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Original Article

Cognitive-Enhancing and the Neuroprotective Propensity of Heartwood *Caesalpinia sappan* against D-Galactose-Induced Cognitive Impairments on Wistar Rats

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ABSTRACT

Background: We suggest evaluating heartwood *Caesalpinia sappan's* capacity to both protect neurons and improve cognition in Wistar rats by protecting them from the cognitive deficits caused by D-galactose. **Objectives:** The purpose of investigation was to identify *Caesalpinia sappan's* neuroprotective and cognitive-enhancing effects against D-galactose-induced cognitive impairments on Wistar rats. **Materials and Methods:** Cognitive impairment in rats was induced by injecting 2 mg/kg of d-galactose. Wistar rats received pretreatment with 150, 250 and 500 mg/kg of *Caesalpinia sappan* 1 hr after the d-galactose challenge. Behavioral impairment was assessed. Oxidative status and neurotransmitter levels were evaluated. Histopathology of the brain and other organs was done. Additionally, Immunoblotting assays were performed to assess apoptosis markers. **Results:** *Caesalpinia sappan* treatment effectively reduced the cognitive impairment problem in the rats. The levels of AchE, H_2O_2, ROS, NO, Nos and 5-TH synthesis were effectively reduced in brain tissue and showed improved cognitive problems in rats by hydroethanolic of heartwood *Caesalpinia sappan* treatment. *Caesalpinia sappan* attenuates the free radical index and ameliorates the antioxidant index in the brains of rats. The levels of neurotransmitters were increased in *Caesalpinia sappan*. The results of histopathological and immunoblotting analysis demonstrated the neuroprotective effects of hydroethanolic of heartwood *Caesalpinia sappan.* **Conclusion:** According to the current findings, *Caesalpinia sappan* pretreatment reduced cognitive impairment in rats by acting on their neurotransmitters and antioxidant levels. Consequently, it is feasible to conclude that hydroethanolic of heartwood *Caesalpinia sappan* could be a useful treatment for cognitive deficits.

Keywords: Alzheimer's disease, Behavioural parameters, *Caesalpinia sappan*, Histopathology analysis, Immunoblotting studies, *In vivo* studies, Neurotransmitters.

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INTRODUCTION

Cognitive impairment is a reciprocal kind of dementia, taking into consideration an estimated 60-80% of cases globally.[1] Brain condition that affects many elderly persons, Alzheimer's Disease (AD) gets worse with time.[2] Since the disease typically affects those 65 years of age and beyond, it poses a severe public health risk as the world's population ages.[3] Understanding the prevalence and characteristics of cognitive impairment is critical to developing thorough care plans, early detection methods and effective treatments for those who have the illness and their family.[4] Amyloid-β (Aβ) aggregation formation and tau protein hyperphosphorylation are frequently associated with the pathogenesis of AD, leading to Neurofibrillary Tangles (NFTs) and synaptic dysfunction.[5] Acetylcholine shortage was suggested as the major etiology of AD by early studies that formed the cholinergic deficit theory of the disease.[6] One of the earliest acetylcholinesterase inhibitors used to increase acetylcholine levels was tacrine. Galantamine, rivastigmine and donepezil-drugs in the same class-have since undergone more research.[7] Unfortunately, the safety profile of these drugs is poor and they have a multitude of undesirable side effects along with limited palliative benefits. The medication donepezil is approved to treat cognitive impairment.[8] Research has demonstrated that donepezil enhances cognitive abilities and overall functioning in individuals suffering from mild-to-moderate AD, with sustained benefits lasting up to 50 weeks.[9] Flavonoids are found in many plant-based foods and offer a variety of health advantages.[10] Each offers unique health benefits, highlighting the significance of a well-rounded diet for optimal intake of these beneficial compounds, both of which are important in triggering the

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pathogenesis of AD.[11] Flavonoids have the potential to prevent neurodegenerative disorders because can cross the blood-brain barrier.[12] *Caesalpinia sappan* (CS), called Brazil or Sappan wood, belongs to the Leguminosae family. *Sappan Caesalpinia* Heartwood, a dark red tint admired for its rich crimson hue, is often used for furniture, musical instruments and decorative things made of wood. Heartwood decoction can be used to treat a range of ailments, such as heart difficulties, obesity, stomach aches, burning sensations, high blood pressure, cataracts, ear infections and traumatic diseases. There are numerous chemicals in heartwood, including those known to possess anti-inflammatory and antioxidant qualities are flavonoids, triterpenoids, tannins, linoleic acid, phenols, saponins, brazilin and Brazilein. With its combination of therapeutic and decorative qualities, sappan wood heartwood is an important natural resource. Numerous reports of activity exist.[13] Nonetheless, the beneficial effects of hydroethanolic extract of heartwood *Caesalpinia sappan* on D-galactose have not been studied yet. Thus, the present effort was aimed at discovering the neuro-protective Propensity properties of *Caesalpinia sappan* against D-galactose-induced cognitive impairment in Wister rats.

