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Original Article

DOSE-DEPENDENT INFLUENCES OF VANILLIC ACID ON COGNITIVE FUNCTION AND REDOX HOMEOSTASIS STATUS IN N-NITROSODIETHYLAMINE TREATED *DROSOPHILA MELANOGASTER*

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ABSTRACT

Objective: To assess the dose-dependent effect of vanillic acid on cognitive behavior and indices of redox homeostasis in the fruit fly, *Drosophila melanogaster*.

Methods: In this study, flies were divided into five groups-group 1 control, group 2-treated with 0.01% NDEA through drinking water for 15 w, group 3-treated with NDEA and vanillic acid (VA) (0.005%) in culture medium, group 4-treated with NDEA and VA (0.01%) and group 5-treated with NDEA and VA (0.05%). Cognitive-behavioral assays and assessment of redox homeostasis indices were performed.

Results: Behavioural abnormalities (negative geotaxis, phototaxis, smell and taste chemotaxis, hypotaxis and thermotaxis) were quantitatively deviated in NDEA treated flies compared to control but were tend to be normalized in VA treated flies. 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 VA (0.01%) 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 VA treated (0.01%) groups.

Conclusion: Vanillic acid, a bioactive phytochemical could act as a potent antioxidant and as well exhibit antiproliferative characteristics. The dose lower than 0.01% could not be effective as the dose is low. Nevertheless, at 0.01% the maximum benefits could have been achieved, and beyond this saturation point, higher doses, such as 0.05% could not be effective.

Keywords: Cancer, *Drosophila melanogaster*, Vanillic acid, Behavioural assays, Free radical, Dose dependency

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INTRODUCTION

Cancer is a multistep disease determined by the activation of precise oncogenic pathways concurrently with the loss of activity of tumour suppressor genes that regulate cell growth and division [1]. Most of the signaling 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 availability of main recombination techniques enable precise characterization of the key functions of conserved oncogenes and tumour suppressor genes in *D. melanogaster* [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 unattainable currently. It is in this context; more studies are necessary.

The fruit fly, *Drosophila melanogaster,* is frequently as a model organism to study research areas 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 exhibit 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 [1]. Reasonable investigations between the fly and human genomes have recognized robust evolutionary conservation in between *Drosophila* to mammals at sequence and pathway levels [5]. Flies respond behaviourally to numerous stimuli in an environment for instance, 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, reliant of the nature and strength of the stimulus [7]. Further, tumorigenesis is known to cause neurochemical, endocrine, immune and behavioural modulations signifying stress and immune impairment in rodent model systems [8]. Rodents and humans bearing tumor are known to suffer from cognitive disturbances. Few studies already indicated that oxidative stress could induce abnormalities in behaviour [9, 10]. However, a systematic investigation on cognitive behavioural functions is lacking in an important experimental model system, viz. *D. melanogaster*. Vanillic acid (VA), a phenolic antioxidant has been evaluated for its protective influences. In addition, indices of redox homeostasis have also been investigated in the present study.

N-nitrosodiethylamine (NDEA) belongs to the nitrosamine family and is well established as an effective carcinogen [11] and it could promote tumour primarily in the liver and in several organs of numerous animal model systems, [12]. This carcinogen is found in a wide range of foods such as soya beans, fish (smoked, dried and salted) cheese, meat and alcoholic beverages [13]. NDEA is also found in cigarette smoke, buns, rolls, muffins, ham and oysters [14]. NDEA is known to cause oxidative and cellular damages by promoting the synthesis of free radicals [15]. The metabolic conversion of NDEA by cytochrome P450 enzymes leads to the formation of ethyl-acetoxyethyl-nitrosamine, which is further conjugated by the phase II enzymes [16] to a non-toxic compound. This activation of NDEA by P450-catalyzed-hydroxylation is known

to produce unstable metabolites that could alkylate the DNA and therefore cause tumour formation [17].

