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Original Article

PROTECTIVE FEATURES OF *LOBOPHORA VARIEGATA* METHANOLIC EXTRACT (LVME) ON COGNITIVE BEHAVIOUR AND REDOX HOMEOSTASIS IN N-NITROSODIETHYLAMINE TREATED *DROSOPHILA MELANOGASTER*

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ABSTRACT

Objective: The protecting influence of methanolic extract of seaweed (*Lobophora variegata*) on cognitive as well as biochemical indices in N-Nitrosodiethylamine (NDEA, a potent carcinogen) treated *Drosophila melanogaster* is evaluated**.**

Methods: In this study, Flies are divided into four groups; group 1–(control), group 2-flies were treated with 0.01% NDEA in food medium, group 3–with 0.01% NDEA and 0.01% *Lobophora variegata* methanolic extract (LVME) and group 4-with 0.01% LVME alone.

Results: Behavioural abnormalities (negative geotaxis, phototaxis, smell and taste chemotaxis, hygrotaxis and thermotaxis) were quantitatively deviated in NDEA treated flies compared to control but were tend to be normalized in LVME 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 LVME treated groups. Further, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPX) and reduced glutathione (GSH) were decreased in NDEA treated group and were significantly increased ($p<0.05$) in LVME treated groups.

Conclusion: It is well known that seaweed extracts contain numerous beneficial phytochemicals in abundantly. From our investigation, we found that the 0.01% LVME is efficient in reverting the abnormal behaviours and restoring the redox homeostasis of NDEA induced carcinogenesis in *Drosophila melanogaster*. Our investigation indicates that these phytochemicals could prevent the abnormalities in behaviour and redox homeostasis during carcinogenesis in *D. melanogaster.*

Keywords: Cancer, *Drosophila melanogaster*, *Lobophora variegata*, Methanolic extract, Behavioural assay, Free radical

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INTRODUCTION

The term cancer designates the disease that results when cellular changes cause the uncontrolled growth and division of cells. Mutations in genes can lead to cancer by quickening cell division frequencies or suppressing normal regulations on the system, for instance, cell cycle arrest or programmed cell death. Carcinogenesis is 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 signalling cascades control cell growth and development in mammalian systems and have conserved functions in flies mimicking the 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, the 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 have 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*. Methanolic extract of seaweed (*Lobophora variegata*). 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 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 tumour-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.

The marine algae (sea weeds) are rich natural resources of various biologically active compounds, for instance, polyunsaturated fatty acids (PUFAs), proteins, sterols, antioxidants, bioactive polysaccharides and pigments. They possess more than 65 trace elements at a noticeably higher concentration than terrestrial plants, [22, 23]. They also contain protein, iodide, bromide, several vitamins, and substances of antibiotic nature, [22, 23]. As many sea weed algae live in habitats in extreme conditions and, as a consequence of in adaption to these adverse environmental surroundings, they synthesize a wide range of secondary metabolites having significant pharmacological properties [22-24] which cannot be found in other organisms. *Lobophora variegata* is a common brown alga that is widely distributed in shallow water ecosystems of tropical and subtropical areas, including coral reefs of the Caribbean, the Indian Ocean, and the Red Sea. In coral reefs, *Lobophora variegata* is an abundant organism of the marine ecosystem and contains rich concentration of phenolic compounds, chiefly bromophenols [22-24].

Secondary metabolites like phlorotannis are produced by the polymerization of phloroglucinol and they are abundantly present in marine brown algae, including *Lobophora variegata* which are known to exhibit numerous biological activities such as, antidiabetic, anti-inflammatory, antimicrobial, antihypertensive and radioprotective properties [25, 26] Bioactive peptides are synthesized as a consequence of enzymatic hydrolysis in sea weeds[22]. These bioactive peptides predominantly have antimicrobial, antioxidant and anticoagulant properties and in addition, they play a key role in the amelioration of several cardiovascular diseases [22-27].