MATERIALS AND METHODS

Experimental Design

Male Wistar Rats 180- 200 g of 36-week aged animals were used. Standard laboratory conditions provided for the animals, including a 12 hr light / dark phase, a temp of 25±2°C and a moisture level 55±5%. Normal pellets and water *libitum* were obtainable to them. After being acclimatized, the rats were alienated into six groups (n=6) and administered different treatments for five consecutive weeks. d-gal, donepezil and CS were all dissolved 0.9% in Normal saline. Group I received: 0.9% NaCl solution as a control. Group II received: 2 mg/kg·bwt standard drug donepezil after 1 hr of the challenge. Group III received: d-galactose 300 mg/kg·bwt dissolved in 0.9% saline and was administered intraperitoneally. Group IV received: 150 mg/kg·bwt *Caesalpinia sappan* orally 1 hr after receiving the d-galactose treatment and Group V received: 250 mg/kg·bwt *Caesalpinia sappan* orally 1 hr after receiving the d-galactose treatment. Group VI received: 500 mg/kg·bwt *Caesalpinia sappan* orally 1 hr after receiving the d-galactose treatment. Following the d-galactose challenge for 30 min, the rats were carefully observed to identify external behavioral changes. On the last day of the experiments, the rats were numb with a 1% ketamine injection to isolate brain tissues. Brains were dissected, washed in ice-cold NaCl solution and stored at -80°C for biomolecular searches but left-brain samples remained fixed in formalin solution 10% for Haematoxylin plus Eosin, crystal staining, immunoblotting study.

Brain Homogenates and staining assay

A hippocampal brain soft tissue homogenate with a 10% concentration was made. Centrifugation at 3500 rpm for 10 min at 40°C, Brain soft tissue supernatant was kept for use in a variety of biochemical tests and examinations tissues were paraffin-embedded, 4 mM parts were stained and histo-pathology examinations were made. An optical microscope was then used to observe the necrosis cells in the soft brain tissues highlighted by H and E stain.

Cognitive-Behavioral Parameter Measurement

Actophotometer assessment: The exploratory behavior of the animal was observed in an explained four-sided-shaped arena 50 cm×50 cm. Hanging assessment: assessed to evaluate the holding and forelimb muscles of animals. Rotarod assessment: Grip strength and balance are evaluated using the Rota rod apparatus. Morris water maze assessment: assesses mainly depressive-like behavior characteristics in animals. Noval object recognition assessment: asses for understanding deficits. The Plexiglas box that comprised the NOR device measured 75x75 cm in height, 40 cm in base and 40 cm in measurement. Discrimination Index.[14,15]

Measurement of Biochemical Estimations

Acetylcholinesterase enzyme activity

Briefly, 2.6 mL of 0.1M inorganic Na_3PO_4 pH 8.0 mixed with 0.1 mL of 0.01M DTNB. After adding 0.04 mL of brain soft tissue supernatant to the mixture above, it was kept for 5 min. Subsequently, the reaction mixture was supplemented with 0.04 mL of substrate 0.075 Macetylthiocholine Iodide. For 5 min, measurements at 420 nm were obtained continuously at intervals of one minute.[16]

Free Radical Scavenging

Reactive oxygen species

Briefly, 1 mL of PBS, pH 7.0 and 50 mg of brain soft tissue supernatant were homogenized. 15 min at 4°C were spent centrifuging the brain tissues were homogenized at 10,000 g. 1.25 mM DCFH-DA in ethanol added toward 190 µL of supernatants (10 µg protein) in methanol. Every sample underwent a 15 min dark incubation period at 37°C.

Hydrogen peroxide

Briefly, 0.2 mL of sample at varying concentrations of 12.5-150 µg/mL, 0.2 mL of EDTA 1.04 mmol L^{-1}, 0.2 mL of $FeCl_3$ 1 mmol L^{-1} and 0.2 mL of 2-deoxy-d-ribose 28 mmol L^{-1} were mixed. The mixture was kept at 37°C hot bath and the action was initiated by adding 0.2 mL of CS and 0.2 mL of H_2O_2 10 mmol L^{-1}. After 1 hr, 1.5 mL of cold TBA 10 g L^{-1} and 1.5 mL of HCl 25% were added, then intense at 100°C for 15 min and cooled. Absorbance intensity determined at 532 nm.[17]

Catalase

Briefly, 0.95 mL of 10 mM H_2O_2 in 60 mM Na_3PO_4 pH 7.0 and 50 µL of brain soft tissue supernatant was combined. The H_2O_2

degradation rate was monitored at 240 nm/min. Catalase activity was calculated using k=2.303/t×log(A1/A2) s^{-1}, with one unit described as the amount that decomposes 1 mole of H_2O_2 per min at pH 7.0 and 25°C.

Glutathione

Briefly, 2 mL of DTNB to 0.5 mL of soft brain tissue supernatant, Na_3PO_4 was added to get the amount up to 3 mL. The sulfhydryl mixtures reduced DTNB to produce a yellow-colored compound, which was detected at 412 nm.

Superoxide dismutase

Briefly, 0.8 mL of CO_3^{2-} buffer pH 10.2 and 100 µL of brain soft tissue supernatant, incubate the mixture for 15 min. add 100 µL of epinephrin solution 1 mM. For 5 min, note the modification in absorbance at 295 nm.

Advanced oxidation protein products

Briefly, Potassium iodide 1.15 M was added to 200 mL of phosphate-buffered saline 1:3 dilution. 200 mL of acetic acid was added after 2 min. With a blank consisting of 2000 mL of Phosphate buffer solution, 100 mL of PI and 200 mL of acetic acid, the absorbance was measured at 340 nm.

Malondialdehyde

Briefly, 1 mL of the aforementioned brain soft tissue supernatant and 2 mL of TCATBA-HCL was well combined, heated for approximately 30 min in a hot water bath, cooled for 10 min in an ice-chilled bath and then centrifuged 10 min at 6000 rpm. At 532 nm, the supernatant's absorbance was calculated.

Glutathione S-Transferase

Briefly, Sulfosalicylic acid 4% was blended with brain soft tissue supernatant in a 1:1 ratio. After an hour next to 4°C, the test was centrifuged at 5000 rpm for 10 min. The assay comprised 100 µL of supernatant, 100 µL of DTNB and 550 µL of 0.1 M phosphate buffer, with absorbance measured at 412 nm.