Oxidative stress is a key step involved in almost all aspects of cancer, from carcinogenesis to the tumor-bearing state and from treatment to prevention [18]. Many reactive oxygen species (ROS) defence systems have evolved in organisms to control intracellular and extracellular ROS levels The *Drosophila* ROS defence system comprises of several subsystems consisting of enzymatic and non-enzymatic antioxidants. Cancer and oxidative stress form a vicious cycle; when oxidative stress surpasses the capacity 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 [19]. The tumor-bearing state is under oxidative stress tightly linked with active oxygen synthesis by tumour cells and irregular oxidation-reduction regulation [20]. Though tumour bearing tissues bear reduced free radical load due to uncontrolled and higher number of cell division, the oxidative stress is elevated [21] 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 [22]. Vanillic acid (4-hydroxy-3-methoxybenzoic acid) (VA) is an oxidized form of vanillin. The highest concentration of VA in plants thus far is noticed in the root of *Angelica sinensis* (female ginseng), an herb native to China, which is used in traditional Chinese medicine. Recently, lot of research has been performed on VA owing to its antiproliferative cytoprotective, [23], apoptotic [24], neuroprotective, antidiabetic and antioxidant [25, 26] properties.

VA contains two hydroxyl groups. It is documented that the-OH groups are involved in the reactions with free radicals, the mechanisms of action of VA are not clearly understood [26]. 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 an imbalance between the production of the reactive oxygen species and the ability to defend against them cellular damage and harmful events initiate [27]. VA is documented as a protective agent, againis hyperinsulinemia, hyperglygemica, hyperlipidemia, hepatic insulin resistance and inflammation [28]. Further, VA is known to prevent uncontrolled human lymphocyte proliferation [29], chronic liver injury [30] and chemically induced oral carcinogenesis [31]. In a study, it is reported that VA has the capacity to prevent benzopyrene induced lung tumorigenesis [32]. VA could positively control diverse essentially by preventing the free radical-induced cellular damages and by exhibiting cytoprotective action [32].

Drosophila has been employed for nearly a decade to investigate cognition and intellectual disability, which has provided a significant amount of disease-relevant information [33]. 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 [34]. In *D. melanogaster* age-associated impairment in cognitive functions has also been documented [35].

There are numerous studies showing the dose-dependent effect of various drugs [36, 37]. Dose-dependent drug reactions occur because of biological variability. For a variety of reasons, including heredity, coexisting diseases, and age, different individuals can require different doses of a drug to produce the same therapeutic effect. Hence, there is a necessity to decide an optimum dose of VA in experimental model systems. In this context, the present study has been carried out. In addition, as the behavioral abnormality and oxidative stress indices during carcinogenesis/treatment with vanillic acid 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 Centre 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]. Flies were divided into five groups-group 1 control, group 2-treated with 0.01% NDEA through drinking water for 15 w, group 3-treated with NDEA and vanillic acid (VA) (0.005%) in culture medium, group 4-treated with NDEA and VA (0.01%) and group 5-treated with NDEA and VA (0.05%). 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 VA were administrated in food medium for 21 d.

Collection of haemolymph and tissue homogenate

Suitable holes in a 0.5 ml Eppendorf tube were made and placed into 1.5 ml Eppendorf 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 haemolymph 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 established methods with minor modifications [40, 41].

Negative geotaxis

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 were expressed as percent flies crossed beyond the distance of 13 cm in 60 s of an interval [10, 42]. Each assay was repeated for all the four groups of flies and mean±SD was calculated (fig. 1a).

Phototaxis

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 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 four groups and mean±SD was calculated (fig. 1b).

Smell chemotaxis

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 a percentage. The test was repeated for separate three sets of flies (fig. 1c).

