

***Benincasa Hispida* Reversed D-galactose-induced oxidative Stress And Neurodegeneration-Mediated Cognitive Impairment In Aged Rats.**

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Research Article

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Abstract

Introduction: Age-related cognitive impairment is a common issue that is frequently brought on by oxidative stress and neurodegeneration in the brain. D-gal is a sugar moiety that has been linked to aging and cognitive disorders. This disease's prevalence is expected to rise linearly by 2050.

Material & methods: *Benincasa hispida*, commonly known as wax gourd or winter melon, is a widely consumed vegetable with various medicinal properties. It belongs to the family of Cucurbitaceae. This study evaluates the neuroprotective role of a hydro-ethanolic seed extract of *Benincasa hispida* (BH) against D-gal-induced aging in rats. Healthy male rats were divided into six groups: The control group received normal saline, donepezil (2 mg/kg b.w.t., i.p.); D-gal (300 mg/kg b.w.t., i.p.); D-gal + BH (150 mg/kg b.w.t., o.p.); D-gal BH (250 mg/kg b.w.t., o.p.); and D-gal BH (300 mg/kg b.w.t.).

Results and discussion: Behavioural, brain biochemical, and histopathological changes were assessed after treatment. The results of the behavioral study depicted that D-gal significantly reduces spontaneous alternation and locomotor activity, indicating behavioral and cognitive impairment. Biochemical studies showed that D-gal significantly increases oxidative stress in rat brains. Histopathological study showed that D-gal disturbs the normal architecture of hippocampal and cortical cells, indicating degeneration in these brain areas. D-gal and BH co-treatment for 42 days attenuated the behavioral, biochemical, and neuroanatomic damages caused by D-gal; it markedly suppressed the D-gal-induced oxidative stress and AChE activity in the brain and maintained the normal cellular architecture in hippocampal and cortical areas.

Conclusion: Thus, this study shows that BH can protect the brain from the adverse effects of D-gal (e.g., memory loss and cognitive impairment) by modulating oxidative stress.

1. INTRODUCTION

The natural aging process and neurodegenerative diseases are both thought to be significantly mediated by Cognitive impairment (CI), and oxidative stress [1]. Oxidative stress and neuroinflammation have been linked to different neurodegenerative illnesses like serious and mental diseases, such as AD (8.2%), stroke or cerebrovascular diseases (5.7%), and alcohol abuse (1.5%), Parkinson's (PD), Huntington's disease (HD), neuronal loss, and host defense in the brain [2]. According to data from a 2011 Indian report, the number of older people aged 60 or over will grow from 962 million to nearly 2.1 billion by 2050 [3]. D-galactose (d-gal) is a reducing sugar or monosaccharide found in milk, fruits, and vegetables that is normally transformed into glucose by galactose-1-phosphate uridylyl transferase and galactokinase [4]. Long-term d-gal treatment can result in an enzymatic excess that compromises the body's natural ability to convert galactose into glucose, increasing galactitol levels and activating aldose reductase. Also, in high concentrations, scientists believe that d-gal may react with long-lived proteins to create amino-fructose at an early stage, resulting in irreversible protein cross-linking to advanced glycation end products (AGEs) [5]. AGEs bind to their AGE receptors (RAGEs), activating intracellular inflammatory

signaling pathways and generating reactive ROS [6]. To fully comprehend the underlying mechanisms and precise specifics of apoptosis control, it is essential to comprehend the complicated interactions between the Bax and Bcl-2 proteins. Understanding the signaling pathways that activate and interact with these proteins may be helpful in developing specific drugs that might encourage apoptosis in increase cell survival in neurological diseases [7]. A few researchers have covered related similar species with different methodologies Scientists Mandana B, Russly AR, et al., 2012 explained the effect of different solvents including ethanol, n-hexane, and ethyl acetate on antioxidant activity and TPC of winter melon (*Benincasa hispida*) seeds extract was investigated using conventional Soxhlet extraction [8]. Bharti V, et al., In 2013 investigated only the memory and learning-improving effects of *Benincasa hispida* seeds in mice using the young animals treated with aqueous and methanol extract (200 and 400 mg kg⁻¹) [9]. Ambikar DB, Mohanta GP, In 2013 they investigated the BHM and BHA 400 mg/kg showed a significant (P < 0.01) [10]. Roy C., and Mazumdar D., In 2014 explained the aqueous pulp *Benincasa Hispida* of BH (400 mg/kg body weight) containing vitamin-A, C, and E [11]. Roy C, and Guha D., In 2017 explain the role of pulp extract of *Benincasa hispida* L. (BH) on hippocampal cell morphology in a colchicine-induced experimental rat model of Alzheimer's disease [12]. Rapaka D et al., In 2021 explain about AlCl₃ induction altered the behavioral profile and hippocampal regions of the brain and the treatment with *Benincasa hispida* extract at doses of 250 mg/kg/day (p < 0.001) in treatment groups [13]. Ambikar DB, and Mohanta GP, In 2021 investigated Co-treatment of D-galactose with BHP, BIM, and BIA (100 mg/kg, p.o) reduced the fluorescence product accumulated in the cerebral cortex [14]. Anbarasu U, in 2022 explained the effect of *Benincasa hispida* fruit dry powder on behavior changes and antioxidant levels in adult Wistar rats [15]. Additionally, the D-Galactose-induced rat model has been recognized worldwide and has been widely used in the study of aging and other fields. the Chronic intraperitoneal administration route, like the systemic method, can reverse cognitive deficits in a D-galactose model of aging/cognitive, thus the protective effects of this sugar may well be concentration or administration route dependent. Elevated D-galactose levels cause ROS formation and alteration in antioxidant enzyme activity in the brain, further inducing cognitive dysfunction, brain senescence, weakened motor function, and shortened lifespan to mimic natural aging in rodents. Since donepezil is a reactive metabolite that is metabolized by the CYP 450 isoenzymes 2D6 and 3A4 and undergoes glucuronidation with a well-known mechanism [16], D-galactose is thought to accelerate aging. Therefore, there is no controversy surrounding the use of d-gal via the intraperitoneal route. In recent years, natural compounds with potential aging/cognitive properties have gained attention in the scientific community. *Benincasa hispida*, commonly known as ash gourd, wax gourd, or winter melon, is a widely consumed vegetable with various medicinal properties. It belongs to the Cucurbitaceae family. *Benincasa hispida* is medicinally used in India, China, Indochina, and Malaya. Various wax gourd parts such as seed peel, core, and pulp show different levels of antioxidation capacity, and fresh seeds appeared to have the highest antioxidant activity. According to the Sanskrit texts, it is useful in insanity, epilepsy, nervous diseases, dyspepsia, and other constipation. Some scientific studies have been carried out to reveal anti-inflammatory, antioxidant, and anti-convulsant. The major constituents of these seeds are triterpenoids, flavonoids, glycosides, saccharides, proteins, carotenes, vitamins, minerals, β -sitosterol, and uronic acid [17]. This study was aimed at investigating the hydro-ethanolic seed extract that readily from fresh fruit seeds and performed antioxidant activities and

western-blotting assay with apoptosis markers, which have a main role in aging. The latter effect is the first such study on the fruit seed. The investigation of the antioxidant activity and biochemical assay includes DPPH, ABTS, H₂O₂, FRAP assay, and different free radicals including AchE, CAT, GSH, SOD, AOPP, MDA, GST enzyme, TT, NO, and GPx, respectively, analyzed CA1 region using H & E staining. as well as Bax/Bcl-2 marker studies have been covered. Recently, we have found the beneficial effects of *Benincasa hispida* (Indian, Budkumlikai) against hydro-ethanol-induced oxidative stress and aging, Hydro-ethanol neuronal apoptosis via Bax and Bcl₂ receptor intracellular signaling in the rat's hippocampus region. Therefore, in this study, we investigated the *Benincasa Hispida* reversed D-galactose-induced oxidative stress & and neurodegeneration-mediated cognitive impairment in aged Male rats. The study conclusions will contribute to the expanding figure of knowledge on natural compounds as therapeutic interventions for age-related cognitive decline.

2. Material and Methods

2.1. Material

2.2. Chemical and reagents

D-galactose, Sodium dodecyl sulfate, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Nitric oxide, Hematoxylin, and eosin stain, Hydrogen peroxide, Acetylcholinesterase, 5, 5'-di thio bis 2-nitro benzoic acid, Glutathione, Advanced oxidation protein products, Malondialdehyde, Glutathione S-Transferase enzyme, Total thiol, Glutathione Peroxidases, Diethyl ether, all chemical are purchased from Sigma-Aldrich.

2.3. Collection of plant specimen

Benincasa hispida fruit was collected in an area of the Nelamangala market in the month of May. The specimen was authenticated by a Pharmacognosiste professor Dr. Nandeesh, Sree Siddaganga College of Pharmacy, B.H. Road, Tumkur. specimen number SSCP/No/15/22.

3. Preparation of plant specimen

The fresh 200g seed of *Benincasa hispida* was ground using a mixer. The collected homogenate paste was mixed with (70:20 v/v) Hydroethanolic solvent for 72 hours by cold maceration process. The homogenate was filtered via muslin cloth followed by Whatman No. 1 filter paper and lyophilized to obtain a dry powder of seed extract. The lyophilized powder extract will be stored in air-tight amber-color bottles [18].

Yield percentage (w/w) from the dried extracts was calculated as: Yield % = $(W1 \times 100) \div W2$

4. Methods

4.1. Housing Condition & Ethical Statement

Thirty-six-week-old Wistar male rats (180–200 g) were used. They were maintained under standard laboratory conditions (temperature $25 \pm 2^{\circ}\text{C}$), relative humidity $55 \pm 5\%$, and a 12 h/12 h light/dark cycle. The animals were kept in standard vivarium conditions with free access to food and water *ad libitum* all the procedures are done according to OECD 423 guidelines [19]. The experimental animals were given proper care and handling according to the institutional animal ethics committee of the National Research Centre SACCP-IAEC/2022-02/69. This experiment was performed in Sri Adichunchanagiri College of Pharmacy, BG. Nagar-571448, Karnataka, India.

4.2. Selection dose

The dose range was selected from previous acute-chronic toxicity studies [20].

5. Experimental design/structure

The section content is available in Figure 1.

6. Experiment procedure

The study was performed on thirty-six, 10–12 weeks old male Wistar rats of weighing 230–260 g. The experimental animals were divided into six groups:

Group 1 – a control group (n = 6);

Group 2 – a group with Donepezil 2 mg/kg·b.wt. (n = 6);

Group 3 – a group with D-galactose 300 mg/kg·b.wt. (n = 6);

Group 4 – a group with *Benincasa hispida* 150 mg/kg·b.wt. + D-galactose 300 mg/kg·b.wt. (n = 6);

Group 5 – a group with *Benincasa hispida* 250 mg/kg·b.wt. + D-galactose 300 mg/kg·b.wt. (n = 6);

Group 6 – a group with *Benincasa hispida* 500 mg/kg·b.wt. + D-galactose 300 mg/kg·b.wt. (n = 6); After one week of acclimatization, the rats were randomly divided into six groups (n = 6) and administered with various doses as shown above for five consecutive weeks. In between behavioural parameters were assessed and at the end of the experimentation rat's brains were removed, rinsed in with ice-cold saline then isolated. Collected brains were kept at -80°C for molecular studies while the remaining brains were fixed in 10% formalin for H and E staining [21].

