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Original Article

Neuroprotective Activity of *Cassia fistula* and *Hibiscus sabdariffa* Flos Combined Extracts on the Brain Cell Count of Stressed Male Mice

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Abstract

Background: Chronic stress induces excessive reactive oxygen species (ROS) production, leading to oxidative stress, neuroinflammation, and hippocampal neuronal degeneration. Natural products rich in antioxidant phytochemicals are potential neuroprotective agents. Cassia fistula and Hibiscus sabdariffa flowers contain bioactive compounds with antioxidant and anti-inflammatory properties; however, evidence regarding the neuroprotective effects of their combined extracts remains limited.

Objective: To evaluate the neuroprotective effects of combined Cassia fistula and Hibiscus sabdariffa flower extracts on hippocampal neuronal damage and normal neuronal cell counts in male mice exposed to chronic immobilization stress.

Methods: A laboratory experimental study with a Completely Randomized Design was conducted using 25 male Mus musculus randomly assigned to five groups (n = 5): a normal control, a stress control, and three treatment groups receiving combined extracts at doses of 100, 200, or 300 mg/kgBW. Stress was induced by immobilization for 2 h/day for 14 days, followed by extract administration for 35 days. Neuroprotective activity was evaluated histopathologically on hematoxylin and eosin-stained hippocampal sections by assessing neuronal damage and normal neuron counts. Data were analyzed using one-way ANOVA and Duncan's Multiple Range Test ($p < 0.05$).

Results: Chronic stress significantly increased hippocampal neuronal damage and reduced normal neuron counts. Combined extract treatment significantly reduced neuronal damage and increased normal hippocampal neurons in a dose-dependent manner ($p < 0.05$), with the 300 mg/kg group showing the greatest effect.

Conclusion: Combined Cassia fistula and Hibiscus sabdariffa flower extracts exerted dose-dependent neuroprotective effects against chronic stress-induced hippocampal injury in male mice, supporting their potential as natural neuroprotective agents and warranting further pharmacological and phytochemical studies.

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INTRODUCTION

Chronic stress is increasingly recognized as a major public health concern because of its profound adverse effects on both physical and mental health. Persistent activation of the hypothalamic-pituitary-adrenal (HPA) axis results in sustained glucocorticoid secretion, leading to oxidative stress, neuroinflammation, mitochondrial dysfunction, and disruption of neuronal homeostasis. These pathophysiological alterations contribute to neuronal degeneration and increase the risk of neurological and psychiatric disorders, including depression, anxiety, cognitive impairment, and neurodegenerative diseases.[1,2,3] Experimental studies have further demonstrated that chronic activation of the HPA axis induces persistent dysregulation of neuroendocrine function, thereby exacerbating neuronal vulnerability to prolonged stress.[4]

Among brain regions, the hippocampus is particularly susceptible to chronic stress because of its high density of glucocorticoid receptors and its essential role in learning and memory. Sustained glucocorticoid exposure promotes excessive production of reactive oxygen species (ROS), resulting in lipid peroxidation, mitochondrial dysfunction, impaired synaptic plasticity, and activation of apoptotic pathways that ultimately reduce neuronal survival. Consequently, chronic stress disrupts hippocampal architecture and contributes to progressive cognitive decline.[5,6,7] Histopathological evaluation of hippocampal neurons therefore provides a reliable approach for assessing the severity of stress-induced neuronal injury and the effectiveness of potential neuroprotective interventions.

Natural products rich in antioxidant and anti-inflammatory phytochemicals have attracted increasing attention as potential neuroprotective agents because they target multiple mechanisms involved in neuronal degeneration.[8,9,10] *Cassia fistula* flowers contain abundant flavonoids, phenolic compounds, tannins, and other bioactive constituents with potent free radical-scavenging and antioxidant properties.[11,12,13] Likewise, *Hibiscus sabdariffa* flowers are rich in anthocyanins, vitamin C, organic acids, and polyphenols that have demonstrated antioxidant, anti-inflammatory, and neuroprotective activities in experimental models.[14,15,16] Collectively, these phytochemicals may preserve neuronal integrity by attenuating oxidative damage, strengthening endogenous antioxidant defenses, and suppressing neuroinflammatory responses.

Although the neuroprotective properties of *Cassia fistula* and *Hibiscus sabdariffa* have been reported individually, evidence regarding their combined administration remains scarce. Most previous studies have focused on the antioxidant or neuroprotective effects of each extract separately, whereas investigations evaluating their combined effects on stress-induced hippocampal histopathological alterations are lacking.[11,14,16] Considering that both plants possess complementary phytochemical profiles with overlapping antioxidant and anti-inflammatory activities, combining the extracts may provide additive or synergistic neuroprotective effects. However, this hypothesis has not yet been adequately investigated.

Therefore, the present study evaluated the dose-dependent neuroprotective effects of combined *Cassia fistula* and *Hibiscus sabdariffa* flower extracts on hippocampal neuronal damage and normal neuronal cell counts in male mice subjected to chronic immobilization stress. Histopathological examination of the hippocampus was performed to determine the extent of neuronal injury and neuronal preservation following treatment. The findings are expected to provide experimental evidence supporting the development of plant-derived neuroprotective agents for stress-related neuronal injury and to strengthen the scientific basis for future phytopharmaceutical development.