Total thiol

Briefly, 0.5 mg/mL EDTA, 2% SDS and 1.5 mL of Na_3PO_4 0.08 M pH 8 mixed. Then, 0.1 mL of 0.1 M Na_3PO_4 pH 8 with 0.01% DTNB was added. After, 15 min at room temperature, measure at 412 nm.

Nitric oxide

Briefly, 0.5 mL of sulphadiazine solution mixed with 0.5 mL of each brain soft tissue supernatant and the nitrite standards and it was then incubated in the dark for 5-10 min. After that, the mixture turned purple magenta after the addition of 0.5 cc of NED Solution. Within thirty min, the absorbance at 535 nm was observed.

Glutathione Peroxidases

Briefly, one unit of GPx is specified as the enzyme number required toward react 1.0 mmol of NADPH to NADP+ in one tiny min at 25°C. The relationship between NADPH consumption and absorbance at 340 nm was assessed.[18,19]

Measurement of Neurotransmitters (Dopamine, Serotonin, GABA, MAO-A and MAO-B)

After slaughtering the rats on day forty-three, brain samples were immediately collected and placed on ice. Following cleaning in a cold buffer, the samples were weighed within 5 min. Serotonin levels were assessed and dopamine content was identified. MAO activity was measured spectrophotometrically with minor adjustments. Protein content was determined using the Lowry method.[20-22]

Immunoblotting Studies

Immunoblotting tests will be used to confirm the protein expression levels of selected proteins based on the gene expression profile that was generated from the experiment. In line with our previous research, whole mitochondrial and cytosolic protein fractions were detached on SDS-PAGE and then relocated onto a nitro-cellulose tissue using an electroblotting device. Following a transfer, the membranes were incubated at 37°C for a whole night while specific proteins from the genes were probed at a 1:1,000 dilution. The tissues were kept at room temperature for 2 hr with secondary antibodies at 1:10,000 and were diluted after being cleaned four times in TBST for 15 min. After another wash, the membranes were created with an improved chemiluminescence sensing method. Film with X-rays was subjected to developed membranes.

RESULTS AND DISCUSSION

Cognitive-behavioral parameter

Measurement of Actophotometer, Hanging wire and Rota-rod, Morris's water maze

The analysis of heartwood from HEE of *Caesalpinia sappan* treated with different doses significantly mitigated cognitive impairments in the treated animal groups compared to those treated with d-galactose. These results indicate that HEE of *Caesalpinia sappan* has potential cognitive protective effects similar to the standard drug donepezil in rats administered d-galactose. The treated group 250 and 500 mg/kg showed significantly improved cognition compared to 150 mg/kg when compared to the d-gal group. Additionally, it was observed that the d-galactose-treated group exhibited significant reductions in all behavioral measurements, as illustrated in Figure 1.

Measurements of Free radical ROS, H_2O_2

The study measured ROS and H_2O_2 concentration levels in soft brain tissues in control and treated Wister rats, as explained in

Figure 2. Effects of heartwood from *Caesalpinia sappan* were examined at different doses levels. The d-gal-treated group had higher amounts of ROS and H_2O_2 in their brain tissues. In contrast, all doses of heartwood from HEE of *Caesalpinia sappan* significantly reduced ROS and H_2O_2 concentration in the brains of the cognitively impaired. Furthermore, donepezil lowered levels of ROS and H_2O_2, indicating a supporting activity like that seen with *Caesalpinia sappan*. At 150 mg/kg, effects may start to show through a reduction in free radical ROS and H_2O_2 but recovered observed at 250 mg/kg. The highest dose 500 mg/kg could increase free radical ROS and H_2O_2 levels, leading to significant decreases in oxidative stress. This suggests potential protective may be benefits against oxidative stress in the cognitive.

Measurement of Biochemical estimations

Results from an analysis of the effect of the HEE of *Caesalpinia sappan* treated group, AchE, CAT, APPO, MDA, GSH, SOD, GPx, TT and GST levels in the experimental rats are presented in Figure 3. d-gal treated group decreases the antioxidant level in brain tissues compared to the control group. HEE of *Caesalpinia sappan* group statistical data are comparable to d-gal rats in cognitive impairment. Whereas, NO and Nos levels in rat's brains

