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Original Article

PROTECTIVE INFLUENCE OF FISETIN ON COGNITIVE AND BIOCHEMICAL INDICES IN N-NITROSODIETYHLAMINE TREATED *DROSOPHILA MELANOGASTER*

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ABSTRACT

Objective: The current investigation is intended to investigate the protecting influence of fisetin on cognitive, as well as biochemical indices in N-Nitrosodiethylamine (NDEA, a potent carcinogen), treated *Drosophila melanogaster.*

Methods*:* *D. melanogaster* is used as a model organism for this investigation. Experimental flies are divided into four groups. Group 1–control, group 2-flies were treated with 0.01% NDEA in food medium, group 3–flies treated with 0.01% NDEA and 0.01% fisetin and group 4-flies were treated with 0.01% fisetin alone. Behavioural abnormalities (negative geotaxis, phototaxis, smell and taste chemotaxis, hygrotaxis and thermotaxis) were quantitatively observed to be deviated in NDEA treated flies compared to control but were tend to be normalized in fisetin treated flies.

Results: The contents of protein carbonyl, thiobarbituric acid reactive substance (TBARS), protein thiol and lipid peroxides were noticeably augmented in NDEA treated flies than control flies and correspondingly tend to normalize in fisetin treated groups. Further, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidise (GPX) and reduced glutathione (GSH) were decreased in NDEA treated group and were significantly increased ($p<0.05$) in fisetin treated groups.

Conclusion: Fisetin, a bioactive phytochemical could act as a potent antioxidant and as well exhibit antiproliferative characteristics. Our investigation indicates that this could prevent the abnormalities in behaviour and redox homeostasis during carcinogenesis in *D. melanogaster.*

Keywords: Cancer, *Drosophila melanogaster*, Fisetin, Behavioural assays, Free radical

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INTRODUCTION

Cancer is a multistep disease determined by the stimulation of specific oncogenic pathways concurrently with the loss of activity of tumour suppressor genes that control cell growth and division [1]. Most of the signalling cascades control cell growth and development in mammalian systems and have conserved functions in flies mimicking biology of tumours in a simple model organism like *Drosophila melanogaster* [2]. The combinations of genetic screens with the accessibility of predominant recombination techniques enable a swift characterisation of the key functions of conserved oncogenes and tumour suppressor genes in the fly model system [3]. While the development of diagnostic techniques, advanced treatment strategies, and cancer awareness programs lead to a notable drop in cancer mortality [4], still an effective strategy for cancer management is unavailable at present. It is in this context; more investigation is desirable.

The fruit fly, *Drosophila melanogaster,* is frequently as a model organism to study disciplines varying from genetics, circadian biology and developmental biology. *Drosophila* genome is 60% homologous to that of human beings, less redundant, and around 75% of the genes accountable for human disease have homologs in flies [5]. *D. melanogaster* genome-specific BLAST indicates that the fruit fly protein sequences which share identical or more than 20% sequence identity, covers equal or more than 15% of target (human) sequence and comprise identical functional domain(s) were considered as encouraging hit for the homology of respective human genes [6]. These characteristics, along with a short multiplication time, low maintenance costs, and the availability of authoritative genetic tools, permit the fruit fly a competent model organism to investigate complex pathways appropriate in biomedical research, including cancer [7]. Reasonable investigations between the fly and human genomes have recognized robust evolutionary conservation in between *Drosophila* to mammals at sequence and pathway levels. Flies respond behaviourally to numerous stimuli in an environment such as light, temperature, humidity, gravity, sound and chemicals. The sensing of these stimuli is carried out, respectively, by vision, smell of volatile chemicals, non-volatile chemicals, thermosensors and sensors of humidity, gravity and hearing in the fly. The response to stimuli can be attraction or repulsion, depending of the nature and strength of the stimulus [8]. Further, tumorigenesis is known to induce neurochemical, endocrine, immune and behavioural modulations suggesting stress and immune impairment in rodent model systems [9]. Humans and rodents bearing tumor are known to suffer from cognitive disturbances. Few studies already indicated that oxidative stress could induce abnormalities in behaviour [10, 11]. However, a systematic investigation on cognitive behavioural functions is lacking in the key experimental model system, viz. *D. melanogaster*.

N-nitrosodiethylamine (NDEA) belongs to the nitrosamine family and is known to be a potent carcinogen [12] and it could promote tumours in several organs of numerous animal model systems [13]. This carcinogen is found in a wide variety of food such as soya beans, fish (smoked, dried and salted) cheese, meat and alcoholic beverages [14]. NDEA is also found in cigarette smoke, buns, rolls, muffins, ham and oysters [15]. NDEA is known to cause oxidative and cellular damages by promoting the synthesis of free radicals [16]. The metabolic conversion of NDEA by cytochrome P450 enzymes leads to the formation of ethyl-acetoxyethyl-nitrosamine, which can be further conjugated by the phase II enzymes [17] to non-toxic compound. This activation of NDEA by P450-catalyzed-hydroxylation, is known to generate unstable metabolites that could alkylate the DNA and therefore cause tumour formation [18].