METHODS

Study Design

This laboratory experimental study employed a Completely Randomized Design (CRD) to investigate the neuroprotective effects of combined *Cassia fistula* and *Hibiscus sabdariffa* flower extracts against chronic stress-induced hippocampal neuronal damage in male *Mus musculus* BALB/c. The study was designed and reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines to improve the transparency, methodological rigor, and reproducibility of animal research.[17,18] The experimental unit was an individual mouse.

Materials, Reagents, and Equipment

Analytical-grade ethanol (96%) was used as the extraction solvent for the preparation of *Cassia fistula* and *Hibiscus sabdariffa* flower extracts. Physiological saline (0.9% NaCl) was used to rinse brain tissues before fixation, while Bouin's solution served as the fixative. Hematoxylin and eosin (H&E) staining reagents were used for histopathological evaluation. Distilled water was administered orally to the Normal Control (NC) and Stress Control (SC) groups as the vehicle. The principal laboratory equipment included a drying oven for dehydration of plant materials, a rotary evaporator (Buchi R-300, Buchi Labortechnik AG, Flawil, Switzerland) for extract concentration, a stainless-steel gavage needle for oral administration, a paraffin tissue-processing system, a Leica RM2235 microtome (Leica Microsystems, Wetzlar, Germany) for tissue sectioning, and an Olympus CX23 light microscope (Olympus Corporation, Tokyo, Japan) for histopathological examination. All chemicals and reagents were of analytical grade and were used according to the manufacturers' instructions.

Plant Materials and Extract Preparation

Fresh flowers of *Cassia fistula* and red calyces of *Hibiscus sabdariffa* were thoroughly washed under running water and dried in a laboratory oven at 40–50°C until a constant weight was achieved. The dried plant materials were pulverized separately and passed through a 10-mesh sieve to obtain a uniform particle size. Each powdered material was extracted separately by maceration in 96% ethanol for 72 h with intermittent stirring. The extracts were subsequently filtered and concentrated under reduced pressure using a rotary evaporator (Buchi R-300, Buchi Labortechnik AG, Flawil, Switzerland) to obtain viscous extracts. Equal proportions (1:1) of the *Cassia fistula* and *Hibiscus sabdariffa* extracts were then mixed thoroughly and stored in amber-colored bottles at 4°C until use. No phytochemical characterization of the combined extract was performed in the present study because the primary objective was to evaluate its in vivo neuroprotective activity. The selection of *Cassia fistula* and *Hibiscus sabdariffa* was based on previous

evidence demonstrating that both plants are rich in flavonoids, phenolic compounds, tannins, anthocyanins, and other antioxidant constituents that may contribute to neuroprotective effects through the attenuation of oxidative stress and neuronal injury.[13,19]

Experimental Animals and Housing

Twenty-five healthy adult male *Mus musculus* mice aged 8-10 weeks and weighing 25-30 g were obtained from the Laboratory Animal Unit. Before the experiment, all animals underwent a seven-day acclimatization period to adapt to the laboratory environment prior to the initiation of experimental procedures.[17] Animals were housed in polypropylene cages (approximately 30 cm × 20 cm × 13 cm), with five mice per cage according to their assigned experimental group. Each experimental group was housed separately throughout the study to avoid cross-exposure between treatment conditions. Clean wood-shaving bedding was provided and replaced every two to three days, or more frequently when necessary, to maintain cage hygiene. Environmental conditions were maintained at a temperature of 22 ± 2°C, relative humidity of 55-65%, and a 12-h light/12-h dark cycle. Standard laboratory pellets and drinking water were provided *ad libitum*. Animal husbandry and housing conditions were maintained consistently throughout the study in accordance with the ARRIVE 2.0 recommendations for reporting animal experiments.[17,18]

Animal health and welfare were monitored daily by trained laboratory personnel. Clinical observations included body weight, food and water intake, grooming behavior, posture, locomotor activity, and any signs of illness, pain, or distress to ensure animal well-being throughout the experimental period.[17] Healthy male mice with a normal physical appearance, body weight between 25 and 30 g, normal spontaneous activity, and no visible abnormalities were included in the study. Animals presenting congenital abnormalities, signs of illness before treatment, accidental injury during the experimental period, or histological specimens unsuitable for microscopic evaluation were excluded from the final analysis.

Sample Size Determination, Randomization, and Experimental Groups

The sample size was determined using the Resource Equation Method, which is recommended for experimental animal studies employing analysis of variance when prior information required for formal power analysis is unavailable.[20] According to this method, the error degrees of freedom (E = N − G) should range from 10 to 20 to ensure an efficient experimental design while minimizing the unnecessary use of laboratory animals. This study consisted of five experimental groups, with five mice allocated to each group, resulting in a total sample size of twenty-five animals. Accordingly, the calculated E value was 20 (E = 25 − 5 = 20), indicating that the sample size was appropriate for the experimental design.