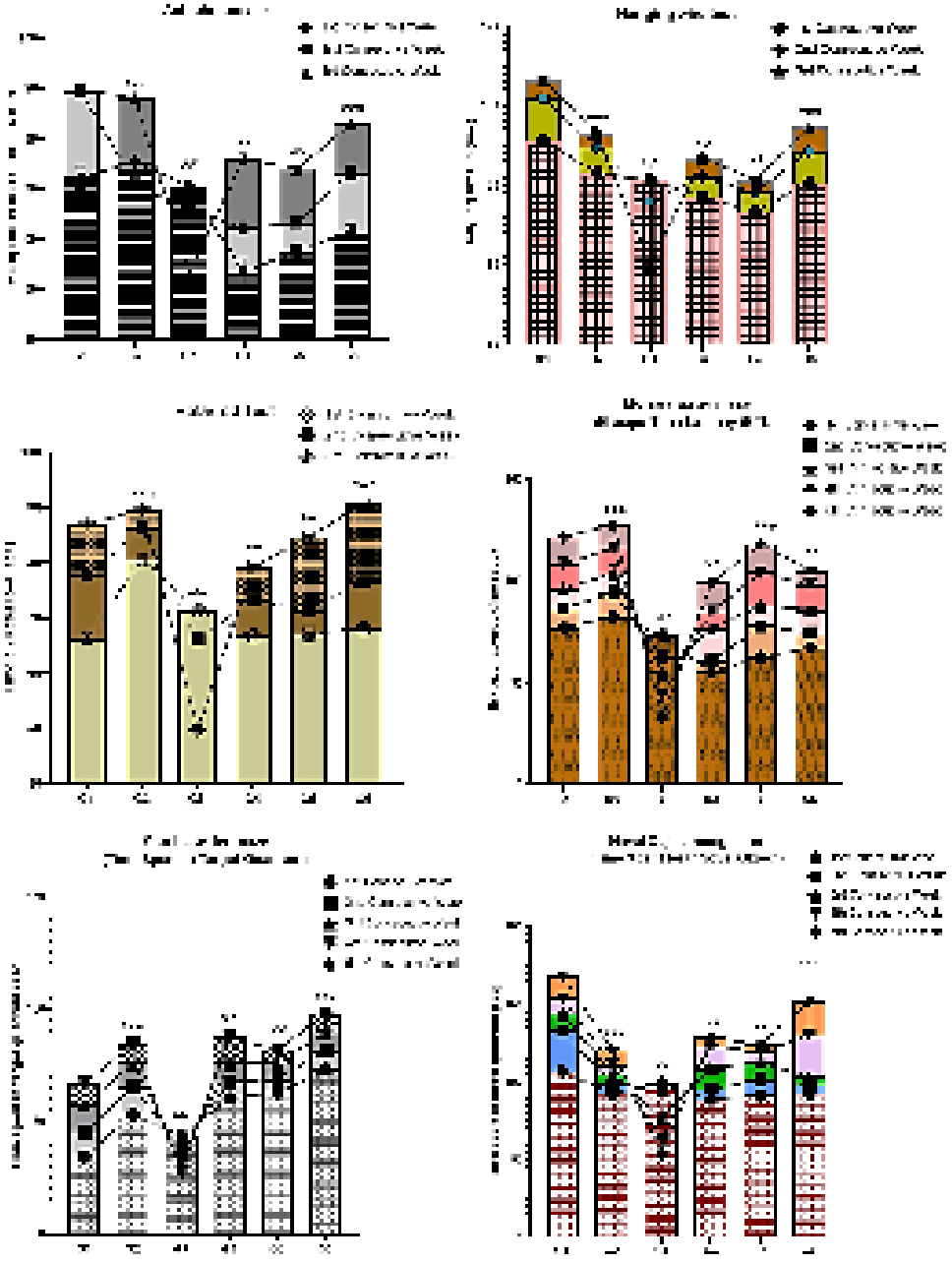

Figure 1: Actophotometer, wire handling, Rotar-rod, Morris water maze and Noval objective recognition test results after HEE of Caesalpinia sappan treatment in D-gal-induced cognitive impairment was examined using two-way ANOVA and Tukey's test, with data expressed as Mean±SEM for six participants. D-gal model and control groups (#p<0.05, ##p<0.01, ###p<0.001, *p<0.05, **p<0.01, ***p<0.001).

increased significantly in d-gal-induced rats. However, the 150, 250 and 500 mg/kg of HEE of *Caesalpinia sappan* noticeably reduced the NO and Nos rat brains. Donepezil rats group decreased NO and NOS levels in cognitive rats, at 150 mg/kg, effects may start to show through a reduction in oxidative stress markers, but improved benefits are likely observed at 250 mg/kg. The highest dose of 500 mg/kg could make a strong antioxidative response, leading to significant declines in markers of oxidative stress, like malondialdehyde MDA levels and improved endeavors of antioxidant enzymes (e.g., APPO, GSH, SOD, CAT, etc.,). which supports the activity of HEE of *Caesalpinia sappan* (Figure 3).

Measurement of neurotransmitters and Monoamines (DA, GABA, 5-HT and MAO-A, MAO-B)

Results from an analysis of the effect of HEE of *Caesalpinia sappan* treatment on DA, GABA and 5-HT levels are presented in Figure 4. D-gal group significantly decreases in DA, GABA, etc. but at 150 mg/kg, effects may start to show attenuated in neurotransmitters but enhanced likely observed at 250 mg/kg. The utmost dose of 500 mg/kg could make an efficient improvement in the levels of neurotransmitters such as 5-HT, DA and GABA. For mood modulation, cognitive function and general brain health, these neurotransmitters are critical. By restoring or enhancing their levels, *Caesalpinia sappan* may help to counteract the impacts of D-galactose-induced impairment. But the 5-HT concentration in the d-gal rats treated rats significantly increased likened to d-gal rats in Figure 4.

Histopathological analysis

In the normal rats, the group exhibited round nuclei and pyramidal cells were tightly packed. In the d-gal rats' group, an increase in intracellular space, shrunken and non-uniform neurons are seen and extensive cell damage is observed. The HEE of *Caesalpinia sappan* and donepezil-treated rats exhibited improved intracellular spaces; neurons are uniformly arranged and morphologically intact.

At 150 mg/kg, effects may start to show decreased cell damage, but improved cell damage is likely at 250 mg/kg. The highest dose of 500 mg/kg could make a strong improvement in cell damage. An optical microscope was used 150 µm and 250 µm to assess histologic damage using both hematoxylin and eosin and crystal violet stain observed in the CA1 part of the brain (Figure 5) and the heart, spleen, kidney and liver (Figure 6).

Immunoblotting studies

Immunoblotting analysis showed a significant reduction in BAX (pro-apoptotic protein levels) and an increase in BCL-2 (anti-apoptotic protein levels) in brain tissues of Wistar rats treated with *Caesalpinia sappan* compared to the D-galactose-only treated group. This shift in the BAX/BCL-2 ratio would suggest a bias towards cell survival, as a higher BCL-2 to BAX ratio is associated with reduced apoptosis. In the case of expression of BAX, brain tissue samples showed upregulation by 1.98, 7.43 and 1.59. There was downregulation in the treatment group of HC/CSH 500 mg/kg by 0.30. When compared to the control rats. BCl-2 to Bax ratio was highest in the tissue HC/CSH 500 mg/kg 12.16, followed by MC/ CSM 250 mg/kg 8.15 and standard (donepezil) 2.34. The inhibitor showed a BCL-2 to Bax ratio of 0.85 data are presented in Figure 7. The treated group and d-gal rats group data are presented in the raw blot in Figure 8.