Oxidative stress is a key process involved in almost all aspects of cancer, from carcinogenesis to the tumour-bearing state and from treatment to prevention [19]. Many reactive oxygen species (ROS) defense systems have evolved in organisms to control intracellular and extracellular ROS levels The *Drosophila* ROS defense system consists of several subsystems consisting of enzymatic and non-enzymatic antioxidants. Cancer and oxidative stress institute a

vicious cycle; when oxidative stress surpasses the capability of the oxidation-reduction system of the body, gene mutations could result and intracellular signal transduction and transcription factors could be affected directly or through antioxidants, leading to carcinogenesis [20]. The tumour-bearing state is under oxidative stress tightly linked with active oxygen synthesis by tumour cells and irregular oxidation-reduction regulation [21]. Though tumour bearing tissues bear reduced free radical load due to uncontrolled and higher number of cell division, the oxidative stress is elevated [22] systemically in the tumor-bearing host.

Flavonoids exhibit antioxidant properties and are able to remove the reactive oxygen and nitrogen species and reactive oxygen (ROS and RNS) by scavenging free radicals. This feature is connected with their capacity to switch their phenolic H atom to a free radical [23]. Fisetin (3, 3', 4', 7-tetrahydroxyflavone) is a bioactive flavonol molecule found in vegetables and fruits (for instance, apple, orange, strawberry, grape, cucumber, bean, onion etc.) [24, 25]. The uppermost concentration of fisetin was reported in strawberries (160 μg/g) [26]. Recently, lot of research has been performed on fisetin owing to its presence in numerous foods and its antiproliferative cytoprotective, [27], apoptotic [28], neuroprotective and antioxidant [29, 30] properties.

The 3′-OH group of fisetin possesses the lowermost estimated BDE (bond dissociation energy) value (O-H covalent bond) succeeded by its 3'-and 4'-OH groups. A lower BDE value is accredited to a higher ability to donate a hydrogen atom from free radicals. Fisetin and its derivatives could augment intracellular glutathione (GSH) level and preservation of GSH level is linked with cell survival with an array of cellular processes [31]. The oxygen radicals damage almost all the cellular molecules such as carbohydrates, amino acids, proteins, lipids, DNA and RNA. When an inequity arises between antioxidants and reactive oxygen species, it leads to oxidative stress and as a consequence of a imbalance between the production of the reactive oxygen species and the ability to defend against them cellular damage and harmful events initiate [32]. Fisetin could positively control diverse cellular events in cancer initiation and progression for instance cellular matrix remodelling, apoptosis, epithelial to mesenchymal transition, cancer-linked inflammation, and oxidative stress [33, 34].

Drosophila has been employed for nearly a decade to investigate cognition and intellectual disability, which has provided a significant amount of disease-relevant information [35]. An assortment of assays has been standardized to evaluate cognitive behaviour in *D. melanogaster*, for instance, negative geotaxis, phototaxis, smell and taste chemotaxis, thermotaxis and hygrotaxis. Several types of cancer are known to damage cognitive functions [36]. In *D. melanogaster* age-associated impairment in cognitive functions has also been documented [37]. However, as the behavioural abnormality and oxidative stress indices during carcinogenesis/treatment with fisetin in *D. melanogaster* have not been performed earlier, the present study has been done to throw light on these lines.

MATERIALS AND METHODS

Fly maintenance and chemicals

D. melanogaster flies, Wild type (WT) was obtained from Center for cellular and Molecular biology (CCMB), Hyderabad, India. The flies were maintained in normal culture medium at room temperature (21-23 °C) in 12:12 h light: dark cycle. [38]. Both types flies were divided into four groups: (1) control, (2) 0.01% NDEA alone, (3) 0.01% NDEA with 0.01% fisetin and (4) 0.01% fisetin. Chemicals and biochemicals used in the present investigation were purchased from Genei Laboratories Pvt. Ltd. (Bangalore, India), S. D Fine-chem Ltd. (Mumbai, India) and Sigma Chemical (St. Louis, USA). NDEA and fisetin were administrated in food medium for 12 d.

