II fed batch under OS was 2305, which was significantly increased when compared to control with OS induced ones.

DISCUSSION

In Ayurveda *W. somnifera* have a wide range of therapeutics values and considered as best medicine for male infertility. It has been proved that WS enhance spermatogenesis and sperm related indices in male (39). The current study shows different soil conditions have a significant impact on the phytoconstituents variability and therapeutic potential of *W. somnifera*. This study made attempts to prove that the existence of regional differences in some biochemical characteristics of *W. somnifera*. The findings evidently indicated that *W. somnifera* plants from Gadag have the highest levels of total antioxidant activity, total phenolics and total flavonoids when compared to plants from Mysore region. BWS extract increases the total antioxidants by 27%, total phenolics increases by 10.20%, while total flavonoids increases by 12.16%. Further, major bioenergetics molecules were estimated in WS extract fed flies. All the three major molecules were found be significantly more in BWS than RWS fed flies.

WS extract reduces the oxidative stress and increases the stress resistance ability. It has been reported that the treatment with Ashwagandha effectively reduced oxidative stress (40). Paraquat induced WS fed flies showed greater survivability in oxidative stress (41). In the present study, BWS extract fed flies exhibited more resistance ability than RWS extract fed groups. It was increased by 2 fold in low dose treatment batch, 2.41 fold increase in high dose treatment fed batch. This shows that black soil nutrients and phytochemicals responsible for developing more resistance ability in the flies.

Oxidative stress can damage the membrane of sperm, affecting motility, morphology. The nutritional supplements become potential remedies for infertility problem. Perusal of literature proves that the administration of natural biomolecules have a positive impact on male infertility (42-45). The amount and quality of nutrients intake by organisms have a strong impact on testis size. Hence, preliminary male reproductive parameter has been carried out in BWS extract fed flies due to its high antioxidant property. In the present study it was noticed that the size of testis was also effected by WS extract, even under stress condition. BWS extract fed flies increases the size by 28.72 % in length and increases the width of testis by 27.17 %.

Sperm function, are essential component for male fertility,that are influenced by both genetic and environmental factors (46). Normally severe stress experiences the decreased testosterone level, lower sperm count, abnormal sperm production, and decreased sperm motility, can negatively affect on overall male fertility. Thermal stress leads to fertility reduction, can cause temporal sterility (47). Further, it has been proved that the oxidative stress can affect sperm production in the testes, leading to reduced sperm (48). Antioxidants become notable potential in counteracting the negative effects of oxidative stress on sperm, antioxidants like vitamin C, L-carnitine, and glutathione have shown potential benefits (49). In the present study it was observed that the total sperm count was also affected by PQ induction in control flies. Further, high dose treatment of BWS extract increases the sperm count in the flies by 21.58 %, while under stress condition with the supplementation of BWS increases the sperm count by 11.35 %. This was due to antioxidants present in the WS. These results are in the line of Kaltsas (2023). Organic fertilizers significantly influence the yield and quality of Ashwagandha. Root yield and bioactive compounds were remarkably increased with the application of organic nutrient sources. These biofertilizer enhanced withanolide content in Ashwagandha (50-51). Based on the obtained result, the present study revealed that WS grown in black soil has greater influence in increasing bioenergetics molecules and reduce the oxidative stress, also has more impact in the size of testis in the flies than WS grown in red soil. In conclusion, the studies summarizes that the supplementation of BWS increases bioenergetics molecules and increases the resistance ability against oxidative stress molecule. Further it increases testis size in male *D. melanogaster*. This is due to high degree of antioxidants and natural polyphenols in *Withania somnifera* grown in black soil of Gadag

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