Measurements of Free radical ROS, H_2O_2

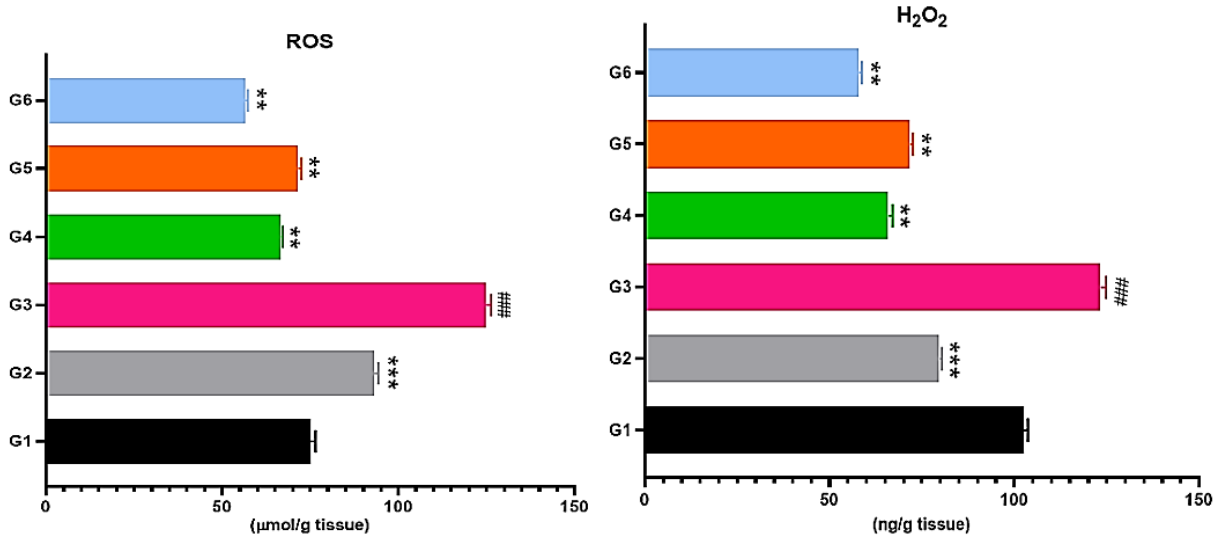

Figure 2: HEE of *Caesalpinia sappan*, ROS and H_2O_2 effects on D-gal-induced cognitive impairment was examined using two-way ANOVA and Tukey's test (n=4). Normal control p-values were #p<0.05, ##p<0.01, ###p<0.001; D-gal model showed **p<0.01, ***p<0.001.

