ORIGINAL ARTICLE

Neuroprotective and Antioxidant Effects of *Benincasa hispida* Ethanolic Extract in D-Galactose-induced SH-SY5Y Cells

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Abstract

Aim: The present study aimed to evaluate the neuroprotective and antioxidant potential of *Benincasa hispida* ethanolic extract (BHE) against d-galactose-induced neurotoxicity in human neuroblastoma SH-SY5Y cells, an *in vitro* model relevant to Alzheimer's disease. **Materials and Methods:** SH-SY5Y cells were cultured and differentiated using retinoic acid, followed by induction of neurotoxicity with d-galactose (300 µM). Cells were pretreated with different concentrations of BHE. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. Oxidative stress markers and antioxidant enzymes, including reactive oxygen species, malondialdehyde assay, reduced glutathione (GSH), superoxide dismutase, catalase, Glutathione peroxidase (GPx), and neurotransmitter-related enzymes, were estimated using enzyme-linked immunosorbent assay-based methods. **Results:** D-galactose significantly reduced cell viability, increased LDH leakage, and elevated oxidative stress markers. Pretreatment with BHE dose-dependently improved cell viability, reduced LDH release, restored antioxidant enzyme levels, and reduced oxidative damage. Conclusion: BHE demonstrated significant neuroprotective and antioxidant effects against d-galactose-induced neuronal damage, suggesting its potential as a therapeutic candidate for neurodegenerative disorders.

Key words: Alzheimer's disease, *Benincasa hispida*, cell viability, *in vitro,* lactate dehydrogenase, MTT estimation

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative condition caused by the accumulation of myloid β (Aβ) and hyperphosphorylated Tau proteins. It is becoming increasingly prevalent globally. This disrupts synaptic connections and causes neuronal death..[1,2] Along with dementia, patients with AD also have memory loss, trouble doing daily tasks, and psychological problems like depression. Amnesia and cognitive decline are pathophysiological features of AD.[3] Neurons with aberrantly elevated phosphorylation neurofibrillary tangled structures and extracellular Aβ plaques have lesions in the brain's cortex and hippocampus.[4,5] Senile plaque formation and the buildup of neurofibrillary tangles are two of these AD signs that are thought to be triggers in neurodegenerative diseases. Senile plaques are actually frequently caused by Aβ deposition in glial and neuronal cells. Aβ aggregation can result in DNA damage, protein folding errors, oxidative damage, and disruptions to the cell membrane and cell cycle.[6] Aging is a contributing factor to the majority of neurodegenerative illnesses, and aging may be linked to oxidative damage, which increases with age and contributes to the pathophysiology of neurodegeneration. When redox equilibrium disturbs, oxidatively changed chemicals accumulate in neuronal cells and cause malfunction.[7] Cell death may come from weak defensive mechanisms and failure in highly intelligent cells such as neurons.[8] Another significant factor in the pathogenesis of AD is the rise in chronic neuroinflammation. By increasing Aβ aggregation and hyperphosphorylating Tau, overexpression of proinflammatory cytokines was also linked to neuronal death.[9] Furthermore, aberrant Aβ buildup speeds up the inflammatory process by boosting proinflammatory

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cytokine production and microglial cell proliferation and activation.[9,10] The scientific world has recently become more interested in natural substances that may have aging or cognitive benefits. BH, also called wax gourd, is a popular vegetable with a number of therapeutic uses. The Cucurbitaceae family includes it. Fresh seeds seemed to have the maximum antioxidant activity among the various portions of the wax gourd, including the pulp, core, and seed peel. Sanskrit writings claim that it helps with dyspepsia, epilepsy, insanity, neurological disorders, and other ailments. Some scientific examinations have been conducted to reveal anti-inflammatory, antioxidant, and anticonvulsant qualities. The primary components of the seeds include proteins, carotenes, vitamins, minerals, glycosides, saccharides, ß-sitosterol, uronic acid, and triterpenoids.[11]

