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The pro-oxidant effect of dextran sodium sulphate on oxidative stress biomarkers and antioxidant enzymes in *Drosophila melanogaster*

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This study sought to evaluate the behavioural and biochemical effects of Dextran Sodium Sulphate (DSS) on the oxidative stress biomarkers and antioxidant enzymes. *Drosophila melanogaster* (both sexes) of 3 days old were exposed to various DSS incorporated diets at concentrations, 0.5 - 3.0% for a period of 14 days (Survival rate). The second phase comprised of 3 groups; Group I- Control (normal diet), Group II-0.5% DSS, and Group III – 1.0% DSS and fed for 5 days. Climbing activity and biochemical assays were then determined. The survival rates of the flies with concentrations above 1.0% were highly reduced. The induced oxidative stress caused by DSS showed an impaired climbing activity, a significant ($p<0.05$) increase in the catalase enzymatic activity and malondialdehyde content in both Groups II and III in relation to the flies in Group I (control). Also there was significant ($p<0.05$) inhibition of GST activity and reduction of total thiol contents in group III in comparison to the control. In conclusion, the DSS dose- dependent toxicity effect was revealed by the increase in the malondialdehyde contents and catalase enzymatic activity.

Key words: *Drosophila melanogaster*, oxidative stress, pro-oxidant, antioxidant enzymes, dextran sodium sulphate.

INTRODUCTION

Drosophila melanogaster is a dipteran insect with two wings; typical characteristic of the “true flies”. Since one hundred years ago, these flies have been introduced as a research model in the studies of genetics and other related aspects of molecular biology. Of recent, Drosophotoxicology has been introduced, which is a toxicology study in which parts or whole of the fly is used (Rand, 2010; Chifiriuc et al., 2016). It meets the standard of the European Centre for the Validation of Alternative Methods (ECVAM): Reduction, Refinement and Replacement (3Rs) of laboratory animal usage (Festing et al., 1998). The use of *D. melanogaster* in toxicity studies addresses the problem of obtaining ethical clearance for the animals, and has many advantages over vertebrates, such as; it is 3-4 mm long in size, wholesome presentation for toxicological testing, smaller required reagents for assays, and has 75% of homologs of human disease genes, with about 90% nucleotide

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sequence identified in some of its species (Festing et al., 1998; Chifiriuc et al., 2016). One of the tools in toxicological testing in *D. melanogaster* involves an assessment of the oxidative stress biomarkers and its antioxidant enzymes activity.

Invertebrates and vertebrates have no control over the challenges posed to them from the environment, so they have evolved mechanisms to control their metabolic pathways as to manage the oxidative stress and other challenges (Hermes-Lima et al., 2001; Costantini, 2018). Oxidative stress is an imbalance between the free radical levels (both from endogenous and exogenous sources) and the antioxidant system of any living organism (Valko et al., 2006, Halliwell, 2007). Pro-oxidants can be endobiotic or xenobiotic that is capable of inducing oxidative stress. This they do by either generating reactive oxygen/ nitrogen species; or by their mechanism of expulsion or inhibition of antioxidant enzymes activity (Halliwell, 1991; Sies, 2018). Antioxidant enzymes are the first and primary defense system from cellular attack by free radicals. The resultant molecular mechanism of pro oxidants – oxidative stress is the basis for many life threatening diseases, which includes, diabetes, cardiovascular, inflammation, cancer and neurodegenerative disease conditions. Reactive oxygen species (ROS) a by-product of the oxidative phosphorylation that takes place in the electron transport chain of the mitochondria and this is the main producer of energy (ATP) (Valko et al., 2007; Sies, 2018). These signalling molecules are important at physiological concentration, but at a higher concentration they overwhelm the antioxidant defense system. Consequently, this may result in DNA damage, protein carbonylation and lipid peroxidation of biomolecules (Valko et al., 2007; Sies, 2018).

