International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 27-09-2022, Accepted: 14-10-2022, Published: 31-10-2022
Volume 7, Issue 10, 2022, Page No. 172-181

Influence of vanillic acid on behavioral and biochemical indices in circadian clock mutant (*cry^b*) of *Drosophila melanogaster* treated with N-nitrosodiethylamine

Akshaya Kani, Perumal Subramanian*
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Chidambaram, Tamil Nadu, India

Abstract
Our experimental *cry^b* (cryptochrome defective) mutant flies were segregated into four groups. Group I - control, group II - treated with 0.01% NDEA via culture medium for 21 days, group III- administered with NDEA, and vanillic acid (0.01%) and group IV - with vanillic acid. Behavioral irregularities in taste chemotaxis, geotaxis, thermotaxis, phototaxis, chemotaxis and hygrotaxis were quantitatively observed in NDEA administered *cry^b* flies but were found to be near normal in vanillic acid administered flies. The levels of protein thiol, protein carbonyl, lipid peroxides and thiobarbituric acid reactive substances (TBARS) were significantly increased in NDEA administered flies and in the same way have a tendency to stabilize to normal level in vanillic acid treated groups; reduced glutathione (GSH), superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), and were diminished in NDEA administered group, and were noticeably elevated in vanillic acid administered groups. Histopathological studies of intestine and brain indicate the tumor in NDEA treated flies and vanillic acid tend to normalize the cellular architecture. Vanillic acid, a biologically active phytoconstituent is known to possess antiproliferative and antioxidant properties and our study indicates that this could prevent the process of carcinogenesis in *cry^b* mutant of *Drosophila melanogaster*.

Keywords: *cry^b*, cognitive function, NDEA; vanillic acid, carcinogenesis, circadian

Introduction

Over nearly a century, *Drosophila* has turned out to be a standing out system to study the molecular basis or diseases of drug action. Nevertheless, the final frame of a fly and a human vary to a great extent [1]. Further, the sequencing (structure) and pathway (functional) levels, comparative investigations of the fly and human genomes have revealed significant evolutionary preservation from insects to mammalian systems [2]. A preliminary relative investigation of the fly proteome indicated that it is greatly similar to the mammalian proteome than to the unicellular or larval proteomes; further, above 60% of around 290 human genes have homologous counterparts in the fly. Human genes have functional homologs for the majority (77%) of *Drosophila* genes. Laboratory-based experimental data utilizing *Drosophila* is valuable to provide information that can be used to extrapolation to higher mammals[3]. In this background, a relatable conclusion is that the fruit flies are similarly susceptible to toxicants as other mammalian systems. Hence, *D. melanogaster* have been comprehensively employed as a useful model system to know the basis of pathophysiology and genetics of circadian rhythms and numerous human diseases such as diabetes, cancer, and neurodegenerative disorders [4].

The circadian clock mutant (cryptochrome deficient *cry^b)* flies have a malfunctioning CRYPTOCHROME (CRY) protein and are faulty to convey the clock's photic input [5]. CRY protein is implicated by synchronization of the biological clock by photo transduction with blue light (420 nm), and it is also involved in the circadian timing core oscillator loop [6]. The blue light-sensitive protein CRY in *Drosophila* transmits photic cues to biological clock [5]. *Cry^b*, a powerful hypomorphic mutation causes an abnormal lack of light input, while flies over expressing *cry* demonstrate greater receptivity to photic cues [7]. This shows that CRY affects the photic input to the circadian clock. Currently, circadian clock paradigm emphasizes CRY's direct influence on the circadian system's molecular elements. It fluctuates on daily basis, which is in period to light-dark (LD) phase, which serves as a foundation [8]. Furthermore, CRY impacts the circadian system and directly modifies neuronal activity through processes that are unrelated to the clock's fundamental components. CRY is known to assemble in neurons projections, where it is expressed, which supports these findings are binds to phototransduction mechanisms in photoreceptors of retina [9]. However no studies have been carried out in NDEA induced tumorogenesis in *cry^b* mutant flies. In the present study in *cry^b* mutant flies bearing tumor in intestine and brain tissue, cognitive and biochemical indices were investigated to comprehend whether circadian clock mutant is more prone to carcinogenesis than wild type flies.

Recent molecular genetics approaches combining *Drosophila* circadian clock mutants with antioxidant/reactive oxygen species (ROS) production discriminating mutants have aided in understanding the relationships between cellular oxidative stresses, and circadian clock. Twenty four hour rhythm of free radical induced damages has been documented in *D. melanogaster* [10]. It is imperative that these rhythms may lend a hand to the organism from uneven levels of ROS, by maintaining circadian regulation of ROS homeostasis, and from the significant damages to biomolecules including nucleic acids and proteins [11].

Further, *Drosophila* has been widely used to study cognitive function and/or intellectual ill health, which has given a huge quantum of disease-related scientific findings [12]. A series of analysis were employed to evaluate the cognitive

actions in *Drosophila* including hygrotaxis, negative geotaxis, thermotaxis, taste and smell chemotaxis, and phototaxis. There are various cancers and their types, which well known to weaken the cognitive behavioral function [13]. In *Drosophila*, age-linked defects in behavioral characteristics have also been documented. It is well known that organism with cancer could experience psychological and oxidative stress. Several researchers investigated the molecular and cellular mechanisms of emotional/cognitive distress during malignancy growth [14].

All facets of malignancy, from oncogenesis to the tumor-bearing phase, with healing and preclusion, are linked to the effect of oxidative stress. When oxidative stress exceeds the ability of the body's oxidation/reduction mechanism, it possible that gene mutation could occur, or intracellular transcription and signal transduction factors may be impacted unswervingly or indirectly through antioxidants, resulting in cancer [15]. Under oxidative stress, the cancer-prone status has been taken into account, which is linked to tumor cells producing active oxygen and improper oxidation-reduction regulation [15]. Even if tissues with tumor have a lower free radicals due to an unregulated and larger digit of cells, oxidative stress is enhanced in the body of the tumor-bearing mass [16]. Oxidative stress is tightly linked to every stage of cancer with carcinogenic factor to the tumor-bearing condition and also, as of remedy to avoidance. Oxidative stress cancer and plays a crucial cycle; when oxidative stress surpasses the capability of the body's oxidation/reduction system, genetic changes might also additional arise and/or cellular signal transduction and transcript elements could be influenced immediately or else via antioxidants, leads in the direction of cancer [15]. Likewise, the tumor formation is documented to be oxidative stress related with excessive energetic oxygen fabrication and irregular oxidation/reduction regulation [15]. The oxidative stress is upraised, despite the fact that tumor manner tissues endure lower free radical stack because of out of control and excessive production of new cells, within the body of host with tumor [16]. Free radicals are reactionary chemical genus with a particular unpaired electron into one of their surface orbits [17]. This unbalanced arrangement releases energy throughout reaction among nearby molecules such proteins, carbohydrates, lipid and nucleic acids. These oxidants can able to harm the cells by initiating chemical chain reactions, such as damaging DNA or lipid peroxidation, and proteins. Both during normal metabolism and under stress, oxidative stress act a crucial role in regulating *Drosophila* lifespan. For example, the metabolic rate of these flies housed under varied settings, assessed as O_2 consumption, is negatively related to their lifetime [18].