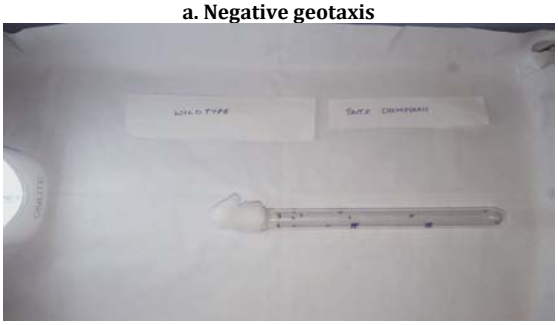
Taste chemotaxis

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

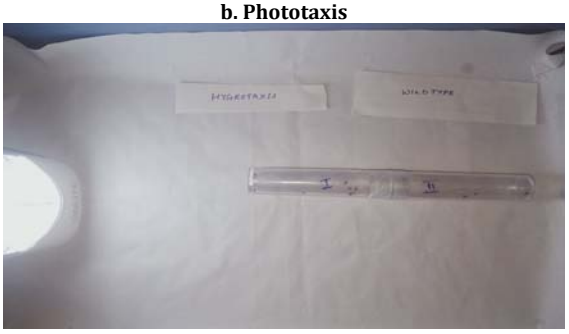
counted and the result was expressed as a percentage. The test was repeated for separate three sets of flies (fig. 1d).

a. Negative geotaxis

b. Phototaxis

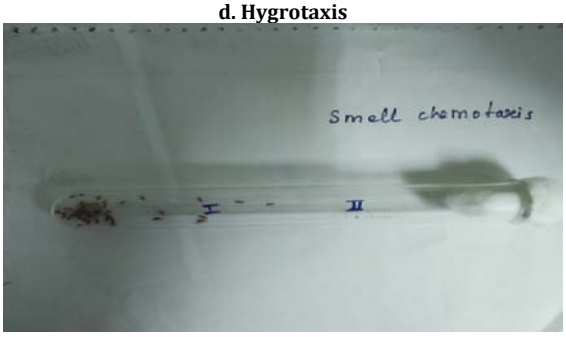


c. Taste chemotaxis



d. Hygrotaxis

e. Thermotaxis



f. Smell chemotaxis

Fig. 1: Behavioural assays in *D. melanogaster*. Negative geotaxis (a), phototaxis (b), smell chemotaxis (c), taste chemotaxis (d), thermotaxis (e) and hygrotaxis (f)

Thermotaxis

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

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 the percentage of total flies present. The test was repeated for separate three sets of flies and mean±SD was calculated (fig. 1f).

Biochemical parameters

Indices of redox homeostasis

The protein carbonyl content was assayed [43]. The sample (haemolymph/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. The levels of TBARS in haemolymph/tissue homogenate were estimated [44]. Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid (TBA) in acidic condition to produce a pink coloured chromophore, which was read at 530 nm**.**

Assay of free protein thiol groups is carried out by derivatization with 5,5′dithiobis (2-nitrobenzoic acid) (DTNB) [45]. 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 sulphate (SDS) [45]. 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 [46].

Glutathione-S-transferase (GST) was assayed in hemolymph/tissue homogenate by an increase in absorbance at 350 nm using CDNB as substrate [47]. 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 in hemolymph/tissue homogenate was measured [48]. 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.

The activity of catalase in haemolymph/tissue homogenate was measured [49]. To 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate/haemolymph 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 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. The activity of GPx in haemolymph/tissue homogenate was assayed [50]. To 0.2 ml of tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide, 0.5 ml of tissue homogenate/haemolymph 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/haemolymph. 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 [45]. The activity was expressed as µmol of GSH consumed/min/mg of protein. The amount of glutathione was expressed as mg/dl haemolymph and mg/100g tissue.

RESULTS

Behavioural assays

The negative geotaxis value is decreased significantly after NDEA treated compared to control flies ($p<0.05$) (table 1). In NDEA+VA (0.01%) treated group the value is increased to the maximum extent compared to NDEA treated group (table 1). Other VA treated groups insignificant and lesser extend of changes as compared to NDEA group. More percentage of flies tends to move closer to the light source (phototaxis) (compartment I, table 2). However, this response was noticeably decreased ($p<0.05$) in group 2 (compartment I). NDEA+VA (0.01%) group showed a higher percentage of flies ($p<0.05$) compared to other VA treated groups.

