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NEUROPROTECTIVE POTENTIAL OF METHANOLIC LEAF EXTRACTS OF CELOSIA CRISTATA AND CALLISTEMON CITRINUS ON SCOPOLAMINE-INDUCED AMNESIA IN SWISS ALBINO MICE

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ABSTRACT
Background: The primary reason for memory loss is Alzheimer's disease, a progressive neurodegenerative condition in specific brain parts. This study aims to illustrate the relative enhancement of memory, along with the neuroprotective and antioxidant properties of methanolic leaf extracts from *Celosia cristata* and *Callistemon citrinus* in scopolamine-induced amnesia in mice. **Methodology:** Methanolic extracts of the leaves of *Celosia cristata* and *Callistemon citrinus* were evaluated for their effects on scopolamine-induced impaired learning and memory in Swiss albino mice using behavioral animal models, including the Morris water maze (MWM), elevated plus maze (EPM), and object recognition task (ORT). Antioxidants such as Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Thiobarbituric acid reactive substance (TBARS), and acetylcholinesterase (AChE) were also assessed at different doses, i.e., 200 and 400 mg/Kg of methanolic extracts of *Celosia cristata* and *Callistemon citrinus*, as well as their combinations. **Results and Discussion:** The various doses of *Celosia cristata* and *Callistemon citrinus* methanolic leaf extracts significantly modified scopolamine effects in experimental animals. Extracts significantly decreased escape latency (ELT) in the MWM test. Inflexion ratio (IR) in the EPM test was significantly raised by extracts, as well as the discrimination index (DI) in ORT. The SOD and GPx levels were significantly enhanced whereas TBARS significantly reduced by extracts. The significant reduced level of AChE was reported in extract treated mice. The extracts from both plants exhibited significant results at different doses (200 mg/kg and 400 mg/kg) and combination of both plant extracts (MCel+MCal 400) at 400mg/kg dose showed most significant result. **Conclusion:** The results revealed that methanolic leaf extracts of *Celosia cristata* and *Callistemon citrinus* hold potent antiamnesic effects.

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INTRODUCTION

Memory refers to a person's capacity to capture sensory stimuli, events, knowledge, and other information, store it for a short or long time, and then retrieve it later when necessary. Memory impairments, characterized by poor retention and slow recall, are increasingly prevalent concerns [1]. The gradually progressing neurodegenerative condition, Alzheimer's disease (AD), is characterized by neuronal deterioration in specific regions of the brain, a major cause of memory impairment. Central cholinergic pathways hold a significant role in cognitive functions, and antimuscarinic medications, such as scopolamine, impair this cognitive function in both animals and humans [2-4]. Epidemiological research on the Indian population indicates that dementia is predominantly an overlooked issue, and the frequency of dementia occurrences escalates significantly as age increases [5-7]. Mitigating oxidative stress through the use of antioxidants, safeguarding against brain inflammation with anti-inflammatory medications, and enhancing cholinergic neurotransmission in the brain with anticholinesterase agents are several beneficial strategies for the management of AD. Current standard medications for AD primarily consist of acetylcholinesterase (AChE) inhibitors. Unfortunately, these medications demonstrate limited effectiveness, and they can produce significant adverse effects. A large number of herbs have been deployed to improve memory and treat conditions associated with dementia. The mechanisms through which phytochemicals operate include exhibiting antioxidant and other neuroprotective effects, inhibiting cholinesterase, restoring mitochondrial energy, and facilitating clearance of aggregated proteins [1, 8]. Ayurveda, an Indian medical system, employs several herbs that have yielded positive results. Vata, Pitta, and Kapha are the three primary doshas that form the foundation of the Ayurvedic medical system. An overactive mind and poor neurotransmission are results of an imbalanced Vata [1, 2]. Approximately 65–80% of the world's population living in developing countries relies essentially on plants for primary healthcare. Herbal medicines serve as a source for approximately 25% of all modern medicines. Numerous studies have indicated that several phytochemicals, including steroids, terpenoids, alkaloids, flavonoids, phenolic compounds, and carbohydrates, exhibit a remarkable neuroprotective effect in neurodegenerative disorders [9-11].