Measurement of Biochemical estimations

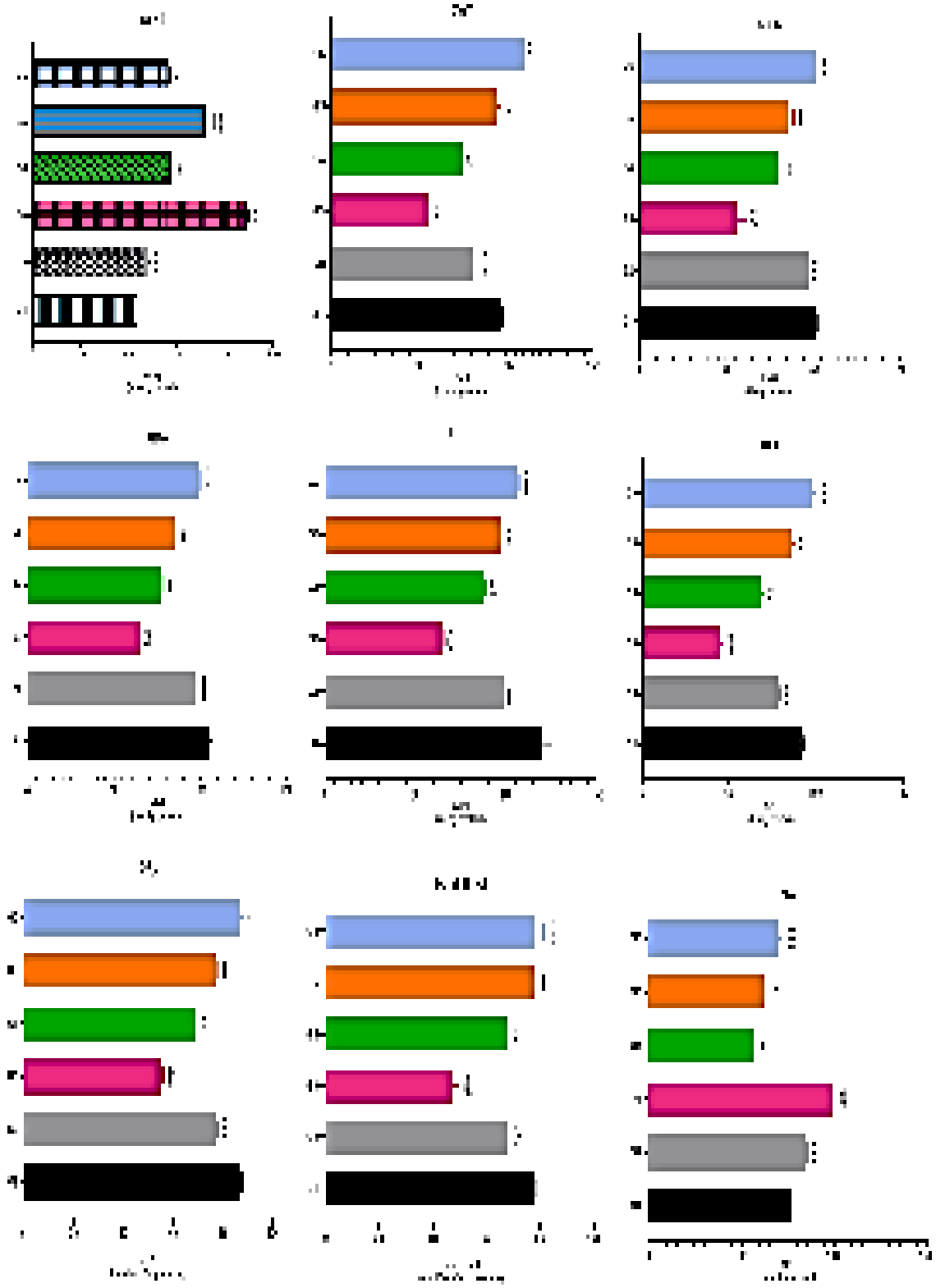

Figure 3: Effect of HEE of *Caesalpinia sappan* on AchE, CAT, APPO, MDA, GSH, SOD, GPX, TT, NO, NOS and GST activities in D-gal-induced. Mean±SEM (n=6) data and examined via one-way ANOVA and Tukey's test. *p<0.05, **p<0.01, ***p<0.001 vs. normal control; #p<0.05, ##p<0.01, ###p<0.001 vs. D-gal model group.

Measurement of neurotransmitters and Monoamines (DA, GABA, 5-HT and MAO-A, MAO-B)

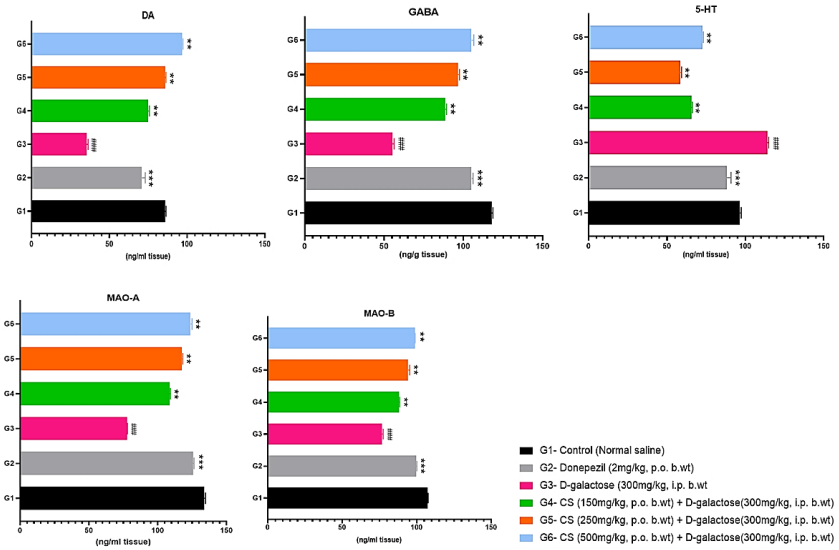

Figure 4: Effects of HEE of *Caesalpinia sappan* on GABA, DA and 5-HT during D-gal-induced group using one-way ANOVA and Tukey's test (mean±SEM, *n*=6). **p*<0.05, ***p*<0.01, ****p*<0.001 vs. D-gal; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 vs. control.

Histopathology studies (CA1 Region) and Crystal violet staining

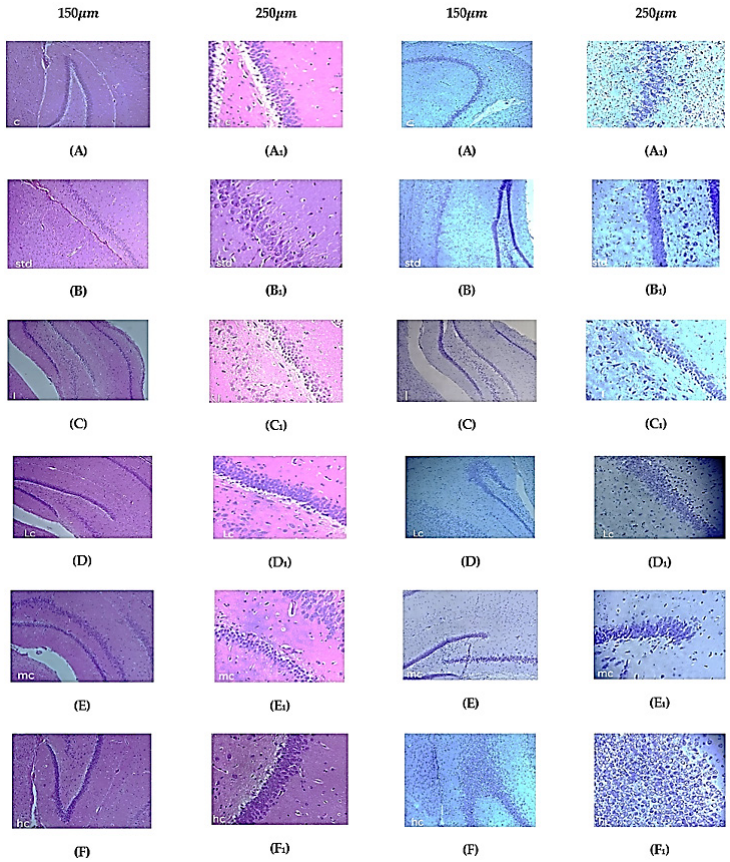

Figure 5: Hematoxylin and eosin and Crystal violet staining: (A: A_1):(G1): the control group; (B: B_1) (G2): STD (donepezil 2mg/kg); (C: C_1) (G3): I (d-galactose 300 mg/kg); (D: D_1) (G4): CSL (150 mg/kg); (E: E_1) (G5): CSM (250 mg/kg); and (F: F_1) (G6): CSH (500 mg/kg).

