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Modulatory Effect of *Decalepis hamiltonii* on Ethanol-Induced Neurotoxicity in *Drosophila melanogaster*

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Abstract

Introduction: T *Drosophila melanogaster* is a suitable in vivo model organism to study the neurotoxic effects of ethanol. In the present study, the behavioral responses, levels of oxidative markers, and activity of antioxidant enzymes were investigated in ethanol-exposed *Drosophila* flies.

Methods: Adult flies were exposed to absolute ethanol using ethanol exposure chamber. Then, their resistance against ethanol vapor was measured by monitoring their locomotion. Biochemical assays were applied to quantify antioxidant enzymes activity in the brains of ethanol exposed flies.

Results: The results showed that acute ethanol exposure led to the hyperactivity of flies. If alcohol exposure was prolonged, then sedation could occur in the experimental flies. The levels of reactive oxygen species (ROS) and lipid peroxidation (LPO) as cellular markers of oxidative stress considerably increased in ethanol-exposed flies while glutathione (GSH) level declined. Higher activities of catalase (CAT) and superoxide dismutase (SOD) were observed in the ethanol-exposed flies. Ethanol neurotoxicity became more evident when a remarkable decrease in acetylcholine esterase (AChE) activity was seen in the ethanol-exposed flies. Treatment with *Decalepis hamiltonii* aqueous extract increased the time of ST50 and decreased the RC50 values of ethanol-exposed flies. Moreover, we demonstrated that *D. hamiltonii* pre-treatment diminished ROS and LPO levels in the ethanol-exposed flies. *D. hamiltonii* treatment resulted in augmentation of GSH level and activity of CAT and SOD enzymes.

Conclusion: This is the first report on the protective effects of *D. hamiltonii* natural antioxidants in *D. melanogaster* on oxidative stress induced by ethanol.

Keywords: *Drosophila melanogaster*, *Decalepis hamiltonii*, Ethanol, ST50, RC50, Oxidative stress

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Introduction

The use of *Drosophila melanogaster* as an in vivo model to learn the role of oxidative stress in neuronal dysfunction is beneficial over the other animal models for several reasons. From evolutionary point of view, there is a high level of homology and conservation between human genome and fly genome. Moreover, fruit fly possesses several signal transduction pathways homologous to that of human being, which makes this insect a suitable model. The fly central nervous system (CNS) has much smaller quantity of cells and interestingly works in the same manner as it in mammalian systems.

Excessive consumption of ethanol is associated with degrees of dementia, liver injury, compromised immunity, hypertension, enhanced cancer risk, and neurobehavioral harms. In utero exposure to alcohol may lead to smaller brain size and lifelong neurobehavioral disturbances, which are commonly referred to as fetal alcohol syndrome (FAS).[1] High alcohol concentration grounds immediate destruction via solubilizing cell membranes and radically changing tertiary structure of proteins.[2] At lower concentrations, regulated forms of cell demise can occur. The mediators considered imperative for ethanol-induced cytotoxicity comprise both oxidative and non-oxidative metabolites. Extreme ethanol consumption persuades oxidative stress and causes brain damage. Neurotoxicity of ethanol is associated with augmentation in generation of reactive oxygen species (ROS) and decline in endogenous antioxidant defenses.[3]

ROS are molecular entities that have reactions with cellular components leading

to detrimental effects on cellular functions. The major parts of cellular ROS are produced during incomplete reduction of oxygen to water.[4] In aerobic organisms, the energy needed for biological functions is produced in the form of ATP via electron transport chain (ETC). Therefore, mitochondrial functional compromise has major effects on oxidative homeostasis.[5] Decreased activity of complex I of mitochondrial ETC has been observed in Parkinson disease (PD) patients, cybrid cell lines, and platelets.[6] This inhibition may result in the production of ROS which when generated in the vicinity, could affect the respiratory chain resulting in further inhibition by subsequent ROS generation and finally mitochondrial damage.[7]

Antioxidants play a major role in scavenging ROS, removal of free radicals, and inhibition of ROS generation. They are classified into two main groups: enzymatic and nonenzymatic. Enzymatic antioxidants consist of a number of proteins including superoxide dismutase (SOD), catalase (CAT) as well as glutathione (GSH) peroxidase along with some supporting enzymes. Nonenzymatic antioxidants are classified into two groups: directly acting and indirectly acting antioxidants. Directly acting antioxidants are really essential in defense against ROS. Most of them are lipoic acid and ascorbic acid, carotenoids and polyphenols which are derived from dietary sources. The cell itself produces some of these antioxidants. The second group mostly comprises chelating agents that bind to redox metals to prevent the generation of free radicals.[8] GSH, the major thiol-containing antioxidant with low molecular weight is made from cysteine, glutamate, and glycine.