The medicinal plants *Celosia cristata* and *Callistemon citrinus* are rich sources of steroids, terpenoids, alkaloids, flavonoids, phenolic compounds, etc. [12]. Our current research focuses on exploring the effects of leaf extracts in methanol from *Celosia cristata* and *Callistemon citrinus*, as well as their combinations, on the neuroprotective potential against scopolamine-induced amnesia in Swiss albino mice. We conducted MWM, EPM, and ORT to examine memory function and quantify SOD and GPx. The TBARS activity was also evaluated in mice brain sample. Additionally, levels of AChE in the mouse brain were also measured.

MATERIALS AND METHODS

Drugs and Chemicals: All drugs and chemicals were acquired from a local vendor of Lucknow, U.P., India.

Animals: Either sex Swiss albino mice of 25g to 35g body weight were taken from the animal house facility, Faculty of Pharmacy, BBDNIIT, Lucknow, and maintained with adequate water and mice feed pellets and kept at 26±1°C under a controlled 12 h light/dark cycle as per guidelines of IAEC with registration no. BBDNIIT/IAEC/JUNE/2023/08.

Plant material and extraction: Leaves of *Celosia cristata* and *Callistemon citrinus* were taken from Sitapur and Lucknow district (U.P., India). N.B.R.I. authenticated plant materials. Lucknow (U.P., India). Specimens were deposited in the N.B.R.I. herbarium with reference numbers LWG109049 (*Celosia cristata*) & LWG109050 (*Callistemon citrinus*). Cleaned leaves were dried in the shade & ground mechanically. Then, the powder was sieved & stored in an airtight container at 25°C. Leaf powders were defatted in petroleum ether for 24 hours. About 10-15 grams of defatted dried powders extracted with Soxhlet apparatus in 250 ml chloroform, methanol, and distilled water as extraction solvent till the complete exhaustion of sample material at 61°C, 65°C, and 100°C, respectively [13, 14]. Extracts were dried in a rotary flash evaporator and stored in a refrigerator for further evaluations.

Phytochemical analysis: A phytochemical investigation of leaf extracts from *Celosia cristata* and *Callistemon citrinus* was conducted to identify the principal phytochemicals responsible for addressing neurodegenerative disorders [15, 16].

Test for alkaloids: Dragendorff's reagent was introduced to the extract solution. The precipitate, of a reddish-brown color, revealed the presence of alkaloids.

Test for phenolic compounds: On addition of lead acetate 10% solution to the extract, white color precipitation revealed the presence of phenolic compounds.

Test for flavonoids: To extract solution, a small amount of magnesium turnings was introduced & then conc. HCl was added. The appearance of Crimson red color revealed the presence of flavonoids.

Test for steroids and terpenoids: To extract, add a few drops of acetic anhydride, then boil and cool. Then, conc. H_2SO_4 was added. A brown ring developed at the intersection of two layers, and the upper layer became green, indicating the presence of steroids. The formation of a deep red color signified the presence of triterpenoids.

Experimental design: All animals were divided into 9 groups (n=5), & treatment was administered daily for 8 days as follows:
Group I (Control): 0.3% Carboxymethyl cellulose, p.o.
Group II (Scopolamine): Scopolamine 0.4mg/kg, i.p.
Group III (Piracetam): Piracetam 200 mg/kg, p.o.
Group IV (MCel 200): Methanolic extract of *Celosia cristata* 200 mg/kg p.o.
Group V (MCel 400): Methanolic extract of *Celosia cristata* 400 mg/kg p.o.
Group VI (MCal 200): Methanolic extract of *Callistemon citrinus* 200 mg/kg p.o.
Group VII (MCal 400): Methanolic extract of *Callistemon citrinus* 400 mg/kg p.o.
Group VIII (MCel+MCal 200): Combination of Methanolic extract *Celosia cristata* 100 mg/kg + Methanolic extract *Callistemon citrinus* 100 mg/kg p.o.
Group IX (MCel+MCal 400): Combination of Methanolic extract *Celosia cristata* 200 mg/kg + Methanolic extract *Callistemon citrinus* 200 mg/kg p.o.

Behavioural Models: Training was provided to each mouse daily for 15-30 minutes to perform tasks. Subsequently, all behavioral models were conducted to examine cognitive functions (i.e., learning and memory) in mice.

