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N-Nitrosodietyhlamine induced experimental carcinogenesis: Protective effect of fisetin on cognitive and biochemical variable in *vestigial* mutant of *Drosophila melanogaster*

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Abstract
The current examination is intended to N-Nitrosodietyhlamine induced experimental carcinogenesis: Behavioural and biochemical indices on fisetin induced vestigial mutant of drosophila melanogaster. For this study D.melanogaster used as a model organism particularyvestigial mutant ofD. melanogaster is employed as a sample organism for the present study.Mutant (vg) flies are grouped into four; they are a) control b) 0.01% NDEA treated flies c) 0.01% fisetin and 0.01% NDEAwere treated flies in food medium and group e)only 0.01% fisetin treated flies. Behavioural abnormalities like response on gravity, light, smell, taste, humidity and temperature were measured quantitatively. Dissociation between NDEA treated flies with control flies but nearly standardized in fisetin administered flies. The quantity of protein thiol, thiobarbituric acid reactive substance (TBARS), lipid peroxides and protein carbonyl also done in vg mutant fly. The biochemical compounds were notably increased in NDEA administered flies than normal flies and respectively tends to return to normal in fistien treated group. In additional, catalase (CAT), glutathione peroxidise (GPX), superoxide dismutase (SOD), reduced glutathione (GSH), and glutathione-S-transferase (GST) were reduced in cancergroup and there was sharp increase ($p<0.05$) in the fisetin treatment group. SEM differentiated the organs like head and intestine between NDEA treated and control vg mutant flies. Fisetin, is a bioactive phytoconstituentthat can function as a powerful antioxidant and demonstrate antiproliferative properties. Our study suggests that this may thwart abnormalities in cognitive behaviour and redox hemostasis for the duration of carcinogenesis in the vg variant of D. melanogaster.

Keywords: cancer, *drosophila melanogaster*,*vestigial* mutant, fisetin, ndea, ros

Introduction
The “fruit flies” is a type of fruit insect, most commonly used in genetics, molecular biology and developmental biology as a research organism. In Drsophilideafamily,one species of *Drosophila*, namely, *Drosophila melanogaster*. *D. melanogaster* and its associated species have been extensively studied available in the literature or decades [1]. General knowledge of genetics and molecular biology of *D. melanogaster* and the long term experimental skill with this organism collectively with widespread genetic homology to mammals has made it of exclusive usefulness in mutation research and toxicology.Many*Drosophila* genes are homologous to human genes, and this proteins is being studied to gain an enhanced perceptive of what role it plays in human [2].
The *vestigial* (*vg*) gene of *D. melanogaster* encodes a protein in nucleus which has vital functions in wing development and in other developmental processes including cell proliferation [3]. Besides the well-defined role in the wing, *vg* is also implicated in the differentiation of other tissues. Vestigial loss, causing the wings to fall off [4] and ectopic expression of *vg* lead to ectopic wing tissueeffcts known *vg* mutations exhibit a range of temperature-sensitive expression patterns. Failure of expression of *vg* cause a damaged embryonic muscle phenotype with reduced connection and muscle deficit in the ventral lateral muscles resulting in elevated oxidative stress which could promote tumorigenesis in the dividing cells in intestine and other tissues of the fly. Further, *vg* is essential for the production of the not direct flight muscles, where Notch-dependent *vg* signalling is necessary for appropriate muscle arrangement and could result in defective cognitive behavioural activities. Furthermore, deficiency of *vg* through a *vg*null allele leads to apoptotic deprivation of the muscle fibres causing transition to a straight flight muscle-like cell destiny enhancing oxidative stress and defective behavioural functions. Oxidative stress investigation in *vg* mutant indicates that, this mutant is highly susceptible to damages as compare to wild type of *D.melanogaster* [5].
Most of organisms are responding behaviourally to different stimuli in their surroundings like brightness, temperature, volatile and non-volatile chemicals, moisture, gravity, and sound. The recognising of these stimulants is performed in the fly, appropriately, by various cognitive senses such as smell of volatile chemicals (olfaction), vision, taste of nonvolatile chemicals (gustation), sensors of heat, moisture, hearing and gravity. The reaction to stimuli can be affinity or disgust, based on the character and the energy of the stimulants [6]. A

recentreports concludes that adult *D. melanogaster* are behaviourally responsive to bright light[7], chemicals: odour [8] and tastings [9], moisture [10] , temperature [11] , gravity [12].

Cancer is a multi-stage process that includes initiation, promotion and progression.Most signal pathways that control cell growth and incursion in mammals have a protective function in flies allowing their alteration into tumour biological mimics in simple specimen organism such as *Drosophila* [13]. The amalgamation of expressible genes with the accessibility of powerful recombination methodologies enables quick classification of the primary function of gene that suppresses cancer and tumour protection throughout the animal [14]. In addition, a current study with *Drosophila* imaginary discs examines the growth and development of epithelial tumours and their communication with local TME (Tumour Micro Environment) and stromal cells including some steps in attaching the immune cell (macrophages) to the tumour mass [15]. Antioxidant pressure is said to be associated with multiple metabolic disorders, the development of chronic disorders or cancer [16].

N-nitrosodiethylamine (NDEA) is a nitrosamine that is N-ethylethanamine replaced by a nitroso group at the N-atom. It has a role as a mutagen, a hepatotoxic agent and a carcinogenic agent and could develop tumour in numerous organs of various animal model. NDEA is present in a extensive range of food items such as soybeans, cheese, dried, salted and smoked fish, cooked meat and alcoholic drinks. Most of the remedial drugs are metabolized to generate N-nitrosodethylamine by the activity of cytochrome p450 enzymes [17]. Antioxidant pressure is considered to be an important mechanism causative to NDEA-treated liver toxicity and the application of antioxidant drugs reduces bioavailability [18].