Collection of hemolymph and tissue homogenate

Suitable holes in a 0.5 ml eppendorf tube were made and placed into 1.5 ml eppendrop tube with removed lid. Flies (30 nos.) were dissected by removing legs and wings. The tubes (1.5 ml containing 0.5 ml tube) were centrifuged for 2500 rpm for 15 min. The hemolymph was collected in the bottom of 1.5 ml tube and was mixed with ice-cold PBS (phosphate-buffered saline) and stored in freezer [39]. The dissected head and intestine tissues using 0.1 M sodium phosphate buffer (pH 7.4) and centrifuged (2500 rpm for 15 min) at 5 °C and used for biochemical assays.

Cognitive behavioural functions of *D. melanogaster*

The cognitive behavioural functions including negative geotaxis, phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis and hydrotaxis were assessed in all groups of flies by the methods of Vang and Neckameyer and Bhatt [8, 40] with minor modifications.

Negative geotaxis: response to gravity

About 30 flies from WT were anesthetised and positioned in a vertical glass column (12 cm X 1.5 cm) sealed at one end with cotton. After a short recovery period of five minutes, flies were softly trapped to the bottom of the column. Following one minute, flies that touched the top of the column and flies that continued to remain in the bottom were counted separately. Data was expressed as percent flies crossed beyond the distance of 13 cm in 60 s of interval [41, 11]. Each assay was repeated for all the four groups of flies and mean±SD was calculated (fig. 1a).

Phototaxis: response to light

The vial was segmented into 3 compartments, in a dark room vial containing about 30 flies plugged by cotton and the test tube were left separately for 30 min. And hence flies were allowed to adapt to darkness. The vial with flies was softly pounded down to keep the flies at the away from the cotton, then the cotton was detached, and the vial was attached to the test tube by a connector. This set-up was horizontal and perpendicular to the horizontal light source kept at 15 cm distance. The light was then turned on. The flies were counted every minute for each quarter of the apparatus. In a control set-up, the apparatus was kept 15 cm away from and parallel to the light source. Each assay was repeated in all the four groups and mean±SD was calculated (fig. 1b).

Smell chemotaxis: response to volatile compound

Volatile repellent benzaldehyde has been used in the study. About 20 flies were placed into two vials (15 × 1 cm) connected together with a transparent tape and is divided into 3 equal compartments (I, II and III). The cotton plug was drenched in 1 ml of benzaldehyde (100 mmol) and was plugged in the test tube (compartment III adjacent to cotton plug). After one minute, the number of flies, present in each compartment was counted and the result was expressed as percentage. The test was repeated for separate three sets of flies (fig. 1c).

Taste chemotaxis: response to nonvolatile compound

Sucrose (a non-volatile compound standardly used in taste chemotaxis) has been used in this assay. About 20-25 flies were placed in a test tube (18 cm × 1 cm) and are divided into 3 equal compartments. The cotton plug was soaked in 1 ml of 0.1% sucrose and plugged in the test tube. After one minute, the number of flies, present in each compartment was counted and the result was expressed as a percentage. The test was repeated for separate three sets of flies (fig. 1d).

Thermotaxis: response to temperature

Two vials (15 × 1.5 cm) were used in the study. One vial was heated to a temperature of 45 ° C and was instantly connected to a vial by means of transparent tape comprising of 20-25 flies. The connected vials were compartmentalized into three equal zones (I, II and III–compartment III heated zone). After one minute, the number of flies present in each compartment was counted and the result was expressed in the percentage of total flies present. The test was repeated for separate three sets of flies (fig. 1e).

Hygrotaxis: response to humidity

A vial (15 × 1.5 cm) was filled with 1 ml of distilled water, covered with parafilm and was kept overnight. After about 12 h, another vial (15 × 1.5 cm) with 20-25 flies was taken. After removing parafilm and water from the first vial, two vials were connected with a help of transparent tape. The connected vials were compartmentalized into three equal zones (I, II and III and compartment I moisturized zone).

After one minute, the number of flies present in each compartment was counted and the result was expressed in percentage of total flies present. The test was repeated for separate three sets of flies and mean±SD was calculated (fig. 1f).