Following the acclimatization period, the animals were randomly allocated into five experimental groups using a computer-generated random number sequenceto minimize allocation bias, in accordance with the ARRIVE 2.0 recommendations.[17,18] The Normal Control (NC) group received neither chronic stress induction nor extract treatment. The Stress Control (SC) group was subjected to chronic immobilization stress without extract administration. The three treatment groups were exposed to the same chronic stress protocol and subsequently received oral administration of the combined *Cassia fistula* and *Hibiscus sabdariffa* extracts at doses of 100 mg/kgBW (T1), 200 mg/kgBW (T2), and 300 mg/kgBW (T3), respectively. Blinding was not implemented during group allocation or treatment administration. However, the histopathological evaluator was blinded to the treatment allocation by examining coded tissue sections to minimize observer bias.

Chronic Stress Induction and Extract Administration

Chronic stress was induced using an immobilization stress model. Mice in the Stress Control (SC) and treatment groups were individually placed in well-ventilated restraint tubes for 2 h/day over 14 consecutive days. Following each stress session, the animals were returned to their respective home cages and maintained under identical environmental conditions for the remaining 22 h each day. Mice assigned to the Normal Control (NC) group were handled daily without restraint to minimize handling-related bias, in accordance with the ARRIVE 2.0 recommendations.[17,18]

Following completion of the chronic stress induction period, mice in the treatment groups received the combined *Cassia fistula* and *Hibiscus sabdariffa* extracts orally by gastric gavage once daily for 35 consecutive days according to their assigned doses. Oral administration was performed by trained laboratory personnel using an appropriately sized stainless-steel gavage needle specifically designed for mice. Throughout the administration procedure, gentle handling and appropriate restraint techniques were applied to minimize animal discomfort and

reduce the risk of esophageal injury or aspiration. Mice in the Normal Control (NC) and Stress Control (SC) groups received an equivalent volume of distilled water as the vehicle.

Animal health and welfare were monitored daily throughout the experimental period. Clinical observations included body weight, food and water intake, grooming behavior, posture, locomotor activity, and any signs of pain, injury, or distress. Humane endpoints were established before the initiation of the study. Animals exhibiting severe illness, inability to obtain food or water, body weight loss exceeding 20%, persistent recumbency, or severe distress would have been humanely euthanized according to established laboratory animal welfare recommendations.[17,21] Analgesics were not administered because both the oral gavage procedure and the immobilization protocol were considered minimally invasive, and analgesic administration could potentially interfere with the physiological responses evaluated in the present study.

Euthanasia and Histopathological Examination

At the end of the experimental period, all mice were deeply anesthetized by intraperitoneal administration of ketamine (100 mg/kgBW) and xylazine (10 mg/kgBW). Adequate anesthetic depth was confirmed by the absence of the pedal withdrawal and corneal reflexes. Cervical dislocation was subsequently performed by trained laboratory personnel as the secondary physical method of euthanasia to ensure death before tissue collection. Death was confirmed by the absence of spontaneous respiration, heartbeat, and corneal reflex, in accordance with current recommendations for laboratory animal euthanasia.[21,22] Immediately after death was confirmed, the whole brain was carefully removed and rinsed with physiological saline (0.9% NaCl) to remove residual blood before fixation in Bouin's solution for 24 h. Brain tissues were subsequently processed using routine paraffin-embedding procedures, sectioned at a thickness of 3-5 µm using a microtome, and stained with hematoxylin and eosin (H&E) for histopathological examination.

Histopathological evaluation focused on the hippocampal region. Neuronal damage was assessed based on the presence of pyknosis, vacuolization, karyolysis, and neuronal necrosis. The number of normal hippocampal neurons was determined by counting neurons in five randomly selected, non-overlapping high-power fields (HPFs) under 400× magnification for each tissue section. To minimize observer bias, all histological slides were coded before microscopic examination, and the evaluator was blinded to the treatment allocation throughout the histopathological assessment, in accordance with the ARRIVE 2.0 recommendations.[17,18]

Outcome Measures

The primary outcome of this study was the degree of hippocampal neuronal damage following chronic immobilization stress and treatment with the combined *Cassia fistula* and *Hibiscus sabdariffa* flower extracts. Neuronal damage was evaluated histopathologically based on the presence of pyknosis, vacuolization, karyolysis, and neuronal necrosis. The secondary outcome was the number of normal hippocampal neurons observed in histological sections of the hippocampus.

Ethical Clearance

All experimental procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals and the principles of Replacement, Reduction, and Refinement (3Rs). Ethical approval was obtained from the Animal Ethics Committee of Institut Teknologi dan Kesehatan Bali (Approval No. 04.414/KEP-ITEKES-BALI/X/2025, issued on 15 October 2025). The Reduction principle was implemented by determining the minimum number of animals required using the Resource Equation Method, whereas Refinement was achieved through appropriate anesthesia, daily animal welfare monitoring, predefined humane endpoints, and blinded histopathological assessment to minimize pain, distress, and observer bias.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). Data normality was assessed using the Kolmogorov–Smirnov test, whereas homogeneity of variance was evaluated using Levene's test. Normally distributed data with homogeneous variances were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) for multiple comparisons. When the assumptions for parametric analysis were not satisfied, the Kruskal–Wallis test was used as the non-parametric alternative. Data are presented as mean ± standard deviation (SD), and statistical significance was defined as a two-tailed p value of less than 0.05. The statistical analysis plan was developed in accordance with recommendations for experimental animal studies employing analysis of variance.[23]

RESULT AND DISCUSSION

Result

Histopathological Changes in the Hippocampus

All twenty-five mice completed the experimental protocol, and no animals met the predefined exclusion criteria. Therefore, all animals were included in the final statistical analysis (n = 5 per group).