N-nitrosamines may be carcinogenic to humans, according to evidence from various animal trials (IARC 1987; NTP2013). It is a well-known fact that the incidence of various types of human malignancies is highly linked to geographical and environmental factors, including food. N-nitrosamines in the diet are perhaps the most thoroughly researched exposure condition for any class of geotaxis carcinogen chemical in human diet. N-nitrosodiethylamine (NDEA) is a known cancer-causing chemical that has been shown to induce tumor growth in a variety of organs in animal models [19]. This carcinogen can be found in soy beans, smoked salted seafood, pork, cheese and a variety of alcoholic beverages. N-nitrosamines may be carcinogenic to various organisms, according to evidence from various animal trials (IARC 1987; NTP2013). As a result, N-nitrosamines are classified as the human carcinogens, and their usage should be limited [20]. N'-Nitrosodiethylamine (NDEA) is a toxin and carcinogen with a high potency. It has been employed as a cancer initiator agent in various experimental trials. N'-Nitrosodiethylamine (NDEA) is an organic compound. A member of the nitrosamines is carcinogenic and mutagenic [21]. Cosmetics, processed meats, cigarette smoke, and special concern products all include nitrosamines and their precursors. Cosmetics contain nitrosating agents like diethanolamine (DEA) and triethanolamine (TEA), in addition to amines like triethanolamine (TEA) and diethanolamine (DEA) [22]. Furthermore, alkanolamides analogues used as pH adjusters or emulsifiers, for example TEA and fatty acid alkanolamides, cause nitrosamine production under specific conditions, (which is generally in acidic environment). Ingesting food substances (such contaminants, pesticides, and drugs) are mistakenly present in food and added to food for processing purposes is referred to as dietary exposure. Chronic exposure occurs when a person is continually or repeatedly exposed to a material (such as acrylamide, mycotoxins, or nitrosamine) over a lengthy period time. N-nitrosamine is a subgroup of N-nitroso compounds, which have been detected in variety foods [23].

Vanillic acid (VA), (4-hydroxy 3-methoxy benzoic acid) is in nature happening bioactive molecule with extensive assortments of pharmacological activities, such as free radical scavenging and anticancer activity [24]. In chronic liver injury, vanillic acid may help to prevent hepatic fibrosis. VA is known to exhibit antibacterial, anticancer, antidiabetic, anti-inflammatory, antiobesity, and antioxidant properties. Despite its therapeutic potential and favourable safety profile, it has received little attention as a nutraceutical or therapeutic component [24]. It has been suggested that vanillic acid could be used to treat sickle cell anaemia [25]. Vanillic acid is known to repress DNA-dependent protein kinase with increased malignant cell susceptibility toward cisplatin. Vanillic acid is an antimicrobial phenolic derivative found in edible plants and fruits, antibacterial [26] antifilarial [27] and free radical scavenging activities and chemopreventive effect. In several models, vanillic acid has been shown to suppress mutagenesis caused by mutagens (physical and chemical) [28].

Vanillic acid protects biological membranes and reduces lipid peroxidation in cells, according to research. Vanillic acid has been shown to have the ability to scavenge or remove ROS such as lipid peroxy radicals and hydroxyl radicals. It targets the mTOR/Ras pathway, HIF-1 inhibition, NF-B, and Nrf2, which govern cell growth, survival, proliferation, and adaption to the cancer microenvironment and further was known to treat in colon cancer [29]. Previously we have studied the defensive effect of vanillic acid during NDEA induce carcinogenesis in the wild type (WT) *D. melanogaster* [30]. However the response in cry^b mutant (circadian clock mutant) to NDEA induced carcinogenesis is unknown at present. In current study we are comparing the protective effect of carcinogenesis of VA in cry^b mutant.

Materials and methods

Maintenance of flies and study group

Flies were cultured in maize powder, yeast and sucrose aliment with nepagin (antifungal compound) at 21 ± 2°C in a 12:12 (light: dark) cycle [31]. *Cry*^{b} mutant flies were received from the Centre for cellular and Molecular biology (CCMB), Hyderabad, India. The flies were kept at ambient temperature (21-23^{o} C). The investigational flies were segregated into 4 groups, WT (group I – control, group II – NDEA (0.01%), group III – (vanillic acid + NDEA treated) and group IV – vanillic acid (0.01%). NDEA and vanillic acid was delivered throughout food medium for 21 days. After the completion of 21st day, flies (n = 30/group) were gathered from all four groups and were used for further analysis.

Collection of the Hemolymph and Tissue Homogenate

Appropriate perforations in 0.5ml microfuge tube were placed into a 1.5ml microfuge tubes with the cover detached. Thirty flies were dissected by removing their wings. The tubes were subjected to centrifuge at 2500rpm for 15minutes (1.5ml containing smaller 0.5 ml tube). The hemolymph was collected in the bottom of a 1 ml tube, mixed with ice cold PBS (phosphate buffered saline), and frozen [32]. The dissected head and intestine tissues were spun (3000 rpm for 10 min) at 5^{o}C and further utilized for biochemical tests in 0.1M sodium phosphate buffer (pH 7.5).

Histopathological Studies

Tissues (brain and intestine) were dissected out was preserved in 10% formalin for 48 hours, then dehydrated with ethyl alcohol-water mixture, which is rinsed in xylem and then fixed in paraffin. Flies were dissected (5-7 m thick), stained with hematoxylin and eosin dye (H/E stain), and kept in DPX (distyrene, a plasticizer and xylene) medium (pH 7.0) for microscopic examination.

Behavioral Functions of *D. melanogaster*

The standard methodologies with slight modifications [33] were used to analyze the cognitive behavioral variables such as negative geotaxis, taste chemotaxis, smell chemotaxis, phototaxis thermotaxis and hygrotaxis in all fly groups.

Negative Geotaxis

About 25 flies from *cry*^{b} were anesthetized by used diethyl ether and in the form of a vertical glass tube (12 cm × 1.5 cm). Flies were carefully transferred to a fresh culture vial after a five-minute resting interval. After one minute, flies, which reached the upper portion of the column and those that remained at the base were counted individually. The data were reported as a percentage of flies crossing a distance of 13cm in 60seconds. Each assay was done in triplicate, the mean± SD was computed for each of the four groups of flies (Fig 1a).

Phototaxis

The apparatus consists of a vial connected by a connector. A light gradient that acted as an attractor for the flies was developed as a visible light source (Emergency Lamp, Philips, India). A vial containing approximately 25 flies plugged with cotton was left separately for 30 minutes in a dark room. The flies were held in the dark after that. The apparatus were horizontal and 15cm away from the light source. The light was turned on, a timer started, and the number of flies that reached compartment I (nearest to the light source) were counted. Each assay was carried out three times in all the groups and the mean± SD were computed (Fig 1b).

Smell Chemotaxis

The investigation employed the volatile repellent benzaldehyde. About 25 flies were placed in two vials (15x1 cm) that were taped together and separated into 3 analogous compartments (I, II and III). The cotton plug was soaked in 1-2ml of 100mM benzaldehyde and inserted into tube for testing (compartment III was closest to the cotton plug). Flies in the compartments were counted outcomes were displayed the percentage after one minute. The assay was performed in triplicate (Fig 1c).