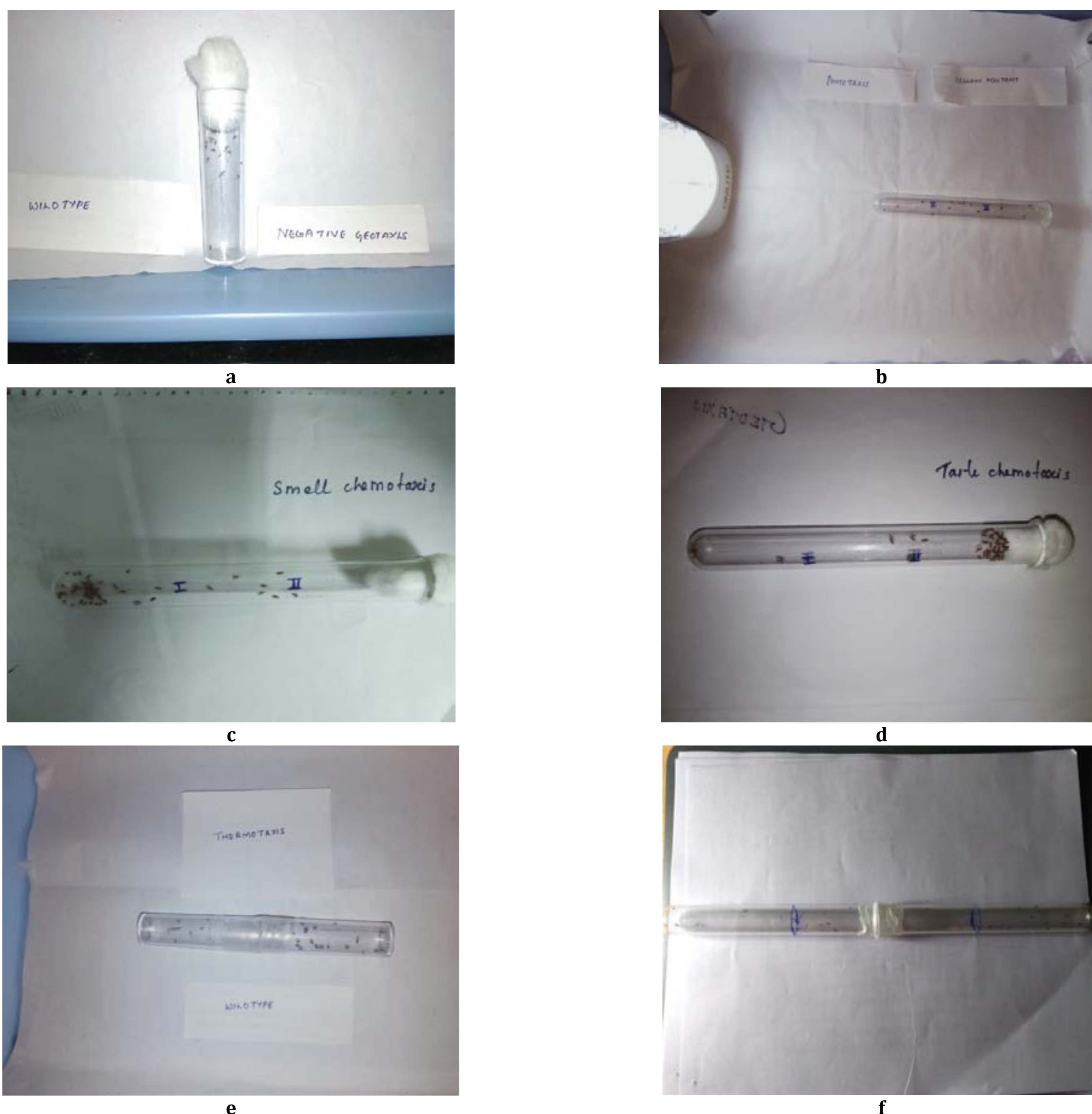

Fig. 1: Behavioural assays in *D. melanogaster*. Negative geotaxis (A), phototaxis (B), smell chemotaxis (C), taste chemotaxis (D), thermotaxis (E) and hygrotaxis (F)

Biochemical parameters

Protein carbonyl content

The protein carbonyl content was assayed [42]. The sample (hemolymph/tissue homogenate) was divided into 2 portions containing 1-2 mg protein each. To one portion, an equal volume of 2 N HCl was added and incubated at 36 °C for 60 min at room temperature. After incubation, the mixture was precipitated with 10% TCA and centrifuged. Precipitate was mixed with ethanol ethyl acetate (1:1) and 1 ml of 6 M guanidine HCl was added. Centrifuged at 1000 rpm for 5 min and the supernatant was taken. The difference in absorbance between the DNPH treated and HCl treated sample was determined at 366 nm and the results were expressed as μ moles, of carbonyl groups/mg of protein.

Thiobarbituric acid reactive substances (TBARS)

The levels of TBARS in hemolymph/tissue homogenate were estimated [43]. Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid (TBA) in acidic conditions to produce a pink coloured chromophore, which was read at 530 nm**.**

Protein thiol

Assay of free protein thiol groups is carried out by derivatization with 5,5′dithiobis (2-nitrobenzoic acid) (DTNB) [44]. The measurement is based on the formation of a coloured thiolate ion complex that can be detected spectrophotometrically at 410 nm. The thiol group assay is often performed on soluble protein fractions, by homogenization in a buffer containing a detergent such as sodium dodecyl sulfate (SDS) [44]

Lipid peroxides

This lipid peroxidation assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) at 40 °C. MDA or 4-HNE reacts with R1 to produce a stable chromophore with an absorbance at 580 nm [45].