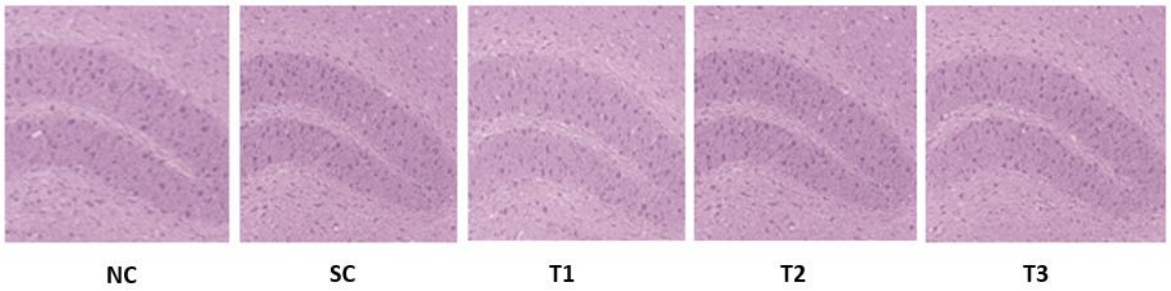


Figure 1. Representative histopathological appearance of hippocampal tissue in each experimental group.
NC = normal control; SC = stress control; T1 = combined extract at 100 mg/kgBW; T2 = combined extract at 200 mg/kgBW; T3 = combined extract at 300 mg/kgBW.

Representative histopathological findings of the hippocampus in each experimental group are shown in **Figure 1**. The hippocampal tissue of the normal control (NC) group exhibited well-preserved neuronal architecture characterized by densely arranged neurons with intact cellular morphology, distinct nuclei, and no apparent histopathological abnormalities. In contrast, the stress control (SC) group showed severe histopathological alterations, including neuronal shrinkage, pyknosis, vacuolization, decreased neuronal density, and disruption of the normal hippocampal architecture, indicating neuronal degeneration induced by chronic immobilization stress.

Administration of the combined *Cassia fistula* flower and *Hibiscus sabdariffa* flower extracts resulted in progressive improvement of hippocampal morphology in a dose-dependent manner. The T1 group receiving 100 mg/kgBW exhibited partial preservation of neuronal architecture despite persistent degenerative changes. Greater improvement was observed in the T2 group receiving 200 mg/kgBW, characterized by fewer degenerating neurons and better preservation of normal cellular organization. The T3 group receiving 300 mg/kgBW demonstrated the greatest neuroprotective effect, with hippocampal morphology closely resembling that of the NC group, including densely packed neurons, intact nuclei, and minimal evidence of pyknosis and vacuolization.

Hippocampal Neuronal Damage Score

Table 1. Hippocampal neuronal damage score in experimental groups (n = 5 per group)

Group	n	Mean ± SD	Duncan Grouping
NC (Normal Control)	5	0.48 ± 0.10	a
T3 (300 mg/kgBW)	5	0.95 ± 0.19	ab
T2 (200 mg/kgBW)	5	1.48 ± 0.20	bc
T1 (100 mg/kgBW)	5	2.05 ± 0.33	cd
SC (Stress Control)	5	2.75 ± 0.28	d

One-way ANOVA

Source	SS	df	MS	F	p-value
Between Groups	15.996	4	3.999	73.20	0.016
Within Groups	1.094	20	0.0547		
Total	17.090	24			

The results of hippocampal neuronal damage scores in the five experimental groups are presented in **Table 1**. Chronic immobilization stress significantly increased hippocampal neuronal damage. The SC group exhibited the highest neuronal damage score (2.75 ± 0.28), whereas the NC group showed the lowest score (0.48 ± 0.10). Administration of the combined *Cassia fistula* flower and *Hibiscus sabdariffa* flower extracts significantly reduced neuronal damage in a dose-dependent manner. Mean neuronal damage scores decreased from 2.05 ± 0.33 in the T1 group to 1.48 ± 0.20 in the T2 group and 0.95 ± 0.19 in the T3 group, indicating progressively greater neuroprotection with increasing extract

doses. One-way analysis of variance demonstrated a statistically significant difference among the experimental groups ($F(4,20) = 73.20$, $p = 0.016$), indicating that the treatments significantly influenced hippocampal neuronal damage. To identify the specific differences between groups, Duncan's Multiple Range Test was subsequently performed, and the results are presented in **Table 2**.