7. Parameter for Cognition

7.1. Actophotometer (ATPM) test

ATPM) test, Locomotion, and exploratory Behavior of rats were observed in a brightly illuminated square-shaped arena (50 cm × 50 cm). The animal was placed in the center and allowed to explore the open field for 5 min. The locomotor activity (path length, resting time) was assessed using an automated tracking system for animal locomotor activity. After each test, the arena was cleaned with 70% ethanol [22].

7.2. Rotar-rod (RR) test

In RR test animals were evaluated for grip strength and balance. Each rat was given a prior training session 1 week before the initialization of therapy to acclimatize them to the Rota rod apparatus. Each animal was placed on the rotating rod at a speed of 25 rpm. Three separate Trials were performed for each rat at 5-minute intervals, and the cutoff time of 180 s was maintained throughout the experiment. The average results were recorded as a fall in time [23].

7.3. Hanging wire (HW) test

The HW test was used to measure the gripping and forelimb strength of rats. In this test, animals were suspended by the forelimbs on a wire (60 cm long and 0.3 cm diameter) stretched between two posts 40 cm above a foam pillow. The length of time the rats were able to hold the wire was recorded. The cut-off time was taken as 90 s [24].

7.4. Novel object recognition (NOR) test

The NOR test was carried out for cognition deficits. The NOR device was made up of a Plexiglas box with dimensions of 75 × 75 cm, base × 40 cm, and height. Two different-shaped (cylindrical and square) objects were used for the object discrimination test. The objects were made up of plastic, between heights of 10 and 12 cm. One day before the trials, the rats were familiarized for 5 min in the device prior to the first NOR trial. During the acquisition trial, 5 min were given to rats to explore two similar cylindrical (A1 and A2) or familiar objects. After the acquisition trial, a delay of 30 min was observed before the retention trial. During the retention trial, one familiar object (A2) was substituted with a new object termed novel object (B) (square), and another 5 min was given for the rats to explore both objects. In order to minimize errors and biases, the novel objects were always placed at identical places to the previous familiar objects, and sides were also interchanged between experiments to avoid side preference. Object exploration was defined as the rat's nose being placed close to the objects at a distance ≤ 2 cm or the rat sniffing, licking, or touching the objects with forepaws while sniffing. The time taken by the rats to explore the two objects during the acquisition and retention phases of the test was recorded manually and separately with two stopwatches (by a trained observer). Its Discrimination Index (DI) was then calculated for the retention trial as $DI = B - A1 / B + A1$ (B = novel object, A1 = familiar-object) [25].

7.5. Forced swim (FS) test

The FS test is a common test to assess depressive-like behaviour. Therefore, the time the animals are immobile and not struggling to escape from the water is considered to be a correlate of depressive-like behaviour. The test consists of a training and a test session on two consecutive days. The rats were placed in a translucent plastic cylinder (20 cm in diameter and 45 cm in height filled with water up to a 33

cm level at a temperature of 25°C). The rats were placed in the cylinder for 10 min during the training and 5 min during the test session. Movements in the cylinder were recorded. The time (s) the animals spent immobile during training (FSIMTR) and during test (FSIMT) were used for the analysis [26].

8. Estimation for Cognition

8.1. Estimation of Acetyl cholinesterase (AChE) content in rats brain homogenate

The principle of the method is the measurement of the rate of production of thiocholine as acetyl thiocholine is hydrolysed. briefly, 0.1ml of 0.01M DTNB was added to 2.6ml of 0.1M phosphate buffer (pH 8.0). 0.04ml of brain homogenate was added to the above mixture followed by incubation for 5min. Then 0.04ml of substrate (0.075M acetylthiocholine iodide) was added to the reaction mixture. The readings were taken at 420nm continuously for 5min at 1min intervals. The results were expressed in $\mu\text{mol}^{-1} \text{ min}^{-1} \text{ mg protein}^{-1}$ using a molar extinction coefficient $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [27].

8.2. Estimation of Catalase (CAT) content in rats brain homogenate

The estimation is based on the property of Catalase to catalyses the decomposition of hydrogen peroxide to water. Briefly, to 0.95 ml of 10 mM H_2O_2 60 mM phosphate buffer (pH = 7.0), 50 μl of the brain tissue supernatant was added and the rate of degradation of H_2O_2 was followed at 240 nm per min. Catalase content in turns of U/mg of protein was estimated from the rate of decomposition of H_2O_2 using the formula $k = 2.303 / t \times \log (A_1/A_2) \text{ s}^{-1}$ (A unit of catalase is defined as the quantity which decomposes 1.0 mole H_2O_2 of per min at pH 7.0 and temperature 25°C [27].

$$\text{CAT (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.2. Estimation of Glutathione (GSH) content in rats brain homogenate

Determination of glutathione (GSH) was estimated by the method of Moron et al. (1979). To 0.5 ml of the brain homogenate tissue, 2 ml of 5, 5'-di thio bis 2-nitro benzoic acid (DTNB) was added and the volume made up to 3 ml with phosphate buffer. DTNB was reduced by the sulfhydryl compounds to form a yellow-coloured complex which was measured using a spectrophotometer (UV-1700, Shimadzu Ltd., India) at 412 nm. The concentration of GSH was calculated from the molar absorptivity value and the level was expressed as mmol/mg protein [28].

$$\text{GSH (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.3. Estimation of Superoxide dismutase (SOD) content in rats brain homogenate

SOD activity was measured based on the ability of SOD to inhibit spontaneous oxidation of epinephrine to adrenochrome at pH 10.2 we are measuring the concentration of adrenochrome at 295 nm Briefly, to 100 µl of brain homogenate add 0.8 ml of carbonate buffer (pH 10.2), incubate the above solution for 15 min initiate by adding 100 µl of adrenaline solution (1 mM). Record the change in absorbance at 295 nm (UV-1700, Shimadzu Ltd., India) for 5 min. One unit of SOD produces approximately 50% of auto-oxidation of adrenaline. Results were expressed as U/mg protein [28].

$$\text{SOD (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.4. Estimation of Advanced oxidation protein products (AOPP) content in rats brain homogenate

AOPP level was analyzed by the method of Witko-Sarsat et al. (1996). Brain homogenate (200ml) was diluted with phosphate buffered saline in a ratio of 1:3 and 100 ml of 1.15 M potassium iodide was added. The sample was incubated for 2 min followed by the addition of 200 ml of acetic acid. The absorbance of the reaction mixture was immediately read on a UV-vis Spectrophotometer at 340 nm (UV-1700, Shimadzu Ltd., India) against the blank containing 2000 ml of PBS, 100 ml of potassium iodide, and 200 ml of acetic acid. The concentration of AOPP was calculated using the extinction coefficient of 26 M/cm and the result was expressed as mM/mg protein [29].

$$\text{AOPP (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.5. Estimation of Malondialdehyde (MDA) content in rats brain homogenate

The assay is based on TBARS and malondialdehyde (MDA) a break down product of lipid peroxidation that reacts to give a chromogen (light pink color) species measured at absorbance maximum at 535 nm. The Estimation of lipid peroxidation (LPO) is based the on TBARS method by mix 1ml of the above supernatant with 2ml of TCATBA-HCL mixed thoroughly and heating for about 30min in a boiling water bath then cooled in an ice bath for 10min followed by centrifugation for a t6000rpm for 10min. The absorbance the of supernatant was measured at 532nm (UV-1700), Shimadzu Ltd., India). The amount of LPO was determined by using $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ as moles MDA/mg protein. Determined by using $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and the results were expressed as moles MDA/mg protein [29].

$$\text{MDA (in units)} = \frac{\text{Absorbance at 532 nm} \times 105}{1.56}$$

8.6. Estimation of Glutathione S-Transferase (GST) enzyme content in rats brain homogenate

The GST content was estimated calorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow et al. (1974). The supernatant was precipitated with 4% sulfosalicylic acid (4%) in the ratio of 1:1. The samples were kept at 4 C for 1 h and then subjected to centrifugation at 5000 rpm for 10 min at 4 C. The assay mixture consisted of 550 ml of 0.1 M phosphate buffer, 100 ml of supernatant and 100 ml of DTNB. The OD was read at 412 nm (UV-1700, Shimadzu Ltd., India) and the results were expressed as mmol of GST/g tissue [29].

$$\text{GST (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.7. Estimation of Total thiol (TT) content in rats brain homogenate

This assay is based on the reduction of 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, which gets oxidized to disulphide producing a yellow derivative (TNB) whose absorption is measured using a spectrophotometer at 412 nm. The concentration of thiol groups was determined by the method of Habeeb et al., 1972. To 0.2 ml of mitochondrial fraction, 1.5 ml of sodium phosphate buffer (0.08 M; pH 8), 0.5 mg/ ml of EDTA and 2% sodium dodecyl sulphate were added. After vortexing, 0.1 ml of 0.01% 5,5'-di thio bis (2-nitro benzoic acid) DTNB in sodium phosphate buffer (0.1 M; pH 8) was added and mixed. This was incubated at room temperature for 15 min and the absorbance of the resulting solution was measured on a UV-Vis spectrophotometer (UV-1700, Shimadzu Ltd., India), at 412 nm with equivalent concentration of bovine serum albumin (BSA) as standard. The concentration of thiol groups was calculated from the molar absorption co-efficient (13600 M⁻¹ cm⁻¹) and the final concentration was expressed as nM/mg of protein [29].

$$\text{TT (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.8. Estimation of Nitric oxide (NO) content in rats brain homogenate

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was estimated by using the principle of Griess reaction. The Griess Reagent System is based on the chemical reaction which uses sulphanilamide and *N*-1-naphthylethylenediamine di- hydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO₂ in a variety of biological and experimental liquid

matrices such as plasma, serum, and tissue homogenate. The Sulphanilamide Solution and NED Solution were allowed to equilibrate at room temperature (15–30 minutes). 0.5 ml of each experimental sample was taken for the test. 0.5 ml of the Sulphanilamide Solution was dispensed in to the respective test tubes containing all the experimental samples and varied concentrations of Nitrite Standard solutions (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μM). Incubated for 5–10 min at room temperature and protected from light. Using a pipette, 0.5 ml of the NED Solution was dispensed to all test tubes. A purple/magenta colour will begin to form immediately. Absorbance was measured within 30 min at 535 nm (UV-1700, Shimadzu Ltd., India) using UV-Visible spectroscopy. Construct the standard calibration curve Absorbance v/s Concentration of standard nitrite. The concentration of nitrite was measured by using straight line equation ($Y = mx + c$) and expressed as $\mu\text{M/g}$ of tissue [30].

$$\text{NO (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.9. Estimation of Glutathione Peroxidases (GPx) content in brain homogenate

The GPx activity assay was based on the method of Paglia and Valentine. tert-Butyl hydroperoxide was used as substrate. The assay measures the enzymatic reduction of H_2O_2 by GPx through consumption of reduced glutathione (GSH) that is restored from oxidized glutathione GSSG in a coupled enzymatic reaction by GR. GR reduces GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance at 340 nm due to NADPH consumption was measured in (UV-1700, Shimadzu Ltd., India). GPx activity was computed using the molar extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of GPx was defined as the amount of enzyme that catalysed the oxidation of 1.0 mmol of NADPH to NADP + per minute at 25.8°C [30].

$$\text{GPx (in units)} = \frac{\text{Absorbance (A-B)} \times 50}{\text{Absorbance (A)}}$$

8.10. Hippocampus tissues homogenates procedure

after the behavioural tests, all of the rats were anesthetized and sacrificed. The hippocampi of 6 rats from each group were excised and immediately frozen individually in liquid nitrogen. Then the hippocampus of each rat was dissolved on ice and immersed in ice-cold physiological saline. A 10% homogenate of the hippocampus was prepared. The supernatant was collected after centrifugation (3500 rpm for 10 min at 4°C) for various biochemical assays. All of the surgeries were performed under diethyl ether anaesthesia, and all effects were made to minimize suffering [31].