Glutathione-S-transferase

Glutathione-S-transferase (GST) was assayed in hemolymph/tissue homogenate by an increase in absorbance at 350 nm using CDNB as substrate [46]. Phosphate buffer, reduced glutathione and CDNB (30 mmol) was prepared in 95% ethanol. The level of GST was expressed as µmoles of CDNB-GSH conjugate formed/min/mg protein.

Superoxide dismutase

Superoxide dismutase in hemolymph/tissue homogenate was measured [47]. The measurement is based on the inhibition of the synthesis of NADH-phenazinemethosulphate, a nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH. After incubation for 90 sec, the addition of glacial acetic acid ceases the reaction. The color developed was extracted into n-butanol layer and measured at 520 nm.

Catalase

The activity of catalase in hemolymph/tissue homogenate was measured [48]. To 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate/hemolymph and 0.4 ml of hydrogen peroxide were added. The reaction was stopped after 15, 30, 45 and 60 s by adding 2.0 ml of the dichromate-acetic acid mixture. The mixture was kept in a boiling water bath for 10 min, cooled and the colour developed was read at 610 nm. The specific activity was expressed as µmol of H_2O_2 consumed/min/mg of protein for tissues or µmol.

Glutathione peroxidase (GPx)

The activity of GPx in hemolymph/tissue homogenate was assayed [49]. To 0.2 ml of tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide, 0.5 ml of tissue homogenate/hemolymph were added. To this, 0.2 ml of GSH and 0.1 ml of H_2O_2 were added. The contents were incubated at 37 °C for 10 min, along with a control containing all reagents except homogenate/hemolymph. After 10 min, the reaction was stopped by the addition of 0.5 ml of 10% TCA. The contents were centrifuged and the supernatant was assayed for GSH [44]. The activity was expressed as µmol of GSH consumed/min/mg of protein. The amount of glutathione was expressed as mg/dl hemolymph and mg/100g tissue.

RESULTS

Behavioural assays

The negative geotaxis value 91.2±14.8 % is decreased significantly after NDEA treated (80.4±13.7%) compared to control flies ($p<0.05$). In NDEA+fisetin treated group the value is increased 83.8±12.3 compared to NDEA treated group. In group 4 (fisetin) the value is more or less similar (91.2±14.8 %) to control group within ($p>0.05$) compared to control group. More percentage of flies tends to move closer to the light source (phototaxis) (compartment I, table 1). However, this response was noticeably decreased ($p<0.05$) in group 2 (compartment I). NDEA+fisetin group showed higher percentage of flies ($p<0.05$) compared to group 2.

Larger number of control flies were seen to move away from the pungent chemical benzaldehyde from compartment I to compartment III compared to NDEA treated flies ($p>0.05$ table 1). Significantly augmened movement was noticed in fisetin+NDEA treated flies to compartment III ($p>0.05$). Higher percentage of control flies were found to move nearer to cotton-plug soaked with sucrose solution (compartment I) compared to the movement of NDEA treated flies towards compartment I ($p<0.05$). The response of NDEA+fisetin treated flies is closer to the movement of control flies ($p>0.05$, table 1).

As for the thermotaxis assay a higher number of control flies tent to move away from the warm surface (compartment III) to a relatively cooled surface (compartment I) as compared to NDEA treated flies ($p<0.05$). On the contrary the trend appeared reversed in NDEA+fisetin treated flies compared to group 2 flies ($p<0.05$). At the same time an in the significantly noticed percentage of fisetin alone treated flies were observed in compartment III compared to control flies ($p>0.05$). Hygrotaxis assay showed a higher percentage of movement of control flies to compartment I compared to NDEA treated flies ($p<0.05$, table 1). Significantly higher percentage of NDEA+fisetin treated flies were move to compartment I as compared to NDEA treated flies ($p<0.05$). Invariably, fisetin-treated flies demonstrated behavioral responses of negative geotaxis, photo, smell and taste chemotaxis, thermotaxis and hygrotaxis similar to the control group ($p>0.05$, table 1).