Dextran Sodium sulphate (DSS), $C_6H_7Na_3O_{14}S_3$, is a polyanionic sulphated derivative of a selected Dextran fraction. DSS acts as a chemical incitant for the inducement of inflammation (colon) because it is toxic to the mucosa epithelial cells. It also causes damage to its barrier integrity. DSS because of its polyanionic nature is pro-oxidant in its mechanism of action. It complexes with biomolecules and forms a nanometer sized vesicles which will activate inflammatory signal pathways (Amchelslavsky et al., 2009; Jianming et al., 2010; Liberti et al., 2017). The activated inflammatory signal pathways bring about the production of reactive oxygen species. During the process of inflammation, the phagocytes such as neutrophils and macrophages generates a large amount of ROS, and reactive nitrogen/chlorine species due to the rolling and frictional forces produced by the movement of these signalling molecules (Feany and Bender, 2000; Mittal et al., 2014). DSS has been used to induce inflammation in the intestinal stem cells of the *D. melanogaster* (Amchelslavsky et al., 2009; Apidianakis and Rahme, 2011; Hairul et al., 2014), but there is paucity of any research work done on the dose-dependent pro-toxicant effect of DSS in *D. melanogaster.* So we sought to investigate the DSS pro-oxidant induced toxicological effect on the oxidative stress biomarkers and antioxidant enzymes in *D. melanogaster*.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in this research work were of analytical grade and the water used was glass distilled (Milli-Q Direct 8/16 System, Molsheim, France). Dextran sodium sulphate was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Drosophila melanogaster strain and culture

D. melanogaster (Harwich strain) from National Species Stock Center (Bowling Green, OH, USA), was obtained from department of Biochemistry, College of Medicine, University of Ibadan, Nigeria. Flies were maintained at constant temperature and humidity (23 ± 1°C; 60% relative humidity, respectively) under 12 h dark/light cycle conditions in the *D. melanogaster* fly laboratory of the African Center of Excellence in Phytomedicine Research and Development, (ACEPRD), University of Jos, Nigeria.

Drosophila melanogaster feed formulation and its handling

The flies were fed with the standard formulated diet corn meal medium, which contained brewer's yeast (1%w/v), sucrose (2%w/v), powdered milk (1%w/v), agar (1%) and Nipagin (0.08%). The water used for making the diet was double distilled water (Milli-Q water system). Flies were randomly selected from vials containing 1-3 days old. Caution was taken when counting the flies and an appropriate brush with soft ends was used. Much care was taken in handling the flies as to prevent "handling stress".

Behavioural experiments

Survival rate analysis

For the determination of the concentration to be used in the research, an initial experiment was carried out using various concentrations of Dextran Sodium sulphate (DSS %). This was done to observe the sum of dead and surviving flies during the fourteen days period. For the survival assay, flies (both genders) of 1-3 days old were divided into seven groups, with each group having 3 vials each. Each vial contained 50 flies.

Group I – Control (Normal diet), Group II – 0.5% DSS, Group III – 1.0% DSS, Group IV -1.5% DSS, Group V – 2.0% DSS, Group VI – 2.5% DSS, Group VII -3.0% DSS

The survival assay was carried out in three replicates of each concentration. The diet was changed every four days, during the period of this experiment. The survival rate was determined with all the concentrations, and both the live and dead flies were recorded daily. By the end of this experiment (14 days), the data obtained were 2ulphate and plotted as percentage of live and dead flies. Two concentrations were then selected Group II and III (0.5% and 1.0%), this is because their survival rate was comparable with that of the control group.

Climbing activity

The climbing performance of the flies was carried out using

negative geotaxis method in the second phase, last (5^{th}) day (Adedara et al., 2016). Briefly, ten flies from the two selected DSS concentrations and control were put in a mild static position, by placing it in a dry fitted filter paper on a petri dish, with dried ice underneath it. Consequently, they were placed respectively in empty labelled vertical glass columns measuring, length 11 cm and diameter 3.5 cm. Within a period of 15 to 20 mins, flies recovered from the mild anaesthesia and the bottom of the column was tapped gently to return them back to the bottom. The number of flies that climbed above the 6 cm mark of the column in 6 s, as well as those that remained below the 6 cm mark was recorded. This procedure was repeated three times at 1 min interval. The scores represent the mean of the number of flies at the top which was expressed as a percentage of the total number of flies.