Taste Chemotaxis

Sucrose was utilized in this experiment, which (a non-volatile chemical standard used for taste chemotaxis). In a test tube (18 cm x 1 cm), about 20-25 flies were distributed into three equal sections. After soaking the cotton plug in 1.5 ml of 0.2 percent sucrose, it was inserted into the test tube. Following a minute, the numbers of flies in all compartments were counted and the percentage was calculated. The experiment was performed with thrice different sets of flies (Fig 1d).

Thermotaxis

In the experiment, two vials (15 x 1.5 cm) were employed. One vial was heated to 45^{o} C and attached to another vial with transparent tape containing 20-25 flies in a matter of seconds. Zones equal size (3 Nos. I, II, and III – heated compartment III) were formed by connecting the vials. Each compartment's fly number was counted after one minute, all flies were present in compartment I (as %) were calculated. The experiments were performed separately with three sets of flies (Fig 1e).

Hygrotaxis

1 ml of distilled water was placed in a 15 x 1.5 cm vial, which was then covered with parafilm and maintained overnight vertically. Another vial (15 x 1.5 cm) with flies (25 No.) was taken without water overnight vertically. Then, water was removed and the vial with flies has been connected with the vial with a transparent tape. Three zones of equal size were marked for the two connected vials (I, II and III among which compartment I is the most the humid zone). After one minute, the flies number in each one compartment were counted, and the % were calculated. The experiment was repeated three times and mean±SD was measured (Fig 1f).

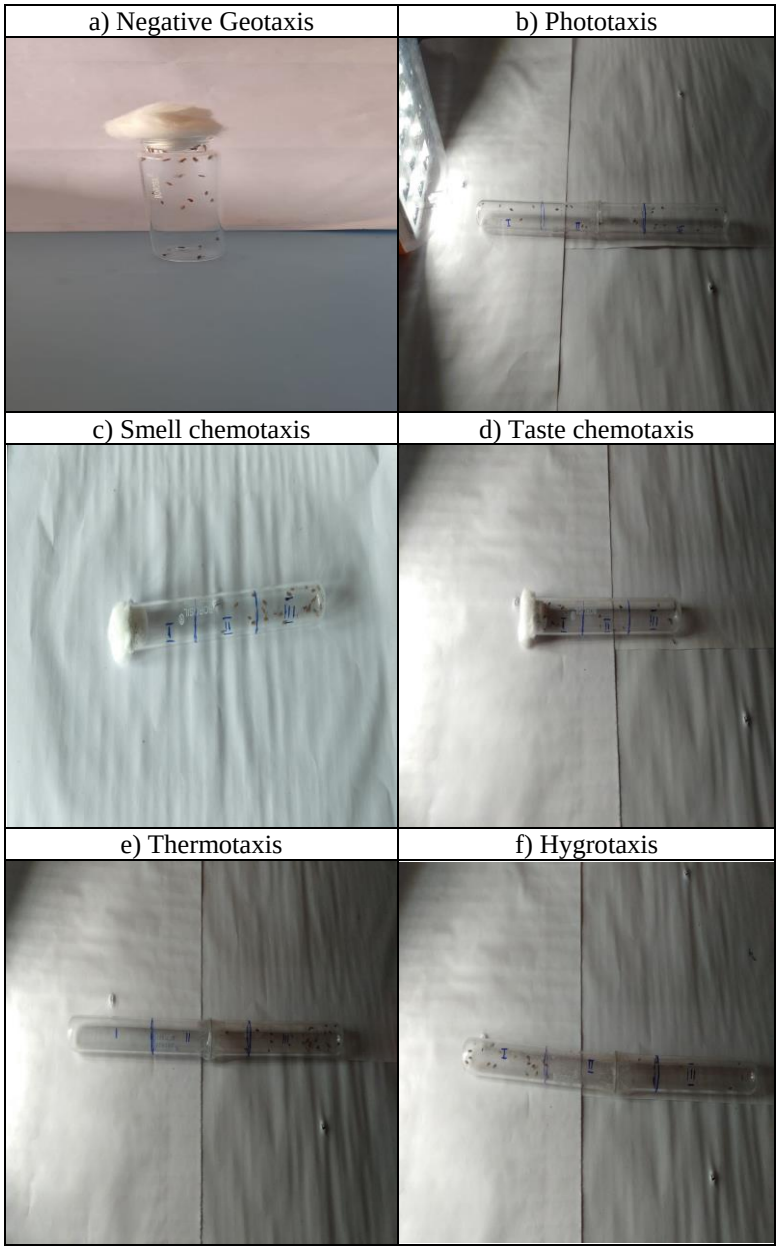


Fig 1: Behavioural assays in *cry*^{b} mutant flies. Negative geotaxis (a), phototaxis (b), smell chemotaxis (c), taste chemotaxis (d), thermotaxis (e) and hygrotaxis (f).

Biochemical Assay

Estimation of Protein Carbonyl Content

The protein carbonyls were quantified by the method [34]. Homogenated *cry*^{b} mutant samples (tissue homogenate and hemolymph) were segregated into 2 sections, while every part contains 1-2mg of standard protein. One part of an equivalent amount of 2N HCl were added and maintained at 25 °C for 45 minutes. Then, the mixture was added with 10% TCA and centrifuged. Ethanol: ethyl acetate (1:1) and 1ml of 6M guanidine HCl were added. The mixtures were centrifuged at 1500 rpm for 4 minutes and collected the supernatant. The absorbance variation among DNPH (dinitro phenylhydrozine) and HCL added samples were measured at 360nm and the values were mentioned as µ moles, of carbonyl groups/mg of protein.

Estimation of Thiobarbituric Reactive Substances (TBARS)

The TBARS levels in tissue homogenate and in hemolymph was estimated [35]. In this technique, malondialdehyde and other TBARS were calculated based on their activity with thiobarbituric acid (TBA) in low pH condition to form a pink colored compound, which can be examined at 535nm**.**

Protein Thiol

Derivatization of 5,5′dithiobis (2-nitrobenzoic acid) were used to measure free protein thiol groups (DTNB). The detection is based upon the creation respecting colored thiolate ion compound at 410 nm, which can be detected spectrophotometrically. The protein fractions were mixed with buffer containing detergent, for example sodium dodecyl sulphate [36].

Lipid Peroxides

The reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), to and 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) at 42°C is involved in this lipid peroxidation assay. R1 interacts with MDA/4-HNE, which forms chromophore with a 580nm absorbance maximum [37].

Assay of Glutathione-s-Transferase

Activity of glutathione-S-transferase (GST) in the tissue homogenate/hemolymph was determined by elevation absorbance at 340nm using CDNB as the substrate [38]. The reagents *viz*. reduced glutathione, phosphate buffer and CDNB – 30 mM were all made in 90% ethanol. Activity of GST was measured in moles of CDNB-GSH, which binds for the production at every minute per milligram of protein.

Estimation of Reduced Glutathione

The method of estimating reduced glutathione was used [36]. Production of yellow color when 5, 5'-dithiobis (2-nitrobenzoicacid) (DNTB) was mixed to the components with sulphydryl groups formed the basis for this approach. Glutathione levels were measured in mg/dl plasma and mg/100g tissue.

Assay of superoxide Dismutase

The presence of superoxide dismutases in tissues and hemolymph were determined [39]. The nitroblue tetrazolium formazon assay is based on the suppression of NADH-phenazinemethosulphate synthesis. The addition of NADH kicked off the reaction. After 90 seconds, the process is terminated by addition of glacial acetic acid. The chromogen formed after the completion of process was hauled out into a layer of n-butanol OD was taken at 520 nm.