Table 1: Negative geotaxis, phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis and hygrotaxis in *D. melanogaster*. Percentage of flies in control, NDEA treated, NDEA+fisetin treated or fisetin treated are shown. Values were mean±SD of three experiments of each group. p≤0.05 were compared with all groups. Abbreviation: Wild type (WT), N-nitrosodiethylamine (NDEA)

% of flies present in compartment Behavioural assay	Compartment I (mean±SD)	Compartment II (mean±SD)	Compartment III (mean±SD)
Phototaxis			
WT (control)	90.2±3.2[a]	24.6±4.1[a]	7.8±1.8[a]
NDEA treated	76.3±4.9[b]	28.3±3.2[b]	11.2±2.8[b]
NDEA+fisetin	83.1±3.6[a]	27.4±3.6[a]	8.8±2.1[a]
fisetin only	92.2±3.1[a]	24.6±4.6[a]	7.9±1.4[a]
Smell chemotaxis			
WT (control)	2.8±0.8[a]	14.5±4.3[a]	92.5±3.5[a]
NDEA treated	13.4±2.3[b]	27.6±3.8[b]	68.3±4.2[b]
NDEA+fisetin	3.4±1.6[a]	15.6±3.3[a]	85.5±4.7[a]
fisetin only	2.1±0.6[a]	12.5±4.1[a]	90.5±2.5[a]
Taste chemotaxis			
WT (control)	89.5±9.6[a]	19.4±5.3[a]	10.4±4.7[a]
NDEA treated	64.2±7.8[b]	26.1±5.5[b]	16.3±6.1[b]
NDEA+fisetin	84.2±6.8[a]	20.7±4.4[a]	11.5±5.2[a]
fisetin only	83.5±9.1[a]	15.4±5.1[a]	10.4±4.2[a]
Thermotaxis			
WT (control)	76.9±7.4[a]	7.4±1.1[a]	1.2±0.6[a]
NDEA treated	62.6±6.5[b]	20.9±4.1[b]	8.5±2.9[b]
NDEA+fisetin	78.1±6.2[a]	5.3±0.9[a]	1.5±0.8[a]
fisetin only	75.2±7.2[a]	7.3±0.9[a]	1.1±0.5[a]
Hygrotaxis			
WT (control)	93.1±8.4[a]	21.6±6.8[a]	7.3±2.3[a]
NDEA treated	70.5±6.7[b]	26.3±5.2[b]	9.8±2.1[b]
NDEA+fisetin	85.6±7.1[a]	21.7±5.4[a]	5.5±1.8[a]
fisetin only	91.5±8.3[a]	19.6±5.8[a]	6.8±2.1[a]

Values are not sharing a common superscript alphabet vary significantly at $p<0.05$ by Duncans Multiple Range Taste (DMRT)

Biochemical assays

The levels of protein carbonyl, TBARS, protein thiols and lipid peroxides were predominantly elevated in hemolymph significantly ($p<0.05$) whereas these values were significantly decreased in head and intestine tissues in NDEA treated flies as compared to controls (table 2). In NDEA+fisetin treated groups the values were decreased in hemolymph and elevated in tissues (head and intestine) compared to NDEA treated flies. Group 4 flies (fisetin treated) showed more or less closer values to control flies. The levels of SOD, CAT, GST, GPx and GSH were predominantly decreased in hemolymph significantly ($p<0.05$) whereas these values were significantly increased in head and intestine tissues in NDEA treated flies as compared to controls (table 2). In NDEA+fisetin treated groups the values were increased in hemolymph as well as in tissues (head and intestine) compared to NDEA treated flies. Group 4 flies (fisetin treated) showed more or less closer values to control flies.

DISCUSSION

Flies treated with NDEA that to develop oxidative stress during tumorigenesis [50]. This, in turn, could inhibit normal negative geotaxis behaviour [51] and to shorten sleep duration [52]. However, possibly, this is the first study showing the improvement of cognitive function by fisetin in NDEA treated *D. melanogaster*. The physiological, molecular and signalling mechanisms underlying for the abnormalities in behavioural indices are to be investigated. However, the normalization of ROS levels and inhibition of carcinogenesis under fisetin treatment could normalize the behaviour in flies.

Our results clearly suggested that during tumorigenesis the behaviours (negative geotaxis, phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis and hygrotaxis) are clearly altered. Defects in cognition are reported widely in wide range of cancers [36]. Our findings also added additional evidences that the cognitive behaviors could have been affected owing to carcinogenesis in flies. Our results also indicted that fisetin could nullify the harmful effects of NDEA and thus tend to bring back the flies' behaviours to near normal. Fisetin, a bioactive flavonoid has been documented widely to inhibit the proliferation of various types of tumors in animals [53] and in various cancer cell lines [54]. The antitumor effects of fisetin could be mediated by modulating different signalling pathways in diverse frameworks [55].