MATERIALS AND METHODS

Method of cell culturing human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (ATCC) (USA) and cultivated according to the manufacturer's instructions. In a 5% carbon dioxide incubator, the cells were cultured in sterile 25 cm^2 flasks using Dulbecco's modified eagle medium, which contains 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin streptomycin. When the cells filled 80% of the flask, they were passaged using trypsin/ethylene diamine tetraacetic acid. 10 µM all-trans retinoic acid was used to differentiate the cells for 6 days before allicin treatment. Commercially available lyophilized Aβ1-42 (Sigma Aldrich) was produced utilizing the recommended preincubation approach to obtain the peptide aggregates required for neurotoxicity research. In 167 µL of molecular biological water, dissolve the lyophilized peptide to create peptide aggregates. The vial contained 833 µL of sterile PBS with a final concentration of 1 mg/mL–1 mL. Neuroblastoma cells in culture medium were treated with allicin at concentrations of 10, 50, and 100 µL.[12,13]

MTT assay

The vitality of the cells was examined using the MTT assay. In compliance with the kit protocol, MTT solution (Sigma-Aldrich) was added to every well. A spectrophotometer was used to measure the absorbance at 480 nm after formazan precipitate was dissolved in 150 µL of dimethyl sulfoxide following the incubation period.

LDH assay

Cytotoxicity was determined using the lactate dehydrogenase (LDH) test. LDH levels were determined using the manufacturer's instructions. The absorbance was determined at 450 nm.

Evaluation of oxidative stress

Human neuroblastoma SH-SY5Y cells were seeded in 75 cm2 flasks and treated as described previously. The cells were collected and lysed by sonication in ice-cold 50 mM potassium phosphate buffer, pH 7.4 containing 2 mM EDTA and 0.1 % Triton X-100. The cell homogenate was centrifuged at 13,0009g for 10 min at 4 C to remove cell debris. The resulting supernatants were analysed for total protein contents by Lowry's method (1951). Activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidise (GPx).

RESULTS

Invitro an analysis of ethanolic Benincasa hispida using SH-SY-5Y Cells

Estimation of cell viability in vitro assessment of MTT cell assay

From the figure 1. SH-SY-5Y cell lines were examined by the MTT assay. Dose dependent response was obtained between the range 75-150 µg/ml for plant extracts and donepezil (standard), expenses decreasing number of viable cells with increasing concentration of BHE as well as donepezil.

Protective effect of *Benincasa hispida* ethanolic (BHE) on d-galactose -induced cell viability in SH-SY-5Y cells

From figure 2. The SH-SY-5Y cells was treated with the increasing concentration of d-galactose (0.25, 0.5, 1.0, 2.0, 3.0 µg/ml) for 24 h. The d-galactose-induced toxicity by decreasing cell survival in a dose-dependent manner. The cell survival rate of 49.50 % was observed at 300 µM d-galactose insult and the same concentration was used in subsequent experiment. Additionally, the cells were pre-treated with 25–100 µg/ml of BHE for one hour in order to ascertain the protective effects of BHE against d-galactose-induced cognitive deficits. BHE pretreatment significantly reduced d-galactose-induced cell mortality, and after 100 µg/ml of BHE, viability was restored to 88.12% of control.

Graphically represented protective effect on BHE on d-galctose-induced in SH-SY-5Y

Protective effect of BHE on d-galactose-induced LDH leakage in SH-SY-5Y cells

From figure 3. LDH leakage assay results further corroborated these findings. Increasing concentrations of d-galactose (0.25,

0.5, 1.0, 2.0, and 3.0 µg/ml) were applied to the SH-SY-5Y cells for a duration of 24 hours. In a dose-dependent rise in LDH leakage, d-galactose caused toxicity by lowering cell survival; at 300 µMd-galactose, 31.75 % LDH leakage. On the other hand, cells pretreatment with 125 µg/ml BHE exhibited a reduction in LDH leakage from 31.75% of the total to 21.94 % of the group treated with d-galactose.

Estimation of LDH assay

LDH leakage was observed, Figure 2a. Conversely, cells pre-treated with 125 µg/mL BHE showed a decrease in LDH leakage from 31.75% of the total to 21.94% of the group treated with d-galactose. The results of the study suggest that BHE has neuroprotective effects because it corrected the d-galactose-induced cognitive impairment, shown in the Figure 3.

In vivo antioxidant assay

BHE antioxidant status of cells on d-galactose on Ache, CAT, and SOD

Illustrated at Figure 5. When exposed to d-galactose, the antioxidant enzymes that are present in cells, including Ache, CAT, SOD, MDA, GSH, GPX, GSH, ROS, and H_2O_2, demonstrated a drop-in activity of 5.850 ± 0.814, 0.525 ± 0.660, 0.650 ± 0.669, 11.790 ± 2.715, respectively. Significant protection was obtained after pre-treatment with three doses of BHE ($P < 0.05$). d-galactose toxicity significantly decreased the whole antioxidant state and cellular decline ($P< 0.05$).