Morris water maze (MWM) test: MWM has various benefits, including: (a) elimination of intramaze cues like odor trains in pool; (b) ability to train animals in a shorter duration (2–7 days); and (c) ability to conduct larger dose–response studies in a matter of weeks [17-20]. It consisted of a circular water tank (35 cm height and 1.5 m diameter), filled with water (at 26±1℃). The water maze was divided into 4 equal divisions by two threads that intersected at right angles. A circular platform (25 cm in height) was placed in one quadrant, 1 cm above and 1 cm below the water level during the acquisition phase and the retention phase, respectively. Each animal underwent four successive trials each day, permitted to step onto the platform and stay there for 10 seconds. When the animal could not find the platform within a 120-second timeframe, it was gently guided and empowered to remain there for 10 seconds. Escape latency time (ELT) was documented as a quantitative metric for finding the platform in the water maze, a sign of learning. Every animal underwent 4 trials (acquisition) daily for three successive days. On day four, after 30 minutes of different administrations, 0.4mg/kg of scopolamine was administered intraperitoneally (i.p.) to all groups except the control group. Thirty minutes post-administration of scopolamine, the platform was hidden, and the time spent by the animal searching for the hidden platform (ELT) was documented as the retention phase [19, 20].

Elevated plus maze (EPM) test: The EPM consisted of 2 enclosed arms (25cm × 6cm × 15cm) and 2 open arms (25cm × 6cm), elevated to 40 cm above the ground. On the 8th day, 0.4 mg/kg scopolamine was administered intraperitoneally to different groups. After thirty minutes of respective treatments and thirty minutes post-administration of scopolamine, mice were placed one by one at the extremity of an open arm. The duration a mouse uses all its legs to enter any enclosed area is documented as the transfer latency (TL). If the animal failed to enter within 90 seconds, it was softly pushed into the enclosed arm, and TL was noted as 90 seconds. The animal was empowered to investigate the maze for the next 2 minutes. At day 8, transfer latency was documented as L0 (indicated learning). Retention of the acquired task was evaluated after 24 h (day 9), TL was noted as L1. Inflexion ratio (IR) represented the effect on TL and was determined as follows [19].

$$\mathrm{IR} = \frac{(\mathrm{L0} - \mathrm{L1})}{L1}$$

Object recognition task (ORT): Instrument is a wooden box (40 × 40 × 30 cm). The object to be discriminated was made up of wood in two dissimilar shapes. On day 8 in T1 (the first trial), two similar objects were placed in the corners of the box, and the time it took for the mice to explore the wooden object was recorded. During T2 (the second trial, 24 hours after the first trial), a newly introduced object replaced one of the items from the first trial, and each mouse was retained inside the instrument for five minutes. Time taken to explore F (familiar) and N (new) objects was documented, and DI (discrimination index) was obtained by (N–F) / (N+F) [19].

BIOCHEMICAL ASSAY

Supernatant Preparation: Following the completion of the test, the mice were euthanized; the brains from each mouse were meticulously extracted and washed with chilled 0.9% NaCl. A phosphate buffer was used to prepare the brain homogenates, and centrifugation was performed at 10,000 rpm and 4 °C for 10 minutes. All biochemical assays were performed on the collected supernatant.

Superoxide dismutase (SOD) activity: The photoreduction of riboflavin generated a superoxide radical. The 100 µL of supernatant was mixed with 700 µL of reactive, then it was made up to 1.5 mL with a solution containing 2 µM riboflavin, 100 mM Tris/HCl (pH 7.8), 6 mM EDTA, and 75 mM nitro blue tetrazolium dye. At 560 nm, absorbance was taken. Reports were documented as U/mg of protein [19].

Glutathione Peroxidase (GPx) activity: GPx activity was measured using diagnostic kits. GPx catalyzed the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. Absorbance was measured at 340 nm, and the findings were presented as Units per Milligram of protein [21].

Thiobarbituric Acid Reactive Substance (TBARS) activity: 700 µL phosphoric acid (9%), and 250 µL of TBA were added to the supernatant (250 µL). Following stirring, the blend was heated (01 h), then cooled. Then, 1,250 µL of butanol was added, the blend was vortexed for 20 seconds, and then centrifuged at 25°C for 20 minutes. Absorbance was taken at 534 nm. Findings were reported as mmol/mg protein [22].