Assay of Catalase

Catalase action was measured in tissues and hemolymph [40]. Phosphate buffer was used to make tissue homogenate (0.01M, pH 7.0). To 1 ml phosphate buffer, 0.15 ml tissue homogenate and hemolymph 0.45 ml hydrogen peroxide were added. By the addition of 2.0ml of dichromate-acetic acid mixture after 15, 30, 45, and 60 seconds, the processes were stopped. The tubes were cooled after 10 minutes of incubation in hot water, and the chromogen produced was read at 620 nm. The test was preceded by aliquot of (25-125 mmole). For tissues, the values are expressed in mmol of H_2O_2 consumed/minute/mg of protein of tissue.

Assay of Glutathione Peroxidasese (GPX)

The action of GPx was assessed in tissues and hemolymph using the method [41]. Tris buffer was used to homogenise the tissue. 0.25 mL tris buffer, 0.25mL EDTA, 0.2mL sodium azide, and 0.7 mL tissue homogenate and hemolymph were added to 0.3 mL tris buffer, 0.3mL EDTA, 0.2 mL sodium azide, and 0.7 mL tissue homogenate and hemolymph, 0.3 ml GSH was added to the mixture, followed by 0.2 ml H_2O_2. The contents, together with a control with all reagents except homogenate, were thoroughly mixed and kept at 37°C for 10 minutes. Following 10 minutes, the reaction was stopped by adding 0.6 mL of 10% TCA. The tubes were centrifuged, and the supernatant was tested for GSH [36]. The level was expressed as µmol of GSH utilized/min/mg of protein. The level of glutathione was expressed as mg/dl of hemolymph.

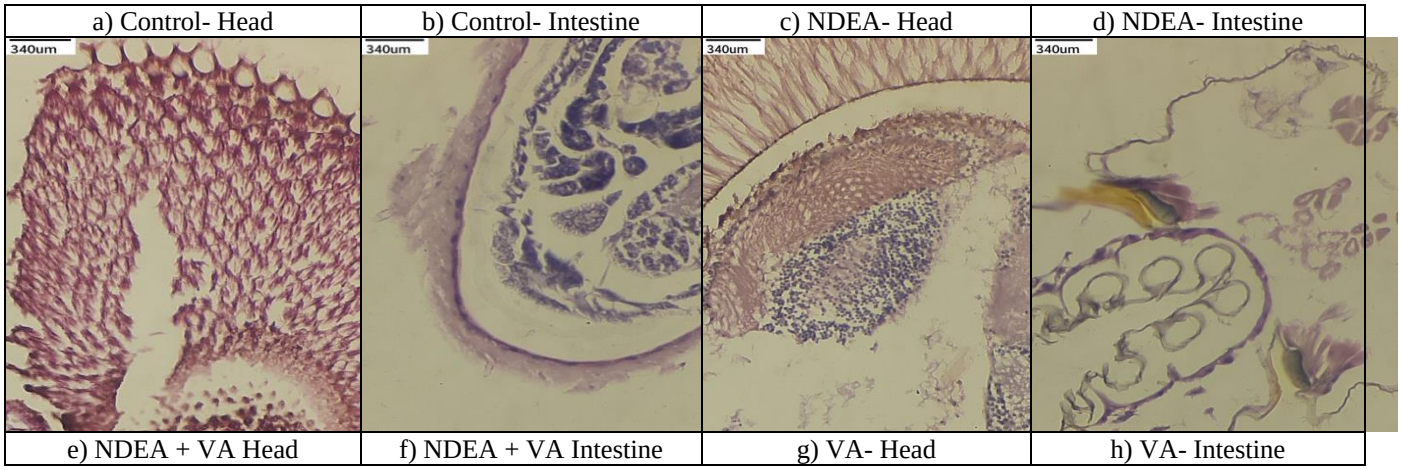
Statistical Analysis

All data were represented as mean ± SD. The statistical significance were assessed using one-way of analysis of variance (ANOVA) by employing SPSS version 17 (SPSS, Cary, North Carolina, USA), and the evaluation of data was carried out by Duncan's multiple range test (DMRT) and p value < 0.05 was considered as significant.

Results

Histopathological changes

Fig 2 shows the results of histological investigations performed on *cry*^{b} mutant flies brain and intestine. The regular histological architecture (group 1), granulated cytoplasm, tiny and uniform size nuclei and nucleoli are seen in Fig 2a and b (brain and intestine). The tissues of group 2 (NDEA) flies (Fig 2 c and d) were disordered, while tumour cells smaller than regular cells and big hyperchromatic nuclei. Tumour Island with a "Keratin pearl" in the centre, stratified epithilia migrating tumour cells with dysregulated cell division, and hyperplasia. Changes in the brain, tubule-shaped structure enclosing lumen, occur especially in brain neoplastic overgrowth tissues. The tissues in group III (NDEA + vanillic acid) had less architecture and a lower ability to multiply (Fig 2 e and f). Group 4 demonstrated that tissue propagation is almost under control (Fig 2 g and h).



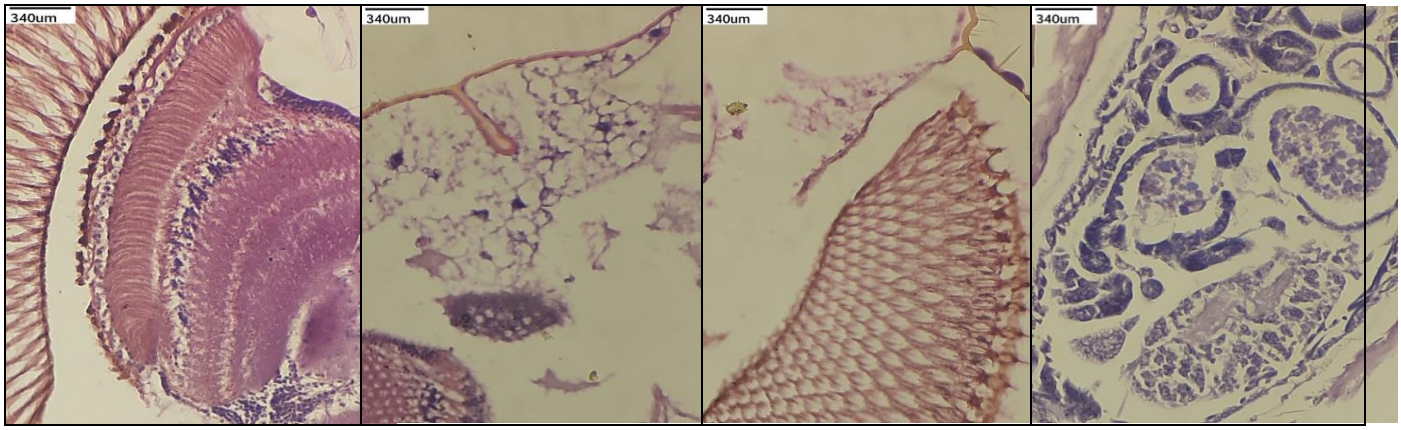


Fig 2: Histopathological studies in *cry*^{b} mutant flies. Control- head (a), control- intestine (b), NDEA- head (c), NDEA intestine (d), NDEA+ vanillic acid – head (e), NDEA+ vanillic acid- intestine (f), vanillic acid- head (g), and vanillic acid- intestine (h). Abbreviation: Cryptochrome (*cry*^{b}) and N-nitrosodiethylamine (NDEA).