Decalepis hamiltonii is a monotypic climbing shrub with aromatic tuberous roots that belongs to the Asclepiadaceae family and grows in scrub forest of the southern part of peninsular India. Its tuberous roots are being used in the ancient Indian Ayurveda as an appetizer, for flatulence relief, and as a general tonic. Its roots are also used as general vitaliser and blood purifier in folk medicine.[9] It has been shown that *D. hamiltonii* roots have antimicrobial, antifungal, antidiabetic, antioxidant, cytoprotective, chemoprotective, and neuroprotective activities.[10] In this connection, the edible roots of *D. hamiltonii* were selected for the present study because they have strong antioxidant potential. It is worth to note that a number of novel antioxidant compounds have been isolated and characterized from the aqueous extract of *D. hamiltonii* roots.[9,11]

Earlier studies have revealed the antioxidant and neuroprotective potential of *D. hamiltonii* root extract in the albino rat.[12,13] The neuroprotective effect of *D. hamiltonii* has been shown by in vivo and in vitro studies in rats.[13] To provide evidence for the involvement of oxidative stress in ethanol-induced behavioral impairments, the neuromodulatory action of antioxidant molecules of aqueous extract of *D. hamiltonii* on antioxidant defense system of wild-type *Drosophila* was evaluated. We investigated the sedation time and recovery time as neurofunctional parameters to explore ethanol-mediated locomotory dysfunction in flies.

Materials and Methods

Fly Stocks

The *Drosophila* stocks used in the present study were fed on standard wheat cream agar medium supplemented with dry yeast granules at 22 ± 1°C, 70%-80% relative humidity, and 12 h light/12 h dark cycle in a vivarium.

Negative Geotaxis Assay

The locomotory function of the flies was determined by negative geotaxis assay as described by Feany and Bender.[14] Thirty adult male flies were placed in a graduated plastic tube (25 cm length, 2 cm diameter). After a 10-minute rest period, the experimental flies were gently tapped down to the bottom of the tube and were allowed to climb up. The number of insects that climbed up to 20 cm mark in 1 minute was quantified. The test was presented as average of 4 repetitive experiments.[14]

Biochemical Assays

The fly samples for biochemical assays were prepared as follows: the adults were decapitated on ice cold PB (phosphate buffer). Heads from 100 flies were suspended in homogenizing buffer, centrifuged at 3000 g for 10 minutes at 4°C and then the supernatant was used as a sample for assaying antioxidant enzymes and oxidative markers.[15] To calculate GSH, LPO and ROS levels, a standard curve of the core component was prepared. To calculate the specific activity of CAT and AchE enzymes, the following formula was employed:

Specific enzyme activity = Δ Absorbance per minute / Molar extinction coefficient (ε) × total protein content (mg).

Catalase Assay

CAT activity was determined by the method of Aebi.[16] The activity of the enzyme was measured based on the decrease of absorbance at 240 nm which occurs as a consequence of H_2O_2 utilization. The reaction was initiated by adding 50 µL of sample to 1 mL of reaction mixture containing 50 µL diluted H2O2 (8.8 mM) in 900 µL sodium phosphate buffer. The changes in absorbance were monitored at 240 nm for 3 minutes. The molar extinction coefficient of H_2O_2 was 44.1 mM-1 cm-1 and the enzyme activity was expressed as µM of H_2O_2 utilized/min/mg protein. Blank solution was a mixture of 50 µL sample and 1 mL PB.

Superoxide Dismutase Assay

SOD activity was determined based on SOD-mediated inhibition of pyrogallol autoxidation following the method of Marklund and Marklund.[17] Pyrogallol (1,2,3-benzenetriol) is known to be autoxidized quickly especially in alkaline solutions. Molecular oxygen