Acetylcholine esterase (AChE) activity: In a 96-well plate, 10 µl of samples were mixed with 70 µl of reaction buffer and 10µl of 5, 5-dithiobis (2-nitrobenzoate) (DTNB) in each well. Then, 10 µL of acetyl-thiocholine iodide (15 mM) was added to the reaction mixture. Absorbance was measured at 412 nm, and the results were presented as units per milligram of protein [23, 24].

Statistical analysis: Data analysis was performed using GraphPad Prism version 9 and documented as the mean ± SEM of two-way and one-way ANOVA tests, followed by Tukey's multiple comparisons test.

RESULTS

Phytochemical analysis

Phytochemical test indicated the presence of flavonoids, phenolic compounds, alkaloids, steroids, and terpenoids in methanolic leaf extracts of *Celosia cristata* and *Callistemon citrinus.*

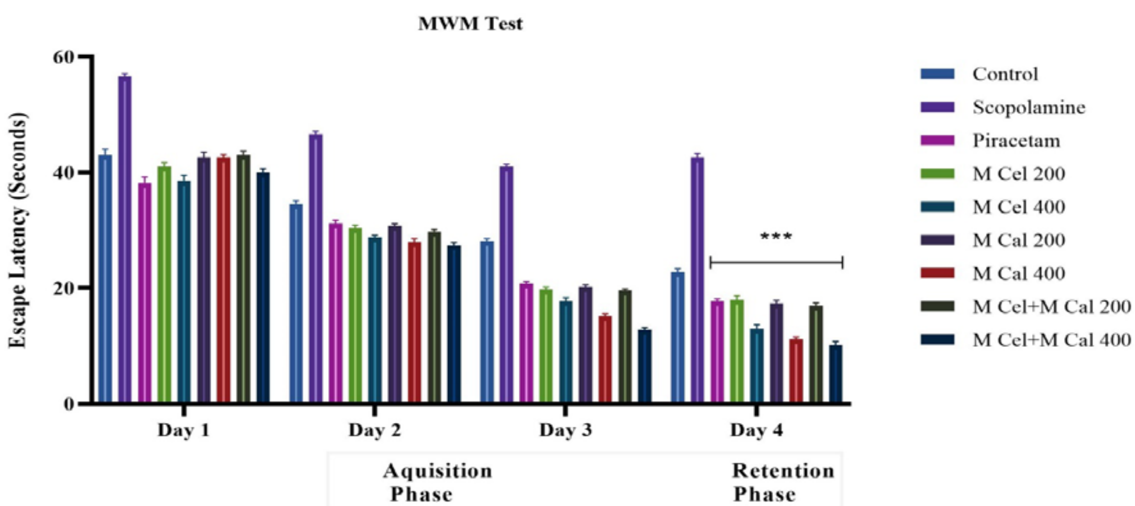

Figure 1: MWM test: ELT (seconds) for all groups. Data given as mean ± SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 as compared with scopolamine-treated group, Two- way ANOVA tests, followed by Tukey's multiple comparisons test.

Morris water maze (MWM)

Following the acquisition phase for three consecutive days, scopolamine administration on the fourth day (retention phase) significantly increased the ELT in the scopolamine-treated group compared to mice from the control group. Various doses of methanolic extracts of *Celosia cristata* and *Callistemon citrinus* leaves were decreased considerably by ELT induced by scopolamine. Piracetam also significantly reduced ELT (Figure 1).

Elevated plus maze (EPM)

Mice receiving scopolamine showed significantly increased TL and decreased IR when compared to the control group (Figure 2). Mice treated with *Celosia cristata* and *Callistemon citrinus* methanolic leaf extracts showed a significant drop of TL and an increase of IR in a dose-dependent manner in comparison with scopolamine-treated groups. Maximum IR was reported at treatment of MCel+MCal 400 (0.86±0.07). The TL reported at treatment MCel+MCal 400 was 14.20 ± 0.66 s. Piracetam treatment also decreased TL and increased IR significantly against the scopolamine group.