The phytochemical constituents of *L. variegata*, such as phenolics, tannins, glycosides, saponins, terpenoids, anthraquinones, flavonoids, and alkaloids play a vital role against pathogens [22]. These compounds also have noteworthy potential as antioxidant, antitumor and anticoagulant properties due to their cytoprotective, anti-proliferative and other activities [28, 29]. Brown algae or Phaeophyceae are the main sources of fucoxanthin since, the predominant pigment of brown seaweeds is fucoxanthin and gives the seaweeds brown colour. Fucoxanthin contains an allenoic moiety and some other functional groups containing oxygen like epoxy, alcohol and ester. Further, carotenoids, polysaccharides, namely alginates, laminarins, fucans, and cellulose are present. The bioactive substances of brown seaweeds stop the uncontrolled division of blood cells [30, 31]. It is observed that phlorotannins such as fucodipholoroethol G and phlorofucofuroethol A of phlorotannins are active against allergic pathway on basophilic leukaemia cell lines [22-31].

Drosophila has been employed for nearly a decade to investigate cognition and intellectual disability, which has provided a significant amount of disease-relevant information [32]. 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 [33]. In *D. melanogaster* age-associated impairment in cognitive functions has also been documented [34]. However, as the behavioral abnormality and oxidative stress indices during carcinogenesis/treatment with LVME 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 a normal culture medium at room temperature (21-23 °C) in 12:12 h light: dark cycle [10]. Both types flies were divided into four groups: (1) control, (2) 0.01% NDEA alone, (3) 0.01% NDEA with 0.01% LVME and (4) 0.01% LVME. The dose of LVME is selected based on a dose-dependent study conducted in our laboratory (unpublished observations). 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 LVME were administrated in food medium for 12 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 hemolymph was collected in the bottom of 1.5 ml tube and was mixed with ice-cold PBS (phosphate buffered saline) and stored in freezer [35]. 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 *Drosophila melanogaster*

The cognitive behavioural functions including negative geotaxis, phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis and hygrotaxis were assessed in all groups of flies by the methods of [35] and [36] with minor modifications.

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 was expressed as percent flies crossed beyond the distance of 13 cm in 60 s of interval [10-37]. Each assay was repeated for all the four groups of flies and mean±SD was calculated (fig. 1a).

Fig. 1a: Negative geotaxis

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 the away from the cotton, then the cotton was detached, and the vial was attached to the test tube by a connector. This set-up was horizontal and perpendicular to the horizontal light source kept at 15 cm distance. The light was then turned on. The flies were counted every minute for each quarter of the apparatus. In a control set-up, the apparatus was kept 15 cm away from and parallel to the light source. Each assay was repeated in all four groups and mean±SD was calculated (fig. 1b).

Fig. 1b: Phototaxis

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

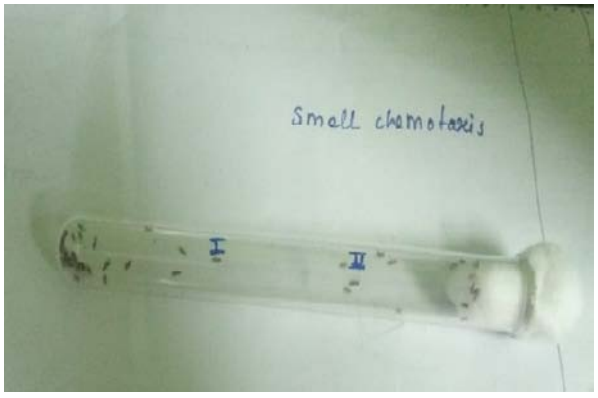

Fig. 1c: Smell chemotaxis

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 three separate sets of flies (fig. 1d).

Fig. 1d: Taste chemotaxis

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 three separate sets of flies (fig. 1e).