carrying 2 unpaired electrons with parallel spins has a tendency for univalent reduction. SOD enzyme rapidly dismutases univalently reduced oxygen and superoxide anion radicals. Therefore, SOD enzyme can be a useful probe to study the participation of free radicals in pyrogallol autoxidation. To perform the experiment, a known volume of sample homogenate (200 µL), Tris HCl (2 mL), and distilled water (1.8 mL) were added into the quartz cuvette. The reaction was initiated by adding 0.5 mL of pyrogallol solution. The inhibition of pyrogallol autoxidation was monitored at 412 nm for 3 minutes, and the optical density (OD) values at 1-minute intervals were measured. Pyrogallol autoxidation was calculated by monitoring the change in absorbance of a reaction mixture containing 2 mL Tris-HCl buffer, 0.5 mL pyrogallol (2 mM) and 1.4 mL distilled water and the corresponding value was used as 100% pyrogallol autooxidation. The OD values of 100% autoxidation were subtracted from the OD values of each experimental sample, and the obtained final score indicated the inhibition of pyrogallol autooxidation. The specific activity of the enzyme was expressed as units in which 1 unit represented 50% inhibition of pyrogallol autooxidation. Hence, the corresponding score divided by total protein content was presented in 200 µL of sample and expressed as unit/mg protein. The blank solution for this assay consisted of all the reagents except pyrogallol.

Acetylcholinesterase Enzyme Assay

Acetylcholinesterase (AChE) activity was determined by the standard method of Ellman et al.[18] AChE proficiently hydrolyzes acetylthiocholine (ATCI), a sulfur analog of its natural substrate, acetylcholine. Following hydrolysis, the substrate analog, ATCI, produces acetate and thiocholine. Thiocholine in the presence of 5, 5'-dithiobis 2-nitrobenzoicacid (DTNB) generates 5-thio-2-nitrobenzoate anion (a yellowish substance). The yellow color obtained can be quantified using a spectrophotometer. The assay reaction mixture consisted of PB (920 µL), ATCI (25 µL), DTNB (25 µL), and head sample (30 µL) that was added into a 1 mL quartz cuvette. The reaction was initiated by adding acetylcholine iodide and absorbance was recorded at 412 nm for 3 minutes. The blank was prepared by mixing DTNB (25 µL) and PB (975 µL). Molar extinction coefficient value (ε) for DTNB was 1.36 × 104 M-1 cm-1 and the enzyme activity was expressed as nmoles of DTNB hydrolyzed/min/mg protein.

Glutathione Assay

GSH level was deliberated according to the method of Hissin and Hilf [19] using O-phthalaldehyde (OPA). In the assay, OPA that possesses a very low fluorescence background reacts with GSH to produce strong fluorescence signals and for that reason, the GSH content can be specifically quantified. For this assay, 50 µL of sample homogenate was added to 0.1 M formic acid and spun at 5200 g for 10 minutes to precipitate the protein. After 30 minutes staying at room temperature to react with OPA (1 mg/mL in methanol), excitation and emission were at 345 nm and 425 nm, respectively. The GSH level was calculated from a standard curve and expressed as µg GSH/mg protein. The standard curve of GSH was prepared by dissolving different concentrations (from 5 µg to 40 µg) of GSH in 1 mL distilled water. The corresponding fluorescent signal values of each solution were documented at excitation of 345 nm and emission of 425 nm. The standard plot was drawn and the line equation was used for calculation of the unknown amount of GSH in each experimental sample.

Reactive Oxygen Species

The ROS levels were calculated by means of a fluorometric method using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) probe described by Black and Brandt.[20] In presence of ROS, the non-fluorescent DCFH-DA probe is rapidly oxidized to the highly fluorescent 2′, 7′-dichlorofluorescein (DCF) which can be detected via fluorometric measurements. The heads of 50 flies were homogenized in 1 mL ice-cold Tris-HCl buffer (0.1 M; pH 7.4) and centrifuged at 2000 g for 10 minutes at 4°C. Afterward, 100 µL of filtered supernatant was transferred to each well of the microtitre plate containing 15 µL of diluted DCFH-DA and finally, the total volume was made up to 200 µL by adding a Tris-HCl buffer. After 1 hour incubation at room temperature, the conversion of DCFH-DA to DCF was quantified at 489 nm excitation and 525 nm emission wavelengths. The fluorescent unit corresponding to each sample was recorded. The blank solution was prepared by mixing 185 µL of homogenizing buffer and 15 µL DCFH-DA. The concentration of DCF present in each sample was determined by the standard curve of DCFH-DA. The standard curve for DCFH-DA was prepared by dissolving different concentrations (from 1.6 µg to 8 µg) of the chemical in 200 µL of Tris-HCl buffer. The corresponding fluorescent units of each solution were documented at 489 nm excitation and 525 nm emission wavelengths. Using the values obtained, the standard plot was drawn and the line equation was used for calculation of ROS level in each experimental sample.