Tissue homogenate preparation for biochemical assay

For the determination of biochemical assays, a second group experiment was carried out, were DSS of 0.5 and 1.0% were introduced to the flies' diet relatively for a period of five days. Each group had five vials containing 50 flies (both gender). At the end of the treatment period, flies were anaesthetized in ice, weighed, and homogenized in cold 0.1M phosphate buffer, Ph 7.0 (1:10 w/v), and centrifuged at 4000 x g for 10 min at 4°C (Allegra X-15R Centrifuge, Beckman Coulter, USA). Then the supernatants were separated into labelled Eppendorf tubes, and used for the various biochemical assays. All the assays were carried out in five replicates for the three groups and relative absorbance read using Jenway spectrophotometer 7315, by Bibi Scientific Ltd, UK.

Determination of oxidative stress biomarkers

Determination of total thiol concentration

Total thiol content was estimated by the method of Ellman (1959). Briefly, the reaction mixture was made up of 170 µl of 0.1 M potassium phosphate buffer (Ph 7.4), 20 µl of sample, and 10 µl of 10 Mm 5', 5'- dithios – 2-nitrobenzoic acid (DTNB). This was followed by 30 min incubation at room temperature, and the absorbance was measured at 412 nm. A standard curve was plotted for each measurement using GSH as standard (expressed as µmol/mg protein).

Thiobarbituric acid reactive substances (TBARS)

Using the Ohkawa et al., (1979) method, briefly fly samples were homogenized (50 flies per vial) in cold 0.1 M phosphate buffer at Ph 7.4 in a ratio of 1:5 (w/v). The stock reagent contained equal volume of TCA (10% w/v), and 2- Thiobarbituric acid (0.75% w/v) in 0.1M HCl. 100 µl of tissue supernatant and 200 µl of stock reagent were incubated at 95°C for 60 min. After cooling for a period of 30 min, they were centrifuged at 8000 x g for 10 min and the absorbance measured at 532 nm. The TBARS levels were expressed as mmol MDA/ mg tissue.

Determination of antioxidant enzyme levels

Determination of catalase enzymatic activity

Catalase activity was measured according to a modified method of Aebi (1984) and Abolaji et al. (2015) by monitoring the clearance of H_2O_2 at 240 nm at 25°C in a reaction medium containing 1800 µl of 50 Mm phosphate buffer (Ph 7.4), 180 µl of 300 Mm H_2O_2, and 20 µl of sample. The kinetics mode was used and monitored for 120 s (2 min) at 10 s intervals, at 240 nm, (expressed as U/mg protein).

Determination of glutathione-S-transferase enzymatic activity

Glutathione-S-transferase (GST; EC 2.5.1.18), activity was estimated by the Habig and Jakoby (1981) method. Briefly, 1-chloro- 2, 4-dinitrobenzene (CDNB) was used as the substrate. The assay reaction mixture was made up of 270 µl of a solution containing (20 µl of 0.25 M potassium phosphate buffer, Ph 7.0, with 2.5 Mm EDTA, 10.5 µl of distilled water, and 500 µl of 0.1 MGSH at 25°C), 20 µl of sample, and 10 ml of 25 Mm 1-chloro-2,4-dinitrobenzene (CDNB). The reaction was monitored for 5 min (30 s intervals) at 340 nm and the data were expressed as mmol/min/mg protein using the molar extinction coefficient (ε) of 9.6 $Mm^{-1}cm^{-1}$ for CDNB conjugate.

Protein determination

Protein concentrations in the whole fly homogenates were determined as described by Lowry (1951), using bovine serum albumin (BSA) as the standard.

Statistical analyses

All data were expressed as mean ± standard deviation. The statistical analysis used was one-way ANOVA, followed by the post hoc Tukey's multiple comparison tests, where $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS

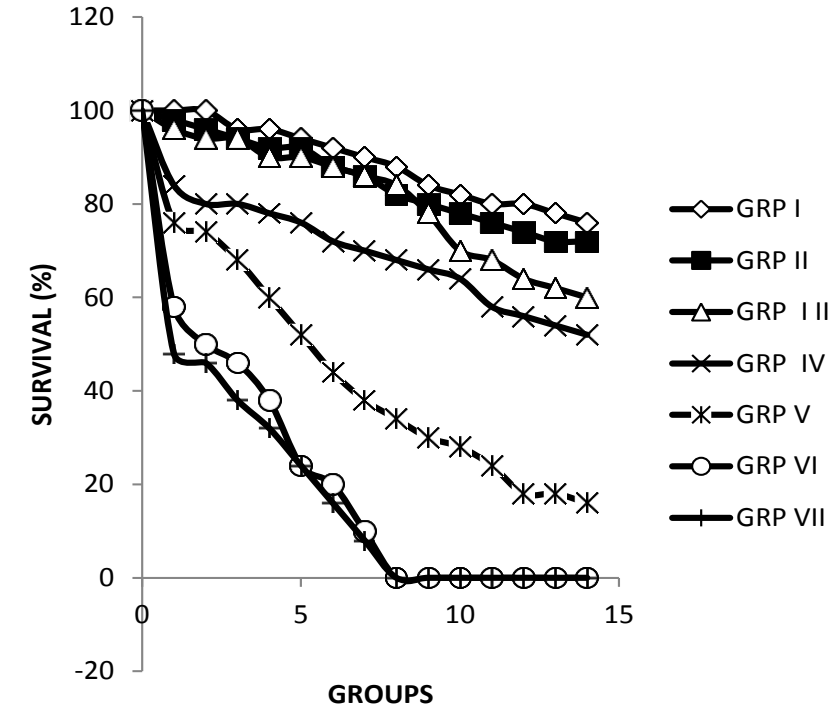
Effect of Dextran sodium sulphate (DSS) incorporated into the diet on survival rate and alive score in *D. melanogaster*

Figure 1A shows the survival rate of the seven Groups I – VII (0.5-3.0%). It revealed that 0.5% DSS incorporated into the feed revealed that, Group II is not significantly ($p>0.05$) different from the control Group (I). Groups III – VII is significantly ($p<0.05$) lower than the control (Group I). Also, DSS incorporated into the diet caused a drastic fall in the survival rate of Groups VI and VII (DSS 2.5 and 3.0%).

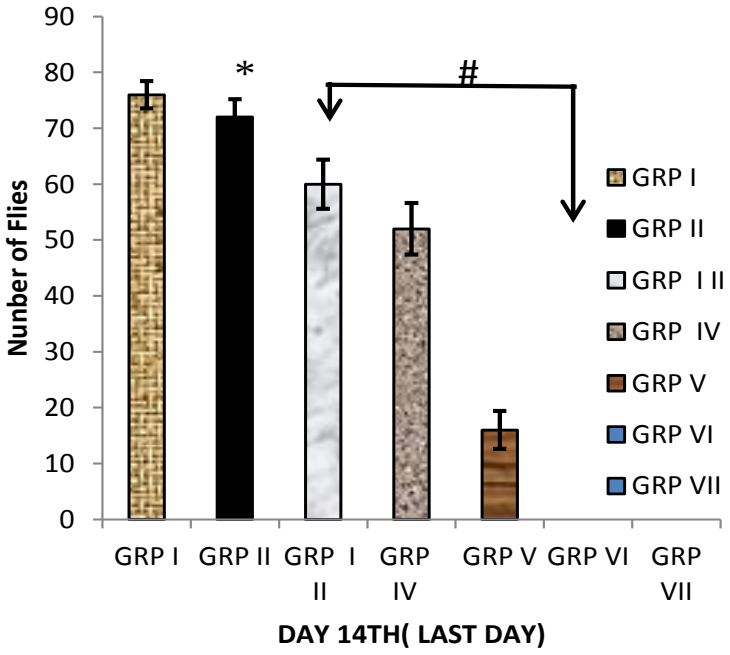
Figure 1B shows a representation of all the alive flies on the last (14^{th}) day of the experiment. The effect of DSS incorporated into the diet at (0.5%) Group II on the flies, was not significantly ($p>0.05$) different from the control group. Groups III- VII (1.0 - 3.0%) flies were significantly reduced ($p>0.05$) in comparison to the control. The reduction was more in the Groups VI and VII treated with 2.5 -3.0% DSS respectively.

Effect of Dextran sodium sulphate (DSS) incorporated into the diet on negative geotaxic (climbing) activity of *D. melanogaster*

Figure 2 represents the climbing activities of the two selected Groups I and II (0.5-1.0% DSS), it revealed that



(A)



(B)

Figure 1A and B. Dextran sodium sulphate incorporated into the diet caused reduction in survival rate of *D. melanogaster* after 14days (A) Survival curve analysis and (B) number of alive flies (both sexes) after 14 days of administration to *D. melanogaster* flies to Groups I – VII. $^{\#}p<0.05$ vs control and $^{*}p>0.05$ vs control. Data are presented as mean ± SD (n=3). Group I-Control, Group II-0.5%DSS, Group III-DSS 1.0%, Group IV- 1.5%DSS, Group V-2.0% DSS, Group VI-2.5% DSS, Group VII-3.0% DSS.