Histopathology of various organ

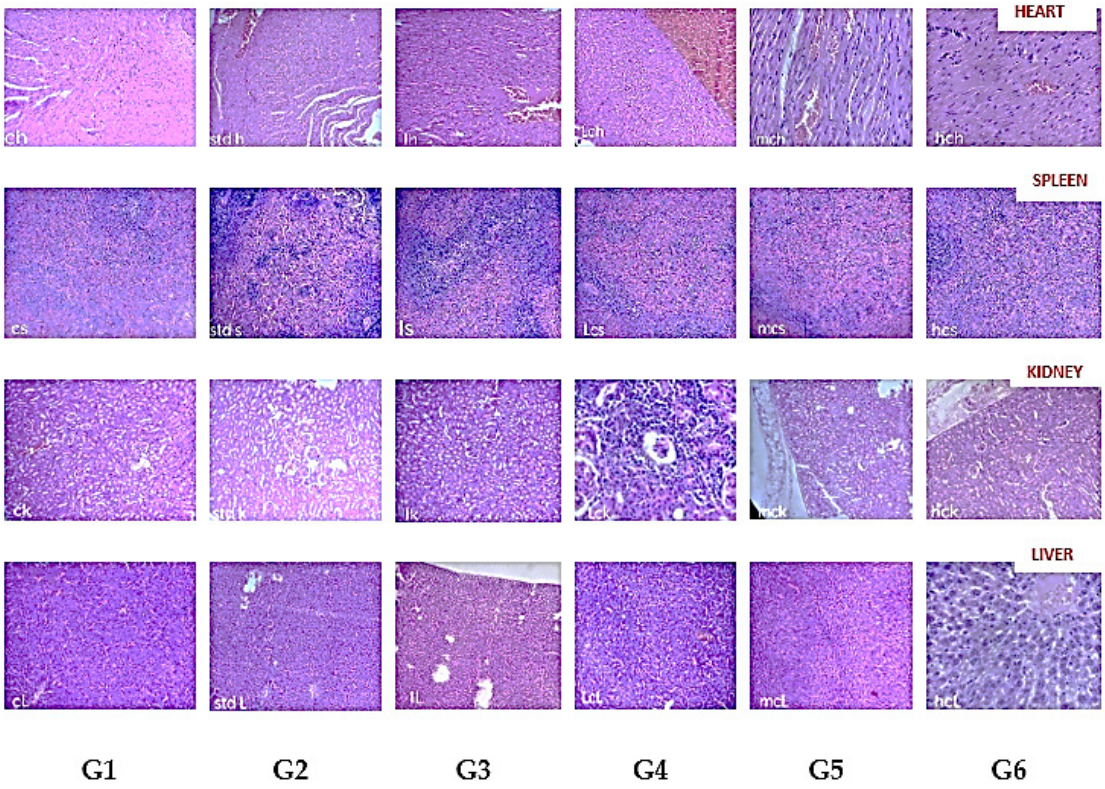

Figure 6: Following D-galactose model using Haematoxylin and eosin staining on various organs. Note: G1: the control group; G2: STD (donepezil 2mg/kg); G3: I (d-galactose 300 mg/kg); G4: CSL (150 mg/kg); G5: CSM (250 mg/kg); and G6: CSH (500 mg/kg).