Fig. 1e: Thermotaxis

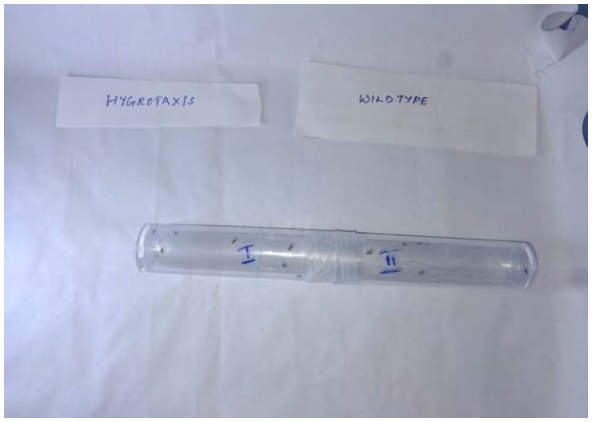

Fig. 1f: Hygrotaxis

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 the help of transparent tape. The connected vials were compartmentalized into three equal zones (I, II and III and compartment I moisturized zone). After one minute, the number of flies present in each compartment was counted and the result was expressed in percentage of total flies present. The test was repeated for separate three sets of flies and mean±SD was calculated (fig. 1f).

Biochemical parameters

Indices of redox homeostasis

The protein carbonyl content was assayed [38]. 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 hemolymph/tissue homogenate were estimated [39]. 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) [39]. The measurement is based on the formation of a coloured thiolate ion complex that can be detected spectrophotometrically at 410 nm. The thiol group assay is often performed on soluble protein fractions, by homogenization in a buffer containing a detergent such as sodium dodecyl sulfate (SDS) [39]. 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 [40].

Glutathione-S-transferase (GST) was assayed in hemolymph/tissue homogenate by an increase in absorbance at 350 nm using CDNB as substrate [41]. Phosphate buffer, reduced glutathione and CDNB (30 mmol) were 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 [42]. 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 [43]. 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 hemolymph/tissue homogenate was assayed [44]. 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 was 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 [39]. 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 89.1±12.7 % is decreased significantly after NDEA treated (74.2±12.4) compared to control flies ($p<0.05$). In NDEA+LVME treated group the value is increased 79.7±11.1 compared to NDEA treated group. In group 4 (LVME only) the value is more or less similar (92.2±14.3 %) to control group. More percentage of flies tends to move closer to the light source (phototaxis) (compartment I, table 1). However, this response was noticeably decreased ($p<0.05$) in group 2 (compartment I). NDEA+LVME group showed higher percentage of flies ($p<0.05$) compared to group 2.

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

As for the thermotaxis assay a higher number of control flies tend to move away from the warm surface (compartment III) to a relatively cool surface (compartment I) as compared to NDEA treated flies ($p<0.05$). The trend appeared to be followed in NDEA+LVME treated flies when compared with group 2 flies ($p<0.05$). At the same time an insignificant percentage of LVME alone treated flies were observed in compartment III similar 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+LVME treated flies were move to compartment I as compared to NDEA treated flies ($p<0.05$). Invariably, LVME treated flies demonstrated behavioural responses of negative geotaxis, photo, smell and taste chemotaxis, thermotaxis and hygrotaxis similar to the control group ($p>0.05$, table 1).

Negative geotaxis, phototaxis, smell chemotaxis, taste chemotaxis, thermotaxis and hygrotaxis in *D. melanogaster*. Percentage of flies in control, NDEA treated, NDEA+LMVE treated and only LVME treated were shown. Values were mean±SD of triplicate experiments (n=30 in each group and in each triplicate). Values not sharing a common superscript alphabet vary significantly at $p<0.05$ by Duncans Multiple Range Test (DMRT). Abbreviation: Wild type (WT), N-nitrosodiethylamine (NDEA).

Biochemical assays

The levels of protein carbonylation, TBARS, protein thiols and lipid peroxides were predomitly 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 2). In NDEA+LVME treated groups, the values were decreased in haemolymph and elevated in tissues (head and intestine) compared to NDEA treated flies. Group 4 flies (LVME treated) showed more or less closer values to control 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 2). In NDEA+LVME treated groups, the values were increased in haemolymph as well as in tissues (head and intestine) compared to NDEA treated flies. Group 4 flies (LVME treated) showed more or less closer values to control flies.