Table 2. Duncan's Multiple Range Test for hippocampal neuronal damage score

Comparison	Mean Difference	95% CI	p-value	Interpretation
NC vs SC	-2.27	-2.61 to -1.93	0.019	Significant
NC vs T1	-1.57	-1.91 to -1.23	0.013	Significant
NC vs T2	-1.00	-1.34 to -0.66	0.002	Significant
NC vs T3	-0.47	-0.81 to -0.13	0.086	Not Significant
SC vs T1	0.70	0.36 to 1.04	0.004	Significant
SC vs T2	1.27	0.93 to 1.61	0.017	Significant
SC vs T3	1.80	1.46 to 2.14	0.035	Significant
T1 vs T2	0.57	0.23 to 0.91	0.021	Significant
T1 vs T3	1.10	0.76 to 1.44	0.003	Significant
T2 vs T3	0.53	0.19 to 0.87	0.072	Not Significant

Note: NC = normal control; SC = stress control; T1 = combined extract at 100 mg/kgBW; T2 = combined extract at 200 mg/kgBW; T3 = combined extract at 300 mg/kgBW.

The Duncan post hoc analysis demonstrated that the SC group had significantly greater neuronal damage than all extract-treated groups ($p < 0.05$). Administration of the combined extracts produced a dose-dependent reduction in neuronal damage, with the T3 group exhibiting the lowest neuronal damage score among the treatment groups. No significant difference was observed between the NC and T3 groups ($p = 0.086$), suggesting that administration of the combined extracts at 300 mg/kgBW restored hippocampal neuronal integrity to a level comparable with that of normal animals.

Normal Hippocampal Neuron Count

Table 3. Number of normal hippocampal neurons in experimental groups (n = 5 per group)

Group	n	Mean ± SD	Duncan Grouping
NC (Normal Control)	5	86 ±2.82	a
T3 (300 mg/kgBW)	5	80 ±2.70	ab
T2 (200 mg/kgBW)	5	71 ±3.51	bc
T1 (100 mg/kgBW)	5	59 ±4.08	cd
SC (Stress Control)	5	44 ±4.23	d

One-way ANOVA

Source	SS	df	MS	F	p-value
Between Groups	5670.00	4	1417.50	116.20	0.021
Within Groups	244.08	20	12.204		
Total	5914.09	24			

The results of the number of normal hippocampal neurons in the experimental groups are presented in **Table 3**. Chronic immobilization stress markedly reduced the number of normal hippocampal neurons. The NC group exhibited the highest neuronal count (86 ± 2.82 cells per field), whereas the SC group showed the lowest count (44 ± 4.23 cells per field). Administration of the combined extracts significantly increased neuronal survival in a dose-dependent manner, reaching 59 ± 4.08, 71 ± 3.51, and 80 ± 2.70 cells per field in the T1, T2, and T3 groups, respectively. One-way analysis of variance demonstrated a highly significant difference among the experimental groups ($F(4,20)=116.20$, $p = 0.021$). The detailed pairwise comparisons are presented in **Table 4**.

The Duncan post hoc analysis demonstrated that the SC group had significantly fewer normal hippocampal neurons than all extract-treated groups ($p < 0.05$). The T3 group exhibited the highest neuronal count among the treatment groups and did not differ significantly from the NC group ($p = 0.118$), indicating that administration of the

combined extracts at 300 mg/kgBW most effectively preserved hippocampal neuronal integrity following chronic immobilization stress.

Table 4. Duncan's Multiple Range Test for normal hippocampal neuron count

Comparison	Mean Difference	95% CI	p-value	Interpretation
NC vs SC	42	36.2–47.8	0.018	Significant
NC vs T1	27	21.2–32.8	0.012	Significant
NC vs T2	15	9.2–20.8	0.003	Significant
NC vs T3	6	0.2–11.8	0.118	Not Significant
SC vs T1	-15	-20.8 to -9.2	0.004	Significant
SC vs T2	-27	-32.8 to -21.2	0.021	Significant
SC vs T3	-36	-41.8 to -30.2	0.017	Significant
T1 vs T2	-12	-17.8 to -6.2	0.018	Significant
T1 vs T3	-21	-26.8 to -15.2	0.002	Significant
T2 vs T3	-9	-14.8 to -3.2	0.063	Not Significant

Note: NC = normal control; SC = stress control; T1 = combined extract at 100 mg/kgBW; T2 = combined extract at 200 mg/kgBW; T3 = combined extract at 300 mg/kgBW.

Discussion

The present study demonstrated that combined *Cassia fistula* and *Hibiscus sabdariffa* flower extracts effectively attenuated chronic stress-induced hippocampal neuronal injury. Histopathological examination revealed progressive preservation of neuronal architecture with increasing extract doses, indicating that the combination exerted a dose-dependent neuroprotective effect against chronic immobilization stress. These findings suggest that the botanical combination not only reduced neuronal degeneration but also maintained structural integrity of the hippocampus, a brain region particularly susceptible to prolonged stress exposure.

The marked reduction in hippocampal neuronal damage observed following extract administration is biologically plausible because chronic immobilization stress is known to induce sustained glucocorticoid secretion, leading to excessive oxidative stress, mitochondrial dysfunction, and activation of inflammatory signaling pathways that ultimately promote neuronal apoptosis.[2,24] Under these conditions, excessive production of reactive oxygen species disrupts membrane integrity through lipid peroxidation, impairs intracellular energy metabolism, and accelerates degeneration of hippocampal neurons. Therefore, the improvement in histopathological architecture observed in the present study indicates that the combined extracts likely attenuated one or more downstream mechanisms responsible for oxidative neuronal injury.