Lipid Peroxidation

Lipid peroxidation (LPO) was measured by following the method of Ohkawa et al[21] using thiobarbituric acid (TBA). This assay is based on malondialdehyde (MDA) generation which is an end-product of LPO. The MDA reacts with TBA and forms a chromogenic solution. The reaction mixture contained 1.5 mL of 20% acetic acid (pH 3.5), 500 µL of fly head homogenate, 1.5 mL of TBA (0.8 % w/v) and sodium lauryl sulphate (SDS) (8 % w/v). The mixture was heated in boiling water bath for 50 minutes and adducts formed were extracted into 3 mL of 1-butanol. The absorbance was measured at 532 nm and

quantified as malondialdehyde equivalents. Using molar extinction coefficient (ε) value for tetramethoxypropane which is 15600 M-1cm-1, the MDA contents of samples were quantified.

Estimation of Total Protein Content

Total protein concentrations in sample homogenates were quantified by following the Lowry's method.[22] The principle of this method lies in the reactivity of peptide nitrogen with the copper ions under alkaline condition and succeeding in reduction of Folin-Ciocalteu reagent by copper-catalyzed oxidation of aromatic acids. In this assay, 150 µL of tissue sample homogenate was added to a reaction mixture containing 0.7 mL alkaline cooper solution, mixed well and incubated for 10 minutes in dark at 25°C (room temperature). Next, 0.1 mL Folin-Ciocalteu reagent was added and the mixture was incubated for 20 minutes in dark at 25°C. The absorbance was read at 750 nm. Bovine serum albumin (BSA) was used for preparing standard curve.

Preparation of the Plant Extract

Roots of *D. hamiltonii* were collected from their natural habitat in forests of B. R. Hills, Karnataka, India. The collected roots were washed in water and then were crushed. The fleshy segment was chopped into small pieces and dried at 40°C. Later, the dried plant roots were powdered finely by a grinder. The aqueous extract was prepared by homogenizing the root powder in warm water (45°C), allowed to rest for 24 hours and filtered with Whatman No.1 filter paper.[23] The yield after lyophilization of filtrate was about 70 g/kg of root powder.

Ethanol Exposure

The standard protocol of Maples and Rothenfluh[23] was followed to measure ethanol sensitivity of the flies. For making behavioral observations, 8 flies were collected 1 day before the test and kept in a fresh media vial. To perform the test, selected flies were transferred into the vial wherein the vials plug were flooded with absolute alcohol (0.5 mL). The cotton plug was inserted in a way that ethanol loaded faced sides of the flies. The number of flies sedated every minute were counted and ST50 (the time taken for half of the ethanol-exposed flies to become stationary) was determined accordingly. To measure RC50 (the time taken for half of the sedated flies to be completely recovered), ethanol was added until all the flies become stationary. Afterward, all the sedated flies were transferred to the fresh media vials and the RC50 was determined. Four vials of each experimental group were maintained as a replicate. The obtained scores of 3 replicates were averaged and expressed as mean ± standard error (SE).

Treatment of Flies With Plant Extract

In the initial step, analysis of toxicity of *D. hamiltonii* root extract was carried out. In the next step, the effective doses of *D. hamiltonii* and the time period of *D. hamiltonii* treatment were determined. Climbing ability and oxidative stress (OS) responses of flies were the parameters used for selection of *D. hamiltonii* effective doses and also effective treatment period. Preparation of *D. hamiltonii* -containing media is given in appendix section. Two-day-old flies were placed and allowed to feed on medium containing *D. hamiltonii* root aqueous extract (0.1%, 0.5%). The flies were transferred to fresh food vial every 3 days.

Statistical Analysis

The data (mean ± SE) was analyzed by one-way and two-way ANOVA followed by Duncan post-hoc comparisons using SPSS software version 19.0. The *P* value of 0.05 was considered as the minimum level of significance and represented at 3 levels determined by asterisks in all illustrations (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

Results

Effect of Ethanol on Wild-type Flies

Excessive ethanol (EtOH) consumption induced OS-mediated damage to the brain.