BHE antioxidant status of cells on d-galactose on MDA, GSH, and GPx

In figure 6. After 24 hours of d-galactose exposure, there was a nearly complete drop in the levels of decreased 2.525

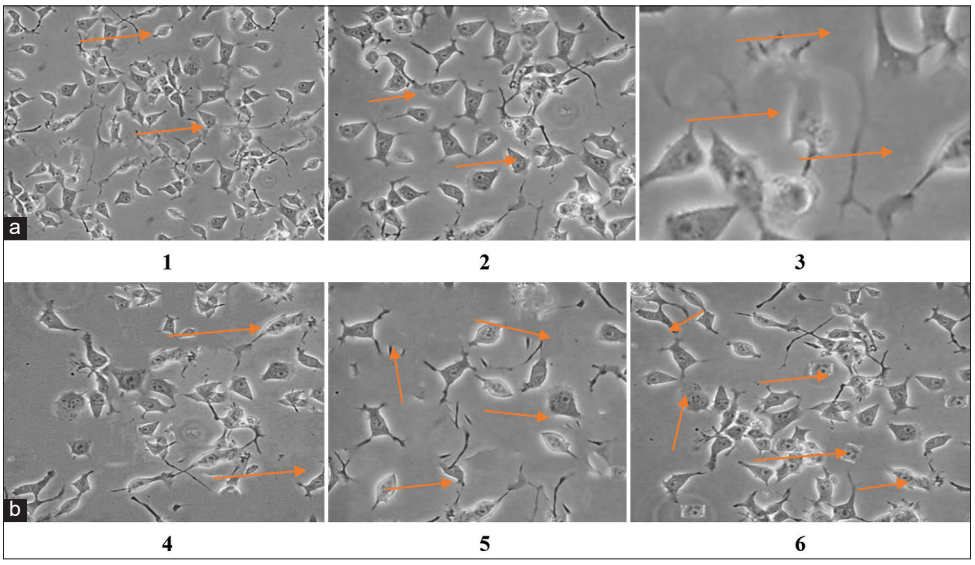

Figure 1: (a and b) Effect of *Benincasa hispida* ethanolic (BHE) on the d-galactose induced cognitive impairment: Representative pictures for each group (×100 magnification). (1) Control, (2) Donepezil, (3,) D-galactose 300 µM (4) BHE 75 µg/mL, (5) 100 µg/mL, (6) 150 µg/mL

Table 1: (a) BHE has a dose-dependent protective effect on SH-SY-5Y cells. (b) BHE dose-dependent protective activity against d-galactose-induced cytotoxicity

a		b	
Concentration	**Cell viability of D-gal**	**Concentration**	**Cell viability of extract**
0	114.023±3.162	0	114.023±1.020
25	87.140±2.641	25	55.160±1.364
50	75.075±1.927	50	66.330±0.413
75	62.455±2.489	75	73.208±0.761
100	56.580±2.171	100	80.258±0.869
125	49.505±1.507	125	88.128±0.628

The data presented are mean ± SEM of three independent experiments. $^{\#}P$ <0.05, $^{\#\#}P$<0.01, $^{\#}P$<0.001 and $^{*}P$<0.05 versus d-gal treated group. Graphical representation of cell viability. BHE: Benincasa hispida ethanolic extract; SEM: Standard error of mean.

Table 2: (a) D-galactose's dose-dependent impact on the viability of SH-SY-5Y cells. (b) BHE's dose-dependent protective activity against d-galactose-induced LDH in SH-SY-5Y cells

a		b	
LDH leakage	**d-gal**	**LDH leakage**	**Extract**
0	4.350±1.173	0	5.575±0.942
0.25	8.950±1.112	25	39.233±3.208
0.5	13.150±2.158	50	19.565±3.014
1	18.450±0.819	75	16.960±1.400
2	22.805±2.531	100	17.998±2.326
3	31.750±3.391	125	21.945±2.844

BHE: *Benincasa hispida* ethanolic extract, LDH: Lactate dehydrogenase

Table 3: Impact of BHE on AchE, CAT, and SOD activities in SH-SY-5Y cells induced by D-galactose