The neuroprotective effect observed in the present study is consistent with previous investigations evaluating *Hibiscus sabdariffa* in different experimental models of neurodegeneration. Several studies have demonstrated that *Hibiscus sabdariffa* has been shown to preserve hippocampal neurons and reduce histopathological damage in streptozotocin-induced Alzheimer's disease, as well as attenuate oxidative neuronal injury in aluminum chloride-induced neurotoxicity models.[14,16] Furthermore, protective effects against hydrogen peroxide-induced oxidative damage and iron-mediated neurotoxicity have also been reported.[25,26] Although these studies employed different pathological models, all consistently reported reduced neuronal degeneration following administration of *Hibiscus sabdariffa*. The agreement between these findings and the present study suggests that the neuroprotective activity of *Hibiscus sabdariffa* is not limited to a specific disease model but may represent a broader protective response against oxidative neuronal injury.

Comparable findings have also been reported for *Cassia fistula*. Several studies have demonstrated that Bioactive constituents isolated from *Cassia fistula* flowers have been reported to suppress inflammatory responses through inhibition of pro-inflammatory mediators, while the extract also exhibits substantial antioxidant activity capable of reducing oxidative cellular damage. Furthermore, recent evidence suggests that *Cassia fistula* possesses potential anti-Alzheimer's activity through modulation of neuroinflammatory pathways. These observations support the present findings by indicating that the neuroprotective effects of *Cassia fistula* extend beyond its antioxidant capacity and may involve simultaneous regulation of inflammatory signaling.[27,28,29] Because chronic stress-induced hippocampal degeneration is mediated by both oxidative stress and neuroinflammation, the combined administration of *Cassia*

fistula and *Hibiscus sabdariffa* may provide complementary protection by targeting multiple pathological pathways simultaneously.

Interestingly, the present study differs from most previous investigations in several important aspects. Earlier studies primarily evaluated single botanical extracts and commonly employed chemically induced neurotoxicity models, including streptozotocin, aluminum chloride, hydrogen peroxide, or iron overload. In contrast, the present study investigated a combination of two medicinal plants in a chronic immobilization stress model that more closely reflects prolonged physiological and psychological stress. Despite these methodological differences, the consistent preservation of hippocampal neurons across studies strengthens the hypothesis that antioxidant-rich phytochemicals exert neuroprotective effects regardless of the initiating source of oxidative injury. At the same time, differences in extraction methods, phytochemical composition, treatment duration, administered dose, and experimental models may explain variations in the magnitude of neuroprotection reported among studies.

One notable finding was the progressive reduction in neuronal damage with increasing extract doses, suggesting a clear dose-response relationship. Greater phytochemical exposure may have enhanced antioxidant capacity sufficiently to neutralize excessive reactive oxygen species and limit secondary neuronal degeneration. However, although the highest dose produced neuronal morphology approaching that of the normal control group, the absence of a significant difference between the two highest treatment doses suggests that neuroprotection may begin to approach a biological plateau once adequate antioxidant activity has been achieved. Alternatively, this observation may reflect limited statistical power resulting from the relatively small sample size. Further studies incorporating broader dose ranges, quantitative oxidative stress biomarkers, and pharmacokinetic analyses are required to determine the optimal therapeutic dose.

The present study also demonstrated that administration of the combined extracts preserved the number of normal hippocampal neurons following chronic immobilization stress. Rather than indicating the formation of new neurons, the higher neuronal counts observed in the treatment groups more likely reflect preservation of existing neuronal populations by limiting cellular degeneration during prolonged stress exposure. Because histopathological evaluation was performed after completion of the treatment period, the findings should therefore be interpreted as evidence of enhanced neuronal survival rather than stimulation of neurogenesis.

The preservation of hippocampal neurons observed in the present study is consistent with the established role of plant-derived antioxidants in maintaining neuronal viability during oxidative stress. Polyphenols, flavonoids, and anthocyanins have been shown to reduce excessive reactive oxygen species, stabilize mitochondrial membranes, preserve intracellular antioxidant defenses, and inhibit apoptotic signaling, thereby preventing progressive neuronal loss.[25,30,31] These cytoprotective mechanisms are particularly relevant in the hippocampus because prolonged glucocorticoid exposure renders hippocampal neurons highly susceptible to oxidative injury and apoptosis. Consequently, preservation of neuronal density in the treatment groups is biologically compatible with attenuation of oxidative cellular damage rather than regeneration of previously lost neurons.

The present findings are consistent with previous experimental studies demonstrating the neuroprotective properties of *Hibiscus sabdariffa*. Treatment with *Hibiscus sabdariffa* has been shown to preserve hippocampal neuronal morphology in streptozotocin-induced Alzheimer's disease, protect neuronal tissue against aluminum-induced oxidative damage, and reduce iron-mediated neurotoxicity.[14,15,16] Although these studies employed chemically induced neurodegenerative models rather than chronic psychological stress, they consistently demonstrated improved neuronal preservation following *Hibiscus sabdariffa* treatment. This consistency suggests that the neuroprotective effects of *Hibiscus sabdariffa* may operate through common antioxidant mechanisms irrespective of the initiating source of neuronal injury.