Our findings are consistent with earlier reports in mammals [56] signifying that elevation of TBARS level in NDEA induced flies. This could be owing to excessive generation of ROS and with an early event associated with hypoxia [57]. This level was decreased in fisetin-treated flies which is owing to the presence of three hydroxyl groups present in fiestin which have a strong ROS scavenging activity [58]. To prevent cellular damage induced by ROS, there is a lot of antioxidative defense system in *D. melanogaster*. The antioxidative defense system could scavenge ROS and play a key role in the inhibition of lipid peroxidation and therefore, play a protective role in cancer development [59]. SOD and CAT comprise an equally protective set of enzymes against ROS [60]. This defence mechanism functions via enzymatic (including SOD, GPx, GST and CAT), and non-enzymatic components [61]. Enzymatic and non-enzymatic antioxidants levels were decreased in NDEA exposed flies.

Table 2: Experimental values of protein carbonyl, thiobarbituric acid reactive substances, protein thiol, lipid peroxides, superoxide dismutase, catalase, glutothion-S-transferase, glutathione peroxidise and reduced glutathione. Percentage of flies in control, NDEA treated, NDEA+fisetin treated or fisetin treated are shown. Values were mean±SD of three experiments of each group. p≤0.05 were compared with all groups. Abbreviation: Wild type (WT), N-nitrosodiethylamine (NDEA)

S. No.	Biochemical parameters	Groups	Hemolymph	Head	Intestine
Redox hemostasis					
1	Protein carbonyl (nmole/mg protein)	WT	5.7±1.03[a]	3.9±0.86[a]	2.3±0.09[a]
		NDEA treated	8.7±2.35[b]	2.7±0.51[b]	1.6±0.07[b]
		NDEA+fisetin	5.0±0.08[a]	3.0±0.65[a]	1.4±0.05[a]
		fisetin only	5.1±1.03[a]	3.1±0.85[a]	2.3±0.09[a]
2	Thiobarbituric acid reactive substances (TBARS)(nmole/mg protein)	WT	12.8±2.63[a]	8.4±1.30[a]	7.2±1.74[a]
		NDEA treated	17.8±3.67[b]	5.4±0.81[b]	4.9±1.14[b]
		NDEA+Fisetin	11.1±2.01[a]	7.5±1.21[a]	5.5±1.24[a]
		fisetin only	19.9±3.73[a]	13.8±3.52[a]	9.4±2.20[a]
3	Protein thiol(mmol/mg protine)	WT	38.7±6.21[a]	28.4±4.81[a]	24.8±3.95[a]
		NDEA treated	48.1±3.10[b]	19.2±2.61[b]	15.8±1.33[b]
		NDEA+Fisetin	34.5±5.11[a]	25.1±2.89[a]	21.5±3.01[a]
		fisetin only	32.7±5.21[a]	22.4±3.81 [a]	20.82.91 [a]
4	Lipid peroxides (nmole/mg lipid)	WT	35.6±5.37[a]	27.9±5.13[a]	26.7±3.73[a]
		NDEA treated	44.6±4.06[b]	16.8±3.10[b]	16.8±2.09[b]
		NDEA+fisetin	33.3±3.29[a]	25.1±3.48[a]	22.3±2.35[a]
		fisetin only	34.8±0.13[b]	21.6±0.95[b]	21.3±0.82[b]
5	Superoxide dismutase (SOD) (Unit[a] nmole/mg protein)	WT	12.1±2.01[a]	7.8±1.21[a]	6.5±1.24[a]
		NDEA treated	11.7±2.18[b]	10.5±2.89[b]	8.3±1.14[b]
		NDEA+fisetin	18.4±3.15[a]	13.5±2.58[a]	9.2±2.10[a]
		fisetin only	20.4±3.33[a]	12.8±1.52[a]	10.2±1.20[a]
6	Catalase(CAT)(Uint[b]/min/mg Protein)	WT	165.3±2.57[a]	140.3±13.59[a]	110.7±10.22[a]
		NDEA treated	133.1±11.86[b]	112.3±9.67[b]	89.3±5.74[b]
		NDEA+fisetin	160.6±18.17[a]	135.3±10.51[a]	105.8±9.11[a]
		Fisetin only	158.1±21.52[a]	141.3±11.54[a]	111.7±11.21[a]
7	Glutothion-S-transferase (Uint/100 mg/Protein)	WT	12.6±0.14[a]	8.5±0.71[a]	6.7±0.53[a]
		NDEA treated	8.2±0.07b	5.5±0.42b	4.3±0.21b
		NDEA+fisetin	11.0±0.95[a]	7.2±0.84[a]	5.6±0.43[a]
		fisetin only	158.1±21.52[a]	140.3±11.54[a]	110.7±11.21[a]
8	Glutothione peroxidise (GPx) (Unit[c] per mg Protein)	WT	11.8±4.32[a]	6.5±0.95[a]	5.6±0.84[a]
		NDEA treated	8.1±1.12[b]	3.6±0.54[b]	3.1±0.41[b]
		NDEA+fisetin	10.0±3.21[a]	5.4±0.87[a]	4.0±0.78[a]
		fisetin only	9.1±3.12[a]	5.5±0.85[a]	5.0±0.74[a]
9	Reduced glutathione (GSH)	WT	16.6±8.14[a] (µl/ml)	10.4±2.11[a] (µl/mg tissue)	9.5±1.54[a] (µl/mg tissue)
		NDEA treated	10.3±3.04[b]	7.4±0.91[b]	6.1±0.45[b]
		NDEA+fisetin	13.3±6.21[a]	9.7±1.32[a]	8.7±0.91[a]
		fisetin only	14.2±7.16[a] (µl/ml)	10.4±1.19[a] (µl/mg tissue)	8.0±1.14[a] (µl/mg tissue)