Acute EtOH exposure induced hyperactivity in flies as judged by the high-speed movement. On the other hand, higher doses of ethanol or long-time exposure induced the loss of postural control and finally sedation. Therefore, to check behavioral impairments induced by acute ethanol exposure, 16-day-old flies were exposed to absolute ethanol (0.5 mL) and the ST50 and RC50 values of their movement behavior were documented. The ST50 and RC50 values were 23 minutes and 38 minutes, respectively. As ethanol induces OS and neurotoxicity due to the reduction of endogenous antioxidant defenses, biochemical assays for CAT, SOD, and AchE enzyme activities and the levels of GSH, ROS and LPO in 16-day-old flies after ethanol exposure were carried out. The results obtained are represented in Figure 1, the perusal of which reveals that ethanol induced elevation in the activity of CAT and SOD enzymes.

Further scrutiny of the figure also shows significant depletion of GSH level in ethanol-exposed flies (12.6 ± 0.6 µg GSH/mg protein) compared to control flies (17.5 ± 1.4 µg GSH/mg protein). The LPO and ROS levels were found to be notably higher in ethanol-exposed flies. The flies exposed to ethanol showed 9.8 ± 0.19 nM MDA/ mg protein and 950 ± 39.6 µM DCF/mg protein for LPO and ROS levels, respectively. The AChE activity was found to be 110 ± 4.2 µM DTNB/min/mg protein in ethanol-exposed flies; while the enzyme showed the activity level of 75 ± 2.9 µM DTNB/min/mg protein in the control flies. Statistical analysis of the data revealed that ethanol induced significant alterations in all the biochemical assays ($P < 0.001$, n = 4). Duncan test also revealed that this level of alterations in the enzyme activities and OS

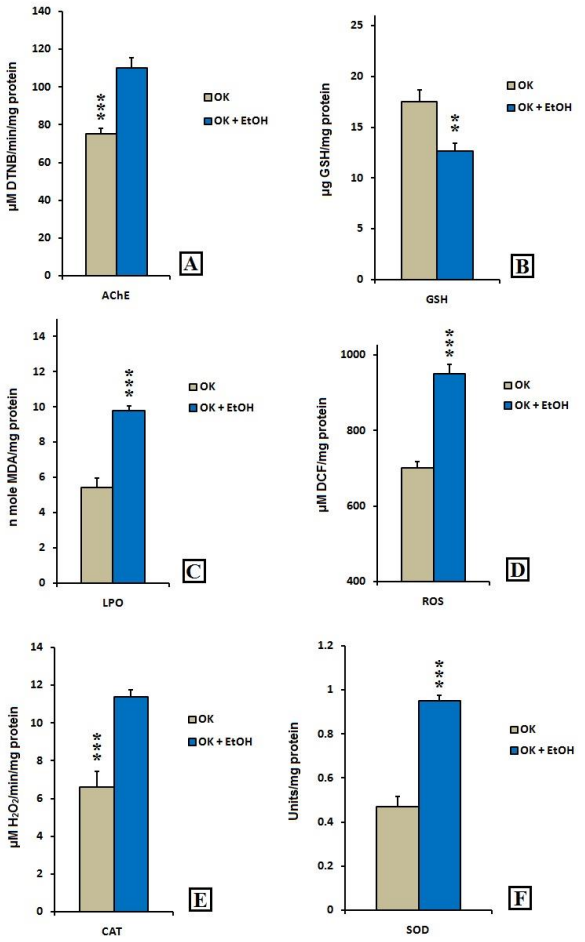

Figure 1. Biochemical Analysis of Antioxidant Enzymes, Oxidative Markers and AchE in Wild-type *D. melanogaster* (Oregon K) Exposed to Ethanol. (A) AchE activity, (B) GSH level, (C) LPO level, (D) ROS level, (E) Catalase activity, (F) SOD activity.

markers after ethanol exposure was significant (P = 0.0009, n = 4).

Decalepis hamiltonii Treatment

The main concern of this study was to investigate the antioxidant and neuroprotective effect of *D. hamiltonii* aqueous extract as potent antioxidant against OS induced by ethanol in wild-type (Oregon K) flies. In the initial step, analysis of toxicity of *D. hamiltonii* aqueous extract showed no mortality at any dose. In the next step, the effective doses of *D. hamiltonii* and the time period of *D. hamiltonii* treatment were evaluated. Climbing ability and OS responses of flies were the parameters used for selection of *D. hamiltonii* effective doses and also effective treatment period. Two concentrations of *D. hamiltonii* (0.1%, 0.5%) were selected based on the initial studies (data are not shown). Moreover, 14 days of *D. hamiltonii* feeding was chosen as the effective treatment period.

The ethanol-induced locomotor deficits of 16-day-old flies pre-fed with *D. hamiltonii* for 14 days were measured by exposure to 0.5 mL absolute ethanol and determination of ST50 and RC50 values.