Evidence supporting the neuroprotective potential of *Cassia fistula* is comparatively limited but generally consistent with the present findings. Rhein isolated from *Cassia fistula* flowers has been shown to suppress inflammatory signaling, while phytochemicals derived from this plant may modulate multiple Alzheimer's disease-related pathways through antioxidant and anti-inflammatory mechanisms.[27,29] In addition, Balinese *Cassia fistula* flowers have been reported to exhibit high antioxidant activity and contain abundant phenolic and flavonoid compounds, further supporting their potential as a source of neuroprotective bioactive constituents.[13] Collectively, these findings strengthen the biological plausibility that *Cassia fistula* contributed substantially to preservation of hippocampal neurons observed in the present study.

An interesting finding of the present study was that neuronal preservation improved progressively with increasing extract doses. The highest treatment dose produced neuronal counts that were statistically comparable to

those of the normal control group, suggesting that prolonged administration of sufficient phytochemical concentrations may effectively counteract chronic stress-induced neuronal loss. Nevertheless, the absence of a significant difference between the two highest dose groups may indicate that maximal neuroprotection had nearly been achieved within the investigated dose range. Similar plateau responses have been described for antioxidant phytochemicals, in which increasing doses beyond a certain threshold produce diminishing biological benefits because endogenous antioxidant systems have already been substantially restored.[32,33]

Despite these encouraging findings, direct comparison among studies should be interpreted cautiously. Previous investigations differed considerably with respect to experimental species, stress or disease induction methods, extraction procedures, phytochemical composition, treatment duration, outcome measurements, and histopathological scoring systems. Such methodological heterogeneity may account for variations in the magnitude of neuronal preservation reported across studies. Nevertheless, the consistent direction of findings supports the growing body of evidence indicating that antioxidant-rich medicinal plants protect hippocampal neurons against diverse forms of oxidative and inflammatory injury.

The neuroprotective effects observed in the present study are most likely attributable to the complementary antioxidant and anti-inflammatory properties of the phytochemicals present in the combined extracts. Although molecular pathways were not directly investigated, the preservation of hippocampal morphology and neuronal survival strongly suggests attenuation of oxidative neuronal injury. Chronic stress is known to generate excessive reactive oxygen species that overwhelm endogenous antioxidant defenses, resulting in lipid peroxidation, mitochondrial dysfunction, DNA damage, and activation of apoptotic pathways. Consequently, reducing oxidative stress represents one of the principal mechanisms by which botanical antioxidants may preserve neuronal integrity. This interpretation is supported by extensive evidence demonstrating that plant-derived polyphenols and flavonoids effectively counteract oxidative neuronal damage through multiple intracellular pathways.[34,35,36]

Among the bioactive constituents, flavonoids, phenolic compounds, and anthocyanins are considered major contributors to neuronal protection because they exert both direct and indirect antioxidant actions. In addition to scavenging reactive oxygen species, these compounds activate endogenous antioxidant defense systems through regulation of the Nrf2/HO-1 signaling pathway, leading to increased expression of superoxide dismutase, catalase, glutathione peroxidase, and other cytoprotective enzymes.[30,33] Simultaneously, numerous phytochemicals suppress activation of the NF-κB pathway and reduce production of pro-inflammatory cytokines, thereby limiting secondary neuronal injury associated with chronic neuroinflammation.[9,36] Although these molecular events were not measured in the present study, they provide a biologically plausible explanation for the histopathological improvements observed following treatment with the combined extracts.

In addition to their antioxidant activity, phytochemicals from *Cassia fistula* and *Hibiscus sabdariffa* may contribute to neuronal preservation through regulation of mitochondrial function and apoptosis. Experimental studies have shown that flavonoids may modulate the balance between pro-apoptotic proteins such as Bax and anti-apoptotic proteins including Bcl-2, thereby reducing activation of caspase-dependent neuronal death pathways.[10,25] Maintenance of mitochondrial integrity is particularly important in hippocampal neurons because prolonged glucocorticoid exposure disrupts mitochondrial energy metabolism and accelerates neuronal degeneration. Therefore, the higher number of preserved neurons observed in the treatment groups may reflect improved resistance of hippocampal neurons to oxidative and apoptotic injury rather than regeneration of neurons lost during stress exposure.

One distinctive aspect of the present study is the use of a combination of two medicinal plant extracts rather than evaluation of each botanical individually. Previous investigations have predominantly examined the neuroprotective properties of *Cassia fistula* or *Hibiscus sabdariffa* as single interventions. Consequently, information regarding the biological effects of their combined administration remains limited. The present findings therefore provide preliminary evidence that concurrent administration of these botanicals may confer substantial protection against chronic stress-induced hippocampal injury.