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 2). Significantly augmented movement was noticed in VA (0.01%)+NDEA treated flies to compartment III ($p>0.05$) as compared to VA treated (0.005% and 0.05%). Higher percentage of control flies were found to move nearer to cotton-plug soaked with sucrose solution (compartment I) compared to movement of NDEA treated flies towards compartment I ($p<0.05$). The response of NDEA+VA (0.01%) treated flies is closer to movement of control flies ($p>0.05$, table 2) than the flies.

Table 1: Negative geotaxis in *D. melanogaster*, percentage of flies in control, NDEA treated, NDEA+vanillic acid treated (0.005%, 0.01% and 0.05%) shown. Values were mean±SD of triplicate experiments (n=30 in each group and in each triplicate)

0.005%		
Negative geotaxis (%±SD) (WT-Control)	NDEA Treated	NDEA+Vanillic acid
91.2±14.8[a]	80.4±15.7[b]	81.8±13.6[a]
0.01%		
Negative geotaxis (%±SD) (WT-Control)	NDEA treated	NDEA+Vanillic acid
91.2±14.8[a]	82.4±11.7[b]	90.8±12.4[c]
0.05%		
Negative geotaxis (%±SD) (WT-Control)	NDEA treated	NDEA+Vanillic acid
92.2±13.9[a]	79.4±12.7[b]	89.9±11.4[a]

Values are not sharing a common superscript alphabet (in a specific assay at a particular compartment) vary significantly at $p<0.05$ by Duncans Multiple Range Test (DMRT)

Table 2: Phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis and hygrotaxis in *D. melanogaster*. Percentage of flies in control, NDEA treated, NDEA+vanillic acid-treated (0.005%, 0.01% and 0.05%) shown. Values were mean±SD of triplicate experiments (n=30 in each group and in each triplicate)

	Behavioral assay								
0.005%				**0.01%**			**0.05%**		
	Compartment I mean±SD	**Compartment II mean±SD**	**Compartment III mean±SD**	**Compartment I mean±SD**	**Compartment II mean±SD**	**Compartment III mean±SD**	**Compartment I mean±SD**	**Compartment II mean±SD**	**Compartment III mean±SD**
Phototaxis									
WT (control)	91.2±3.2[a]	26.6±4.1[a]	8.8±1.8[a]	90.2±3.2[a]	24.6±4.1[a]	7.8±1.8[a]	91.2±3.2[a]	25.6±4.1[a]	9.8±1.8[a]
NDEA treated	86.3±4.9[b]	37.3±3.2[b]	18.2±2.9[b]	76.3±4.9[b]	28.3±3.2[b]	11.2±2.8[b]	56.3±5.9[b]	18.3±5.2[b]	9.2±2.9[b]
NDEA+Vanillic acid	89.1±3.6[a]	38.4±3.6[a]	16.9±2.1[a]	84.1±3.6[a]	28.4±3.6[a]	8.9±2.1[a]	74.1±3.6[a]	22.4±3.6[a]	7.9±2.1[a]
Smell chemotaxis									
WT (control)	1.8±0.9[a]	18.5±4.3[a]	95.5±3.5[a]	2.8±0.8[a]	14.5±4.3[a]	92.5±3.5[a]	1.6±0.4[a]	16.5±6.3[a]	96.5±8.5[a]
NDEA treated	15.4±2.3[b]	37.6±3.4[b]	88.3±4.2[b]	13.4±2.3[b]	27.6±3.8[b]	68.3±4.2[b]	11.4±2.7[b]	25.6±2.8[b]	60.3±4.1[b]
NDEA+Vanillic acid	3.9±1.6[a]	26.8±3.3[a]	89.5±4.1[a]	3.5±1.6[a]	16.8±3.3[a]	88.5±4.7[a]	2.4±1.6[a]	14.8±3.3[a]	81.5±4.7[a]
Taste chemotaxis									
WT (control)	87.5±8.6[a]	15.4±5.3[a]	9.4±4.7[a]	89.5±9.6[a]	19.4±5.3[a]	10.4±4.7[a]	92.5±9.6[a]	21.4±6.3[a]	9.4±4.7[a]
NDEA treated	80.2±7.8[b]	25.1±5.3[b]	22.3±5.1[b]	64.2±7.8[b]	26.1±5.5[b]	16.3±6.1[b]	61.2±3.6[b]	22.1±6.7[b]	12.3±5.1[b]
NDEA+Vanillic acid	79.2±6.2[a]	19.7±4.4[a]	13.5±4.2[a]	86.2±6.8[a]	21.7±4.4[a]	13.5±5.2[a]	89.2±4.8[a]	27.7±4.4[a]	15.5±5.2[a]
Thermotaxis									
WT (control)	80.9±7.4[a]	7.4±1.1[a]	1.2±0.6[a]	76.9±7.4[a]	7.4±1.1[a]	1.2±0.6[a]	86.9±7.4[a]	7.4±1.1[a]	2.2±0.6[a]
NDEA treated	79.6±6.5[b]	18.9±4.1[b]	7.5±2.7[b]	62.6±6.5[b]	20.9±4.1[b]	8.5±2.9[b]	58.6±6.5[b]	19.9±4.1[b]	6.5±2.9[b]
NDEA+Vanillic acid	78.1±6.2[a]	7.3±0.9[a]	1.4±0.8[a]	79.1±6.2[a]	6.3±0.9[a]	1.6±0.8[a]	69.1±7.2[a]	6.5±0.9[a]	1.2±0.8[a]
Hygrotaxis									
WT (control)	91.1±8.4[a]	20.6±6.8[a]	6.3±2.6[a]	93.1±8.4[a]	21.6±6.8[a]	7.3±2.3[a]	91.1±8.4[a]	19.6±6.8[a]	7.1±2.3[a]
NDEA treated	88.5±6.7[b]	24.3±542[b]	8.8±2.1[b]	70.5±6.7[b]	26.3±5.2[b]	9.8±2.1[b]	60.5±6.7[b]	19.3±5.2[b]	7.6±2.1[b]