Cognitive Behavior

The remark of negative geotaxis was shown in (Fig 1 a). Flies were placed at the bottom of a vertical tunnel to be subjected to gravity gradient. When compared to WT [30], the *cry*^{b} mutant's normal geotaxis (negative) activity was significantly reduced. After NDEA treatment, the negative geotaxis value of 79.4 ± 13.7 percent is greatly reduced (74.8 ±12.4). When comparing the NDEA+ vanillic acid treated group to group I, the value increased 74.8 ± 12.4 ($p > 0.05$). When compared to the control group, group IV (vanillic acid) had a value that was more or less equal to group I ($p>0.05$) (Table 1). In general, a higher proportion of flies will attempt to move the light source. However, when compared to WT [30], this reaction was dramatically diminished in *cry*^{b} mutant flies (compartment I, Table 2) (Fig 1 b).Nevertheless these response was significantly reduced ($p>0.05$). Comparatively to *cry*^{b}, a larger number of NDEA-treated flies were detected in compartment II than in compartment I ($p<0.05$).

Conversely, more number of WT flies [30] was observed moving from compartment I to compartment III, away from pungent benzaldehyde than *cry*^{b} flies. A lesser quantity of control flies were seen compared to NDEA-administered flies from compartment I to compartment III ($p>0.05$, Table 2). In vanillic acid + NDEA treated flies, there was a considerable increase in migration to compartment III ($p>0.05$) (See Table 2) (Fig 1 c). Despite the fact that it was not statistically significant, WT flies were shown to migrate closer to cotton-plug treated in sucrose solution in larger numbers (compartment I) [30]. NDEA treated flies migrated more towards compartment III than *cry*^{b} mutant NDEA administrated flies ($p<0.05$).The vanillic acid- administrated flies' response be similar toward that of control flies ($p>0.05$, Table 2) (Fig 1 d).

Whereas in thermotaxis assay, more WT flies [30] than *cry*^{b} mutant flies have a tendency to migrate from hot compartment III to cooler compartment I. *Cry*^{b} mutant flies were treated with NDEA ($p<0.05$). In comparison to group II flies, the tendency appeared to be reversed in NDEA + vanillic acid treated flies ($p<0.05$). Comparing control flies to vanillic acid alone treated flies, a considerably higher percentage of vanillic acid alone treated flies were detected in compartment III ($p>0.05$) (Table 2) (Fig 1 e). In the hygrotaxis assay, there were more control flies than NDEA-treated flies. In compartment I, which had moved towards the humid part ($p>0.05$, Table I) (Fig 1 f). Comparing NDEA + vanillic acid treated flies to NDEA treated flies, the NDEA + vanillic acid treated flies were considerably more in compartment III ($p <0.05$) (Table 2). Vanillic acid-treated flies exhibited near normally in taste chemotaxis, negative geotaxis, smell chemotaxis, phototaxis hygrotaxis and thermotaxis responses alike to control *cry*^{b} group ($p>0.05$).

Table 1: Negative geotaxis in *cry*^{b} mutant flies. Percentage of flies in control, NDEA treated, NDEA+ vanillic acid treated (0.01%) shown. Values were mean± SD of triplicate experiments (n=30 in each group and in each triplicate).

Negative Geotaxis (%±SD) (*cry* ^{b} -Control)	NDEA treated	NDEA + Vanillic acid	Vanillic acid
80.2±10.8	79.4±13.7	74.8±12.4	89.9±13.7

Table 2: Phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis, and hygrotaxis in *cry*^{b} mutant flies. Percentage of flies in control, NDEA treated, NDEA+ vanillic acid treated (0.01%) shown. Values were mean± SD of triplicate experiments (n=30 in each group and in each triplicate).

% of flies present in compartment / Behavioural assay	Compartment I (mean±SD)	Compartment II (mean±SD)	Compartment III (mean±SD)
Phototaxis			
cry ^{b} (control)	80.1±1.8^{a}	24.6±4.1^{a}	7.2±3.2^{a}
NDEA treated	72.3±4.9^{b}	28.3±3.2^{b}	11.2±2.8^{b}
NDEA+ Vanillic acid	70.1±2.6^{a}	18.4±3.2^{a}	8.5±2.1^{a}
Vanillic acid only	76.2±1.1^{a}	23.6±1.4^{a}	7.6±1.3^{a}
Smell chemotaxis			
cry ^{b} (control)	27.8±0.8^{a}	35.5±4.3^{a}	78.5±2.5^{a}
NDEA treated	43.4±2.3^{b}	27.6±1.8^{b}	38.3±5.2^{b}

NDEA+ Vanillic acid	18.5±1.6[a]	16.8±3.3[a]	68.5±4.7[a]
Vanillic acid only	16.2±0.6[a]	23.5±4.1[a]	77.5±2.5[a]
Taste chemotaxis			
cry [b] (control)	81.5±7.6[a]	10.4±6.3[a]	8.4±4.2[a]
NDEA treated	54.2±7.8[b]	36.1±5.5[b]	14.3±4.1[b]
NDEA+ Vanillic acid	76.2±6.2[a]	31.7±4.4[a]	12.5±4.2[a]
Vanillic acid only	79.5±6.1[a]	15.4±4.1[a]	10.4±2.2[a]
Thermotaxis			
cry [b] (control)	74.9±7.4[a]	7.4±1.1[a]	1.2±0.6[a]
NDEA treated	52.6±4.5[b]	20.6±4.1[b]	7.5±2.7[b]
NDEA+ Vanillic acid	69.1±6.2[a]	7.3±0.9[a]	1.4±0.8[a]
Vanillic acid only	72.2±5.2[a]	8.2±0.9[a]	1.2±0.5[a]
Hygrotaxis			
cry [b] (control)	90.1±8.4[a]	19.6±6.8[a]	6.3±2.3[a]
NDEA treated	69.5±6.3[b]	24.3±5.2[b]	8.8±2.1[b]
NDEA+ Vanillic acid	78.6±6.1[a]	21.7±5.4[a]	5.5±1.8[a]
Vanillic acid only	89.5±7.3[a]	20.6±5.4[a]	7.1±2.1[a]

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

Biochemical indices

Cry^b mutant is known to possess higher amounts of protein carbonyl, TBARS, protein thiol, and lipid peroxides in head and intestinal tissues than WT [30] (with significance; Table 3) after NDEA treated. In NDEA treated *cry*^{b} flies TBARS, lipid peroxides protein carbonyl were noticeably higher ($p<0.05$) as compared to group I. In *cry*^{b} mutants head and intestinal tissues, and as well as hemolymph showed a similar trend was detected for antioxidant enzymes such as SOD, CAT, GST, GPx, and as well as GSH, a non-enzymatic antioxidant. When the of intestinal and head protein carbonyl, lipid peroxides, GST and GPx, as well as GSH in hemolymph, were compared to WT [30], the contents of vanillic acid-treated *cry*^{b} mutant flies were consistently considerably reduced ($p<0.05$).