Although synergistic interactions were not directly evaluated, the observed dose-dependent preservation of neuronal morphology supports the possibility that complementary phytochemicals derived from both plants act through multiple converging mechanisms. *Cassia fistula* is particularly rich in flavonoids, phenolic compounds, tannins, and anthraquinones with documented antioxidant and anti-inflammatory activities.[12,13,37] *Hibiscus sabdariffa* contains abundant anthocyanins, phenolic acids, vitamin C, and other polyphenolic constituents that have consistently demonstrated neuroprotective properties.[5,14,16,38-39] Because these phytochemicals possess overlapping yet distinct

biological activities, their combination may simultaneously target oxidative stress, neuroinflammation, mitochondrial dysfunction, and apoptosis more effectively than either extract administered alone.

Nevertheless, the present study was not designed to distinguish between additive and true synergistic effects. Since treatment groups receiving individual extracts were not included, it cannot be concluded whether the observed neuroprotection resulted from pharmacological synergy or from the cumulative activity of both extracts. Future studies comparing single-plant and combined formulations, together with phytochemical standardization and mechanistic analyses, are required to determine whether synergistic interactions contribute to the enhanced neuroprotective response observed in the present study.

Contribution of the present study lies in its investigation of combined *Cassia fistula* and *Hibiscus sabdariffa* flower extracts against chronic stress-induced hippocampal injury. While previous studies have primarily evaluated each botanical separately in chemically induced models of neurodegeneration, the present study employed a chronic immobilization stress model that more closely reflects prolonged physiological stress exposure. Moreover, histopathological evaluation demonstrated dose-dependent preservation of hippocampal neurons following administration of the combined extracts, thereby extending existing evidence regarding the neuroprotective potential of antioxidant-rich medicinal plants. These findings provide preliminary experimental support for the development of combination phytotherapeutic strategies targeting stress-related neuronal injury.

The findings of the present study may have important implications for the development of plant-based neuroprotective interventions aimed at mitigating chronic stress-related brain injury. Chronic stress is increasingly recognized as a contributing factor to cognitive decline and neurodegenerative disorders, highlighting the need for safe preventive strategies that can preserve neuronal integrity before irreversible damage occurs. In this context, medicinal plants rich in antioxidant phytochemicals represent promising candidates because they simultaneously target multiple pathological pathways involved in oxidative stress and neuroinflammation.

From a translational perspective, the combined administration of *Cassia fistula* and *Hibiscus sabdariffa* may provide a basis for future development of standardized phytopharmaceutical formulations or functional botanical products. However, translation into clinical application requires substantial additional evidence, including phytochemical standardization, pharmacokinetic characterization, toxicological evaluation, and well-designed clinical trials to confirm efficacy and safety in humans. Therefore, the present findings should be regarded as preclinical evidence supporting further investigation rather than direct evidence for therapeutic use.

Limitation

Several limitations should be acknowledged when interpreting the findings of the present study. First, oxidative stress biomarkers, including malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase, and total antioxidant capacity, were not measured. Consequently, the proposed antioxidant mechanisms remain biologically plausible but cannot be confirmed directly. Second, inflammatory mediators and apoptosis-related proteins, such as tumor necrosis factor-α, interleukin-1β, Bax, Bcl-2, and caspase-3, were not evaluated, limiting mechanistic interpretation of the observed histopathological improvements. Third, phytochemical characterization and standardization of the combined extract were not performed; therefore, the specific bioactive compounds responsible for the neuroprotective effects remain uncertain. Fourth, behavioral assessments of learning and memory were not included, preventing correlation between structural preservation of hippocampal neurons and functional cognitive outcomes. Finally, the relatively small sample size, although consistent with ethical principles for exploratory animal experiments, may have reduced statistical power to detect subtle differences between treatment doses.

CONCLUSION

The present study demonstrated that the combined extracts of *Cassia fistula* flos and *Hibiscus sabdariffa* flos exerted significant neuroprotective effects against chronic stress-induced hippocampal neuronal damage in *Mus musculus*. Among the tested doses, 300 mg/kgBW produced the greatest reduction in neuronal damage and restored the number of normal hippocampal neurons to levels approaching those of the normal control group. These findings suggest that the combination of both extracts possesses promising neuroprotective properties, potentially through the antioxidant and anti-inflammatory activities of their bioactive constituents. Therefore, this herbal combination may represent a promising natural candidate for the development of neuroprotective interventions targeting stress-related neuronal injury. Nevertheless, further studies are required to elucidate the underlying molecular mechanisms, identify

the optimal therapeutic dosage, evaluate long-term safety, and investigate cognitive and behavioral outcomes before considering translational or clinical applications.

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GENERATIVE AI DISCLOSURE STATEMENT

Generative AI assistance (Grammarly, Paperpal, Trinka AI) was used to enhance the structural clarity, academic grammar checking to improve the English language and phrasing of this manuscript. All AI-assisted suggestions were critically reviewed, evaluated, and revised by the authors. The authors take full responsibility for the accuracy and integrity of the data, analyses, and conclusions presented in this manuscript.

AUTHOR CONTRIBUTION STATEMENT

Kadek Buja Harditya: Conceptualization, Methodology, Data curation, Writing—Original draft preparation, Visualization; **Anak Agung Istri Mas Padmiswari**: Supervision, Validation, Writing—Reviewing and Editing.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest regarding the content of this manuscript.

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