Group	Ache	CAT	SOD
Control (normal saline)	2.725±0.769	1.675±0.769	2.450±0.957
D-galactose 300 µM	5.850±0.814$^{###}$	0.525±0.660$^{###}$	0.650±0.669$^{###}$
Donepezil 2 µg/mL	2.575±0.576***	1.050±0.669***	1.950±0.669***
BHE 75 µg/mL+d-galactose 300 µM	2.925±0.660*	0.600±0.795**	1.200±0.532**
BHE 250 µg/mL+d-galactose 300 µM	3.325±0.721*	0.850±0.447**	1.625±0.721**
BHE 500 µg/mL+d-galactose 300 µM	3.900±0.865**	1.375±0.660**	2.000±0.700**

The data were obtained from three separate experiments mean±SEM was used to represent the data. *P<0.05, **P<0.01, ***P<0.001 in comparison to the d-gal model group and $^{###}P$<0.001 comparison to the control group. CAT: Catalyse, SOD: Superoxide dismutase, BHE: *Benincasa hispida* ethanolic, SEM: Standard error of mean

Table 4: d-galactose-induced MDA, GSH, and GPx activity in SH-SY-5Y cells is inhibited by BHE

Group	MDA	GSH	GPx
Control (normal saline)	26.508±2.285	12.498±3.140	3703.500±23.971
D-galactose 300 µM	11.790±2.715$^{###}$	2.525±0.931$^{###}$	3069.250±18.204$^{###}$
Donepezil 2 mg/kg	23.630±2.955***	10.708±1.594***	918.000±17.624***
BHE 150 µg/mL+D-galactose 300 µM	17.075±2.175**	5.550±0.908**	1699.250±18.263**
BHE 250 µg/mL+D-galactose 300 µM	19.970±2.158**	8.325±0.876**	1981.250±11.764**
BHE 500 µg/mL+D-galactose 300 µM	21.833±2.389**	9.100±1.165**	2649.750±31.449***

The data were obtained from three separate experiments mean ± SEM was used to represent the data. $^{#}P$ <0.05, $^{##}P$<0.01, $^{#}P$<0.001 and *P<0.05 versus d-gal treated group. SEM: Standard error of mean, BHE: Benincasa hispida ethanolic, MDA: Malondialdehyde assay, GPx - Glutathione Peroxidase, GSH - Reduced Glutathione.

Table 5: BHE's response to d-galactose-induced ROS, H_2O_2, and GST activity in SH-SY-5Y cells

Group	ROS	H_2O_2	GST
Control (normal saline)	17.520±2.328	18.080±2.168	87.770±2.014
D-galactose 300 µM	8.750±1.562$^{###}$	9.453±1.451$^{###}$	13.913±2.859$^{###}$
Donepezil 2 µg/mL	11.805±1.250***	16.923±2.268***	86.063±1.733***
BHE 150 µg/mL+D-galactose 300 µM	13.908±2.719**	12.700±1.355**	37.643±3.889**
BHE 250 µg/mL+D-galactose 300 µM	14.488±2.841**	14.610±2.795**	55.305±2.939**
BHE 500 µg/mL+D-galactose 300 µM	16.205±2.169***	13.978±2.594**	66.070±4.082**

The data were obtained from three separate experiments mean±SEM was used to represent the data. *P<0.05, **P<0.01, ***P<0.001 in comparison to the d-gal model group and $^{###}P$<0.001 comparison to the control group. SEM: Standard error of mean, ROS: Reactive oxygen species, GST: Glutathione S-transferase, BHE: *Benincasa hispida* ethanolic

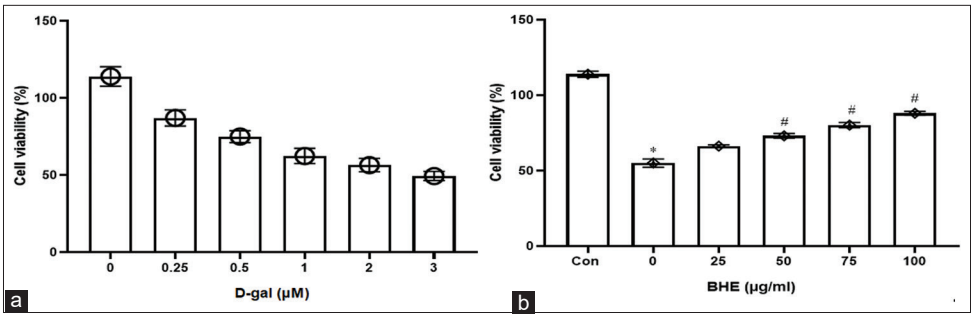

Figure 2: (a and b) d-galactose-induced cytotoxicity in SH-SY-5Y cells in a dose-dependent manner. The data presented are mean ± standard error of the mean of three independent experiments. **P* < 0.05 versus control group and #*P* < 0.05 versus d-gal treated group