When the vanillic acid treated *cry*^{b} mutants were compared to those that were not, significantly higher levels of redox homeostasis parameters were noticed in the hemolymph (GSH), head (protein carbonyl and all antioxidant enzymes and GSH), intestinal (TBARS, lipid peroxides, and all antioxidant enzymes apart from SOD) tissues. Lipid peroxide content of the hemolymph of vanillic acid-administrated mutant was significantly lower than that of untreated mutants (Table 3). The activity of enzymatic and non-enzymatic antioxidants was considerably reduced ($p<0.05$) in group II. The number of flies treated with vanillic acid increased were significantly ($p<0.05$).

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

S. No	Biochemical Parameters	Groups	Hemolymph	Head	Intestine
Redox haemostasis					
1	Protein carbonyl (nmole/mg protein)	*cry* [b]	4.7±1,03[a]	3.4±0.76[a]	1.3±0.09[a]
		NDEA treated	6.7±2.35[b]	2.7±0.51[b]	1.4±0.07[b]
		NDEA + Vanillic acid	4.2±0.08[a]	2.1±0.65[a]	1.5±0.05[a]
		Vanillic acid only	4.2±1.03[a]	2.1±0.85[a]	2.2±0.09[a]
2	Thiobarbituric acid reactive substances (TBARS)(nmole/mg protein)	*cry* [b]	11.3±2.61[a]	6.4±1.20[a]	5.2±1.72[a]
		NDEA treated	16.8±3.67[b]	5.4±0.81[b]	4.9±1.14[b]
		NDEA + Vanillic acid	11.1±2.01[a]	5.8±1.21[a]	5.5±1.24[a]
		Vanillic acid only	19.9±3.73[a]	12.8±3.52[a]	9.4±2.20[a]
3	Superoxide dismutase (SOD) (Unit[a] nmole/mg protein)	*cry* [b]	10.1±2.01[a]	6.8±1.21[a]	4.5±1.24[a]
		NDEA treated	9.7±2.18[b]	9.5±2.82[b]	7.3±1.14[b]
		NDEA + Vanillic acid	17.4±3.15[a]	11.4±2.53[a]	7.4±2.10[a]
		Vanillic acid only	19.4±3.33[a]	11.8±1.62[a]	10.2±1.22[a]
4	Catalase(CAT) (Uint[b]/min/mg protein)	*cry* [b]	153.3±2.57[a]	130.3±13.49[a]	105.7±10.22[a]
		NDEA treated	123.1±11.76[b]	110.3±8.67[b]	78.3±5.74[b]
		NDEA + Vanillic acid	152.6±18.16[a]	130.3±10.41[a]	102.8±9.11[a]
		Vanillic acid only	151.1±21.52[a]	140.3±11.54[a]	110.7±11.20[a]
5	Glutothion-S-transferase(Uint/100mg/protein)	*cry* [b]	11.6±0.14[a]	7.2±0.61[a]	5.7±0.51[a]
		NDEA treated	12.2±0.07[b]	5.7±0.32[b]	4.3±0.21[b]
		NDEA + Vanillic acid	11.0±0.95[a]	6.1±0.84[a]	5.7±0.43[a]
		Vanillic acid only	10.6±0.14[a]	6.8±0.81[a]	6.4±0.48[a]
6	Glutothione peroxidise(GPx) (Unit[c] per mg protein)	*cry* [b]	11.9±4.32[a]	5.5±0.85[a]	5.1±0.74[a]
		NDEA treated	9.1±1.12[b]	3.1±0.54[b]	3.5±0.41[b]
		NDEA + Vanillic acid	10.0±3.21[a]	4.5±0.87[a]	4.4±0.68[a]
		Vanillic acid only	10.1±3.12[a]	4.9±0.85[a]	5.2±0.74[a]
7	Reduced glutathione (GSH) (µl/mg tissue)	*cry* [b]	14.6±8.14[a]	9.4±2.11[a]	8.5±1.34[a]
		NDEA treated	10.3±3.05[b]	6.4±0.81[b]	5.1±0.45[b]
		NDEA + Vanillic acid	12.3±6.11[a]	8.9±1.32[a]	7.7±0.81[a]

		Vanillic acid only	13.2±7.14 [a]	10.4±1.19 [a]	7.2±1.14 [a]
8	Protein thiol(mmol/mg protein)	*cry* [b]	36.7±4.21 [a]	25.4±4.71 [a]	22.8±3.85 [a]
		NDEA treated	45.1±3.10 [b]	12.2±2.61 [b]	11.8±1.13 [b]
		NDEA + Vanillic acid	32.5±4.11 [a]	21.1±2.85 [a]	19.5±3.01 [a]
		Vanillic acid only	30.7±5.11 [a]	20.4±3.61 [a]	18.8±2.81 [a]
9	Lipid peroxidise (nmole/mg lipid)	*cry* [b]	31.6±5.37 [a]	22.9±4.13 [a]	21.7±2.63 [a]
		NDEA treated	40.6±4.06 [b]	14.8±2.10 [b]	15.8±2.04 [b]
		NDEA + Vanillic acid	29.3±3.29 [a]	24.1±3.28 [a]	21.3±2.25 [a]
		Vanillic acid only	33.8±0.13 [b]	21.6±0.95 [b]	20.4±0.82[b]

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

Discussion

The circadian clock relies on rhodopsin and CRY-mediated light transduction pathways (cryptochrome) to provide synchronizing light signals to the biological clock. The photosensitive CRY is involved in the majority of cellular and molecular mechanisms that induce photic entrainment. The light input of circadian clock in *cry*^{b} mutant flies was faulty. The biological clock has the ability to control activities endogenously, allowing for the maintenance of several physiological and behavioral functions under constant settings. *Cry*^{b} mutant flies have a faulty photoreceptor cryptochrome, whereas wild type flies have a normal architecture of the gene encoding the protein cryptochrome [42]. The defective photic input and circadian organization could be responsible to the alteration of cognitive behavioral and biochemical indices in our study in *cry*^{b} flies compared to wild type flies [30]. However, future studies are necessary to confirm this hypothesis. The activities of the alterative in NDEA treated *cry*^{b} mutant flies are higher than the WT flies with NDEA treatment.

Histopathological studies clearly indicate the tumorogenesis in brain and intestine tissues of *cry*^{b} mutant *D. melanogaster*. After the treatment of vanillic acid, the histopathological appearance tends to be reverse to near normally. In this study, we employ simple approaches to demonstrate *Drosophila* reactions to a variety of stimuli. Cognition problems are typical in a large sort of malignancies [13].Our results, suggested that VA could counteract the negative effects of NDEA, restoring the *Drosophila*'s behavior to close to normal levels. In animals, vanillic acid (VA), a pharmacologically active phytochemial has been found to restrain the growth of a number of malignancies [43]. According to the findings of this study, vanillic acid inhibits tumor growth via NDEA indices in experimental carcinogenesis.