Table 1: Behavioural response in WT *D. melanogaster*

Negative geotaxis (%±SD) (WT-control)	NDEA treated	NDEA+LVME	LVME only
89.1±12.7	74.2±12.4	79.7±11.1	92.2±14.3
% of flies present in compartment	Compartment I	Compartment II	Compartment III
Behavioral assay	(mean±SD)	(mean±SD)	(mean±SD)
Phototaxis			
WT (control)	85.2±3.1[a]	23.3±3.9[a]	8.2±1.9[a]
NDEA treated	71.2±3.5[b]	27.6±3.6[b]	12.1±2.8[b]
NDEA+LVME	80.7±2.9[a]	24.9±4.2[a]	8.1±2.1[a]
LVME only	83.6±2.7[a]	22.1±4.9[a]	7.9±3.1[a]
Smell chemotaxis			
WT (control)	2.8±1.2[a]	12.1±3.7[a]	88.2±3.6[a]
NDEA treated	9.8±2.2[b]	26.2±3.8[b]	69.7±4.5[b]
NDEA+LVME	2.6±1.7[a]	15.4±4.1[a]	86.1±3.2[a]
LVME only	2.2±1.3[a]	11.1±4.1[a]	85.7±2.5[a]
Taste chemotaxis			
WT (control)	85.2±7.9[a]	22.8±4.5[a]	12.3±4.9[a]
NDEA treated	67.3±7.1[b]	27.5±4.6[b]	17.9±4.6[b]
NDEA+LVME	83.4±5.2[a]	21.4±5.1[a]	11.2±4.3[a]
LVME only	83.2±5.6[a]	20.2±4.7[a]	11.2±4.7[a]
Thermotaxis			
WT (control)	83.1±5.9[a]	12.5±1.9[a]	3.7±1.2[a]
NDEA treated	67.2±4.7[b]	25.1±4.4[b]	12.3±2.8[b]
NDEA+LVME	79.1±5.1[a]	11.5±2.2[a]	4.1±1.6[a]
LVME only	81.9±6.7[a]	13.1±1.8[a]	4.1±1.2[a]
Hygrotaxis			
WT (control)	91±7.3[a]	20.2±5.1[a]	6.2±2.9[a]
NDEA treated	74.3±5.2[b]	26.2±4.2[b]	9.2±1.9[b]
NDEA+LVME	89.2±6.2[a]	22.3±3.8[a]	5.1±3.1[a]
LVME only	91.3±6.9[a]	19.1±4.3[a]	5.9±2.5[a]