Values are not sharing a common superscript alphabet vary significantly at $p<0.05$ by Duncans Multiple Range Taste (DMRT)

The augmented levels of TBARS and lipid hydroperoxides in hemolymph and tissues (intestine and brain) noticed in this study might be owing to NDEA induced free radical synthesis, membrane damage, and cell lysis; improvement of lipid peroxidation is observed in fisetin treated flies due to enhanced antioxidant activity [62]. The antioxidant nature of the polyphenolic compounds could sustain the fly's defenses against NDEA mediated free radical damages. The chemical structure, position and degree of hydroxylation are the important factors to exhibit the biological and pharmacological properties of flavonoids. The noteworthy elevation in GSH level in fisetin-treated flies implies the ability of fisetin to sustain GSH level by preventing glutamate toxicity and stimulating cystine (GSH precursor and excellent source for thiol group) uptake into brain by its free radical scavenging and cytoprotective properties. The plausible mechanism by which fisetin caused its protective effect could be by its free radical scavenging properties, and by maintaining the cellular integrity of cells in *D. melanogaster*.

The contents of protein carbonyl, TBARS, protein thiols and lipid peroxides (the products of excessive oxidative stress) were higher in hemolymph ($p>0.01$) although they are noticeably lesser in the tissues of head and intestine of flies. The regulation of reactive oxygen species (ROS) levels is a key factor during tumorigenesis as higher levels of ROS can be damaging to cells. Therefore, the tumour cells exhibit a mechanism of actions such as peroxide scavenging system to maintain the balance of ROS to ascertain cells proliferative state [60]. Furthermore, the rapidly dividing tumour cells in head and intestinal tissues were previously reported to utilise high levels of ROS [63]. Together, these could have resulted in the curtailment in the end-products of oxidative stress in these tissues in this study, as the ROS levels were decreased by the tumour cells. In contrast, the above-said indices (protein carbonyl, TBARS, protein thiols and lipid peroxides) may possibly have augmented in the hemolymph of flies, due to the overall tumour load in their system. There is also a consistent decrement in the levels of antioxidants–SOD, CAT, GST, GPx and GSH in hemolymph and the tissues; this could be due to the rapid utilisation of antioxidants by the tumor-bearing host [64]. As stated earlier, fisetin is well known for its antioxidative actions, which includes direct detoxication of reactive oxygen and reactive nitrogen species and indirectly, by stimulating antioxidant enzymes while suppressing the activity of pro-oxidant enzymes [65]. The administered dose of fisetin could have alleviated the oxidative stress, thus, reversing the pro-oxidative effects of fly by representing a significant upregulation in most of the indices of the redox homeostasis. In particular, the noticeable decrement of lipid peroxides in hemolymph of fisetin-treated flies denotes a decrease in lipid peroxidation of lipids. Similar observation has also been reported earlier about the antioxidant activities of fisetin against oxidative stress [66].

The chemical structure, position and degree of hydroxylation are the key factors to exert the biological and pharmacological properties of fisetin. The low molecular weight and highly hydrophobic nature of fisetin helps it to readily move through cell membranes and to accumulate intracellularly, which protect most susceptible brain and intestine tissues of carcinogenic flies. The significant decrease noticed in the activity of SOD, catalase and GPx in carcinogenic flies might be owing to the presence of three hydroxyl groups present if fiestin which have a strong ROS scavenging activity. Most of the enzymes of redox pathway in carcinogenic flies have been evidenced, (including SOD, catalase and GPx) [63]. The noticeable augmentation in GSH level in fisetin-treated flies denotes the capability of fisetin to increase the concentration of GSH in brain by promoting cystine (GSH precursor uptake into the brain and could directly protect from NDEA's actions by its free radical scavenging and cytoprotective effects.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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