8.11. Hematoxylin and Eosin Staining

After the behavioural tests, all of the rats were anesthetized and sacrificed. the hippocampi of 5 rats from each group were fixed in 4% paraformaldehyde solution for 24 h. After routine tissue processing, the

tissues were embedded in paraffin. Then, 4 mm thick sections obtained from each paraffin block were stained with hematoxylin and eosin (HE) stain for histopathological evaluation. Finally, histological features of apoptotic cells in the hippocampi revealed by the HE staining were observed in an optical microscope [31].

9. Western-blotting studies

Processing of Tissue Samples and Protein Isolation

The 100–200 mg tissue harvested for the assay, washed tissue thoroughly in cold PBS and resuspended tissue in 300 µl of ice-cold RIPA buffer. Homogenized tissue with homogenizer until sample is fully homogenized and is completely smooth. The samples were incubated for 30 mins by gentle mixing every after 5 mins at 4°C. Centrifuged the sample at 10,000 x g for 15 minutes at 4°C in a cold centrifuge and supernatant collected and transferred to a clean tube on ice. Determined the sample protein concentration by extracting a portion of the sample.

9.1. SDS-PAGE and Western Blot procedure

The 50-µg protein sample from each cell lysate was mixed with 5X loading dye and heated for 2 mins at 95°C. Protein samples were loaded and separated on 12% and 15% SDS-PAGE gel using Mini protean Tetra cell (Bio-Rad). Nitrocellulose membrane (0.2 µM) was equilibrated in transfer buffer for 10 mins at RT. Protein transfer was done for 15 mins in Turbo Transblot (Bio-Rad) apparatus at 2.5 A and 25 V. Blot was blocked in 3% BSA in TBST for 1hr at RT followed by incubation with respective 1° Ab at appropriate dilutions overnight at 4°C. Blot was washed thrice with TBST for 5 mins at RT. Blot was incubated with 2° Ab (anti-Rabbit or anti-Mouse IgG- HRP) at dilution 1:10000 for 1 hr. at RT. Washed 3 times with TBST for 5 mins at RT. Blot was rinsed with ECL reagent (two component system) for 1 min in dark and image was captured between 0.5 sec to 15 secs exposure in Chemidoc XRS + imaging system (Bio-Rad).

10. Statistical analysis

The Values are expressed as mean ± SEM. Statistical difference in mean was analysed using one-way ANOVA followed by Tukey's multiple comparison tests. $p < 0.05$ was considered statistically significant.

11. Results and Discussion

11.1. *In-vivo* assay in Behavioural effects of the *Benincasa hispida*

11.2. Actophotometer test

Actophotometer provided with a digital counter, photocell and a light source were used to measure locomotor activity (horizontal movement) of animals [32]. The locomotor activity was measured as

actophotometric score per five min, In D -gal alone treated rats shows significantly ($^{###}p < 0.001$) decrease in locomotor activity when compared to normal control group. when treated with *Benincasa hispida* treatments show ($^{**}p < 0.01$, $^{***}p < 0.001$) when compared with the normal group and Donepezil significantly shows ($^{***}p < 0.001$) preserved locomotor activity score. Results are summarized in Table 1.

Table 1

Effect of *benincasa hispida* extract on D -gal induced cognitive impairment in actophotometer. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. $^{\#}p < 0.05$, $^{\# \#}p < 0.01$, $^{\# \# \#}p < 0.001$ compared to the normal control group and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the D -gal model group.

Group	1st consecutive week	3rd consecutive week	5th consecutive week
Control	138.63 $\pm$ 0.77	135.50 $\pm$ 0.61	141.33 $\pm$ 0.92
Donepezil 2mg/kg (Standard)	192.93 $\pm$ 3.35 ***	184.57 $\pm$ 2.69 ***	185.90 $\pm$ 1.71 ***
D -gal 300mg/kg (Inducer)	89.97 $\pm$ 1.21 $^{###}$	76.30 $\pm$ 0.72 $^{###}$	67.13 $\pm$ 0.50 $^{###}$
BH 150mg/kg	178.53 $\pm$ 0.56 **	188.83 $\pm$ 0.50 ***	136.50 $\pm$ 3.85 **
BH 250mg/kg	181.20 $\pm$ 1.20 ***	192.30 $\pm$ 0.74 **	197.00 $\pm$ 0.81 ***
BH 500mg/kg	187.53 $\pm$ 0.58 ***	195.50 $\pm$ 0.76 **	192.77 $\pm$ 0.56 **

11.3. Rotar Rod Test

the rotarod test is widely used to evaluate the motor coordination of rodents, and is especially sensitive in detecting cerebellar dysfunction [33]. In D -gal alone treated rats shows significantly ($^{###}p < 0.001$) drop in motor coordination when compared to normal control group. when treated with *Benincasa hispida* treatments shows restored motor coordination ($^{**}p < 0.01$, $^{***}p < 0.001$) when compared with the normal group and Donepezil significantly shows ($^{***}p < 0.001$) conserved motor coordination. Results are summarized in Table 2.

Table 2

Effect of *benincasa hispida* extract on D-gal induced cognitive impairment in rotor rod. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the normal control group and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the D-gal model group.

Group	1st consecutive week	3rd consecutive week	5th consecutive week
Control	91.6 $\pm$ 0.70	94.93 $\pm$ 0.42	94.90 $\pm$ 0.38
Donepezil 2mg/kg (Standard)	90.1 $\pm$ 0.67***	94.93 $\pm$ 0.59***	96.63 $\pm$ 0.37***
D-gal 300mg/kg (Inducer)	86.1 $\pm$ 0.96###	72.80 $\pm$ 0.4###	64.20 $\pm$ 0.54###
BH 150mg/kg	82.9 $\pm$ 1.11**	87.57 $\pm$ 0.57**	91.73 $\pm$ 0.43***
BH 250mg/kg	81.5 $\pm$ 0.43***	84.60 $\pm$ 0.38***	85.47 $\pm$ 0.25**
BH 500mg/kg	84.0 $\pm$ 0.75***	85.83 $\pm$ 0.42**	87.50 $\pm$ 0.41**

11.4. Grip Strength test

The Grip Strength test is used to evaluate neuromuscular disorders of muscle strength to assess motor function and deficiency [34]. In D-gal alone treated rats shows significantly (### $p < 0.001$) decrease in muscle strength and motor coordination when compared to normal control group. when treated with *Benincasa hispida* treatments shows bring back muscle strength and motor coordination (** $p < 0.01$, *** $p < 0.001$) when compared with the normal group and Donepezil significantly shows (*** $p < 0.001$) well-maintained muscle strength and motor coordination. Results are summarized in Table 3.

Table 3

Effect of *benincasa hispida* extract on D-gal induced cognitive impairment in Grip Strength test. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the normal control group and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the D-gal model group.

Group	1st consecutive week	3rd consecutive week	5th consecutive week
Control	140.83 $\pm$ 0.57	132.17 $\pm$ 0.52	134.03 $\pm$ 0.39
Donepezil 2mg/kg (Standard)	128.50 $\pm$ 0.53***	130.60 $\pm$ 0.41***	131.90 $\pm$ 0.34***
D-gal 300mg/kg (Inducer)	121.47 $\pm$ 0.39###	90.93 $\pm$ 0.59###	73.80 $\pm$ 0.41###
BH 150mg/kg	115.37 $\pm$ 0.70***	119.57 $\pm$ 0.35***	121.53 $\pm$ 0.37***
BH 250mg/kg	114.77 $\pm$ 0.40**	117.80 $\pm$ 0.63**	120.93 $\pm$ 0.57**
BH 500mg/kg	116.13 $\pm$ 0.29**	118.47 $\pm$ 0.45**	120.47 $\pm$ 0.39**

11.5. Noval object recognition (Time Spent Near Novel Object)

The Novel Object Recognition (NOR) task is used to evaluate cognition, particularly recognition memory, in rodent models of CNS illnesses. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one [35]. The D-gal group showed a significant (### $p < 0.001$) deficit in object recognition learning compared with control group. In contrast, the rats with *benincasa hispida* extract showed a significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) improvement in learning and memory in the object-placed recognition task. these results suggest that *benincasa hispida* administration can significantly ameliorate the memory ability of D-gal induced aging rats for time spent near novel object and discrimination index. Results are summarized in Tables 4 & 5.

Table 4

Effect of *benincasa hispida* extract on D-gal induced cognitive impairment in time spent near the object. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the normal control group and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the D-gal model group.

Group	1st consecutive week	2nd consecutive week	3rd consecutive week	4th consecutive week	5th consecutive week
Control	195.07 $\pm$ 0.98	184.60 $\pm$ 0.86	181.30 $\pm$ 1.27	185.53 $\pm$ 0.92	193.13 $\pm$ 1.20
Donepezil 2mg/kg (Standard)	177.83 $\pm$ 0.93***	168.23 $\pm$ 0.83***	157.00 $\pm$ 0.56***	163.83 $\pm$ 0.67***	188.90 $\pm$ 0.76***
D-gal 300mg/kg (Inducer)	180.20 $\pm$ 0.8###	164.20 $\pm$ 0.67###	142.43 $\pm$ 0.66###	135.27 $\pm$ 0.78###	126.07 $\pm$ 1.15###
BH 150mg/kg	178.97 $\pm$ 2.23***	165.43 $\pm$ 1.25***	171.23 $\pm$ 0.50**	165.33 $\pm$ 0.99**	170.43 $\pm$ 1.34*
BH 250mg/kg	170.07 $\pm$ 1.44**	174.20 $\pm$ 1.12**	178.10 $\pm$ 0.83**	184.57 $\pm$ 0.82**	188.47 $\pm$ 1.13**
BH 500mg/kg	185.37 $\pm$ 1.06**	180.50 $\pm$ 1.38**	189.70 $\pm$ 0.4**	191.63 $\pm$ 0.60**	195.70 $\pm$ 0.92***

11.6. Discrimination index

Table 5

Effect of *benincasa hispida* extract on D-gal induced cognitive impairment in discrimination index. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. # p < 0.05, ## p < 0.01, ### p < 0.001 compared to the normal control group and * p < 0.05, ** p < 0.01, *** p < 0.001 compared to the D-gal model group.