NDEA+Vanillic acid	87.4±5.1[a]	20.7±5.2[a]	5.5±1.9[a]	87.6±7.1[a]	22.7±5.4[a]	6.5±1.8[a]	78.6±7.1[a]	19.7±5.4[a]	5.4±1.8[a]

Values are not sharing a common superscript alphabet (in a specific assay at a particular compartment) vary significantly at p<0.05 by Duncans Multiple Range Test (DMRT)

Table 3: Experimental values of protein carbonyl, thiobarbituric acid reactive substances, protein thiol, lipid peroxides, superoxide dismutase, catalase, glutothione-S-transferase, glutathione peroxidise and reduced glutathione. Percentage of flies in control, NDEA treated, NDEA+vanillic acid-treated (0.005%, 0.01% and 0.05%) are shown. Values were mean±SD of triplicate experiments (n=30 in each group and in each triplicate)

Biochemical Parameters	Groups	Hemolymph (0.005%)	Head	Intestine	Hemolymph (0.01%)	Head	Intestine	Hemolymph (0.05%)	Head	Intestine
	WT	2.5±0.09[a]	2.9±0.86[a]	1.3±0.09[a]	5.7±1,03[a]	3.9±0.86[a]	2.3±0.09[a]	2.5±0.09[a]	2.9±0.86[a]	1.3±0.09[a]
Protein carbonyl	NDEA treated	6.7±2.35[b]	1.7±0.51[b]	0.9±0.05[b]	8.7±2.35[b]	2.7±0.51[b]	1.6±0.07[b]	2.5±0.09[a]	2.9±0.86[a]	1.3±0.09[a]
nmole/mg protein	NDEA+Vanillic acid	2.5±0.08[a]	2.1±0.75[a]	0.8±0.04[a]	5.3±0.08[a]	3.1±0.65[a]	1.5±0.05[a]	6.7±2.35[b]	1.7±0.51[b]	0.9±0.05[b]
Thiobarbituric	WT	8.8±1.53[a]	6.9±1.30[a]	4.2±1.84[a]	12.8±2.63[a]	8.4±1.30[a]	7.2±1.74[a]	2.8±0.08[a]	2.0±0.95[a]	1.2±0.07[a]
acid reactive substances	NDEA treated	11.1±3.87[b]	4.4±0.91[b]	2.9±1.14[b]	17.8±3.67[b]	5.4±0.81[b]	4.9±1.14[b]	8.8±1.53[a]	6.9±1.30[a]	4.2±1.84[a]
(TBARS)(nmole /mg protein)	NDEA+Vanillic acid	8.4±1.01[a]	6.8±1.21[a]	3.5±1.04[a]	12.1±2.01[a]	7.8±1.21[a]	6.5±1.24[a]	11.1±3.87[b]	4.4±0.91[b]	2.9±1.14[b]
Superoxide	WT	9.1±2.08[a]	6.8±1.21[a]	4.5±1.34[a]	12.1±2.01[a]	7.8±1.21[a]	6.5±1.24[a]	15.5±2.73[a]	12.8±3.12[a]	7.4±1.20[a]
dismutase (SOD) (Unit[a]	NDEA treated	8.7±2.08[b]	9.5±2.79[b]	6.3±1.04[b]	11.7±2.18[b]	10.5±2.89[b]	8.3±1.14[b]	9.1±2.08[a]	6.8±1.21[a]	4.5±1.34[a]
nmole/mg protein)	NDEA+Vanillic acid	10.4±2.15[a]	10.2±2.48[a]	7.4±2.10[a]	19.4±3.15[a]	13.4±2.58[a]	9.4±2.10[a]	8.7±2.08[b]	9.5±2.79[b]	6.3±1.04[b]
Catalase	WT	115.3±2.37[a]	110.3±13.59[a]	101.7±8.08[a]	165.3±2.57[a]	140.3±13.59[a]	110.7±10.22[a]	16.4±3.03[a]	10.1±1.52[a]	9.4±1.10[a]
(CAT)(Uint[b]/min/mg p	NDEA treated	101.1±10.16[b]	106.3±8.67[b]	79.3±5.74[b]	133.1±11.86[b]	112.3±9.67[b]	89.3±5.74[b]	115.3±2.37[a]	110.3±13.59[a]	101.7±8.08[a]