Vanillic acid is very effectively decreasing the number of early lesions, as induced earlier, during, or later by NDEA treatment. As a result, as shown in this study, their cognitive activities (smell chemotaxis, negative geotaxis, taste chemotaxis, thermotaxis, phototaxis and hygrotaxis) might be tending to normalize. Vanillic acid has been shown to correct cognitive dysfunction and restore normal sleep period and energy in people [44] as well as flies [45]. Negative geotaxis was used to confirm the locomotion, and the results revealed that flies exposed to NDEA had difficulty flying. It heard a moderate reduction in the number of deaths when combined with a specific concentration of vanillic acid, as well as a beneficial influence of vanillic acid on negative geotaxis data. Antioxidant therapy can help to mitigate these detrimental effects. NDEA-treated *cry*^{b} flies' normal geotaxis (negative) activity was drastically reduced when compared to *cry*^{b} mutant flies. Phototaxis, olfactory and gustatory chemotaxis, thermotaxis and hygrotaxis, were all tend to be normalized. In general, a more number of flies move toward the light source. However, the opposite results observed in NDEA -treated *cry*^{b} mutant flies (and as compared to NDEA-treated WT flies) [30] indicate the influence of defective photic input on the cognitive observations. However, future studies are essential to confirm this hypothesis.

Vanillic acid treatment in flies resulted in normalized behavior than tumor-induced flies. As vanillic acid possess all the cyto protective characteristics with antioxidant activity it could suppress the tumorogenesis in *cry*^{b} flies. NDEA treatment could elevate the oxidation process, which results in an increase in ROS and, as a result, a rise in the concentrations of oxidized DNA bases. GPx uses H_2O_2 to mediate the oxidation of GSH to GSSG, effectively neutralizing H_2O_2's negative effects [46]. Reduced GPx action implies cellular accrual of lipid hydroperoxides, which could set off a chain reaction in which additional unsaturated lipids turn into targets for peroxidative product production and ultimately leading to tissue injury. In the current investigation, GPx activities diminished in the neoplastic tissue, which could be due to increased concentrations of hydroperoxides, and vanillic acid treatment retained GPx activity in carcinogen-treated flies (group III & IV), demonstrating vanillic acid's ability to enhance GPx activity in hemolymph, head and intestine. In mammalian cells, GSH is the most significant non-enzymatic antioxidant [47]. Its metabolism is a complicated process that involves GSH breakdown and resynthesis, along with GSH interconversion to GSSG [48]. Additionally there is also a consistent decreased in antioxidants – GSH, SOD, GPx, CAT, and GST– in tissues and hemolymph; this could be due to the tumor-applicability host's rapid utilization of antioxidants [49].

Natural plant derived phenolic photochemical were widely used for the effective prevention of cancer metastasis [30]. Vanillic acid could inhibit and destroy the cancer cell invasion. Excessive reactive oxygen species production (ROS) and a lack of antioxidant capacity have been linked to a variety of diseases, including cancer. A developing characteristic of cancer is the abnormal buildup of reactive oxygen species (ROS). ROS-driven tumorogenesis and development necessitate an abnormal redox equilibrium that promotes onco-signaling while avoiding ROS-induced programmed death through the coordination of antioxidant systems [50]. Induced stress (via NDEA administration) accelerates the production of ROS and lipid peroxidation products. The difference among *cry*^{b} and WT flies is that NDEA-administrated wild-type flies are less prone to oxidative stress, as compared clock mutant (*cry*^{b}) flies [30].

Because of present in the hydroxyl groups in VA it could exhibit a strong ROS scavenging action. There are numerous antioxidative defense systems in place to avoid cellular damage caused by ROS. The antioxidative defense mechanism may scavenge reactive oxygen species (ROS)

that limit lipid peroxidation and hence protect against cancer growth. This protection classification operates throughout enzymatic (include SOD, GST, GPx and CAT), and non-enzymatic components (generally GSH, vitamins-C and E). In NDEA-exposed groups, antioxidant levels, both enzymatic and non-enzymatic were altered. When malignancies are present, the activity of antioxidant enzymes, particularly SOD, is lowered. It has been proposed that the manganese-containing SOD isoforms act as tumour suppressors [50]. Our results have shown reduced actions of SOD and CAT in the NDEA administrated flies as compared control flies and elevation after VA treatment suggesting VA's mode of action. The concentrations of TBARS and lipid hydroperoxides in hemolymph and tissues (intestine and brain) detected in this examination could possibly as a result of NDEA treatment which stimulates cell lysis, production of free radicals, and injury to the cell membranes; in addition, inhibition of lipid peroxidation in vanillic acid administrated *cry*^{b} mutant flies could due to increased antioxidant activity and thereby could protect their tissues. To conclude our study indicates that *cry*^{b} mutant is more susceptible for tumor induction and subsequently elevated stress and a cognitive abnormality as compared to WT flies [30]. Further, the treatment to *cry*^{b} mutant is also less responsive as compared to WT flies indicating the defective photic input and malfunction of biological clock could play a major role in carcinogenesis in *D. melanogaster*. Further investigations are desirable in this line.

Conclusion

In the present cognitive and biochemical studies indicated that, the high hydrophobic nature and low molecular weight of vanillic acid support to guard most liable intestine and brain tissue of tumor flies. This above activates was gradually decrease in mutant (*cry*^{b}) individuals than the winged (WT) individuals.

Acknowledgement

Financial assistance from RUSA 2.0 project (DRD/RUSA/R&I/Project Proposal/Field 2/5/2021, dated 22.02.2022) sanctioned to PS is gratefully acknowledged.

Founding support

The authors declare that there is no funding support for this study.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Sexton T, Yaffe E, Kenigsberg E, Bantignies F, Leblanc B, Hoichman M *et al*. Three-dimensional folding and functional organization principles of the *Drosophila* genome. Cell.,2012:148(3):458-472.
2. Tickoo S, Russell S. *Drosophila melanogaster* as a model system for drug discovery and pathway screening. Curr Opin Pharmacology,2002:2(5):555-566.
3. Gupta SC, Mishra M, Sharma A, Deepak Balaji TG, Kumar R, Mishra RK *et al*. Chlorpyrifos induces apoptosis and DNA damage in *Drosophila* through generation of reactive oxygen species. Ecotoxicol Environ Saf.,2010:73(6):1415-1423.
4. Feany MB. ASIP Outstanding Investigator Award Lecture. New approaches to the pathology and genetics of neurodegeneration. Am J Pathol.,2010:176(5):2058-2066.
5. Busza A, Emery-Le M, Rosbash M, Emery P. Roles of the two *Drosophila* cryptochrome structural domains, in circadian photoreception. Science,2004:304(5676):1503-1506.
6. Yoshii T, Funada Y, Ibuki-Ishibashi T, Matsumoto A, Tanimura T, Tomioka K. *Drosophila cr*^{yb} mutation reveals two circadian clocks that drive locomotor rhythm and have different responsiveness to light. J Insect Physiol.,2004:50(6):479-488.
7. Emery P, Stanewsky R, Hall JC, Rosbash M. A unique circadian rhythm photoreceptor. Nature.,2000:404:456-457.
8. Hunter Ensor M, Ousley A, Sehgal A. Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. Cell.,1996:84(5):677-685.
9. Mazzotta G M, Costa R. Circadian control of visual plasticity in arthropods. Ethol Ecol Evol.,2016:28(1):1-19.
10. Coto-Montes A, Hardeland R. Antioxidative effects of melatonin in Drosophila melanogaster: antagonization of damage induced by the inhibition of catalase. Journal of pineal research,1999:27(3):154-158.
11. Hardeland R, Coto-Montes A, Poeggeler B. Circadian rhythms, oxidative stress, and antioxidative defense mechanisms. Chronobiol Int.,2003:20(6):921-962.
12. Coll-Tané M, Krebbers A, Castells-Nobau A, Zweier C, Schenck A. Intellectual disability and autism spectrum disorders 'on the fly': insights from *Drosophila*. Dis Model,2019:12(5):dmm039180.
13. Pendergrass JC, Targum SD, Harrison JE. Cognitive Impairment Associated with Cancer: A Brief Review. Innov Clin Neurosci.,2018:15(1-2):36-44.
14. Zhang Y, Zanos P, Jackson IL, Zhang X, Zhu X, Gould T *et al*. Psychological stress enhances tumor growth and diminishes radiation response in preclinical model of lung cancer. Radiother Oncol.,2020:146:126-135.
15. Lin Y, Jiang M, Chen W, Zhao T, Wei Y. Cancer and ER stress: Mutual crosstalk between autophagy, oxidative stress and inflammatory response. Biomed Pharmacother,2019:118:109249.
16. Donohoe C, Senge MO, Arnaut LG, Gomes-da-Silva LC. Cell death in photodynamic therapy: From oxidative stress to anti-tumor immunity. Biochem Biophys Acta Rev Cancer,2019:1872(2):188308.
17. Riley PA. Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol.,1994:65(1):27-33.
18. Fleming JE, Reveillaud I, Niedzwiecki A. Role of oxidative stress in *Drosophila* aging. Mutat Res.,1992:275(3-6):267-279.
19. El-Din NKB, Ali DA, Othman R, French SW, Ghoneum M. Chemopreventive role of arabinoxylan rice bran, MGN-3/Biobran, on liver carcinogenesis in rats. Biomed Pharmacother,2020:126:110064.
20. Krul CA, Zeilmaker MJ, Schothorst RC, Havenaar R. Intragastric formation and modulation of N-nitrosodimethylamine in a dynamic *in vitro* gastrointestinal model under human physiological conditions. Food Chem Toxicol.,2004:242(1):51-63.