Group	1st consecutive week	2nd consecutive week	3rd consecutive week	4th consecutive week	5th consecutive week
Control	0.69 $\pm$ 0.13	0.68 $\pm$ 0.09	0.69 $\pm$ 0.10	0.63 $\pm$ 0.12	0.59 $\pm$ 0.09
Donepezil 2mg/kg (Standard)	0.58 $\pm$ 0.12***	0.67 $\pm$ 0.04***	0.55 $\pm$ 0.08***	0.60 $\pm$ 0.09***	0.61 $\pm$ 0.07***
D-gal 300mg/kg (Inducer)	0.55 $\pm$ 0.05###	0.43 $\pm$ 0.06###	0.50 $\pm$ 0.13###	0.37 $\pm$ 0.05###	0.26 $\pm$ 0.05###
BH 150mg/kg	0.47 $\pm$ 0.05***	0.53 $\pm$ 0.05***	0.61 $\pm$ 0.13***	0.54 $\pm$ 0.07**	0.45 $\pm$ 0.06*
BH 250mg/kg	0.63 $\pm$ 0.06***	0.61 $\pm$ 0.08**	0.63 $\pm$ 0.12***	0.63 $\pm$ 0.06**	0.64 $\pm$ 0.09**
BH 500mg/kg	0.72 $\pm$ 0.0***	0.72 $\pm$ 0.04**	0.74 $\pm$ 0.0**	0.69 $\pm$ 0.06**	0.75 $\pm$ 0.06***

11.6. Forced Swim Test (Escape Time Latency (ETL))

Forced Swim Test task was used to evaluate the spatial learning and memory ability of the rats [36]. The D-gal treated rats had impaired cognition and memory as indicated by the extended escape latencies across the successive days (### p < 0.001) compared with control group and spent a significantly decreased time in the goal area (** p < 0.05) compared with those of the control group during the test hidden sessions. However, rats in the D-gal treatment plus *benincasa hispida* administration group displayed remarkable increases in the time spent in the goal area (*** p < 0.001), and reduced escape latencies in the probe test (** p < 0.05) compared to those of the model group. These results indicate that the D-gal treated rats had impaired spatial learning and memory, while the administrated of extracts could recover the aged-related cognitive impairment caused by D-gal. Results are summarized in Tables 6 & 7.

Table 6

Effect of *benincasa hispida* extract on D-gal induced cognitive impairment escape time latency. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the normal control group and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the D-gal model group.

Group	1st consecutive week	2nd consecutive week	3rd consecutive week	4th consecutive week	5th consecutive week
Control	88.17 $\pm$ 0.50	77.80 $\pm$ 0.38	68.47 $\pm$ 1.24	62.17 $\pm$ 1.42	62.17 $\pm$ 1.42
Donepezil 2mg/kg (Standard)	70.74 $\pm$ 0.57***	67.40 $\pm$ 0.72***	60.80 $\pm$ 0.38***	52.93 $\pm$ 0.60***	44.13 $\pm$ 0.58***
D-gal 300mg/kg (Inducer)	86.33 $\pm$ 0.45###	64.47 $\pm$ 0.64###	51.80 $\pm$ 0.63###	42.53 $\pm$ 0.44###	64.47 $\pm$ 0.59###
BH 150mg/kg	78.30 $\pm$ 0.23***	75.50 $\pm$ 1.25**	46.93 $\pm$ 0.42**	30.10 $\pm$ 0.74**	26.63 $\pm$ 0.83**
BH 250mg/kg	71.23 $\pm$ 0.55***	67.87 $\pm$ 0.81***	37.43 $\pm$ 1.32*	42.20 $\pm$ 0.56*	28.50 $\pm$ 0.91**
BH 500mg/kg	60.83 $\pm$ 0.57***	53.00 $\pm$ 0.51**	27.53 $\pm$ 0.63**	22.53 $\pm$ 0.70*	19.87 $\pm$ 0.47*

11.7. Time Spent in Target Quadrant

Table 7

Effect of *benincasa hispida* extract on D -gal induced cognitive impairment time spent in target quadrant. Data are expressed as mean $\pm$ SEM (n = 6) statistical analysis by one-way ANOVA followed by Tukey's test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the normal control group and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the D -gal model group.

Group	1st consecutive week	2nd consecutive week	3rd consecutive week	4th consecutive week	5th consecutive week
Control	79.10 $\pm$ 0.52	74.43 $\pm$ 1.26	76.77 $\pm$ 1.04	80.80 $\pm$ 1.26	81.77 $\pm$ 1.22
Donepezil 2mg/kg (Standard)	69.67 $\pm$ 0.80***	60.17 $\pm$ 0.72***	72.20 $\pm$ 0.59***	85.83 $\pm$ 0.94***	92.17 $\pm$ 0.74***
D -gal 300mg/kg (Inducer)	75.53 $\pm$ 1.02###	64.20 $\pm$ 0.85###	58.00 $\pm$ 1.50###	46.97 $\pm$ 1.11###	20.60 $\pm$ 0.68###
BH 150mg/kg	86.30 $\pm$ 0.48***	70.80 $\pm$ 0.55**	75.63 $\pm$ 0.70***	49.37 $\pm$ 0.84**	56.90 $\pm$ 0.78**
BH 250mg/kg	76.63 $\pm$ 0.82***	59.77 $\pm$ 1.02**	63.83 $\pm$ 0.95**	32.93 $\pm$ 1.17**	46.97 $\pm$ 1.11*
BH 500mg/kg	75.17 $\pm$ 0.98***	54.53 $\pm$ 1.23**	41.53 $\pm$ 0.71**	29.73 $\pm$ 0.94**	27.03 $\pm$ 0.68**

12. Effects of *Benincasa hispida* extract on AchE content

Cholinesterase inhibitors function to decrease the breakdown of acetylcholine molecules once the communication between the neuron and the muscle cell is complete [37]. The D -gal group showed a significant (### $p < 0.001$) increase in AchE level in hippocampus tissues compared with control group. *Benincasa hispida* extracts administration showed significantly reversed the abnormalities in AchE activities compared with those of the control group (Fig. 2). This result indicated that *benincasa hispida* extract might ameliorate dysfunction of the cholinergic system.

12.1. Effects of *Benincasa hispida* extract on anti-oxidation activities

Oxidative stress and ROS have been proposed as being critical causes of aging. The anti-oxidative activity has been reported to be beneficial with respect to slowing this aging process [38]. In the present study, D -gal treatment caused notable oxidative damage, including a decrease in CAT, GSH-Px, SOD, Total thiol, GPx, and AOPP, as well as an increase in AchE, MDA, GST, and NO level in the hippocampus of rats, which was similar to the previous report [39]. CAT decomposes hydrogen peroxide (H_2O_2) into molecular oxygen and water [39] SOD converts superoxide anions into peroxides, which are converted into water by

CAT and GSH-Px. GSH-Px reduces toxic peroxide into nontoxic hydroxyl compound and preserves the structure and function of the cell membrane [39]. AOPP relies on the formation of highly reactive oxidant species, mainly hydroxyl radicals, which can react with harmful compounds until their degradation [40]. Thiol is a very important antioxidant in preventing oxidative stress-induced damage and protects the cell against oxidative stress [41]. MDA is a major biomarker that appears during the final stages of lipid peroxidation initiated by excessive ROS and indicates indirect impairment at the cellular levels [42]. *Benincasa hispida* treated with extracts significantly reserved the changes in CAT, GSH-Px, SOD, Total thiol, GPx, AOPP and MDA levels, suggesting that the neuroprotective effect of *benincasa hispida* scavenged ROS mainly by enhancing antioxidant activity and consequently decreasing lipid peroxidative damage. Moreover, NO plays a role in maintaining “antioxidant homeostasis” and impaired NO bioactivity is a pathogenic factor in aging [43]. We observed that accompanying with the increase of CAT, GSH-Px, SOD, Total thiol, GPx, and AOPP levels in the rat's hippocampal CA1 region, the activities of NO were significantly decrease by *benincasa hispida* treatment. Thise finding indicated that benincasa mobilized its protective mechanism to inhibit the overproduction of NO in the rat's hippocampus region. graphical representation of results is represented in the figure. 3.

12.1 *Benincasa hispida* Protects against d-Gal Induced Pyramidal Cells Loss in CA 1 Subregions of Hippocampus of Rats

The neuropathological features of hippocampal CA1 sections are presented in Fig. 4. The pyramidal cells in the CA1 region were tightly arranged and exhibited round nuclei in normal animals. Pyramidal neuron shrinkage and chromatin condensation of nuclei were also observed in D -gal model animals. Treatment with *benincasa hispida* results in an improvement in the structure of these neurons. Rats in the control group had full hippocampal neuron, which were arranged tightly and were morphologically intact. The pyramidal cells neurons presented round and large nuclei and clean nucleoli (Fig. 4.C & 4.STD). Widespread damage was visible in the hippocampi of the model group treated with D -gal (Fig. 4I). The intercellular space increase in size, and the cells were loosely arranged. The pyramidal neurons either presented a densely stained shrunken appearance with minimal cytoplasm or disappeared. However, when *benincasa hispida* extract was administrated after the D -gal was injected, the neuronal cells in the hippocampus were rescued (Fig. 4Lb-hb) compared with those of the D -gal. the neurons in the hippocampi of the rats treated with *Benincasa hispida* at the dose of 500mg/kg of appeared almost normal (Fig. hb).

12.2. Effect of *Benincasa hispida* in various organ using H & E Staining

The results of tissues stained with hematoxylin and eosin are shown in Fig. 5. The tissues of the kidney, liver, spleen, and heart were observed. These tissues in the control group showed cardiac muscle fibers cell bodies extending to a shuttle shape, a trend of parallel assembling into a beam, structure integrated, intercellular boundaries clear, packing closely, graduation distinct, clearly visible band, and intercalated

disc. However, these tissues in the aging model group showed cardiac muscle fibre plumping, structure fuzzy and twisted shortening, significantly widened interval, and obvious capillary vessels of myocardial interstitial congestion. After *benincasa hispida* treatment, the microstructures of rat's viscera were obviously improved, which was similar to those of the Vc group. These results indicated that D-gal induced changes in the morphology, number of hepatocytes, and neurons. Meanwhile, the *benincasa hispida* extracts has protecting effects on the microstructure of rat's viscera.

12.3. Benincasa hispida Protects against d-Gal Induced Pyramidal Cells Loss in CA 3 Subregions of Hippocampus of Rats

As shown in Fig. 6, the observation of CA 3 sub-region of hippocampus of rats of control group showed cells with well-defined nuclear membrane, clearly visible nucleolus and fewer abnormalities. Noticeable changes were observed in the CA 3 sub-region of hippocampus of rats of the model group, which included cells with indistinct nuclear membrane as well as no prominent nucleolus, besides being darkly stained. Further, the number of normal pyramidal cells were also reduced in the CA 3 sub-regions of the hippocampus in the model group of rats. Interestingly, these pathological changes observed in the hippocampus of model group of rats were altered in groups where D-gal were co-administered with donepezil 2mg/kg·b.wt. or *Benincasa hispida* at doses of 150, 250 and 500 mg/kg·b.wt. the results indicated that *Benincasa hispida* showed protective effect.

12.3. Effects of Benincasa hispida extract on expression levels of apoptosis-related proteins including Bax/Bcl-2, and GAPDH Western blotting analysis

Bcl-2 is an anti-apoptotic protein, and Bax pro-apoptotic proteins and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes used in comparisons of gene expression data [44–45]. It is documented that the Bax/Bcl-2ratio, and the levels are crucial mediators for aging progression. Bcl-2 family members have an effect on cell apoptosis by changing the permeability of the mitochondrial membrane. To further explore the neuroprotective mechanism of *Benincasa hispida* in D-gal treated rats, a western blot was used to detect the protein levels of Bax, Bcl-2 in the hippocampus. The western blot analysis revealed that D-gal led to a significantly increase in the Bax/Bcl-2ratio (Fig. 7A and B), levels in the model group compared with those of the control group. *Benincasa hispida* extract administration remarkably reduced the Bax/Bcl-2 ratio, and the levels compared with those of the D-gal treated rats. These results indicate that *Benincasa hispida* extract has the effects of anti-apoptosis and neuroprotection through regulating the expression of apoptosis-related proteins. The western blot analysis also showed that *Benincasa hispida* extracts has a neuroprotective effect on the rat aging model induced by D-gal.