Flavonoids are the main group (60%) of nutritional polyphenols. Flavonoids are a family of antioxidants commonly found in fruits, vegetables, dried legumes and popular beverages such as chocolate and red wine, coffee and tea. Many studies strongly recommend polyphenols to prevent, cancer, osteoporosis, cardiovascular disease and neurodegenerative disease. Flavonoids in the diet can affect and alter various biochemical processes and pathways that cause cancer. In addition, they support the function of the immune system and act as biological response modifiers that damage cells with free radicals. Natural flavonoids have emerged as the main focus in the search for new antineoplastic therapies [19]. The antioxidant activity of these compounds prevents various degenerative diseases associated with antioxidant pressure, including neoplastic disease, which is considered to be the main mechanism of their action. The flavonoids capacity is to remove free radicals contributeto their noticeable antioxidant function and important biological property. Oxygen radicals are harmful tocarbohydrates, amino acids, nucleic acids and lipids. When an inequality takes place between reactive oxygen species and antioxidants, it directs to antioxidant deficiency and is the result of a disparity associating with the synthesis of reactive oxygen species and their capability to preserve them. It has been involved in the progress of several diseases, such as cancer, senility, diabetes, retinal degeneration and neurodegenerative disease [20].

The antioxidant function of flavonoids and their benefits in human diseases condition have been increasingly documented, but not much is recognized concerning how they alter its effect on lipid droplet biology. In the fly sample, the influence of flavonoids is to reduced fat storage, the number and size of fat droplets and the total area engaged by fat dropletsdroplets[21]. After adding the fly diet with flavonoids, the elevation in fat droplets on the sides of the oenocytes indicates that lipolysis of the body is enhanced.The central nervous system and muscles need more energy, so the amount of lipid droplets in the glial cells and muscles increases after administrationwith flavonoids. Hence, the treatment of flavonoids controls favourably the biological synthesis of fat droplets in a tissue specific fashion and the utilization of flavonoids is an effective treatment for neurological and metabolic diseases [22].

Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a biochemicalflavonoide group of compound present in vegetables and fruits at a concentration ranging from of 2–160 μg/g[23].In recent years, fisetin had been the subject of research in various human diets due to its presence and its antiproliferative, apoptotic and antioxidant functions.Fisetin has been documented as a chemotherapeutic/chemopreventive compound in many types of tumour and as well as a neuroprotective agent. Numerous investigations suggestthat fisetin is a potential antioxidant. Fisetin has strong anti-inflammatory, antioxidant, antineoplastic, anti-viral, antiangiogenic, antidiabetic, and neurosis as well as cardio-protective activities in cell cultures and animal specimens [19].

Reactive oxygen species (ROS) are vital signal molecules in cancer. The amount of ROS will findthe physiological function. High level of ROS can cause damage to tissues and cell death, low level of ROS can cause proliferative effects. While the cellular homeostasis weakens associating the pro-oxidant functions and antioxidant production, our ROS increasesthe production of oxygen species and free radicals [24]. ROS are mainly produced as a by-product of antioxidant phosphorylation in calls in the ETC (electron transport chain) in the mitochondria. The quantities of our ROS productions cause antioxidant pressure, which is concerned in the progress of inflammatory processes that lead to several regenerative diseases and cancer. Flavonoids possess a dual function in relation to ROS homeostasis-they function as antioxidants in standard conditions and stimulate the apoptotic pathways of potent pro-oxidants in cancer tissue [25]. Flavonoids can unswervingly quench ROS, and chelate metal ions owing to their capacity to alleviate free radicals owing to the occurrence of phenolic hydroxyl groups.Not direct flavonoid antioxidant influences have been implicated in the stimulation of antioxidant enzymes, suppression of pro-oxidant enzymes, and activation of the synthesis of detoxification enzymes of phase II metabolism in addition to antioxidant enzymes. Antioxidant and pro-oxidant actionscould play vital roles in exhibiting the anticarcinogenic influences of fisetin[26]. The present study is undertaken whether fisetin could nullify the behavioural abnormalities and markers of oxidative stress in *vg* mutant during the process of carcinogenesis.

Earlier, we have investigated the protective effects of fisetin during NDEA induced carcinogenesis in wild type fruit fly, *Drosophila melanogasterr* [27]. However, the influence of fisetin in the wing mutant, *vg* is unknown at present. It is in this context, the present study is undertaken which highlights the protective efficacy of fisetin in the *vg* mutant.

Materials and methods

Fly culture

D. melanogaster flies, *vestigial* mutant flies (*vg*) acquired from Centre for Cellular and Molecular biology (CCMB), Hyderabad, India. Flies were retained in culture medium in *Drosophila* culture incubator (Rays Scientifics Instruments,Chennai) (21-23 °C) in 12:12 hrs light: dark cycle (Subramanian*et al*., 2016). Flies were grouped into (1) control (untreated), (2) 0.01% NDEAtreated, (3) 0.01% NDEA with 0.01% fisetin treated and (4) 0.01% fisetin treated alone. Biochemicals and chemicals were procured from Genei Laboratories Pvt. Ltd. (Bangalore, India), S. D Fine-chem Ltd. (Mumbai, India) and Sigma Chemical (St. Louis, USA)used in the present investigation. Fisetin and NDEA were treated in culture medium for 21 days.

Hemolymphcollection and tissue homogenate

The appropriate punctures were made in 0.5 ml microfuge tube and it was fixed into 1.5 ml microfuge tube along with removable cap.Around 30 sectioned (removed led and wings) *vg* flies were taken. The 0.5ml eppendorf tube placed within the 1.5ml eppendorf tube and then it was centrifuged for 15min with 2500 rpm. Collect the hemolymph at the bottom of the large (1.5ml) tube and it was added along with chilled phosphate-buffered (PBS) and kept in -20 ° C [28]. The tissue (intestine and head) were centrifuged at the same above rpm for 15 min in a suitable buffer medium containing 0.1M sodium phosphate (pH 7.4) at 5ºC and used for biochemical studies.