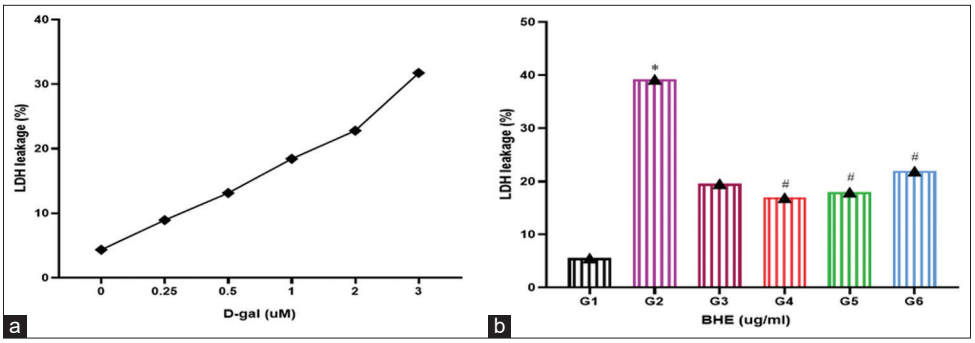

Figure 3: (a and b) d galactose's dose dependent effects and lactate dehydrogenase (LDH) leakage. The dosage of Benincasa hispida ethanolic determines its protective effect against d galactose induced LDH leakage in SH SY 5Y cells. The mean ± standard deviation of three different experiments was used to report the data. The data presented are mean ± standard error of the mean of three independent experiments. **P* < 0.05 versus control group and #*P* <0.05, ##*P*<0.01, #*P*<0.001 versus d gal treated group

± 0.931, and 3069.250 ± 18.204, MDA, GSH, and GPX respectively. After receiving BHE, the reduction in levels and antioxidant status increased. MDA levels in SH-SY-5Y cells treated to d-galactose (300 µM) for 24 hours were noticeably higher than those in the control group. As compared to the group treated with d-galactose, those treated with BHE at 150, 250, and 150 µg/ml showed a considerably lower rate of lipid peroxidation ($P < 0.05$).

BHE antioxidant status of cells on d-galactose on ROS, H_2O_2, and GSH

Shown figure 7. Exposure of SH-SY5Y cells to d-galactose resulted in noticeable morphological alterations in cell shape. Treatment with BHE improved the cellular morphology compared to the d-galactose-treated group. Upon exposure to 75 µg/ml, 100 µg/ml, and 150 µg/ml of BHE, a dose-dependent improvement was observed, with 150 µg/ml showing the most pronounced effect. This highest concentration demonstrated reduced cellular damage and better preservation of structural integrity. Furthermore, cells treated with BHE exhibited increased viability and more normal neuronal-like features. These observations indicate a potential protective role of BHE against d-galactose-induced cellular stress.

BHE antioxidant status of cells on d-galactose on DA, monoamine oxidase (MAO-A), and MAO-B

The Dopamine, MAO-A, MAO-B are treated against d-galactose drugs. We observed that, different range dose showed different effect. But when treated with BHE 75 µg/ml, 100 µg/ml, and 150 µg/ml, they are protected from d-galactose dose. All three-dose, 75 µg/ml, 100 µg/ml, and 150 µg/ml of BHE showed good effect on the SH-SY5Y cells. Additionally, the protective effect appeared to increase in a dose-dependent manner across the tested concentrations. Cellular viability assays further confirmed improved survival compared to untreated controls. These findings suggest that BHE may have potential neuroprotective properties against d-galactose-induced toxicity.