The results of ST50 are shown in Figure 2. The RC50 values of flies pre-fed with *D. hamiltonii* are shown in Figure 3. A scrutiny of these figures reveals that pre-feeding of flies with *D. hamiltonii* increased the acute ethanol ST50 and decreased the RC50 value of flies compared to unfed ones (P = 0.0038, n = 4).

To further substantiate the neuroprotective effect of *D. hamiltonii*, biochemical assays were made on the same flies (16-day-old flies pre-fed with *D. hamiltonii* for 14 days and exposed to 0.5 mL absolute ethanol). The results obtained are compiled in Table 1 and Table 2.

A careful analysis of these results showed that ethanol exposure led to the obvious exhaustion of GSH in flies, and *D. hamiltonii* pre-feeding suppressed the depletion. Flies exposed to EtOH had elevated LPO compared to the untreated flies. Pre-feeding the flies with *D. hamiltonii* extract considerably decreased the LPO near to the basal levels. Ethanol exposure increased activity of SOD, which was restored in *D. hamiltonii* pre-fed flies to the enzyme

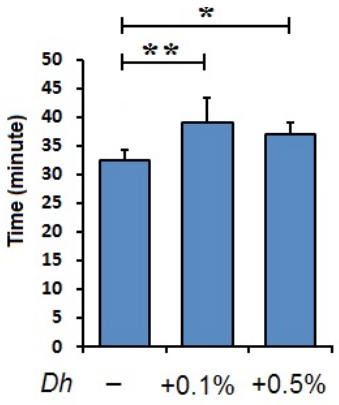

Figure 2. Ameliorative Effect of *D. hamiltonii* on Locomotion Behavioral Impairments Induced by Ethanol. Ethanol-induced sedation measured by ST50 in control flies and *D. hamiltonii* treated ones. ST50 value significantly increased in 0.1% *D. hamiltonii* - (P = 0.0062) and 0.5% *D. hamiltonii* - (P = 0.0047) treated groups in comparison with control untreated flies. Abbreviation: *Dh, D. hamiltonii*.

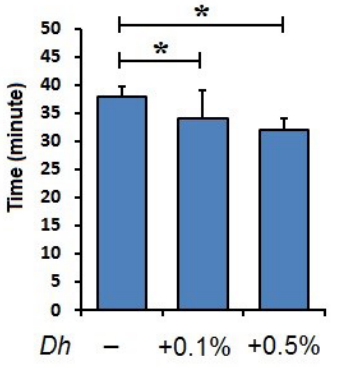

Figure 3. RC50 Value of Flies Exposed to Ethanol and Neuroprotective Effects of *D. hamiltonii*. *D. hamiltonii* treatment significantly decreased RC50 of flies (P = 0.0049 for 0.1% D. hamiltonii and controls, and P = 0.0038 for 0.5% *D. hamiltonii* and controls).

Table 1. Mean ± SE of Endogenous Antioxidant Enzyme Activity, Oxidative Markers and AchE in Wild-type *Drosophila melanogaster* (Oregon K) Flies Exposed to Ethanol Showing Antioxidant Potential of *Decalepis hamiltonii* treatment

Genotype	Diet	AchE	CAT	SOD	GSH	LPO	ROS
Wild-type (Oregon K)	Control	75.0±7.3	6.6±0.26	0.47±0.04	17.5±0.3	5.4±0.65	700±85.3
	100% EtOH	110±6.1	11.4±0.43	0.95±0.04	12.6±0.4	9.8±0.21	950±14.6
	EtOH + 0.1% *Decalepis hamiltonii*	85.0±2.3	7.8±0.86	0.56±0.08	16.4±0.1	6.6±0.37	800±23.2
	P	< 0.01	<0.001	< 0.01	< 0.05	<0.001	< 0.01

ROS, Reactive oxygen species levels (μM DCF/mg protein); AchE, Acethylcholinesterase; CAT, Catalase specific activity (μM H2O2/min/mg protein); SOD, Superoxide dismutase specific activity (units/mg protein); GSH = Glutathione levels (μg GSH/mg protein); LPO = Lipid peroxidation levels (nM MDA/mg protein).