Histropatholgyical studies

Theflies samples were set for 48hrs in 10%formalin, dehydrated in water-ethyl alcohol mixture, washed in xylene and fixed in paraffin. Flies dissection (5-7 µm thick) were ready, stained along with hematoxylin and eosin dye (HandE) and seated in DPX medium (pH 7.0) for microscopic assessment.

Behavioural assays of *D.melanogaster*

The habitual characters like (test on gravitation, light, odour, taste, temperature and moisture) phototaxis, negative geotaxis, smell and taste chemotaxis, hygrotaxis and thermotaxis were assessed in all groups of vestigial flies as reported by Vangand Neckameyer and Bhatt[6,29]with slight alterations.

Response to gravitation-negative geotaxis

Fig.1a shows that the response to gravitational force. The negative geotaxis assay was performed with mutant (*vg*) flies, 30 anesthetized *vg* flies were transferred into glass tube (15 × 1.5 cm) edge was closed with cotton plug. After some revival period, flies were smoothly moved to the base of the tube. After 60 seconds, flies that arrived at the uppermost of the tube and flies that stayed behind at the bottom were numbered individually. Results were measured as percent flies runaway from the length of 13cm in 60 seconds of period. Around 30 *vg*flies for every test were used and the test was done thrice. All the following assays were performed [6,29]with small alterations.

Response to light -phototaxis

Fig.1b indicate that the response to light**.** Two glass tubes (15 x 1.5 cm) used for phototaxis, these two tubes were connected with translucent tape and segregated into 3 equivalent compartments and were named as I, II and III in which I compartment is closer to light source. Around 30 adult vg mutant flies are poured into the tube and sealed with cotton. The experimental *vg* flies were allowed to the blackness for 30 minutes. This permitted adjustment of the flies to the blackness. Then, the vg flies were permitted to the light basis (emergency lamp, Philips, India) put up in 5cm beyond from the test tube. After 60 seconds, the flies maintained in each compartment were noted and the value is denoted as % of flies. The expriment was performed for 3 separated sets of flies and mean±SD was measured.

Response to volatile compound -smell chemotaxis

Fig.1c shows the response to the volatile compound (smell chemotaxis test). The smell chemotaxis was performed with 30 adult *vg* flies using benzaldehyde. The two test tubes (15× 1.5 cm) were joinded with a paraflim and segregated into 3 equivalent segments like I, II and III. The cotton-plug soaked in 1 ml of 100mM benzaldehyde and it was sealed in the test tube maintaining compartment III adjacent to cotton cap. After 60 seconds, flies were present in each segment was counted and result was denoted as percentage. The experiment was triplicated and mean±SD was measured.

Response to sucrose solution-taste chemotaxis

Fig.1d indicated that the response to non-volatile attraction. In taste chemotaxis study, sucrose has been used as a non-volatile attractant. Around 30 vg flies were poured in to test tube (18 cm × 1 cm) and it was segmented into

3 similar segments. The cotton socket was immersed in 1ml of sucrose solution (0.1%) and the cotton was sealed in the test tube. After 60 seconds, the flies that present in each segments and it was denoted in percentage. The test was triplicated and mean±SD was calculated.

Response to temperature – thermotaxis

Fig.1e indicated that the response to heat, two test tubes (15 × 1.5 cm) was used in thetmotaxis. One tube was heated of 45º C and it was straight away connected with another tube containing round 20-30 mutant vg flies, tubes were connected by paraflim. Tubes were segmented into 3 equal parts (I, II and III – segment III heated segment). After 60 seconds, flies that were maintained in each segment were noted and the outcome was denoted in percentage. The assay was frequented three times and mean±SD was calculated.

Response to humidity-hygrotaxis

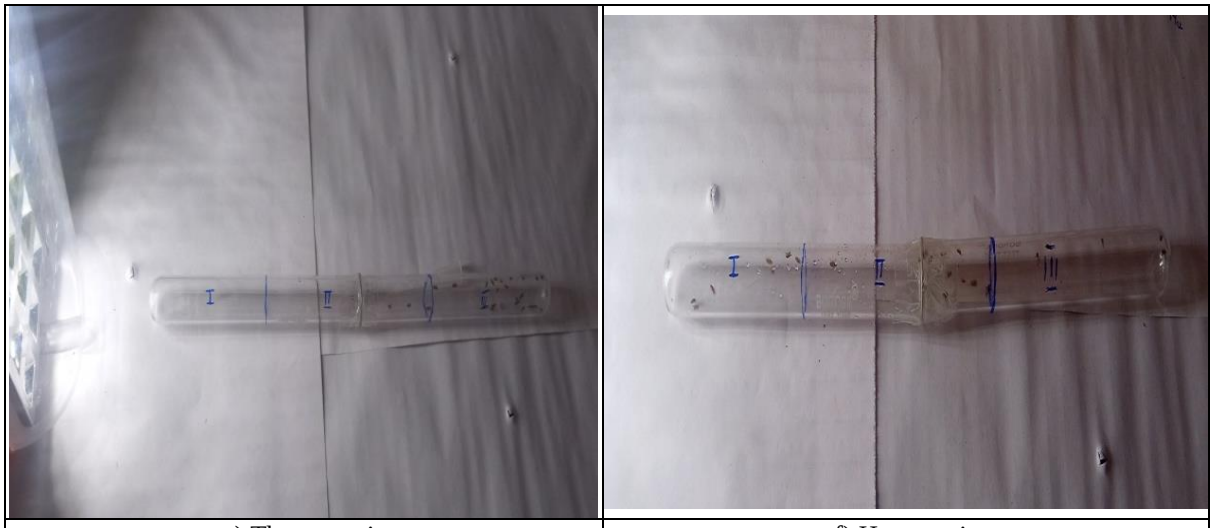
Fig.1f shows that response to moisture test (hygrotaxis). A tube (15 × 1.5 cm) poured 1ml of distilled water for hugrotaxis study, closed with parafilm and was kept overnight. After 12 hours, another (15 × 1.5 cm) tube was taken with 25-30 flies. After taking out the parafilm and water from the first tube, the two tubes were linked by means of transparent tape. The linked tubes were segmented in the 3 equal segments like I, II and III, segment I moisturized. After 60 seconds, the tested flies maintained in each segment were noted and the reported in % of total flies present. The assay was frequented three times and mean±SD was measured.