Conclusion

The findings of this study suggest that *Benincasa hispida* seed extract possesses potential aging properties against cognitive impairment in aged rats. This effect may be attributed to its ability to alleviate oxidative stress, and neurodegeneration. Further research addressing the underlying

mechanisms and clinical trials are warranted to validate the efficacy of *Benincasa hispida* as a therapeutic option for age-related cognitive impairment.

Abbreviations

AD	Alzheimers diseases
PD	Parkinson's disease
HD	Huntington's disease
BH	<i>Benincasa hispida</i>
NADPH	Nicotinamide adenine dinucleotide phosphate
AGEs	Advanced glycation end products
ROS	Reactive oxygen species
TPC	Total phenolic content
FC	Folin-Ciocalteu method
M	Methanol
E	Ethanol
AlCl ₃	Aluminum chloride
AchE	Acetylcholinesterase
CAT	Catalase
GSH	Glutathione
SOD	Superoxide dismutase
AOPP	Advanced oxidation protein products
MDA	Malondialdehyde
GST	Glutathione S-Transferase enzyme
TT	Total thiol
GPx	Glutathione peroxidase
NO	Nitric oxide
H & E	Hematoxylin and eosin staining
NOR	Novel object recognition
HW	Hanging wire test
RR	Rotar-rod test
ATPM	Actophotometer test
FT	Force swim test

Declarations

Ethics Statement

The study was carried out in accordance with CPCSEA guidelines. The protocol was approved by the Institutional Animal Ethics Committee (approval no. SACCP-IAEC/2022-02/69).

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Conflict of interest

the authors declare that they have no conflicts of interest.

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References

1. Li XL, Tao X, et al. Cognitive-exercise dual-task intervention ameliorates cognitive decline in natural aging rats through reducing oxidative stress and enhancing synaptic plasticity. *Exp Gerontol.* 2022 Nov;169:111981. doi: 10.1016/j.exger.2022.111981. Epub 2022 Oct 18. PMID: 36270545.
2. Mani V, Rashed Almutairi S. Impact of levetiracetam on cognitive impairment, neuroinflammation, oxidative stress, and neuronal apoptosis caused by lipopolysaccharides in rats. *Saudi Pharm J.* 2023 Sep;31(9):101728. doi: 10.1016/j.jsps.2023.101728. Epub 2023 Aug 3. PMID: 37583755; PMCID: PMC10424214.
3. Malhotra RK, Manoharan N, Deo SS. The Trend and Prediction of Cervical Cancer Incidence in Delhi, India: An Age-Period-Cohort Analysis. *Asian Pac J Cancer Prev.* 2022 Aug 1;23(8):2787-2795. doi: 10.31557/APJCP.2022.23.8.2787. PMID: 36037135; PMCID: PMC9741874.
4. Umbayev B, Askarova S, Almabayeva A, Saliev T, Masoud AR, Bulanin D. Galactose-Induced Skin Aging: The Role of Oxidative Stress. *Oxid Med Cell Longev.* 2020 Jun 17;2020:7145656. doi: 10.1155/2020/7145656. PMID: 32655772; PMCID: PMC7317321.
5. He X, Yan C, et al. The preventive effects of probiotic *Akkermansia muciniphila* on D-galactose/AICl₃ mediated Alzheimer's disease-like rats. *Exp Gerontol.* 2022 Dec;170:111959. doi: 10.1016/j.exger.2022.111959. Epub 2022 Sep 21. PMID: 36152776.
6. Gill V, Kumar V, Singh K, Kumar A, Kim JJ. Advanced Glycation End Products (AGEs) May Be a Striking Link Between Modern Diet and Health. *Biomolecules.* 2019 Dec 17;9(12):888. doi: 10.3390/biom9120888. PMID: 31861217; PMCID: PMC6995512.

7. Zhang SJ, Su SH, Gao JF. Electroacupuncture improves learning-memory ability possibly by suppressing apoptosis and down-regulating expression of apoptosis-related proteins in hippocampus and cerebral cortex in immature mice with Alzheimer's disease. *Zhen Ci Yan Jiu*. 2020 Aug 25;45(8):611-6. Chinese. doi: 10.13702/j.1000-0607.200080. PMID: 32869569.
8. Mandana B, Russly AR, Farah ST, Noranizan MA, Zaidul IS, Ali G. Antioxidant activity of winter melon (*Benincasa hispida*) seeds using conventional Soxhlet extraction technique. *International Food Research Journal*. 2012 Feb 1;19(1).
9. Bharti V, Kiran SK, Singh S. Evaluation of the Memory and Learning Improving Effects of *Benincasa Hispida* Seeds in Mice. *Pharmacologia*. 2013;4(3):249-53.
10. Ambikar DB, Mohanta GP. Effect Of Dried Fruit Extract of *Benincasa Hispida* on Brain Behaviour in Laboratory Animals. *Journal Of Cell & Tissue Research*. 2013 Jan 1;13(1).
11. Roy C, Mazumdar D. A nootropic effect of *Benincasa hispida* on Ach and ChAT activity in colchicine induced experimental rat model of Alzheimer's disease: Possible involvement of antioxidants. *World Journal of Pharmaceutical Research*. 2014 Aug 25;3(9):1282-96.
12. Roy C, Guha D. Neuroprotective Role of *Benincasa Hispida* L. On Hippocampal Cell Morphology In Colchicine Induced Experimental Rat Model Of Alzheimer's Disease. 2017; 6(15),1067-1077.
13. Rapaka D, Bitra VR, Ummidi R, Akula A. *Benincasa hispida* alleviates amyloid pathology by inhibition of Keap1/Nrf2-axis: Emphasis on oxidative and inflammatory stress involved in Alzheimer's disease model. *Neuropeptides*. 2021 Aug;88:102151. doi: 10.1016/j.npep.2021.102151. Epub 2021 Apr 22. PMID: 33932860.
14. Ambikar DB, Mohanta GP. Neuroprotective Effect of Petroleum Ether, Methanolic and Aqueous Extracts of Fruits of *Benincasa hispida* on Lipofuscin genesis and Fluorescence Product in Brain of D-galactose Induced Aging Accelerated Mice. *Indian Journal of Pharmaceutical Education and Research*. 2021 Apr 1;55(2):S528-34.
15. Anbarasu U, Jayagowri K, Bhuvaneswari K. Effect of *Benincasa Hispida* Fruit Dry Powder On Behaviour Changes And Antioxidants Levels In Adult Wistar Rats: An Experimental Study. *Journal Of Clinical & Diagnostic Research*. 2022 Nov 1;16(11).
16. Zhang N, Gordon ML. Clinical efficacy and safety of donepezil in the treatment of Alzheimer's disease in Chinese patients. *Clin Interv Aging*. 2018 Oct 11;13:1963-1970. doi: 10.2147/CIA.S159920. PMID: 30349215; PMCID: PMC6187917.
17. Islam MT, Quispe C, et al. A Literature-Based Update on *Benincasa hispida* (Thunb.) Cogn.: Traditional Uses, Nutraceutical, and Phytopharmacological Profiles. *Oxid Med Cell Longev*. 2021 Dec 10;2021:6349041. doi: 10.1155/2021/6349041. PMID: 34925698; PMCID: PMC8683187.
18. Yusoff IM, Chua LS, Taher ZM. Valorization of fruit waste from Cucurbitaceae family: Profiling of phytoconstituent of *Benincasa hispida* and *Citrullus lanatus* rinds using ultrasound-assisted extraction. *Food Bioscience*. 2023 Feb 1;51:102190.
19. Tozlu ÖÖ, Türkez H, et al. Assessment of the neuroprotective potential of d-cycloserine and l-serine in aluminum chloride-induced experimental models of Alzheimer's disease: In vivo and in vitro studies.

20. Shakya A, Chaudhary SK, et al. Acute and sub-chronic toxicity studies of *Benincasa hispida* (Thunb.) cogniaux fruit extract in rodents. Regul Toxicol Pharmacol. 2020 Dec;118:104785. doi: 10.1016/j.yrtph.2020.104785. Epub 2020 Sep 22. PMID: 32976857.
21. Feng W, Liu J, et al. Alginate oligosaccharide alleviates D-galactose-induced cardiac ageing via regulating myocardial mitochondria function and integrity in mice. J Cell Mol Med. 2021 Aug;25(15):7157-7168. doi: 10.1111/jcmm.16746. Epub 2021 Jul 6. PMID: 34227740; PMCID: PMC8335675.
22. Tiwari A, Khera R, et al. Neuroprotective Effect of α -Mangostin in the Ameliorating Propionic Acid-Induced Experimental Model of Autism in Wistar Rats. Brain Sci. 2021 Feb 25;11(3):288. doi: 10.3390/brainsci11030288. PMID: 33669120; PMCID: PMC7996534.
23. Rakhunde PB, Saher S, Ali SA. Neuroprotective effect of *Feronia limonia* on ischemia reperfusion induced brain injury in rats. Indian J Pharmacol. 2014 Nov-Dec;46(6):617-21. doi: 10.4103/0253-7613.144920. PMID: 25538333; PMCID: PMC4264077.
24. Adebisi OE, Omobowale TO, Abatan MO. Neurocognitive domains and neuropathological changes in experimental infection with *Trypanosoma brucei* in Wistar rats. Heliyon. 2021 Oct 26;7(11):e08260. doi: 10.1016/j.heliyon.2021.e08260. PMID: 34765779; PMCID: PMC8571699.
25. Wang Q, Dong L, et al. Dammarane Sapogenins Improving Simulated Weightlessness-Induced Depressive-Like Behaviors and Cognitive Dysfunction in Rats. Front Psychiatry. 2021 Mar 26;12:638328. doi: 10.3389/fpsyt.2021.638328. PMID: 33841208; PMCID: PMC8032884.
26. Kelberman MA, Anderson CR, et al. Consequences of Hyperphosphorylated Tau in the Locus Coeruleus on Behavior and Cognition in a Rat Model of Alzheimer's Disease. J Alzheimers Dis. 2022;86(3):1037-1059. doi: 10.3233/JAD-215546. PMID: 35147547; PMCID: PMC9007891.
27. Arya A, Chahal R, et al. Acetylcholinesterase Inhibitory Potential of Various Sesquiterpene Analogues for Alzheimer's Disease Therapy. Biomolecules. 2021 Feb 25;11(3):350. doi: 10.3390/biom11030350. PMID: 33669097; PMCID: PMC7996600.
28. Jiang W, Yan Yu, et al. Single Point Mutation from E22-to-K in A β Initiates Early-Onset Alzheimer's Disease by Binding with Catalase. Oxid Med Cell Longev. 2020 Dec 24;2020:4981204. doi: 10.1155/2020/4981204. PMID: 33425208; PMCID: PMC7775154.
29. Chaâbane M, Koubaa M, et al. *Nitraria retusa* fruit prevents fluconazole-induced kidney injury in adult rats through modulation of oxidative stress and histopathological changes. Pharm Biol. 2017 Dec;55(1):1061-1073. doi: 10.1080/13880209.2016.1278455. PMID: 28198206; PMCID: PMC6130529.
30. Zhang C, Rodriguez C, et al. Age-dependent and tissue-related glutathione redox status in a mouse model of Alzheimer's disease. J Alzheimers Dis. 2012;28(3):655-66. doi: 10.3233/JAD-2011-111244. PMID: 22045490; PMCID: PMC4221633.
31. Pang K, Jiang R, et al. An App knock-in rat model for Alzheimer's disease exhibiting A β and tau pathologies, neuronal death and cognitive impairments. Cell Res. 2022 Feb;32(2):157-175. doi:

- 10.1038/s41422-021-00582-x. Epub 2021 Nov 17. PMID: 34789895; PMCID: PMC8807612.
32. Kaur A, Jaiswal G, Brar J, Kumar P. Neuroprotective effect of nerolidol in traumatic brain injury associated behavioural comorbidities in rats. *Toxicol Res (Camb)*. 2021 Jan 5;10(1):40-50. doi: 10.1093/toxres/tfaa100. PMID: 33613971; PMCID: PMC7885190.
33. Abhishek M, Rubal S, Rohit K, Rupa J, Phulen S, Gurjeet K, Raj SA, Manisha P, Alka B, Ramprasad P, Bikash M. Neuroprotective effect of the standardised extract of *Bacopa monnieri* (BacoMind) in valproic acid model of autism spectrum disorder in rats. *J Ethnopharmacol*. 2022 Jul 15;293:115199. doi: 10.1016/j.jep.2022.115199. Epub 2022 Mar 26. PMID: 35346813.
34. Adebisi OE, Omobowale TO, Abatan MO. Neurocognitive domains and neuropathological changes in experimental infection with *Trypanosoma brucei brucei* in Wistar rats. *Heliyon*. 2021 Oct 26;7(11):e08260. doi: 10.1016/j.heliyon.2021.e08260. PMID: 34765779; PMCID: PMC8571699.
35. Oberman K, Hovens I, de Haan J, Falcao-Salles J, van Leeuwen B, Schoemaker R. Acute pre-operative ibuprofen improves cognition in a rat model for postoperative cognitive dysfunction. *J Neuroinflammation*. 2021 Jul 8;18(1):156. doi: 10.1186/s12974-021-02206-y. PMID: 34238316; PMCID: PMC8265047.
36. Aliphon B, Dai T, Moretti J, Penrose-Menz M, Mulders WHAM, Blache D, Rodger J. A repeated measures cognitive affective bias test in rats: comparison with forced swim test. *Psychopharmacology (Berl)*. 2022 Dec 1. doi: 10.1007/s00213-022-06281-8. Epub ahead of print. PMID: 36450831.
37. Suleman Z, Engwa GA, Shauli M, Musarurwa HT, Katuruza NA, Sewani-Rusike CR. Neuroprotective effects of *Lippia javanica* (Burm.F.) Spreng. Herbal tea infusion on Lead-induced oxidative brain damage in Wistar rats. *BMC Complement Med Ther*. 2022 Jan 4;22(1):4. doi: 10.1186/s12906-021-03471-3. PMID: 34983490; PMCID: PMC8725456.
38. An Z, Yan J, Zhang Y, Pei R. Applications of nanomaterials for scavenging reactive oxygen species in the treatment of central nervous system diseases. *J Mater Chem B*. 2020 Oct 7;8(38):8748-8767. doi: 10.1039/d0tb01380c. PMID: 32869050.
39. Lv R, Dong Y, Bao Z, Zhang S, Lin S, Sun N. Advances in the activity evaluation and cellular regulation pathways of food-derived antioxidant peptides. *Trends in Food Science & Technology*. 2022 Apr 1;122:171-86.
40. Liu Z, Yao X, Jiang W, Li W, Zhu S, Liao C, Zou L, Ding R, Chen J. Advanced oxidation protein products induce microglia-mediated neuroinflammation via MAPKs-NF- κ B signaling pathway and pyroptosis after secondary spinal cord injury. *J Neuroinflammation*. 2020 Mar 20;17(1):90. doi: 10.1186/s12974-020-01751-2. PMID: 32192500; PMCID: PMC7082940.
41. de Souza Cardoso J, Baldissarelli J, Reichert KP, Teixeira FC, Pereira Soares MS, Chitolina Schetinger MR, Morsch VM, Farias Martins Filho AO, Duarte Junior HR, Ribeiro Coriolano FH, Spanevello RM, Stefanello FM, Tavares RG. Neuroprotection elicited by resveratrol in a rat model of hypothyroidism: Possible involvement of cholinergic signaling and redox status. *Mol Cell Endocrinol*. 2021 Mar 15;524:111157. doi: 10.1016/j.mce.2021.111157. Pub 2021 Jan 7. PMID: 33421531.

42. Hu DW, Zhang G, Lin L, Yu XJ, Wang F, Lin Q. Dynamic Changes in Brain Iron Metabolism in Neonatal Rats after Hypoxia-Ischemia. *J Stroke Cerebrovasc Dis.* 2022 Apr;31(4):106352. doi: 10.1016/j.jstrokecerebrovasdis.2022.106352. Epub 2022 Feb 10. PMID: 35152131.

43. Tripathi MK, Kartawy M, Ginzburg S, Amal H. Arsenic alters nitric oxide signaling similar to autism spectrum disorder and Alzheimer's disease-associated mutations. *Transl Psychiatry.* 2022 Mar 28;12(1):127. doi: 10.1038/s41398-022-01890-5. PMID: 35351881; PMCID: PMC8964747.

44. Aslan A, Beyaz S, Gok O, Parlak G, Can MI, Agca CA, Ozercan IH, Parlak AE. Royal jelly protects brain tissue against fluoride-induced damage by activating Bcl-2/NF-κB/caspase-3/caspase-6/Bax and Erk signaling pathways in rats. *Environ Sci Pollut Res Int.* 2023 Apr;30(17):49014-49025. doi: 10.1007/s11356-023-25636-y. Epub 2023 Feb 10. PMID: 36759409.

45. Cornett K, Puderbaugh A, Back O, Craven R. GAPDH in neuroblastoma: Functions in metabolism and survival. *Front Oncol.* 2022 Oct 4;12:979683. doi: 10.3389/fonc.2022.979683. PMID: 36267982; PMCID: PMC9577191.

Figures

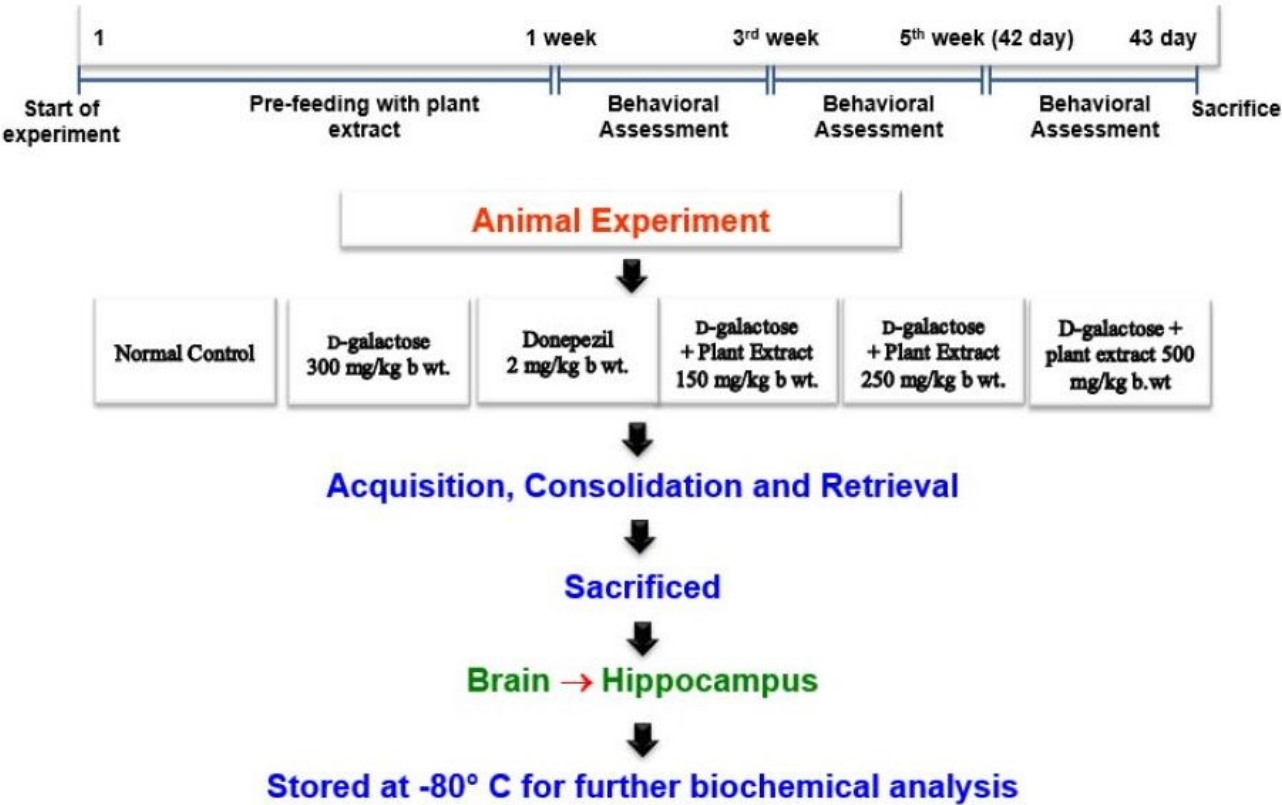


Figure 1

Experimental design

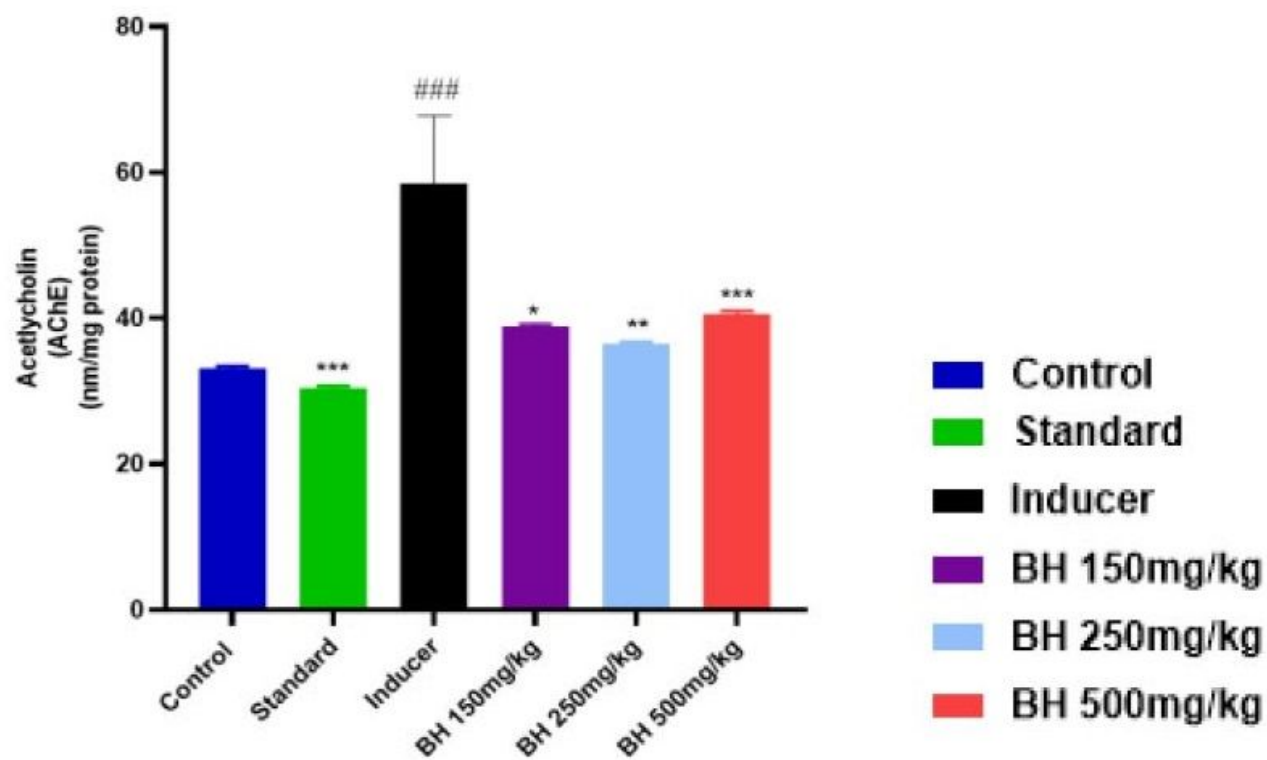


Figure 2

Effect of *benincasa hispida* extract on D-gal induced cognitive impairment acetylcholinesterase. Data are expressed as mean $\pm$ SEM (n=6) statistical analysis by one-way ANOVA followed by Tukey's test. # p <0.05, ## p <0.01, ### p <0.001 compared to the normal control group and * p <0.05, ** p <0.01, *** p <0.001 compared to the D-gal model group. Note: C) Control group; STD) donepezil 2mg/kg; I) D-gal 300mg/kg; BH) Low-dose of *Benincasa hispida* 150mg/kg; BH) Middle-dose of *Benincasa hispida* 250mg/kg; BH) High-dose of *Benincasa hispida* 500mg/kg extract group.