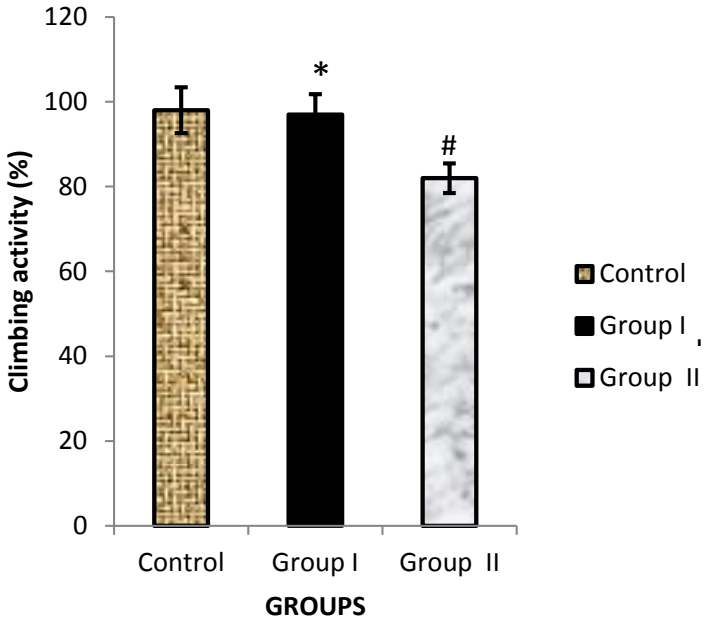


Figure 2. Dextran sodium sulphate incorporated into the diet decreases the climbing rate (%) after 5 days in Control, Group I (0.5%) and Group II (1.0%).

Table 1. The Catalase, Glutathione S-transferase enzymatic activities, total thiol and malondialdehyde contents of control, group I (0.5%) and group II (1.0%) flies.

Group	Catalase activity (U/ mg protein)	GST activity (mmol/min/mg protein)	Total thiol content (µmole/mg protein)	Malondialdehyde content (mmol MDA/mg protein)
Control	49.70 ± 0.82	0.71 ± 0.031	196.68 ± 2.56	2.47 ±0.06
Group II	52.72 ± 1.50*	0.67 ± 0.02*	182.44 ± 2.82*	3.64±0.14*

[a]On the same column, shows there is no significant difference (*p>0.05) from the control, and [b] shows that there is significant ([#]p<0.05) difference from the control.

After 5 days of exposure to the DSS, the effect of 0.5% DSS upon the flies in Group I was not significantly (p>0.05) different from the control group. Whereas the effect of 1.0% DSS upon the flies in Group II climbing activity was significantly (p<0.05) reduced in comparison to the control.

Effect of Dextran sodium sulphate (DSS) incorporated into diet on Total thiol and Malondialdehyde content in *D. melanogaster*

Table 1, also gives a representation of both the total thiol and malondialdehyde contents of the three groups studied, control, I (0.5%) and II (1.0%). The level of total thiols in flies exposed to 1.0% DSS Group II was significantly (p<0.05) reduced in comparison to the control group. The total thiol content in flies in Group I (0.5%DSS) were not significantly (p>0.05) different when compared to the flies in the control group. The quantification of malondialdehyde produced from the activity of thiobarbituric acid reactive species in Group II flies was significantly (p<0.05) increased in comparison to those in control group. Group I also revealed that there was no significant (p>0.05) difference in MDA content in comparison to the control group.

Effect of Dextran sodium sulphate (DSS) incorporated into the diet on catalase, and glutathione S-transferase enzymatic activities in *D. melanogaster*

Table 1 also revealed that the catalase activity was significantly (p<0.05) enhanced in flies exposed to 1.0% DSS in Group II in comparison to the control. While there was no significant (p>0.05) change in Group I (0.5% DSS) in comparison to group control. There was no significant (p>0.5) difference in the GST activity of the

flies in Group I in comparison to those in control group.