a) Negative Geotaxis

b) Phototaxis

c) Smell chemtaxis

d) Tastechemotaxis



e) Thermotaxis | f) Hygrotaxis

Fig 1

Biochemical parameters

Protein carbonyl content

The protein carbonyl was quantified by the method of Levine [30]. The homogenateed*vg* mutant sample (hemolymph/tissue homogenate) was segregated into 2 parts, each part contain 1-2 mg of standard protein. To one part, an equivalent amount of 2N HCl was added and maintained at 25 °C for 1 hour. Then, the mixture was added with 10% TCA and centrifuge. Ethanol ethyl acetate (1:1) and 1ml of 6M guanidine HCl was mixed with precipitation. The mixture was centrifuged at 1000 rpmfor 5 min and the supernatant was removed. The absorbance variation between the HCl and DNPH added sample was measured at 360 nm and the values were mentioned as µ moles, of carbonyl groups/mg of protein.

Thiobarbituric acid reactive substances (TBARS)

In mutant flies the concentration of TBARS was estimated using hemolymph/tissue homogenate *vg* mutant [31]. Melondialdehyde and other thiobarbituric acid reactive substance were estimated by their action with thiobarbituric acid (TBA) in acidic conditions to give a pink coloured chromophore, which was measured at 530 nm.

Protein thiol

This assay is perfomed by the reaction with 5, 5'dithiobis (2-nitrobenzoic acid) (DTNB) [32] in hemolymph/tissue homogenate of *vg* mutant. The assay is on the basis of the synthesis of a thiolate ion coloured compound, this coloured complex can be measured at 410 nmspectrophotometrically. The thiol group measurement is carried out on soluble protein portion, by homogenization in a relevant buffer holding a detergent, sodium dodecyl sulphate (SDS).

Lipid peroxides

This lipid peroxidation assayed using hemolymph/tissue homogenate of *vg* mutant is via the reaction of a chromogenic reagent, N-methyl-2-phenhlindole (R1), with malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) at 42 °C and they react with R1 to produce a steady chromophore which can be measured at 580 nm [33].

Glutathione-S-transferase

Glutathione-S-transferase (GST) was measured using CDNB as substrate [34] in hemolymph/tissue homogenate of *vg* mutant; increased absorbance was measured at 350nm. Phosptate buffer, CDNB (30 mmol) and reduced glutathione was maintained in 95% ethanol. The concentration of GST was expressed as µmoles of CDNB-GST conjugate formed/min/mg protein.

Superoxide dismutase

The measurement of superoxide dismutase is carried out via the suppression of the production of NADH-phenazinemethosulptate, a nitrobluetetrazoliumformazon. It was measured by using hemolymph/tissue homogenate of *vg* mutant [35].

This response was triggered by adding NADH. After incubation for 90sec, adding up of concentrated acetic acid stops the reaction. The coloured compound formed was separated into n-butanol layer and estimated at 520 nm.

Catalase

The catalese activity was measured using hemolymph/tissue homogenate of *vg* mutant [36]. To 0.1ml of tissue homogenate/hemolymph and add 0.9 ml of phosphate buffer, 0.4 ml of hydrogen peroxide were mixed. This reaction was ceased after regular intervals (15, 30, 45 and 60 seconds) by mixing 2.0 ml of the dichromate-acetic acid combination. The combination was maintained at 100 ° C for 15 min, and then kept at room temperature for 20 min. and the colour formed was measured at 610 nm. The definite activity was measured as µmol of H_2O_2 utilized/min/mg of protein of tissue.

Glutathione peroxidase (GPx) and reduced glutathione (GSH)

GPx range in *vg* mutant hemolymph/tissue homogenate was measured [37]. To 0.5 ml tissue homogenate/ hemolymph 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1ml of sodium azide was added. To the mixture, 0.2 ml of GSH and 0.1ml of H_2O_2 were added. The contents were maintained for 10 min at 37°C, together with a control with all reagents without homogenate/hemolymph. After 10 min, 0.5 ml of 10% TCA were mixed, reaction was stopped and centrifuged and the supernatant was used for the assay of GSH [32] (Ellman 1959). The level was expressed as µmol of GSH consumed/min/mg of protein. The level of glutathione was expressed as mg/dl of hemolymph.

Results

Behavioural assays

In negative geotaxis (Fig.1a), rate 70.2±7.8% is significantly reduced after NDEA administered *vg* mutant value (61.7±8.8 %) compare with control flies ($p<0.05$). Group 3 (NDEA+fisetin) assessment is increased 64.8±7.6% compared to NDEA administered group. In fisetin (0.01%) treated group value is nearly (70.2±7.8%) to group 1 ($p>0.05$ Table 1).

In phototaxis (Fig.1b) the higher number of *vg* flies to mobilizenear to the light (phototaxis) (segment I, Table 1). Though, this result was markedly diminished ($p<0.05$)) in NDEA treated (segment 1). Group 3 (NDEA+fisetin) exhibited elevated number of *vg* flies ($p<0.05$) as evaluated with NDEA treated flies. Fisetin(0.01%) treated flies value is more or less similar to group 1 (control). Fig.1c shows the repulsion of *vg* mutant flies by benzaldehyde. The elevated number of group 1 mutant flies were observed to shift aside from the strong chemical benzaldehyde fromsegment I to III compare to group 2 (NDEA) *vg* flies ($p>0.05$ Table 1). Much increased mobilization was observed in group 3 (NDEA+ fisetin) *vg*flies to segment III ($p>0.05$). Group 4 movement is more or less similar to control *vg* flies. (Fig.1d) showed attraction towards the sucrose, higher number of control *vg* flies were start to mobilize closer to cotton-lid immersed with 1% sucrose solution (segment I) as evaluated against the mobilization of NDEA administered*vg* flies in the direction ofsegment I ($p<0.05$). The movement of NDEA + fisetin administered *vg* flies is faster towards the mobilization of group 1 *vg* flies ($p>0.05$, Table 1).