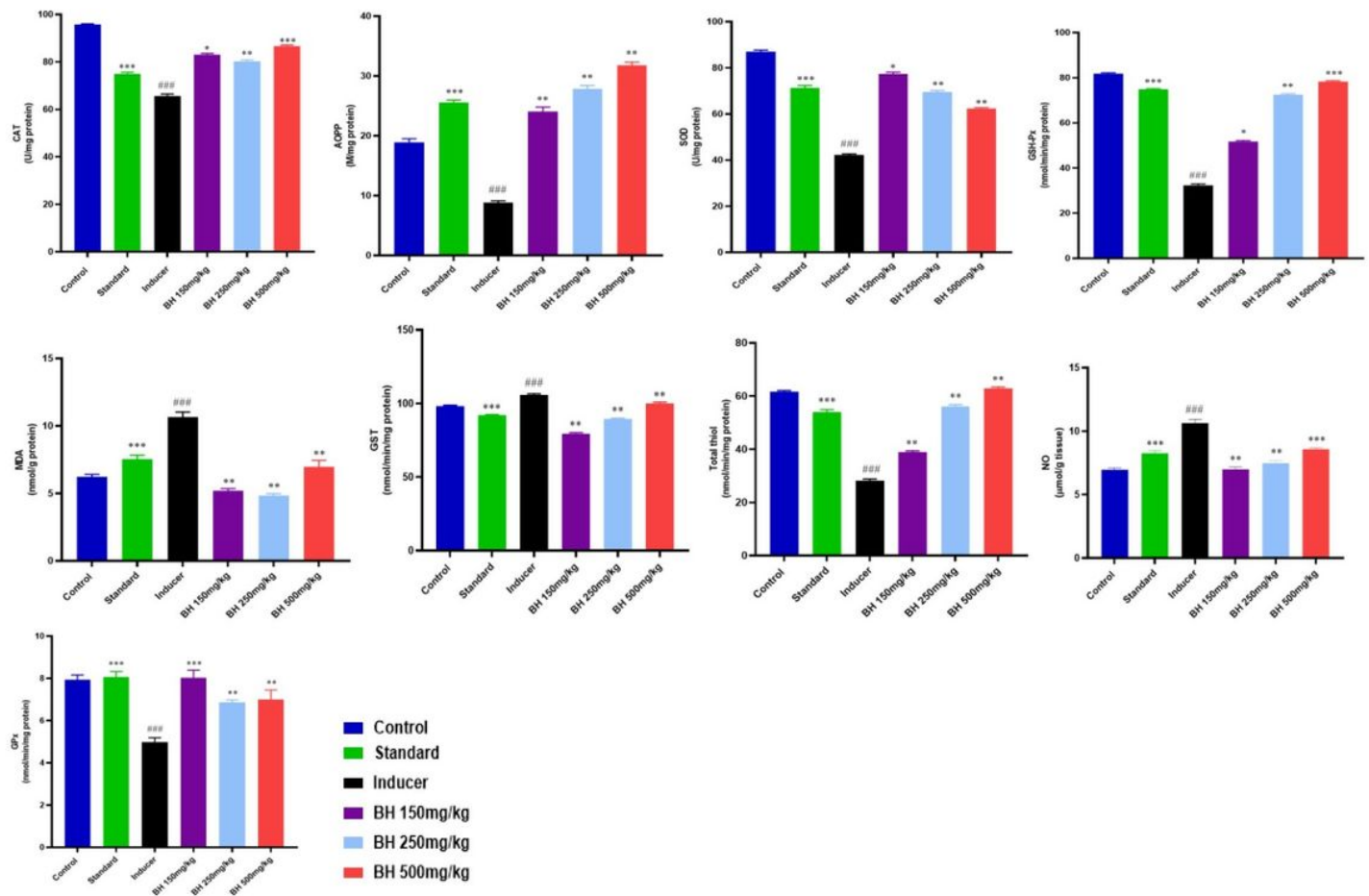


Figure 3

Effect of *benincasa hispida* extract on D -gal

induced cognitive impairment in AchE, CAT, GSH, SOD, AOPP, MDA, GST, TT, NO, and GPx activity. Data are expressed as mean $\pm$ SEM (n=6) statistical analysis by one-way ANOVA followed by Tukey's test. # p <0.05, ## p <0.01, ### p <0.001 compared to the normal control group and * p <0.05, ** p <0.01, *** p <0.001 compared to the D-gal model group. Note: C) Control group; STD) donepezil 2mg/kg; I) D -gal 300mg/kg; BH) Low-dose of *Benincasa hispida* 150mg/kg; BH) Middle-dose of *Benincasa hispida* 250mg/kg; BH) High-dose of *Benincasa hispida* 500mg/kg extract group.

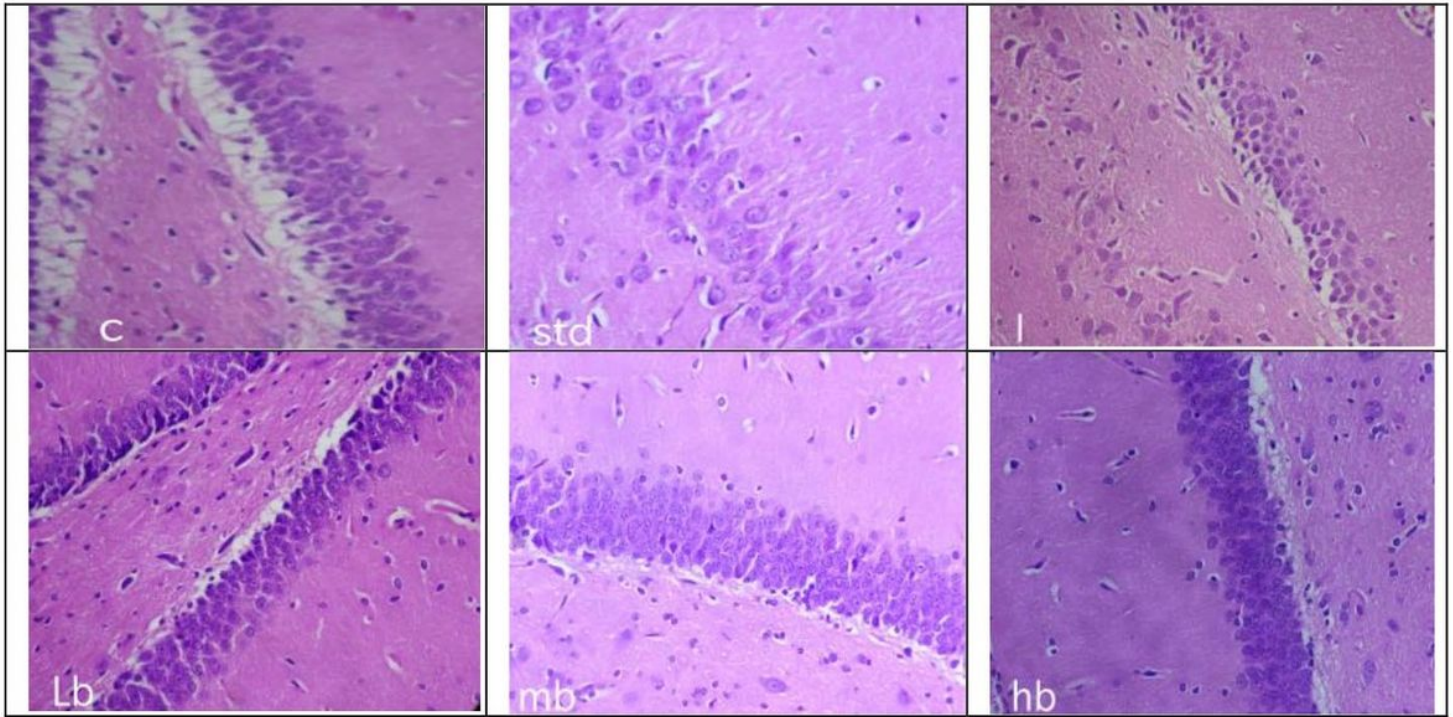


Figure 4

Protective effect of *Benincasa hispida* on the cell loss in the hippocampi of D-gal treated rats revealed by Hematoxylin and Eosin (HE) staining ($\times 200$). Note: C) Control group; STD) donepezil 2mg/kg; l) D-gal 300mg/kg; Lb) Low-dose of *Benincasa hispida* 150mg/kg; mb) Middle-dose of *Benincasa hispida* 250mg/kg; hb) High-dose of *Benincasa hispida* 500mg/kg extract group.