	NDEA+Vanillic acid	113.6±18.27[a]	118.3±10.91[a]	100.8±9.61[a]	163.6±18.17[a]	138.3±10.51[a]	106.8±9.11[a]	101.1±10.16[b]	106.3±8.67[b]	79.3±5.74[b]
Glutothion-S-transferase(Unit/100 mg/protine)	WT	10.6±0.14[a]	7.5±0.91[a]	5.9±0.93[a]	12.6±0.14[a]	8.5±0.71[a]	6.7±0.53[a]	112.1±31.52[a]	123.3±11.64[a]	111.7±11.33[a]
	NDEA treated	6.2±0.07b	4.5±0.72b	3.3±0.21b	8.2±0.07b	5.5±0.42b	4.3±0.21b	10.6±0.14[a]	7.5±0.91[a]	5.9±0.93[a]
	NDEA+Vanillic acid	10.0±0.95[a]	6.4±0.64[a]	4.8±0.33[a]	12.0±0.95[a]	7.4±0.84[a]	5.8±0.43[a]	6.2±0.07b	4.5±0.72b	3.3±0.21b
Glutothione peroxidise(GPx)	WT	9.8±5.32[a]	5.9±0.98[a]	4.6±0.94[a]	11.8±4.32[a]	6.5±0.95[a]	5.6±0.84[a]	11.1±01.52[a]	7.3±10.54[a]	5.7±11.61[a]
(Unit[c] per mg protine)	NDEA treated	7.1±1.02[b]	2.3±0.54[b]	2.1±0.31[b]	8.1±1.12[b]	3.6±0.54[b]	3.1±0.41[b]	9.8±5.32[a]	5.9±0.98[a]	4.6±0.94[a]
	NDEA+Vanillic acid	9.0±2.21[a]	4.3±0.88[a]	3.1±0.68[a]	11.0±3.21[a]	5.5±0.87[a]	4.1±0.78[a]	7.1±1.02[b]	2.3±0.54[b]	2.1±0.31[b]
Reduced glutathione (GSH)	WT	12.6±8.94[a] (µl/ml)	9.4±2.31[a] (µl/mg tissue)	8.5±2.54[a] (µl/mg tissue)	16.6±8.14[a] (µl/ml)	10.4±2.11[a] (µl/mg tissue)	9.5±1.54[a] (µl/mg tissue)	9.1±3.42[a]	4.9±0.95[a]	4.1±0.64[a]
	NDEA treated	8.3±3.06[b]	6.4±0.81[b]	4.1±0.25[b]	10.3±3.04[b]	7.4±0.91[b]	6.1±0.45[b]	12.6±8.94[a] (µl/ml)	9.4±2.31[a] (µl/mg tissue)	8.5±2.54[a] (µl/mg tissue)
	NDEA+Vanillic acid	10.3±4.21[a]	8.9±1.52[a]	6.7±0.81[a]	14.3±6.21[a]	9.9±1.32[a]	8.7±0.91[a]	8.3±3.06[b]	6.4±0.81[b]	4.1±0.25[b]
Protine thiol(mmol/mg protine)	WT	29.7±8.21[a]	18.4±4.81[a]	14.8±4.95[a]	38.7±6.21[a]	28.4±4.81[a]	24.8±3.95[a]	11.2±7.96[a] (µl/ml)	10.4±1.99[a] (µl/mg tissue)	7.2±1.94[a] (µl/mg tissue)
	NDEA treated	37.1±3.10[b]	15.2±3.61[b]	10.8±1.53[b]	48.1±3.10[b]	19.2±2.61[b]	15.8±1.33[b]	29.7±8.21[a]	18.4±4.81[a]	14.8±4.95[a]
	NDEA+Vanillic acid	26.5±4.11[a]	17.1±2.19[a]	12.5±3.01[a]	36.5±5.11[a]	27.1±2.89[a]	22.5±3.01[a]	37.1±3.10[b]	15.2±3.61[b]	10.8±1.53[b]
Lipid peroxidise (nmole/mg lipid)	WT	27.6±4.87[a]	18.9±5.13[a]	16.7±2.93[a]	35.6±5.37[a]	27.9±5.13[a]	26.7±3.73[a]	27.7±3.11[a]	13.4±2.61 [a]	12.8±3.91 [a]
	NDEA treated	30.6±3.08[b]	11.8±3.10[b]	10.8±3.09[b]	44.6±4.06[b]	16.8±3.10[b]	16.8±2.09[b]	27.6±4.87[a]	18.9±5.13[a]	16.7±2.93[a]
	NDEA+Vanillic acid	24.3±2.29[a]	17.1±3.48[a]	13.3±1.55[a]	34.3±3.29[a]	26.1±3.48[a]	23.3±2.35[a]	30.6±3.08[b]	11.8±3.10[b]	10.8±3.09[b]