Fig.1e shows the temperature repulsion, an elevated controls flies tent to mobilize left from the heated area (segment III) to a comparatively chilled surface (segment I) as analysed with group 2 (NDEA administered) *vg* flies ($p<0.05$). On the opposing the movement tend to be upturned in 3nd group (NDEA+ fisetin) *vg* flies analysed with group 2 flies($p<0.05$). Remarkable differences in the percentage of group 4 (fisetin only) *vg* flies were noticed in compartment III analysed with group 1 (control) *vg* flies. Fig.1f shows the attraction towards the humidit, a large percentage of control *vg* flies movement to compartment I analysed with group 2 *vg* flies ($p<0.05$, Table 1). Remarkably large % of group 3 *vg* flies were move to compartment I as analysed with group 2 *vg* flies($p<0.05$). prpetually, group 4 (fisetin administered) *vg* flies exhibited behavioural characteristics of negative geotaxis,phototaxis, taste and smellchemotaxis and hygrotxis comparable to the group 1 flies ($p>0.05$, 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. $p \leq 0.05$ were compared with all groups. Abbreviation: *Vestigial* flies (*vg*), N-nitrosodiethylamine (NDEA)

% of flies present in compartment Behavioural assay	Compartment I (mean±SD)	Compartment II (mean±SD)	Compartment III (mean±SD)
Phototaxis			
vg(control)	42.1±1.8[a]	18.4±1.1[a]	2.8±0.8[a]
NDEA treated	17.3±0.7[b]	12.2±0.6[b]	0.7±0.5[b]
NDEA+ fisetin	39.2±3.6[a]	20.4±1.2[a]	1.8±1.1[a]
fisetin only	41.2±1.6[a]	17.6±1.0[a]	2.4±0.74[a]
Smell chemotaxis			
vg(control)	0.8±0.2[a]	10.5±2.3[a]	96.5±3.5[a]
NDEA treated	0.3±0.2[b]	12.2±1.8[b]	80.3±2.2[b]
NDEA+ fisetin	0.6±1.6[a]	10.3±2.2[a]	89.5±3.4[a]
fisetin only	0.7±0.4[a]	9.5±2.1[a]	95.5±2.5[a]
Taste chemotaxis			
vg(control)	60.5±7.6[a]	40.4±4.3[a]	30.4±3.7[a]

NDEA treated	40.2±4.8[b]	25.1±5.5[b]	20.3±6.1[b]
NDEA+ fisetin	57.2±6.8[a]	38.7±3.9[a]	28.5±3.5[a]
fisetin only	59.5±9.1[a]	39.5±4.0[a]	30.0±3.4[a]
Thermotaxis			
vg(control)	60.8±8.2[a]	33.4±1.0[a]	2.8±0.2[a]
NDEA treated	40.6±6.5[b]	20.9±4.1[b]	18.0±1.9[b]
NDEA+ fisetin	57.1±7.2[a]	29.3±0.9[a]	21.5±0.2[a]
fisetin only	60.2±6.2[a]	32.2±0.8[a]	2.6±0.2[a]
Hygrotaxis			
vg(control)	70.3±8.3[a]	34.6±6.7[a]	10.3±3.5[a]
NDEA treated	55.5±5.7[b]	22.3±5.2[b]	6.8±2.1[b]
NDEA+ fisetin	69.5±7.1[a]	30.7±5.3[a]	9.5±2.8[a]
fisetin only	69.8±8.2[a]	32.6±5.9[a]	9.8±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 concentrations of lipid peroxides, protein carbonyl, TBARS, protein thiol were mainly augmented in hemolymph noticeably ($p<0.05$) while these results were notably diminished in intestine and head tissues in group 2 (NDEA administered) *vg* flies as analysed with group 1 flies (Table 2). In NDEA+ fisetin administered group concentrations were diminished in hemolymph and augmented in both head and intestine analysed with group 2 *vg* flies. Fisetin administered *vg* flies exhibited comparatively similar values to control flies. The level of GSH, GPx, GST, CAT and SOD were commonly reduced in hemolymph appreciably ($p<0.05$). While these concentrations were noticeably augmented in intestine and head tissues in group 2 (NDEA administered) *vg* flies as compared to group 1 flies (Table 2). In group 3 (NDEA+ fisetin) values were augmented in hemolymph in addition to tissues (intestine and head) analysed with group 2 *vg* flies. Group 4 flies (fisetin administered) showed comparatively similar value to group 1 *vg* 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. $p\leq0.05$ were compared with all groups. Abbreviation: *Vestigial* (*vg*), N-nitrosodiethylamine (NDEA).