Effects of SH-SY-5Y cells an antioxidant activity in BHE on d-galactose

Illustrated at Figure 5. When exposed to d-galactose, the antioxidant enzymes that are present in cells, including Ache, CAT, SOD, MDA, GSH, GPX, GSH, ROS, and H2O2, demonstrated a drop-in activity of 5.850 ± 0.814, 0.525 ± 0.660, 0.650 ± 0.669, 11.790 ± 2.715, respectively. Significant protection was obtained after pre-treatment with three doses of

Table 6: BHE's effect on d-galactose-induced DA, MAO-A, and MAO-B activity in SH-SY-5Y cells

Group	DA	MAO-A	MAO-B
Control (normal saline)	15.833±2.475	55.593±3.351	28.078±4.679
D-galactose 300 µM	6.125±1.664###	45.095±2.516###	11.241±2.015###
Donepezil 2 µg/mL	10.668±2.127***	16.613±2.676***	16.608±1.879***
BHE 75 µg/mL+D-galactose 300 µM	13.225±1.791**	34.278±3.845**	19.723±3.386**
BHE 100 µg/mL+D-galactose 300 µM	15.355±2.374**	42.858±3.543**	21.168±2.560**
BHE 150 µg/mL+D-galactose 300 µM	17.010±2.010**	51.130±3.384**	22.530±0101**

The data were obtained from three separate experiments mean±SEM was used to represent the data. *P<0.05, **P<0.01, ***P<0.001 in comparison to the d-gal model group and ###P<0.001 comparison to the control group. SEM: Standard error of mean, BHE: *Benincasa hispida* ethanolic, MAO: Monoamine oxidase

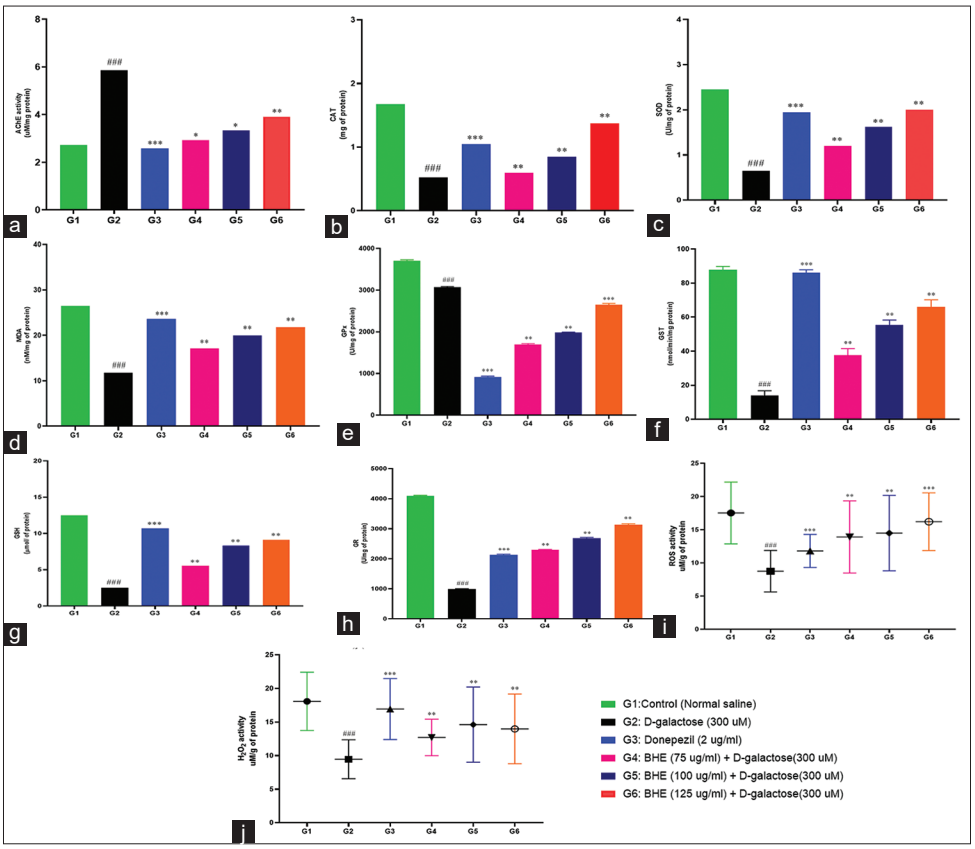

Figure 4: (a-j) Effect of *Benincasa hispida* ethanolic on SH SY 5Y cells against antioxidants produced by d galactose. The data were obtained from three separate experiments mean ± standard error of the mean was used to represent the data. #P < 0.05, ##P < 0.01, #P < 0.001 and *P < 0.05 versus d-gal treated group and *P < 0.05, **P < 0.01, ***P < 0.001 in comparison to the d gal model group and ###P < 0.001 comparison to the control group

BHE (P < 0.05). d-galactose toxicity significantly decreased the whole antioxidant state and cellular decline (P < 0.05).