Object recognition task (ORT)

Different doses of *Celosia cristata* and *Callistemon citrinus* meth. Leaf extracts significantly increase the discrimination index (DI) in scopolamine-treated mice when compared to mice receiving MCel200, MCel400, MCal200, MCal400, MCel+MCal 200 & MCel+MCal 400 treatment. The exploration time for new objects was also significantly elevated by the different treatments of both plant extracts. Both plant extracts significantly decreased the time to explore the familiar object in all treatments. Piracetam increased DI & time to explore new objects & reduced time taken to explore familiar objects significantly (Figure 3).

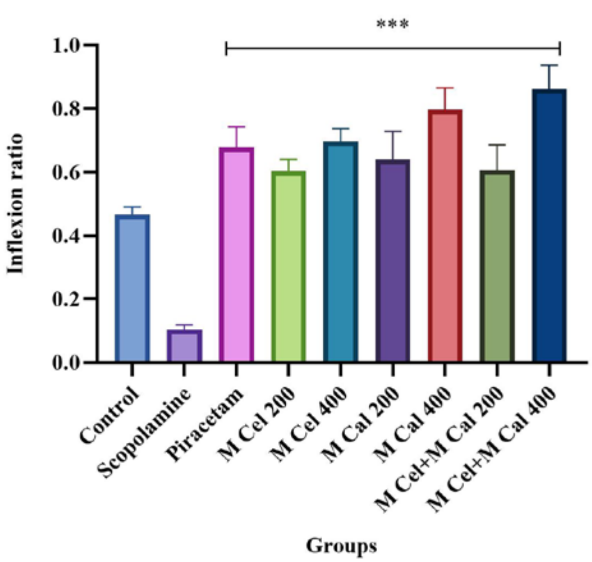

Figure 2: Inflexion ratio (IR) for all groups in the EPM test. Dat given as mean ± SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.00 as compared with the scopolamine-treated group, One-wa ANOVA tests, followed by Tukey's multiple comparisons test.

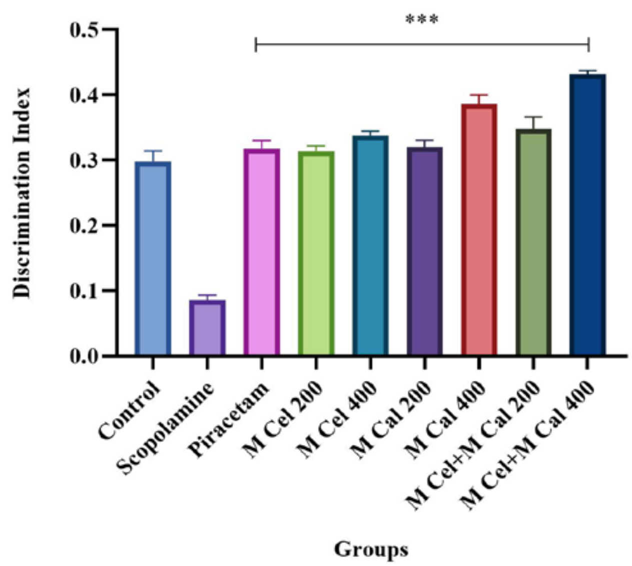

Figure 3: DI for all groups in ORT. Data in mean ± SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 as compared to scopolamine group, One-way ANOVA tests, followed by Tukey's multiple comparisons test.

Superoxide Dismutase (SOD)

A significant decrease in SOD activity was observed in the group treated with scopolamine compared to the control group. The mice treated with different doses of *Celosia cristata* and *Callistemon citrinus* meth. Leaf extracts significantly elevated SOD activity compared to the scopolamine-treated group. SOD activity was also increased in the piracetam-treated group significantly (Figure 4).

Glutathione peroxidase (GPx)

GPx activity was significantly decreased in the Scopolamine-treated group versus the control group. Results indicated a significant increase in GPx activity at various doses of *Celosia cristata* and *Callistemon citrinus* meth. leaf extracts when compared with the scopolamine-treated group. GPx activity in the piracetam-treated group was also increased (Figure 5).