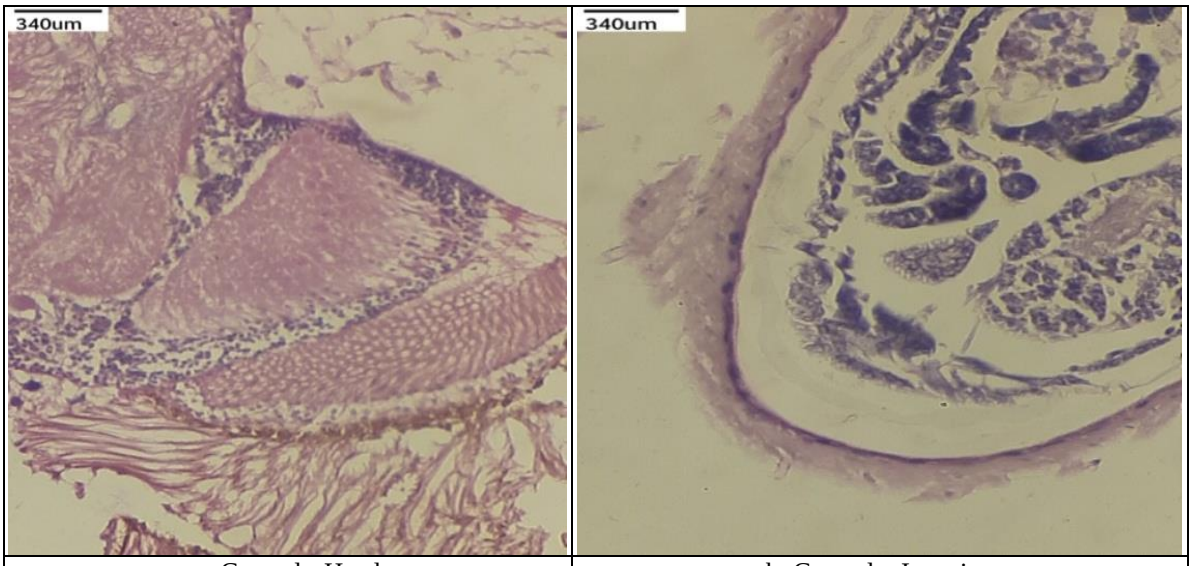
S. No	Biochemical Parameters	Groups	Hemolymph	Head	Intestine
Redox hemostasis					
1	Protein carbonyl (nmole/mg protein)	*Vg*(Control)	7.5±2.04[a]	4.2±0.94[a]	3.2±0.07[a]
		NDEA treated	12.3±1.24[b]	3.3±0.31[b]	2.1±0.11[b]
		NDEA + fisetin	8.1±0.10[a]	5.4±0.42[a]	3.2±0.13[a]
		fisetin only	7.4±2.03[a]	4.0±0.92[a]	3.0±0.09[a]
2	Thiobarbituric acid reactive substances (TBARS)(nmole/mg protein)	*Vg*(Control)	22.7±4.45[a]	15.4±2.20[a]	11.2±2.34[a]
		NDEA treated	31.9±4.22[b]	10.3±0.71[b]	8.9±1.05[b]
		NDEA +Fisetin	28.1±3.01[a]	11.6±2.04[a]	10.5±1.75[a]
		fisetin only	22.9±4.43[a]	15.3±2.22[a]	11.1±2.30[a]
3	Protein thiol(mmol/mg protine)	*Vg*(Control)	43.9±7.35[a]	32.4±3.71[a]	30.5±2.85[a]
		NDEA treated	54.1±5.10[b]	24.2±2.65[b]	21.8±1.33[b]
		NDEA +Fisetin	43.5±5.08[a]	35.1±3.79[a]	31.5±3.11[a]
		fisetin only	43.7±4.2[a]	32.4±3.21[a]	30.4±2.311[a]
4	Lipid peroxides (nmole/mg lipid)	*Vg*(Control)	56.6±6.37[a]	37.9±4.76[a]	25.7±3.73[a]
		NDEA treated	78.6±8.06[b]	26.8±3.10[b]	18.8±2.09[b]
		NDEA +fisetin	63.3±6.29[a]	32.1±3.48[a]	24.3±2.85[a]
		fisetin only	56.8±6.23[b]	37.6±4.74[b]	25.3±3.72[b]
5	Superoxide dismutase (SOD) (Unit[a]nmole/mg protein)	*Vg*(Control)	54.1±4.03[a]	37.8±1.11[a]	26.3±1.14[a]
		NDEA treated	49.6±2.23[b]	45.5±1.89[b]	37.3±1.54[b]
		NDEA + fisetin	65.4±3.15[a]	55.5±1.98[a]	47.2±2.10[a]
		fisetin only	54.4±3.33[a]	37.8±1.12[a]	26.2±1.20[a]
6	Catalase(CAT)(Uint[b]/min/mg Protein)	*Vg*(Control)	197.3±3.59[a]	160.3±12.59[a]	120.7±9.22[a]
		NDEA treated	182.1±10.86[b]	175.3±9.67[b]	140.3±5.74[b]
		NDEA + fisetin	189.8±17.17[a]	182.3±10.51[a]	158.8±7.11[a]
		Fisetin only	196.9±3.60[a]	159.3±11.54[a]	121.7±10.01[a]
7	Glutothion-S-transferase(Uint/100mg/ Protein)	*Vg* (Control)	48.6±2.15[a]	33.5±1.68[a]	26.5±1.53[a]
		NDEA treated	40.5±1.07b	35.5±1.92b	28.3±1.84b
		NDEA + fisetin	55.2±1.95[a]	47.4±1.97[a]	38.6±1.99[a]

		fisetin only	48.6±2.12 [a]	33.3±1.88 [a]	26.3±1.87 [a]
8	Glutothioneperoxidase(GPx) (Unit[c] per mg Protein)	*Vg* (Control)	88.8±4.32 [a]	55.6±2.75 [a]	45.6±1.44 [a]
		NDEA treated	67.1±3.12 [b]	63.6±2.84 [b]	54.1±1.52 [b]
		NDEA + fisetin	78.0±3.21 [a]	74.4±2.87 [a]	60.0±1.78 [a]
		fisetin only	88.7±4.22 [a]	55.5±2.85 [a]	45.0±1.74 [a]
9	Reduced glutathione (GSH)	*Vg*(Control)	95.6±8.14 [a] (µl/ml)	65.4±6.11 [a] (µl/mg tissue)	58.5±1.54 [a] (µl/mg tissue)
		NDEA treated	85.3±3.04 [b]	73.4±5.91 [b]	68.1±2.45 [b]
		NDEA + fisetin	89.3±6.21 [a]	79.7±6.12 [a]	75.7±0.91 [a]
		fisetin only	96.9±8.16 [a] (µl/ml)	65.4±6.19 [a] (µl/mg tissue)	58.0±2.14 [a] (µl/mg tissue)