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

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+VA (0.01%) treated flies compared to group 2 flies (p<0.05). At the same time no significantly noticed percentage of VA (other doses) 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+VA (0.01%) treated flies were move to compartment I as compared to NDEA treated flies (p<0.05). Invariably, VA (0.005% and 0.05%) treated flies demonstrated did not exhibit behavioural responses of negative geotaxis, photo, smell and taste chemotaxis, thermotaxis and hygrotaxis similar to the control group (p>0.05, table 2) as effective that of the VA (0.01%) treated flies.

Biochemical assays

The levels of protein carbonyl, TBARS, protein thiols and lipid peroxides were predominantly elevated in haemolymph significantly (p<0.05) whereas these values were significantly decreased in head and intestine tissues in NDEA treated flies as compared to controls

(table 3). In NDEA+VA (0.01%) treated groups the values were decreased in haemolymph and elevated in tissues (head and intestine) compared to NDEA treated flies. Similar trend has not been observed in VA (other doses) treated flies. The levels of SOD, CAT, GST, GPx and GSH were predominantly decreased in haemolymph significantly ($p<0.05$) whereas these values were significantly increased in head and intestine tissues in NDEA treated flies as compared to controls (table 3). In NDEA+VA (0.01%) treated groups the values were increased in haemolymph as well as in tissues (head and intestine) compared to NDEA treated flies. VA treated (0.005% and 0.05%) groups did not exhibit similar significant changes.