DISCUSSION

D. melanogaster is one of the alternative invertebrate models useful in toxicological testings. It meets the standard of the ECVAM, Reduction, Refinement and replacement (3Rs) of the usage of Laboratory animals (Festing et al., 1998). DSS has been used over a period of time to induce inflammation- pro-oxidative mechanisms (Amcheslavsky et al., 2009; Jianming et al., 2010; Mittal et al., 2014). In this experiment, incorporation of varying concentration of DSS into the diet was used for the first phase, to investigate if its pro-oxidant effect can induce oxidative stress using *Drosophila melanogaster* as a model. From the survival rate and numberof flies alive, 0.5% and 1.0% DSS was chosen for use in the next phase for antioxidant enzyme activities and oxidative stress determination. The result (Figure 1A and B) showed that the flies in Group II survival rate (%) was not significantly ($p>0.05$) different from those in the control Group I. Though Group III flies were significantly ($p<0.05$) reduced in comparison to those in the control group, but 60% survived (in Group III) having more surviving flies when compared to those in Groups IV- VII treated with 1.5 -3.0% DSS. The reduction in their survival rate correlates with the level of dose (toxicity) administered in the diets. This can be attributed to the pro-oxidant activity of DSS because of its ability to induce inflammation and its consequent signalling pathways which brings about the production of reactive oxygen species (Amcheslavsky et al., 2009; Mittal et al., 2014).

Substances that can act as pro-oxidants are generally toxic in nature, expressing their damaging effect by affecting the Redox balance, consequently affecting both the survival rates of the flies and the negative geotaxic (vertical climbing) activity. From Figure 1A and B, the survival rate (%) of flies in Group IV – VIII was reduced by the toxic effect of the high concentration used. On the 7^{th} day the flies in Group VI and VII were all dead. Naturally flies have explorative tendencies which depend basically on the novelty of the situation and the levels by which their motor function has been altered by the toxicant (Durier and Rivault, 2002). DSS, an anionic polymer, has been used as one of the incitants for inflammatory bowel diseases in animal models. DSS because of its polyanionic nature is pro-oxidant in its mechanism of action. It complexes with biomolecules and forms a nanometer sized vesicles which will activate inflammatory signal pathways. The process of inflammation in itself favours a large production of reactive oxygen species; due to the rolling and friction forces produced by the signalling molecules (Mittal et al., 2014, Adedara et al., 2016). The ability to induce oxidative stress is a hallmark for the measurement of the toxicity of any substance in the *D. melanogaster*. This was shown in the survival activity, in which an increase in the dose level of DSS, increased toxicity, consequently a decrease in survival rate (%). This observation agrees with Oboh et al. (2018), which shows a reduction in survival rates observed in flies fed with 0.5-1.0% dietary inclusion of *Garcinia kola*. This showed that an increase in dose level also increases reactive oxygen species (ROS) level would have led to the decrease in survival rate of flies in Groups IV – VII.

Malondialdehyde is one of the products of lipid peroxidation, which is one of the biomarkers for oxidative stress (Habig and Jakoby, 1981; Ghani et al., 2017). When the antioxidant system is overwhelmed, there is a mitochondrial dysfunction which leads to accumulated oxidative damage and an increase in reactive species generation. In this study we observed an increase in the level of oxidative damage (MDA) highest in the homogenate of flies from Group (II) fed with 1.0% of DSS. The increase in ROS levels and reactive nitrogen species is measured by a method based on the ROS–dependent oxidation of 2'7' dichlorodihydrofluorescin diacetate (DCFH-DA) to dichlorodihydrofluorescin (DCF) – a general index of oxidative stress measurement (Pérez-Severiano et al., 2004). In comparison, TBARS assay is known to estimate lipid damage from cells and tissues and an increase in its level is an indirect indication of high ROS production. Some scientific scholars are predisposed about the use of TBARS assay in the assessment of oxidative damage in the Harwich strain of *D. melanogaster* fly. This is because of the interference of the red pigment in its eyes with the generated pink colour of TBARS assay. But in spectrophotochemical readings, the sample blank (contains all the assay constituents except the analyte), and using this practice, the blank is treated as identical to the sample as possible). Thus by taking the sample blank reading, the level of interference is taken into consideration and brought to level zero before other readings are determined (Ingle and Crouch, 1988). Some researchers have determined both the level of malondialdehyde produced from lipid peroxidation (Ohkawa et al., 1979), a standard oxidative damage indicator and level of reactive oxygen species via oxidation of fluorescent dye 2,7-dichlorofluorescein diacetate (DCF-DA) a general index of ROS levels (Pérez-Severiao et al., 2004) using the Harwich strain of *D. melanogaster* (Paula et al., 2016; Saraiva et al., 2018; Poetini et al., 2018). This is supported by our findings from Table 1, in which the inhibition of GST activity may be due to the increase in the toxicity effect of 1.0% DSS. This increase in the malondialdehyde level agrees with the result of Paula et al. (2016), Saraiva et al. (2018) and Colpo et al. (2018) which showed an increase in the MDA levels of the `Harwich strain of *D. melanogaster.*