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

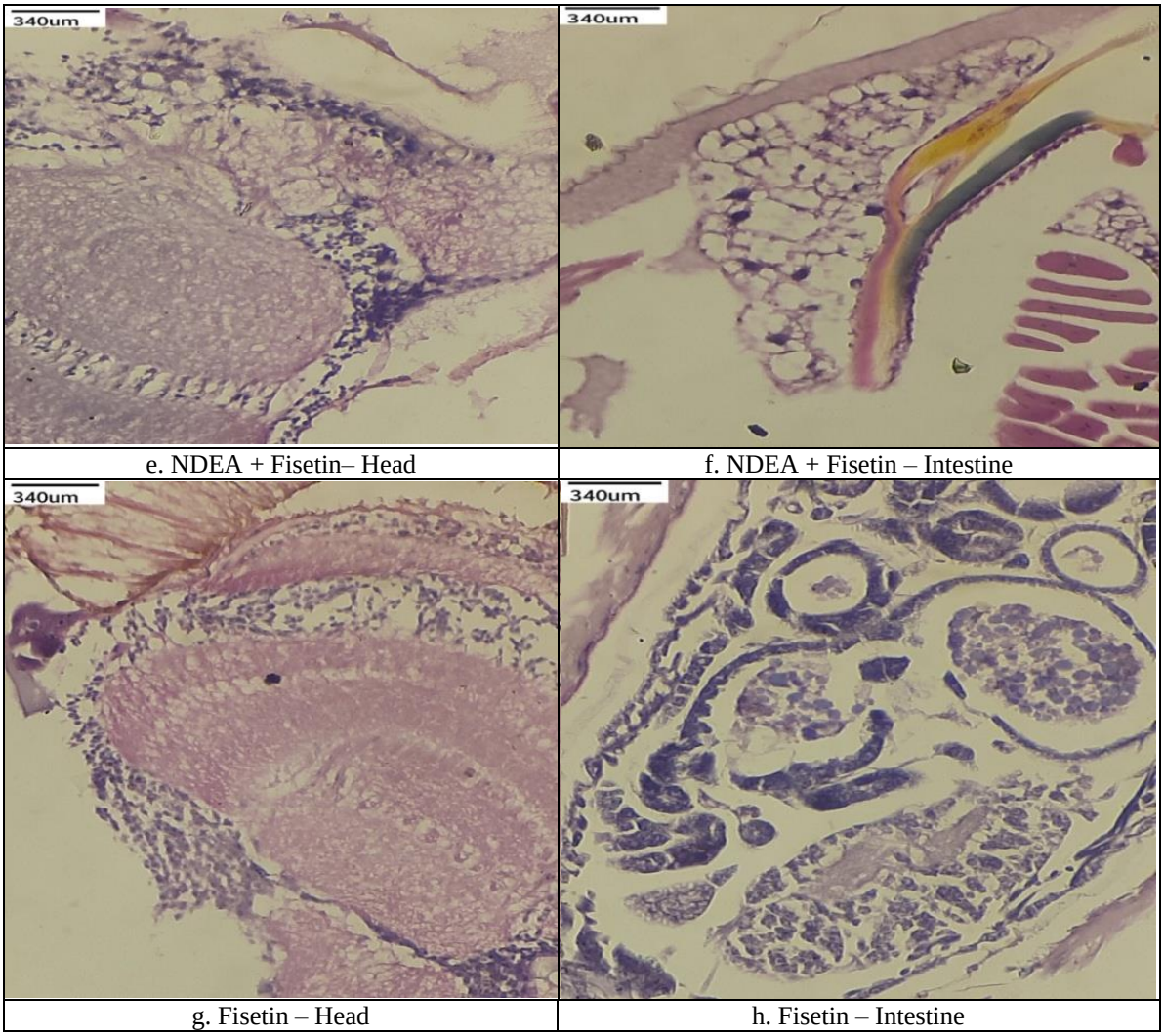
Histopathological change

The histopathological examinations in figure 2 basically exhibit the results obtained from head and intestine of *vg* flies. Fig.2a and b (Head and intestine) shows the normal cellular architecture (group 1) and granulated cytoplasm, small and uniform sized nuclei and nucleoli. Group 2 (NDEA alone) flies tissues (Figure 2c and d) exhibited disorganized architecture and tumour cells are smaller than normal cells, large hyperchromatic nuclei. Tumour Island containing 'Keratin pearl' in its centre, stratified epithilia migrating tumour cells with dysregulated proliferation, hyperplasia. Especially in brain neoplastic overgrowth tissues changes occur in brain, tubule shaped structure encircling lumen. Group III tissues (NDEA + fisetin) showed less architecture, comparative less tendency to spread (Figure 2e and f). Group 4 showed the tissue proliferation is nearly to control (Figure 2g and h).



a. Control - Head | b. Control – Intestine

c. NDEA - Head | d. NDEA – Intestine



e. NDEA + Fisetin– Head	f. NDEA + Fisetin – Intestine
g. Fisetin – Head	h. Fisetin – Intestine

Fig 2

Discussion

The mutant (*vg*) phenotype, when analysed with the winged one, exhibits a significant drawback for its less aggressive capacity. It is elevated through following generations when below condition of strained cohabitation, while it tends to move about in situations where a positive level of isolation is authorized. For these reason*vg* indicate a helpful device to differentiate the cognitive factor connected with thecourtship and mating patterns that are accountable for aggregation or isolation phenomenon[38].