Table 2: Parameters of redox homeostasis

Biochemical parameter	Group	Hemolymph	Head	Intestine
Protein carbonyl (mmole/mg protein)	WT (control)	3.5±1.2[a]	2.3±0.5[a]	1.4±0.01[a]
	NDEA treated	6.4±.1[b]	0.9±0.4[b]	0.9±0.2[b]
	NDEA+LVME	3.4±0.1[a]	1.5±0.51[a]	1.1±0.01[a]
	LVME only	3.5±1.1[a]	1.4±0.92[a]	1.6±0.07[a]
Thiobarbituricacid reactive substance	WT (control)	9.3±1.92[a]	5.1±1.2[a]	4.8±1.42[a]
(TBARS) (nmole/mg protein)	NDEA treated	13.2±3.11[b]	2.1±0.6[b]	3.2±1.3[b]
	NDEA+LVME	8.5±1.14[a]	5.3±1.2[a]	4.5±1.31[a]
	LVME only	16.5±3.12[a]	12.4±2.71[a]	8.2±2.41[a]
Superoxide dismutase (SOD) (Unit[A]/min/mg	WT (control)	16.4±3.51[a]	10.2±2.12[a]	7.2±2.31[a]
protein)	NDEA treated	8.7±1.21[b]	7.7±1.92[b]	5.6±2.21[b]
	NDEA+LVME	15.2±2.07[a]	9.9±2.2[a]	6.5±0.91[a]
	LVME only	17.5±2.37[a]	10.2±1.5[a]	8.6±1.21[a]
Catalase (CAT) (Unit[B]/min/mg protein)	WT (control)	140.8±19.12[a]	126.9±7.892[a]	87.4±9.93[a]
	NDEA treated	104.5±10.52[b]	87.6±9.41[b]	64.5±4.91[b]
	NDEA+LVME	134.6±15.05[a]	124.7±17.91[a]	82.3±8.41[a]
	LVME only	144.2±20.81[a]	122.3±10.41[a]	96.8±12.88[a]
Glutathione-S-transferase (Unit/100 mg	WT (control)	9.5±0.81[a]	5.8±0.81[a]	5.1±0.24[a]
protein)	NDEA treated	4.6±0.01[b]	2.9±0.38[b]	2.8±0.48[b]
	NDEA+LVME	7.3±0.75[a]	4.7±0.87[a]	4.2±0.37[a]
	LVME only	6.6±0.17[a]	4.4±0.58[a]	4.8±0.22[a]
Glutathione peroxidase(gpx) (Unit[B]/mg	WT (control)	16.4±2.32[a]	9.1±1.21[a]	4.7±0.81[a]
protein)	NDEA treated	12.3±1.21[b]	5.3±0.2[b]	2.6±0.41[b]
	NDEA+LVME	15.6±2.32[a]	7.3±1.52[a]	2.6±0.71[a]
	LVME only	14.4±1.99[a]	8.1±1.21[a]	4.6±0.99[a]
Reduced glutathione (GSH)	WT (control)	11.7±6.88[a]	8.7±2.11[a]	7.6±1.55[a]
	NDEA treated	7.1±2.05[b]	5.6±1.22[b]	4.7±0.71[b]
	NDEA+LVME	10.2±3.66[a]	6.9±0.33[a]	6.3±1.11[a]
	LVME only	11.8±5.19[a]	8.3±1.66[a]	7.1±2.11[a]
Protein thiol	WT (control)	31.5±4.31[a]	24.5±3.87[a]	25.3±2.15[a]
(mmol/mg protein)	NDEA treated	42.1±2.36[b]	15.5±3.01[b]	12.1±1.31[b]
	NDEA+LVME	29.5±3.61[a]	22.1±1.82[a]	18.7±2.55[a]
	LVME only	27.2±3.311[a]	19.8±2.18[a]	17.4±2.16[a]
Lipid peroxides	WT (control)	32.5±4.22[a]	28.3±4.47[a]	25.9±2.74[a]
(nmol/mg lipids)	NDEA treated	41.1±2.71[b]	16.7±1.76[b]	17.1±2.71[b]
	NDEA+LVME	31.4±2.18[a]	26.6±3.84[a]	23.6±2.23[a]
	LVME only	32.5±0.56[a]	22.4±0.89[a]	21.7±1.02[a]

Experimental values of protein carbonylation, 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+LMVE treated and LMVE only treated are shown. Values were mean±SD of triplicate experiments (n=30 in each group and in each triplicate). Values not sharing a common superscript alphabet vary significantly at $p<0.05$ by Duncans Multiple Range Test (DMRT). Abbreviation: Wild type (WT), N-nitrosodiethylamine (NDEA). Abbreviation: Wild type (WT), N-nitrosodiethylamine (NDEA).

DISCUSSION

Flies treated with NDEA that to develop oxidative stress during tumorigenesis [45]. This, in turn, could inhibit normal negative geotaxis behaviour [46] and to shorten sleep duration [47]. However, possibly, this is the first study showing the improvement of cognitive function by LVME 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 LVME 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 a wide range of cancers [33]. Our findings also added additional evidences that the cognitive behaviours could have been affected owing to carcinogenesis in flies. Our results also indicted that *Lobophora variegata* could nullify the harmful effects of NDEA and thus tend to bring back the flies' behaviours to near normal. The antitumor effects of LVME could be mediated by modulating different signalling pathways in diverse frameworks [25, 26].