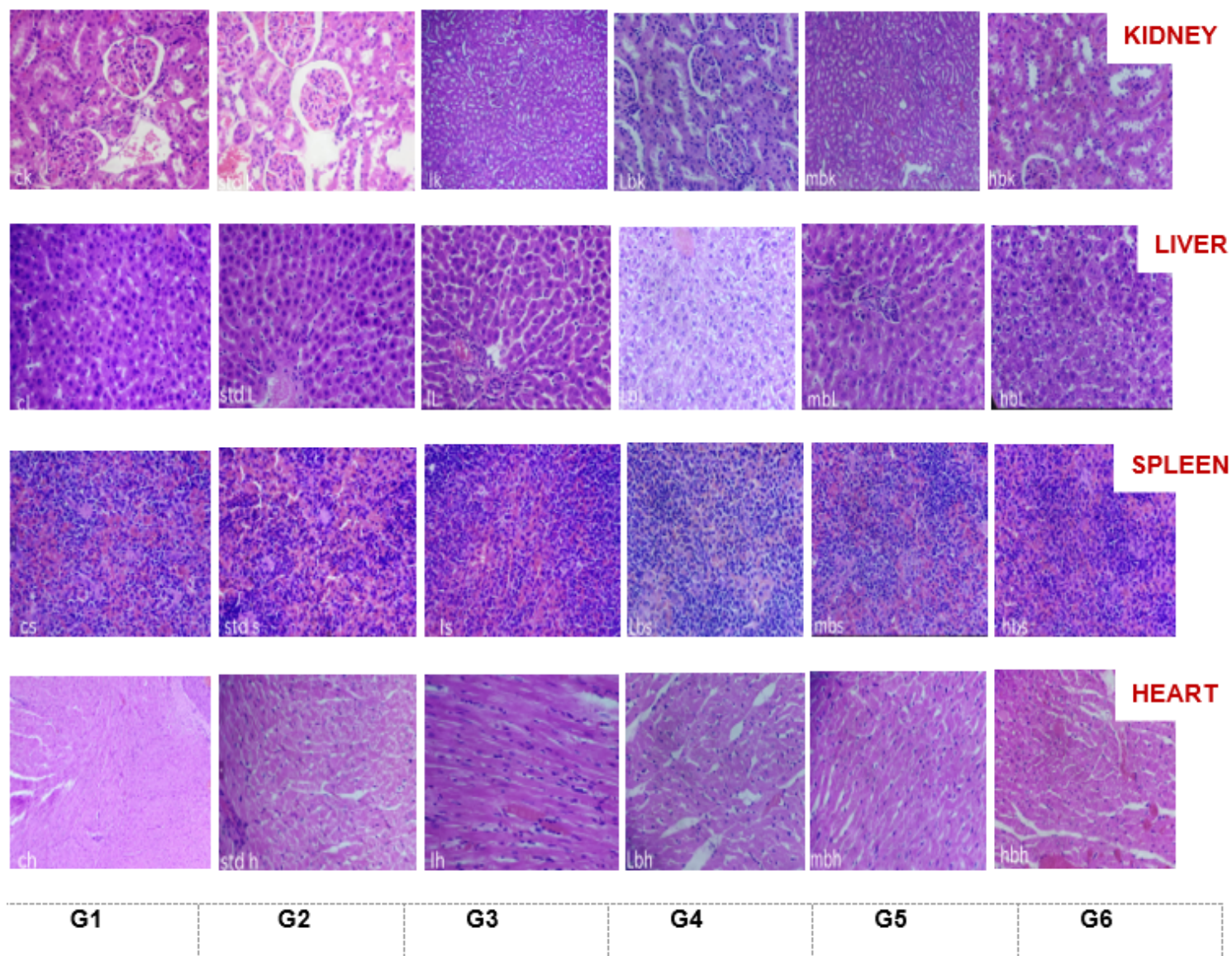


Figure 5

The Organs tissues stained with hematoxylin and eosin obtained from D-galactose-induced in rat: G1- Control group, G2- Donepezil 2mg/kg; G3- D-gal 300mg/kg; G4- Low-dose of *Benincasa hispida* 150mg/kg; G5- Middle-dose of *Benincasa hispida* 250mg/kg; G6- *Benincasa hispida* 500mg/kg extract group.

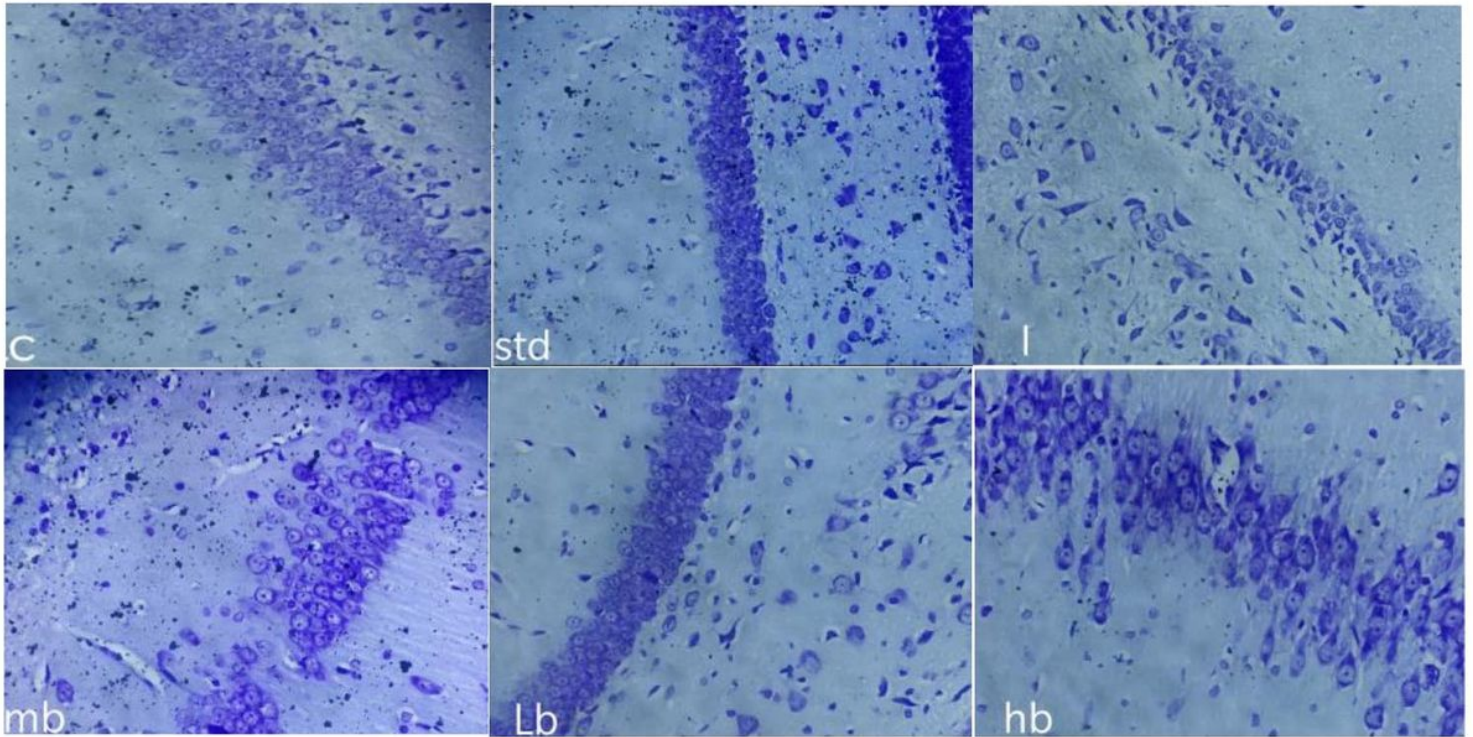


Figure 6

Protective effect of *Benincasa hispida* on the cell loss in the hippocampi of D-gal treated rats revealed by Hematoxylin and Eosin (HE) staining ($\times 200$). Note: C) Control group; STD) donepezil 2mg/kg; l) D-gal 300mg/kg; Lb) Low-dose of *Benincasa hispida* 150mg/kg; mb) Middle-dose of *Benincasa hispida* 250mg/kg; hb) High-dose of *Benincasa hispida* 500mg/kg extract group.

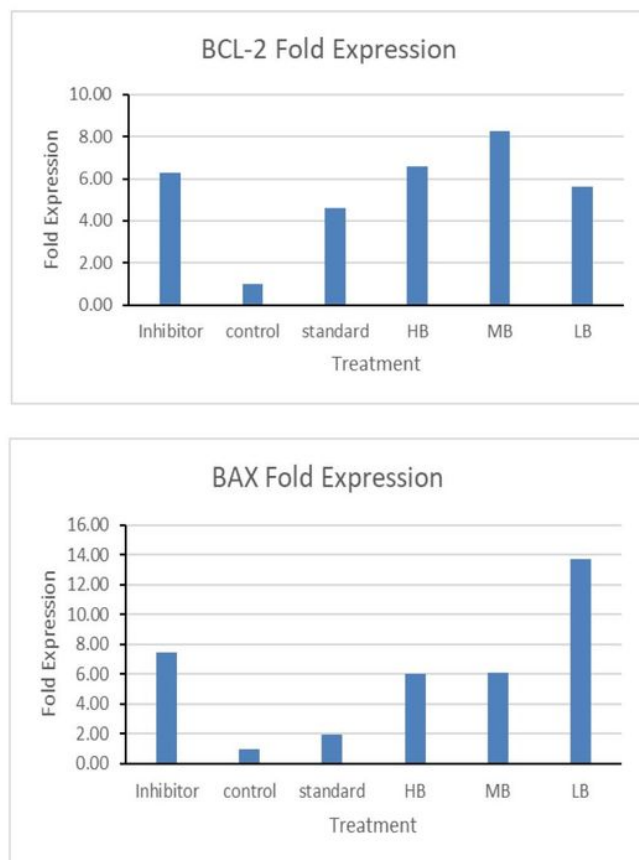
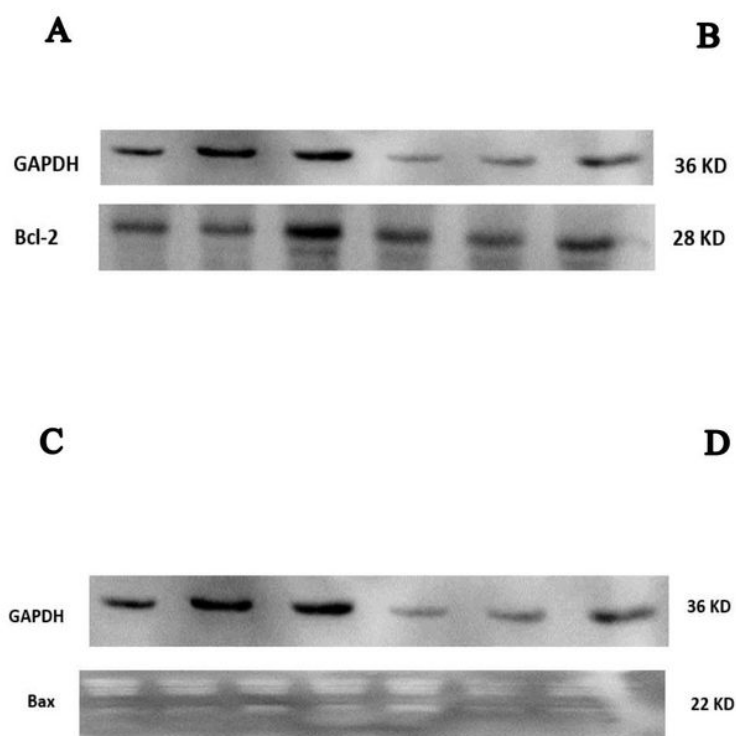


Figure 7

A. The effect of extracts on the expression levels of apoptosis-related proteins. Bax, Bcl-2 ratio (A/C); GAPDH/Bax (C/D); GAPDH /Bcl-2 (C/D) induced by d-gal are expressed as a ratio of GAPDH. Data are expressed as mean $\pm$ SEM (n=6) statistical analysis by one-way ANOVA followed by Tukey's test. # p <0.05, ## p <0.01, ### p <0.001 compared to the normal control group and * p <0.05, ** p <0.01, *** p <0.001 compared to the D-gal model group. NOTE: The band are from a representative blot. Loading details: Lane 1: Inhibitor (D-gal 300mg/kg); Lane 2: Control group; Lane 3: Standard (Donepezil 2mg/kg); Lane 4: HB (*Benincasa hispida* 500mg/kg); Lane 5: MB (Middle-dose of *Benincasa hispida* 250mg/kg); Lane 6: LB (Low-dose of *Benincasa hispida* 150mg/kg).