DISCUSSION

MTT assay was used to evaluate SH-SY-5Y cell lines. The dose-dependent response for plant extracts and donepezil 6 (standard) ranged from 75 to 150 µg/mL. As the concentration of BHE and donepezil increased, the number of viable cells decreased.[14] Figure 1 displays the determined cell viability. SH-SY-5Y cells were exposed to escalating d-galactose concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 µg/mL) for 24 h to test for d-galactose-induced neurotoxicity. The toxicity caused by d-galactose decreased cell survival in a dose dependent way. At 300 µM d-galactose insult, a cell survival rate of 49.50% was noted, and the same concentration was employed in the experiment that followed, Figure 1a and Table 1. To determine the protective effects of BHE against d-galactose-induced cognitive deficits, the cells were also pre-treated with 25–100 µg/mL of BHE for an hour. BHE pre-treatment considerably, as shown in Figure 1b. decreased cell mortality caused by d-galactose, Table 2, and viability returned to 88.12% of control after 100 µg/mL of BHE. These

conclusions were further supported by the LDH leakage assay results. The SH-SY-5Y cells were treated with increasing d-galactose concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 µg/mL) for a full day. By reducing cell survival, d-galactose produced toxicity through a dose-dependent increase in LDH leakage; at 300 µMd-galactose, 31.75% LDH leakage was observed, Figure 2a. Conversely, cells pretreated with 125 µg/mL BHE showed a decrease in LDH leakage from 31.75% of the total to 21.94% of the group treated with d-galactose. The results of the study suggest that BHE has neuroprotective effects because it corrected the d-galactose-induced cognitive impairment, Figure 2b. Antioxidant enzymes found in cells,[15] antioxidant assay such Ache, CAT, SOD, MDA, GSH, GPX, GSH, ROS, and H2 O2 , showed drop-in activity of 5.850 ± 0.814, 0.525 ± 0.660, 0.650 ± 0.669, 11.790 ± 2.715, 2.525 ± 0.931, and 3069.250 ± 18.204, respectively, when exposed to d-galactose. Pretreatment with three doses of BHE resulted in significant protection ($P < 0.05$) .Tables 3, 5 and 6 show that d-galactose toxicity dramatically lowered antioxidant status and cellular deterioration ($P < 0.05$). After 24 h of d-galactose exposure, there was an almost complete drop in reduced CAT, SOD, MDA, GSH, GPX, and total antioxidant status. Following BHE treatment, levels decreased and antioxidant status improved. MDA levels in SH-SY-5Y cells treated with d-galactose (300 µM) for 24 h increased significantly compared to the control group. Treatment with BHE at 150, 250, and 150 µg/mL resulted in significantly decreased rates of lipid peroxidation ($P < 0.05$) compared to the d-galactose group.

CONCLUSION

The present study demonstrates that *Benincasa hispida* ethanolic extract exerts a significant neuroprotective effect against d-galactose-induced oxidative stress and cytotoxicity in SH-SY-5Y human neuroblastoma cells. Exposure to d-galactose markedly reduced cell viability, increased LDH leakage, and disrupted antioxidant defense mechanisms, confirming successful induction of neuronal injury. These changes mimic key pathological features associated with neurodegenerative disorders such as AD. Pretreatment with BHE significantly attenuated d-galactose-induced cytotoxicity in a dose-dependent manner, as evidenced by improved cell viability and reduced LDH release. Furthermore, BHE restored the balance of antioxidant enzymes, including SOD, CAT, GPx, and GSH, while reducing oxidative stress markers such as ROS, MDA, and hydrogen peroxide. The extract also modulated neurotransmitter-related enzymes and MAO activity, indicating a protective role in maintaining neuronal function. The observed neuroprotective effects of BHE may be attributed to its rich phytochemical composition and strong antioxidant properties. Overall, the findings suggest that *B. hispida* has promising therapeutic potential in preventing or slowing oxidative stress–mediated neuronal damage. However, further *in vivo* studies and clinical investigations are required to validate these effects and explore its applicability in neurodegenerative disease management.

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