Our findings are consistent with the report on alleviating oxidative stress by LVME in cell lines of *Drosophila* [48], signifying that elevation of TBARS level in NDEA induced flies could be attenuated by antioxidants such as those present in LVME. This could be owing to excessive generation of ROS and with an early event associated with hypoxia [49]. This level was decreased in LVME treated flies which is owing to the presence of several bioactive phytochemicals present in LVME, which have a strong ROS scavenging activity [25-31] 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 [50]. SOD and CAT comprise an equally protective set of enzymes against ROS [51]. This defence mechanism functions via enzymatic (including SOD, GPx, GST and CAT), and non-enzymatic components [52] 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 LVME treated flies due to enhanced antioxidant activity [30, 31]. 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 [22-31]. The noteworthy elevation in GSH level in LVME treated flies implies the ability of LVME to sustain GSH level by preventing glutamate toxicity and stimulating cystine (GSH precursor and excellent source for thiol group) uptake into brain by its free radical scavenging and cytoprotective properties [30, 31]. The plausible mechanism by which LVME 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 the 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 [50]. Furthermore, the rapidly dividing tumour cells in head and intestinal tissues were previously reported to utilise high levels of ROS [53]. 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 [54] Substances with potent antioxidant activity, such as, ascorbic acid is known to prevent hepatocarcinogenesis [55]. As stated earlier, LVME 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 [22-31]. The administered dose of LVME 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 LVME-treated flies denotes a decrease in lipid peroxidation of lipids. Similar observation has also been reported earlier about the antioxidant activities of vanillic acid (a phytochemical antioxidant) against oxidative stress [48].

Numerous studies afford evidences that LVME could increase the liver/disease marker enzymes in serum during hepatotoxicity (induced by chemicals) by diminishing free radicals and lipid peroxidation and promoting antioxidants [56]. There are many studies on the flavonolic contents of seaweeds [25-31]. Some investigations documented those seaweeds are a rich source of catechins and other flavonoids. Flavonoids such as rutin, quercitin and hesperidin, among others, were detected in species of Rhodophyta, Chlorophyta and Phaeophyceae [26-31] and diverse bioactive phytochemicals have been identified, for instance, hesperidin, kaempferol, catechin and quercetin [30, 31, 57] including in *L. variegata*.

The impact of LVME on modulation on xenobiotic-metabolizing enzymes is obvious from our observation. It also increases the antioxidant status and decreases the deleterious lipid peroxidation due to NDEA ethyl radical metabolites by decreasing toxic products like malondialdehyde (MDA). The abovementioned factors play a significant role in reacting with cellular targets like DNA, thus persuading mutagenicity and carcinogenicity. The reduced activities of antioxidants (enzymatic and non-enzymatic) in NDEA–triggered hepatocellular carcinoma could be owing to over-utilization of the antioxidants to scavenge the lipid peroxidation products, which ultimately leading to the distraction of antioxidant defence mechanisms in hepatic tissue. Our observations corroborate the results of other studies [25-31]. LVME inhibits lipid peroxidation and synthesis of free radicals by promoting antioxidant status as observed by the augmented levels of SOD, catalase and GPx and non-enzymatic antioxidants, such as vitamin E and C, and GSH. LVME was reported to possess rich levels of flavonoids, alkaloids, phenolics, tannins, glycosides, saponins, terpenoids, anthraquinones etc, [23, 30, 31] and natural phytochemicals are found to have significant antioxidant properties as cited by various sources.

Our results suggest that the modulation of the subtle balance between oxidant and antioxidants by numerous natural phytochemicals in LVME is a rational approach to prevent tumour progression. Antioxidant activities of seaweeds have also been demonstrated in cadmium-induced liver toxicity and MNNG-induced gastric carcinogens [23-26, 30]. Treatment with LVME efficiently repressed the NDEA-initiated carcinogenesis by normalizing xenobiotic-metabolizing enzymes (XMEs) and weakened lipid peroxidation through scavenging of free radicals and promoting antioxidant status and normalizing disturbed redox status in the flies.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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