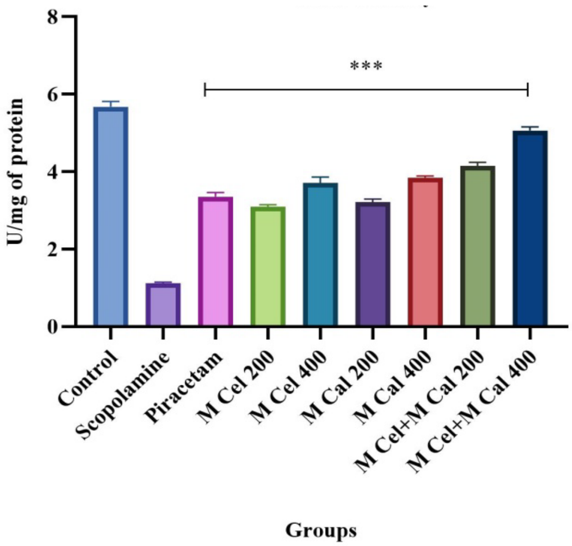

Figure 4: SOD activity: all groups exhibited significant elevation versus the scopolamine-treated group. *Data documented in mean±SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 as compared with the scopolamine-treated group, One-way ANOVA tests, followed by Tukey's multiple comparisons test.*

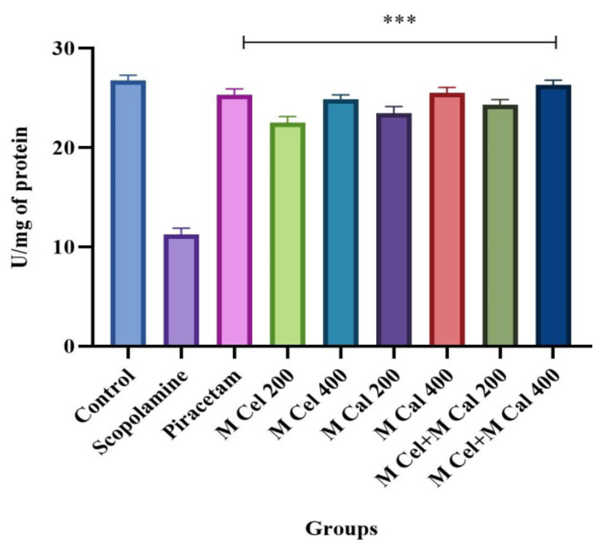

Figure 5: GPx activity: all groups raised significantly versus the scopolamine-treated group. *Data documented in mean±SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 as compared with the scopolamine-treated group, One-way ANOVA tests, followed by Tukey's multiple comparisons test.*

Thiobarbituric acid reactive substance (TBARS)

The scopolamine-administered group exhibited a significant increase in TBARS concentration vs the control group. Different doses of *Celosia cristata* & *Callistemon citrinus*, i.e., MCel 200, MCel 400, MCal 200, MCal 400, MCel + MCal 200, and MCel + MCal 400, significantly decrease the TBARS concentration in comparison to scopolamine-treated mice (Figure 6).

Acetylcholinesterase (AChE) activity

Scopolamine administration showed a significant increase in brain AChE activity. All treatment groups, MCel 200, MCel 400, MCal 200, MCal 400, MCel+MCal 200, and MCel+MCal 400, resulted in a significant decrease in AChE activity when compared to the scopolamine-treated mice group (Figure 7).

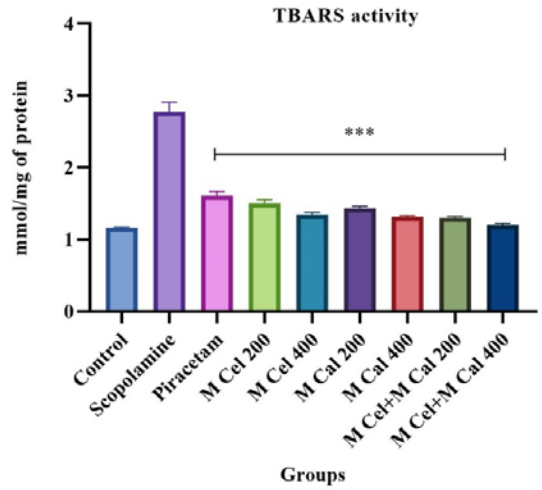

Figure 6: TBARS activity: decreased significantly in every group versus the scopolamine-treated group. *Data documented in mean±SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 as compared with the scopolamine-treated group, One-way ANOVA tests, followed by Tukey's multiple comparisons test.*