The *vestigial* mutant exhibits abnormally decreased wings, is disadvantageous related to the wild phenotype in flight, and therefore, the strain has a cognitive and biochemically significant effect. In this study, mutant flies (*vg*) administered with NDEA that gradually increases their oxidative pressure through carcinogenesis than wild type due to selector gene present in second chromosome. Consecutively this could prevent common negative behaviourand to shorten sleep duration[39]. Nevertheless, to the authors' awareness this is the first published description oftumorigenesis in mutant (*vg*) flies by NDEA treatment and also on the effects of the drug,fisetin. The functional, molecular and signalling mechanical deviation in behavioural codes should be explored. However, standardisation of ROS range and prevention of cancer in physiotherapy may standardise the behaviour of flies.

Our outcome evidently recommended that, the behavioural action (phototaxis, negative geotaxis, taste chemotaxis, smellchemotaxis, hygrotaxis and thermotaxis) of *vestigial* flies during tumorigenesis are cleary changed. Faults in cognition are described usually in a wide assortment of cancers[40]. In addition, our results provide indications that cognitive behaviour alterations are resulted owing to the carcinogenicity of *vestigial* flies. Our reports indicated that, fisetinmay reverse the adverse stuff on NDEA and consequently normalize the behaviour of flies.Fisetin, a biologically active flavonoid has been recognized broadly prevent to the propagation of different types of cancer in animals [41] and in different kinds of cancer cell lines. The anticancer property of fisetin might be moderated by regulating various signalling pathways in various configurations [42].

Our results also corroborate theprevious findings in mammalsthat indicate an increase in TBARS range in NDEA treated flies. This may be due to extreme protection of ROS and the initial occurrence associated with hypoxia. This amount is reduced in fisetin treated *vg* flies due to showing of triplet hydroxyl groups in fisetin, which contain a great ROS quenching action [43]. To avoid cellular destruction influenced by ROS, *D.*

melanogaster has numerous antioxidant production systems.The antioxidant defence system might scavenge ROS and plays an important function in preventing peroxidation of lipid and then, participate in defensive role in carcinogenic development[44]. SOD and CAT are equivalent protection enzymes in opposition to ROS. This protective mechanism works via enzymatic (including CAT, GPx, SOD and GST), and non-enzymatic compounds [45]. Non-enzymatic and enzymatic antioxidants range was reduced in NDEA induced mutant flies.

The levels of lipid hydroperoxides and TBARS in hemolymph and tissues (brain and intestine) detected in this investigation may be due to NDEA treaded free cell lysis, free radical production, and membrane damage; in addition, suppression of lipid peroxidation in fisetin treated *vg* flies owing to enhancement of antioxidant activity[46].The antioxidant properties of polyphenolic agents can protect fly's defences against NDEA regulated free radical destructions. Thedegree of hydroxylation, chemical structure and position are the vital factors in revealing thepharmacological, physiological and biologicalfeatures of flavonoids. Significant increase in GSH range in fisetin treatment flies indicates the capability of fisetin to maintain GSH range by inhibiting glutamate toxicity and elevating cystine (GSH precursor and main provider ofthiol group) aborption into brain through its free radical quenching and cytoprotective behaviour.The most reliable method by which fisetin exerts its defensive influences may be by maintaining its free radical quenching function, and cellular integrity of the cells within the *Drosophila melanogaster*.

The quantity ofprotein thiols, TBARS, lipid peroxides and protein carbonyl (the product of extreme oxidative stress) was high in hemolymph (p>0.01) even while they are noticeably lower in head and intestine of variant flies.Controlling the level of reactive oxygen species (ROS) is ankey feature in carcinogenesis because esteemed levels can harm ROS cells.Tumour cells therefore reveal a method of performance similar to peroxide exploring system to managing the equilibrium of ROS to detect cells proliferation. It has also been previously reported that quickly separating cancer cells in tissues (intestine and head)use top level of ROS [47]. As ROS level reduced by tumour cells, this may be owing to a reduction in final solutions of oxidative stress in those tissues in this research. In disparity, the over-mentioned index (TBARS, proteinthiol, lipid peroxide, and protein carbonyl) might be due to an increase in the hemolymph of *Drosophila*, due to the total cancer weight in their structure. There is also dependable decrease in antioxidants – SOD, GSH, GPx, GST and CAT in tissues and hemolymph; this may be caused by the quick use of antioxidants in the tumour-applicability host[48]. As before, fisetin is recognised for its antioxidant activity of reactive nitrogen and reactive oxygen species and, ultimately, the stimulation of antioxidant enzymes whilst inhibiting the action of antioxidant enzymes[49]. The treated dosage of fisetin may contain relieved the antioxidant pressure, thus, contrary the pro-oxidative activity of fly indicating an important upregulation in top indices of the redox homeostasis.In exacting, fundamentals decrease in lipid peroxide in the hemolymph of fisetin-treated flies mentioned a decrement in lipid peroxide of lipids. Same examination has been documented on the antioxidant activity of fisetin in opposition to antioxidant pressure[50].

The degree and position of hydroxylation and chemical structure are the important reasons in activating the pharmacological and biological characters of fisetin.High hydrophobic nature and low molecular weight of fisetin allow it to easily migrate through cell walls and accumulate within the cell, protecting the intestine and brain tissues of cancer causing flies.Major reduction in the action of GPx,SOD and catalase in malignant flies may be due to the existence of triplet hydroxyl groups infiestin which containa potent ROS quenching function. Maximum enzymes of the redox pathway in malignant flies have been conformed, (including GPx, catalase and SOD)[47]. Significant increasein GSH rangein fisetin induced flies indicates the ability of fisetin to amplifying the amount of GSH in brain tissue by upgrading cystine(GSH precursssor) absorption into brain tissues and its direct protection against NDEA activity by itscytoprotective and free radical scavenging effects.

Conclusion

In the present cognitive and biochemical studies indicated that, the high hydrophobic nature and low molecular weight of fisetin support to guard most liableintestine and brain tissue of tumor flies. This above activates was gradually decrease in mutant (*vg*) individuals than the winged (WT) individuals.

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Conflict of interest

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