Table 2. Mean±SE of Endogenous Antioxidant Enzyme Activity, Oxidative Markers and AchE in Wild-type *Drosophila melanogaster* (Oregon K) Flies Exposed to Ethanol Showing Antioxidant Potential of DHA-I Treatment

Genotype	Diet	AchE	CAT	SOD	GSH	LPO	ROS
Wild-type (Oregon K)	Control	70±4.1	7±0.27	0.33±0.02	16±0.36	5±0.08	620±29.2
	EtOH	106±3.3	12.2±0.4	0.84±0.06	11.4±0.07	8.7±0.05	880±21.4
	EtOH + 0.5% *Decalepis hamiltonii*	80.0±4.7	7.2±0.62	0.51±0.02	17.0±0.9	6.2±0.84	770±48.6
	P	< 0.05	<0.01	<0.01	<0.01	<0.05	<0.01

ROS, Reactive oxygen species levels (μM DCF/mg protein); AchE, Acethylcholinesterase; CAT, Catalase specific activity (μM H2O2/min/mg protein); SOD, Superoxide dismutase specific activity (units/mg protein); GSH = Glutathione levels (μg GSH/mg protein); LPO = Lipid peroxidation levels (nM MDA/mg protein).

activity level observed in control flies. Similarly, exposure to EtOH also boosted CAT activity whilst *D. hamiltonii* treatment inhibited CAT enzyme activity similar to the flies without ethanol exposure. Flies exposed to ethanol showed an elevation in the AChE activity similar to *D. hamiltonii*-unfed flies. *D. hamiltonii* pre-feeding restored the activity of AChE to the levels observed in control flies. Statistical analysis of the results followed by Duncan post hoc comparison revealed that *D. hamiltonii* significantly ameliorated the neurotoxicity induced by ethanol in wild-type flies ($P = 0.0087$, n = 4).

Discussion

For fruit fly, alcohol (EtOH) serves as an environmental indication to locate decomposing fruits. The remarkable association between *Drosophila* and ethanol makes it a gorgeous model organism to elucidate the mechanisms underlying ethanol-induced behavioral responses. Numerous kinds of behavioral responses to ethanol are conserved from flies to mammals.[24] Hyperactivity and sedation are of common responses to acute alcohol exposure in both human and fruit fly.

The results of the present study revealed that ethanol exposure caused significant behavioral impairments, which were investigated by measuring ST50 and RC50 values. The findings of the present study represented significant elevation in the levels of ROS and LPO and marked depletion in GSH level in the flies exposed to ethanol. Moreover, flies exposed to ethanol showed increased activities of CAT, SOD, and AchE. These results imply the role of ethanol in inducing OS in flies as was evident by elevated levels of oxidative stress markers and significant alterations in the activity of antioxidant enzymes.

Excessive ethanol consumption induces OS and leads to damages to the brain. Neurotoxicity of ethanol goes together with production of ROS and a decrease in endogenous cellular antioxidant defense syetem.[3,25] Acute ethanol exposure causes elevation of membrane LPO in the brain which can be suppressed by antioxidant agents.[26] Acute EtOH intake also results in depression of glutathione and protein carbonyl formation which can be used as common markers of OS in the brain.[27,28] As one of the major metabolites in fruit fermentation is ethanol, fruit flies repeatedly encounter ethanol in their natural environment. *Drosophila* is a suitable model organism to explore ethanol neurotoxicity as the enzymatic mechanism of ethanol metabolization.[29] Many types of behavioral responses to EtOH including hyperactivity, sedation, alcohol tolerance, and alcohol preference are well known in fruit flies and mammals. It is evident that acute EtOH exposure induces hyperactivity in flies as evaluated by the higher speed of walking. Furthermore, flies display alcohol tolerance following repetitive exposure.[30] Many of dopaminergic neurons are concerned in ethanol-related mutilations in mammals as well as in *Drosophila*.[31]

As ethanol induces OS in the brain which leads to neurobehavioral impairments, natural antioxidants are helpful to ameliorate OS and therefore, can serve as defensive therapeutic agents for the treatment of neurodegenerative diseases. The roots of *D. hamiltonii* are used as general vitalizer in folk medicine in southern India. The root extract of *D. hamiltonii* is a cocktail of novel natural antioxidants. Several of them that have

been isolated and characterized are DHA-I (4-hydroxy isophthalic acid), DHA-II (14-aminotetradecanoic acid), DHA-III (4-(1-hydroxy-1methylethyl)-1-methyl-1, 2-cyclohexane diol), DHA-IV (2-hydroxy methyl-3-methoxy benzaldehyde), DHA-V (2,4,8-trihydroxy bicyclo[3.2.1]octan-3-one), and ellagic acid which have been shown to possess free radical scavenging activity in vitro.[11,12]