Total thiols contain a sulfhydryl group which are among the major portion of the total body antioxidants and they play a significant role in the defense against reactive

oxygen species (ROS). So the quantification of the total thiols group (indicates the chemical effects in the thiol group of proteins and peptides) is an indirect measurement of oxidative stress biomarkers. Under oxidative/nitrosative stress condition, S-Glutathionylated proteins are reduced to free thiol groups by (thioltransferases) glutaredoxins (McDonagh, 2017). The total thiol level is an indirect marker of oxidative stress biomarker, because it gives an indication of any chemical changes in thiol groups of proteins and peptides (Durier et al., 2002; Abolaji et al., 2014; Ghani et al., 2017; Oboh et al., 2018). The total thiol content in this study was showed that group II was significantly ($p>0.05$) different from the control, this could be as a result of thiol consumption in reaction to the presence of reactive oxygen species.

The antioxidant enzymatic network consist of, superoxide dismutase (SOD), catalase, glutathione peroxidase (Gl-Px), glutathione reductase (Gl-Red) and glutathione-s-transferase (GST) that play an important role in the prevention and management of ROS generated endogenously e.g., during inflammation. They represent the adaptive response to most toxic substances (Weydert and Gullen, 2010). Catalase enzymes react efficiently with hydrogen peroxide by reducing it to water and molecular oxygen. This enzyme also plays an essential role in activating the tolerance level in relationship to adaptive response of cells in oxidative stress. This result agrees with the reported effect of catalase enriched transfected cells that were able to prevent drug induced damage by either destroying the hydrogen peroxide moiety or by interaction with the drug (Mittal et al., 2014). This activity of catalase enzyme is very important as a first line defense antioxidant enzyme. The concept of maintaining low cellular level of hydrogen peroxide (H_2O_2), is gaining increasing recognition because of its damaging effect (Mittal et al., 2014). The result from this study showed that the increasing concentration of DSS (pro oxidant) led to an enhanced catalase enzymatic activity.

Glutathione S-transferases (GST) are phase II detoxification enzyme that catalyse the conjugation of glutathione with electrophilic centres of both endogenous and exogenous electrophiles. They also function in the regulation of some cellular processes involved in oxidative stress in nature (Heydel et al., 2013). The result from this study showed that GST enzymatic activity of flies in Group I were not significantly ($p>0.05$) different from those in the control group. But due to the toxicity level of flies in Group II the antioxidant system was overwhelmed, as revealed in the MDA result in Table 1. The inhibition of the GST activity in the flies in Group II may be due to both the overwhelming nature of toxicity and the *D. melanogaster* GST (DmGSTs) may not have been expressed fully, because it develops much better and effective as the fly matures into the adult stage (Gonzalez et al., 2018). In this experiment the flies used were juveniles (3 days old). This result is in agreement with Abolaji et al. (2014), which showed an inhibition in the activity of GST using 3 days old flies. The findings of this study demonstrated that the administration of 0.5% DSS produced a reduced state of oxidative stress. At higher concentrations of 3.0 -3.5% DSS the flies were not able to survive.

RECOMMENDATIONS AND FUTURE DIRECTIONS

It is recommended that all experiment for the enzymatic assays be done under cold environment and where possible the Eppendorf test-tube holder should be placed on ice. It is necessary for further study to be carried out to determine the relative age and sex factor in the cause of oxidative stress in *D. melanogaster* using DSS as a pro-oxidant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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