DISCUSSION

Flies treated with NDEA tend to develop oxidative stress during tumorigenesis [51]. This, in turn, could inhibit normal negative geotaxis behaviour [52] and to shorten sleep duration [53]. However, possibly, this is the first study showing the improvement of cognitive function in a dose-dependent manner by VA 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 vanillic acid 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 [54]. Our findings also added additional evidences that the cognitive behaviours could have been affected owing to carcinogenesis in flies. Our results also indicted that VA could nullify the harmful effects of NDEA and thus tend to bring back the flies' behaviours to near normal. Vanillic acid (VA), a bioactive flavonoid has been documented widely to inhibit the proliferation of various types of tumors in animals [55] and in various cancer cell lines [23]. The antitumor effects of VA could be mediated by modulating different signalling pathways in diverse frameworks [56].

Our findings are consistent with the report on alleviating oxidative stress by VA in cell lines of *Drosophila* [55] signifying that elevation of TBARS level in NDEA induced flies could be attenuated by vanillic acid. This could be owing to excessive generation of ROS and with an early event associated with hypoxia [57, 58]. This level was decreased in VA treated flies which is owing to the presence of three hydroxyl groups present in VA which have a strong ROS scavenging activity [55]. 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 [57-59]. SOD and CAT comprise an equally protective set of enzymes against ROS ([58, 59]. This defence mechanism functions via enzymatic (including SOD, GPx, GST and CAT), and non-enzymatic components [59]. Enzymatic and non-enzymatic antioxidants levels were decreased in NDEA exposed flies.

The augmented levels of TBARS and lipid hydroperoxides in haemolymph 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 VA (0.01%) treated flies due to enhanced antioxidant activity. The antioxidant nature of the polyphenolic compounds could sustain the fly's defences 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 VA (0.01%) treated flies implies the ability of VA at an optimum dose to sustain GSH level by preventing glutamate toxicity and stimulating cystine (GSH precursor and excellent source for thiol group) uptake into the brain by its free radical scavenging and cytoprotective propeties. The plausible mechanism by which VA (0.01%) 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 haemolymph ($p>0.01$) 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 mechanism of actions such as peroxide scavenging system to maintain the balance of ROS to ascertain cells proliferative state [57]. Furthermore, the rapidly dividing tumour cells in head and intestinal tissues were previously reported to utilise high levels of ROS [60]. 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 haemolymph of flies, due to the overall tumour load in their system. There is also consistent decrement in the levels of antioxidants–SOD, CAT, GST, GPx and GSH in haemolymph and the tissues; this could be due to the rapid utilisation of antioxidants by the tumor-bearing host [61]. Substances with potent antioxidant activity, such as, ascorbic acid is known to prevent hepatocarcinogenesis [62]. As stated earlier, VA 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 [29]. The administered optimum dose of VA (0.01%) 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 haemolymph of VA (0.01%)-treated flies denotes a decrease in lipid peroxidation of lipids. Similar observation has also been reported earlier about the antioxidant activities of VA against oxidative stress [55].

The chemical structure, position and degree of hydroxylation are the key factors to exert the biological and pharmacological properties of VA. The low molecular weight and highly hydrophobic nature of VA 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 presence of three hydroxyl groups present if VA which have a strong ROS scavenging activity. Most of the enzymes of redox pathway in carcinogenic flies are known to be decreased (including SOD, catalase and GPx) [20]. The noticeable augmentation in GSH level in VA (0.01%) treated flies denotes the capability of VA 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.

VA administered at lower dose (0.005%) could not elicit a significant response as the lower dose could lower drug absorption and hence therapeutic response could also be insufficient. On the other hand, VA at a higher concentration (0.05%) could evoke adverse reactions and in addition, the binding of drug with its receptors/components could also reached a saturation point at 0.01% concentration. Our findings clearly indicate 0.01% VA is the optimum dose and this concentration could be used for future investigations in fruit flies.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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