21. Beard JC, Swager TM. An Organic Chemist's Guide to *N*-Nitrosamines: Their Structure, Reactivity, and Role as Contaminants. J Org Chem.,2021:86(3):2037-2057.
22. Sannino A, Bolzoni L. GC/CI-MS/MS method for the identification and quantification of volatile N-nitrosamines in meat products. Food Chem.,2013:141(4):3925-3930.
23. Abdullah ATM, Khan TA, Sharif M, Mazumdar RM, Rahman MM. Determination of dietary exposure and extraction efficiency of nitrosamine from cooked meat. Curr Res Food Sci.,2022:5(491-497):2665-9271.
24. Kaur J, Gulati M, Singh SK, Kuppusamy G, Kapoor B, Mishra V *et al*. Discovering multifaceted role of vanillic acid beyond flavours: Nutraceutical and therapeutic potential. Trends in Food Science & Technology,2022:122:187-200.
25. Abraham DJ, Mehanna AS, Wireko FC, Whitney J, Thomas RP, Orringer EP. Vanillin, a potential agent for the treatment of sickle cell anemia. Blood.,1991:77(6):1334-1341.
26. Rai RP, Maurya MS. Synthesis and evaluation of antibacterial activity of vanillin derivatives. J Sci Technol India,1966:4:275-276.
27. Varma RS, Shukla A, Chatterjee RK. Evaluation of vanillic acid analogues as a new class of antifilarial agents. Indian J Exp Biol.,1993:31(10):819-821.
28. Shaughnessy DT, Setzer RW, DeMarini DM. The antimutagenic effect of vanillin and cinnamaldehyde on spontaneous mutation in Salmonella TA104 is due to a reduction in mutations at GC but not AT sites. Mutat Res.,2001:480-481:55-69.
29. Kaur J, Gulati M, Gowthamarajan K, Vishwas S, Kumar Chellappan D, Gupta G *et al*. Combination therapy of vanillic acid and oxaliplatin co-loaded in polysaccharide based functionalized polymeric micelles could offer effective treatment for colon cancer: A hypothesis. Med Hypotheses,2021:20(156):110679.
30. Kani A, Subramanian P. Dose-dependent influences of vanillic acid on cognitive function and redox homeostasis status in N-nitrosodiethylamine treated *Drosophila melanogaster*. Int J Curr Pharm Res.,2021:13(4):85-92.
31. Subramanian P, Kaliyamoorthy K, Jayapalan JJ, Abdul-Rahman PS, Haji Hashim O. Influence of quercetin in the temporal regulation of redox homeostasis in Drosophila melanogaster. Journal of Insect Science,2017:17(2).
32. MacMillan HA, Hughson BN. A high-throughput method of hemolymph extraction from adult *Drosophila* without anesthesia. Journal of insect physiology,2014:63:27-31.
33. Neckameyer WS, Bhatt P. Protocols to Study Behavior in *Drosophila*. Methods Mol Biol.,2016:1478:303-320.
34. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG *et al*. Determination of carbonyl content in oxidatively modified proteins. In Methods in enzymology,. Academic Pres, 1990:186:464-478.
35. Niehaus WG, Samuelsson B. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem.,1968:6(1):126-130.
36. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys.,1959:82(1):70-77.
37. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem.,1979:95(2):351-358.
38. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem.,1974:249(22):7130-7139.
39. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase (SOD). Indian J Biochem and Biophys.,1984:21(2):130-132.
40. Sinha AK. Colorimetric assay of catalase. Anal Biochem.,1972:47(2):389-94.
41. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. Science,1973:179(4073):588-590.
42. Lindsley D, Zimm G. The Genome of *Drosophila melanogaster*. Academic Press Inc.,1992:15(6):433-434.
43. Taqvi S, Bhat EA, Sajjid N, Sabir JSM, Quershi A, Rather IA *et al.* Protective effect of vanillic acid in hydrogen peroxide-induced oxidative stress in D.Mel-2 cell line, Saudi Journal of Biological Sciences.,2021:28(3):1795-1800.
44. Huang, Jun-qing, Muwen Lu, Chi-Tang Ho. "Health benefits of dietary chronobiotics: beyond resynchronizing internal clocks." Food & Function,2021:12:10:1039.
45. Sun X, Ran D, Zhao X, Huang Y, Long S, Liang F *et al*. Melatonin attenuates hLRRK2-induced sleep disturbances and synaptic dysfunction in a *Drosophila* model of Parkinson's disease. Mol Med Rep.,2016:13(5):3936-3944.
46. Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegeneration. Eur J Biochem.,2000:267(16):4904-4911.
47. Wu G, Fang YZ, Yang S, Lupton JR, Tuner ND. Glutathione matebolism and its implications for health. J Nutr.,2004:134(3):489-492.
48. Thirupurasundari CJ, Padmini R, Devaraj SN. Effect of berberine on the antioxidant status, ultrastructural modifications and protein bound carbohydrates in azoxymethane-induced colon cancer in rats. Chem Biol Interact.,2009:177(3):190-195.
49. Gaya-Bover A, Hernández-López R, Alorda-Clara M, de la Rosa JMI, Falcó E, Fernández T *et al*. Antioxidant enzymes change in different non-metastatic stages in tumoral and peritumoral tissues of colorectal cancer. The International Journal of Biochemistry & Cell Biology.,2020:120:105698.
50. Zuo J, Zhang Z, Li M, Yang Y, Zheng B, Wang P *et al*. The crosstalk between reactive oxygen species and non-coding RNAs: from cancer code to drug role. Mol Cancer,2022:21(1):30.