Immunoblotting Assay

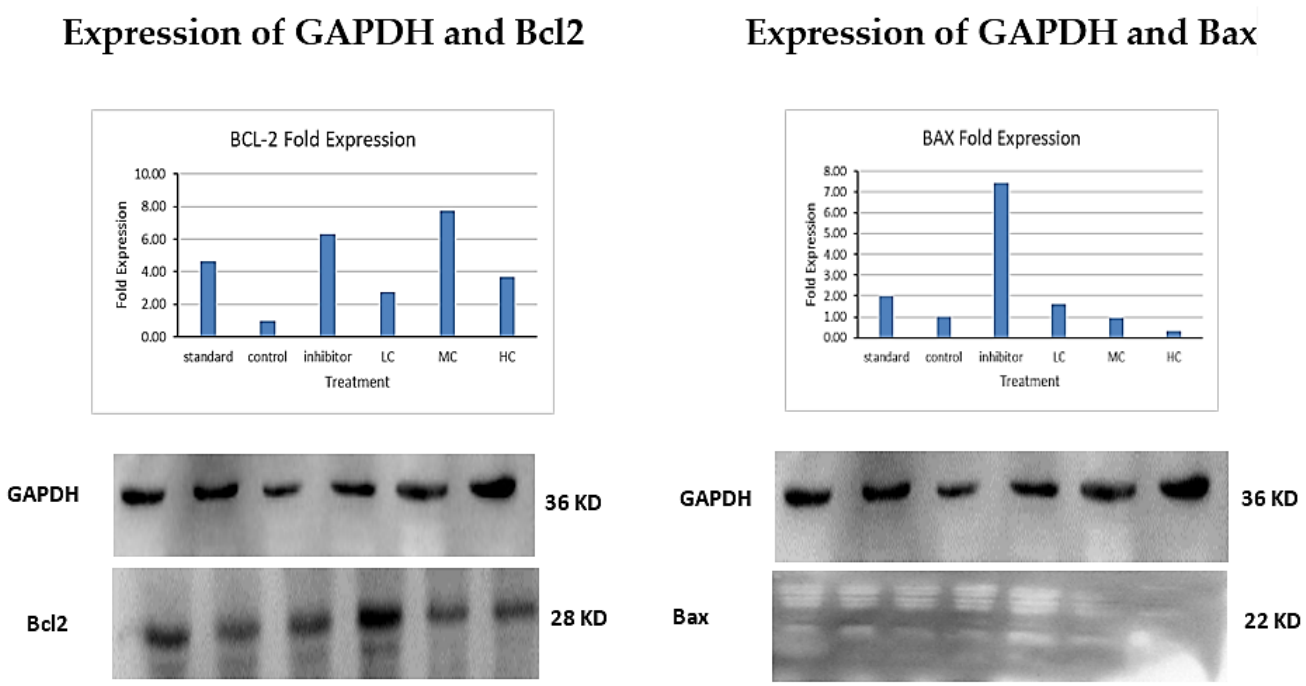

Figure 7: Apoptosis marker used in Immunoblotting Assay; Loading details: Lane 1: C; Lane 2: STD; Lane 3: I; Lane 4: CHL; Lane 5: CHM; Lane 6: CHH. Note: G1: the control group; G2: STD (donepezil 2 mg/kg); G3: I (d-galactose 300 mg/kg); G4: CHL (150 mg/kg); G5: CHM (250 mg/kg); and G6: CHH (500 mg/kg).

Raw Blot images

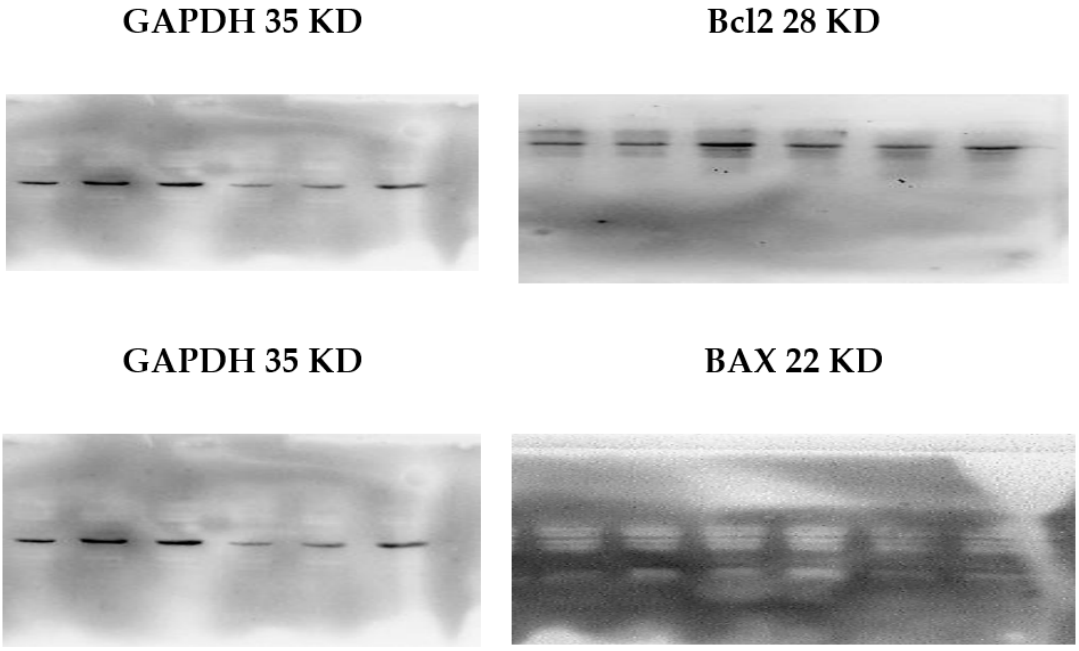

Figure 8: Raw Blot images of Apoptosis marker; Loading details: Lane 1: C; Lane 2: STD (donepezil 2 mg/kg); Lane 3: I/ (d-galactose 300 mg/kg); Lane 4: HC/ CSH (500 mg/kg).; Lane 5: MC/ CSM (250 mg/kg); Lane 6: LC/ CSL (150 mg/kg).

CONCLUSION

This study reveals that D-gal therapy negatively affects brain histology and behavior in rats. However, our research shows that *Caesalpinia sappan* protects against neurodegeneration and cognitive decline induced by D-gal. It also normalizes brain biochemistry and oxidative stress. These consequences imply that *Caesalpinia sappan* improves the cholinergic program and reduces neuroinflammation, apoptosis oxidative damage, supporting memory and cognitive function. While some recent studies have explored the neuroprotective effects of *Caesalpinia sappan* in an AD rat model, this is the first to demonstrate its anti-aging with apoptosis markers properties.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CS: *Caesalpinia sappan*; **HHC:** Heartwood hydroethanolic *Caesalpinia sappan*; **HEE:** Hydroethanolic extract; **AchE:** Acetylcholinesterase enzyme activity; **GST:** Glutathione S-Transferase enzyme; **MDA:** Malondialdehyde; **GSH:** Glutathione; **NO:** Nitric Oxide; **$NaPO_4$:** Sodium Phosphate; **PI:** Potassium Iodide; **H and E:** Haematoxylin and eosin staining; **C:** Control; **STD:** Standard; **I:** Inducer; **LCS:** Low dose; **HCM:** Middle dose; **HCS:** High dose.

ETHICAL STATEMENT

Ethics committee SACCP-IAEC/2024-01/108 of the National Research Center oversaw care.

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