It is obvious from the present study that wild-type flies treated with *D. hamiltonii* aqueous extract exposed to ethanol demonstrated a lower frequency of mortality and marked reduction of behavioral impairments. The neuroprotective effect of *D. hamiltonii* was comparable to that of quercetin, zedoariaerhizoma, curcumin, and *Sanguisorba officinalis* with regard to negative geotaxis assay.[32] The ethanol-exposed flies showed remarkable induction of OS as shown by elevation of LPO and ROS, depletion of GSH, and altered antioxidant enzyme profile of flies. *D. hamiltonii* caused suppression of OS induced by ethanol as was evident by oxidative markers. GSH level in the flies treated with *D. hamiltonii* was considerably reduced, suggesting that ameliorative effect of *D. hamiltonii* can be credited to radical scavenging along with increased GSH synthesis. The activity of CAT and SOD enzymes were induced by ethanol in flies, which were reversed in *D. hamiltonii*-treated ones. These results indicated that *D. hamiltonii* modulated antioxidant pathways in flies by restoring their redox status. In this study, ethanol also caused an elevation in the activity of AchE and it can be considered as a strong evidence for defective dopaminergic neurotransmission. Exposure to *D. hamiltonii* extract decreased AchE activity and then protected *Drosophila* against the neurotoxic effect of ethanol. Therefore, neuroprotective potential of *D. hamiltonii* extract can be attributed to their ability to abrogate OS induced by ethanol in *Drosophila* brain. Since the brain is rich in oxidizable substrates and is poor in antioxidant defenses, it is susceptible to OS. Therefore, the neuroprotective efficiency of *D. hamiltonii* is evident by its ability to attenuate OS induced by ethanol, to restore AchE activity in the fly brain, to significantly improve the locomotion performance, and to raise survival rate after treatment with *D. hamiltonii* extract. To know the biochemical processes involved in behavioral impairments in *Drosophila* can be beneficial to explore new therapies enhancing the antioxidant defenses. The present study was the first report on neuroprotective efficiency of *D. hamiltonii* against OS induced by ethanol in *Drosophila* model organism.

OS has been implicated in the neurological dysfunctions of neurological diseases like PD, AD and Amytropic lateral sclerosis (ALS).[33] In the same scenario, OS has been shown to be a major factor in the onset of PD symptoms,[34] while enhancement of ROS level accompanies α-synuclein aggregation and consequent degeneration of dopaminergic neurons.[35] Intracellular overexpression of α-synuclein gene generates excessive ROS resulting in some damages to the cell membrane, mitochondrial dysfunction, and decline in the GSH level, all of which make the brain defenseless against oxidative damage.[36] Recent therapeutic managements of PD have been based on the exploit of natural antioxidants to diminish OS, besides the use of the therapeutic approaches to increase the activity of dopaminergic neurons to inhibit the cholinergic effects on the striatum.[37] For that reason, a powerful neuroprotective compound can prevent or postpone the onset of age-associated devastating conditions of PD patients.[38,39] Dietetic supplementation of antioxidant-rich plant in humans and animals have illustrated outstanding improvements in neuronal functions probably by the protection of susceptible neurons, raising functions of accessible neurons or promoting regeneration of neurons.[40] Consequently, natural antioxidants are capable of being the choice to modulate the OS; therefore, they are preserved as potential preventative therapeutic agents for the treatment of neurodegenerative conditions.[41,42] Natural antioxidants have been reported to operate as molecular inhibitors of α-synuclein misfolding and aggregation. Therefore, these agents could be achievable protective factors to decelerate the progression of PD. Current studies illustrate that antioxidants induce remodeling effects on α-synuclein aggregates to renovate them to nontoxic species[43]. The neuroprotective efficiency of antioxidant supplementation might be valuable in *Drosophila* model when applied in an earlier age.[44,45]

Conclusion

Our biochemical investigations showed a marked increase in the activity of CAT and SOD enzymes, remarkable reduction in GSH level and a significant increase in the levels of LPO and ROS and activity of AchE in wild-type flies exposed to ethanol. It is evident from the present study that the neuroprotective effect of *D. hamiltonii* against neurotoxicity induced by ethanol in wild-type flies was associated with their potential to modulate changes in the levels of oxidative stress markers and antioxidant defense system. Moreover, *D. hamiltonii* attenuated behavioral impairments induced by ethanol in wild-type flies.

Ethical Approval

Not applied.

Competing Interests

The author declares no competing interests.

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