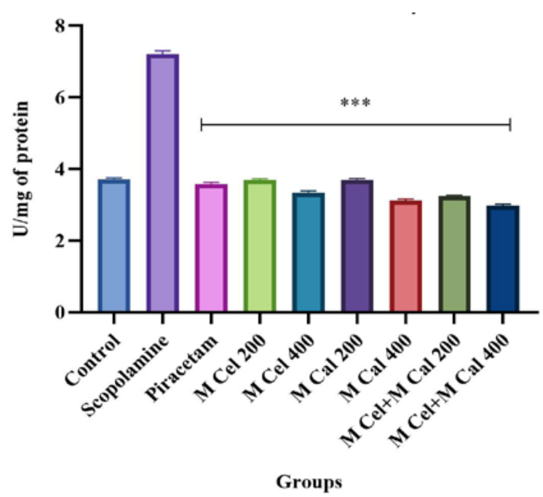

Figure 7: AChE activity: significant decrease in every treatment versus the scopolamine group. *Data given in mean±SEM, n=5. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001 on comparison to scopolamine-treated group, One-way ANOVA tests, followed by Tukey's multiple comparisons test.*

DISCUSSION

MWM is used for the validation of rodent models to evaluate potential neurocognitive treatments [25-27] and to increase ELT in the scopolamine-treated group, which exhibits memory impairment in mice. At different doses, methanolic extracts of leaves of *Celosia cristata* and *Callistemon citrinus* significantly reversed this effect by decreasing the prolonged ELT induced by scopolamine, suggesting the recall of the platform.

A significant decrease in IR and an increase in TL were reported in scopolamine-treated mice, indicating impaired cognitive function in the EPM due to its antimuscarinic action on cholinoceptors [28-30]. Pretreatment with varying doses of methanolic leaf extracts enhanced IR and reduced TL, indicating cognitive function improvement in scopolamine-treated mice. In ORT, scopolamine significantly reduced the DI, showing impaired learning and memory [31, 32]. This effect was counteracted by methanolic leaf extracts from both plants, notably enhancing DI and the time consumed for exploring novel objects, which suggests that the extracts possess memory-enhancing activity through their encouraging effect on recognizing the original object. Reduced serum levels of SOD and GPx after scopolamine administration indicate their consumption due to heightened activity of oxygen-free radicals, leading to impaired memory. Evidence suggests that lipid peroxidation is a crucial mechanism of neurodegeneration in AD, providing significant evidence of learning and memory deficits, as indicated by an elevation in TBARS [33, 34].

Studies from earlier days revealed that TBARS activity increased in both the hippocampus and the frontal cortex region of the brain following scopolamine administration [35, 36]. Malondialdehyde (MDA) is a product of oxidative damage resulting from lipid peroxidation, indicating oxidative stress [37, 38]. Antioxidant enzymes, such as SOD and GPx, are responsible for reducing oxidative stress [39, 40]. In this research work, methanolic leaf extracts of *Celosia cristata* and *Callistemon citrinus* decreased TBARS activity. They increased the level of SOD and GPx in the brain, suggesting neuroprotective effects due to antioxidant properties. Scopolamine administration increases AChE activity, resulting in a greater breakdown of ACh, and subsequently reduces the brain's ACh reserve, which is crucial for learning and memory [41, 42]. We found that the methanolic leaf extracts of *Celosia cristata* and *Callistemon citrinus* significantly reduced AChE activity compared to mice treated with scopolamine. It shows that both plants have a protective function in the breakdown of ACh, leading to an increase in cholinergic neurotransmission and a subsequent neuroprotective effect.

Phytochemical investigation of both plant extracts exhibited the presence of flavonoids, alkaloids, polyphenols, steroids, and terpenoids. Numerous researchers have reported that these phytochemicals primarily exhibit antioxidant, anti-inflammatory, and neuroprotective properties, which are key elements in the treatment of neurodegenerative diseases [43]. Polyphenolic compounds provide neuronal defense against neurotoxin-induced damage by reducing neuroinflammation and may enhance cognitive abilities [44]. Flavonoids have antioxidant effects, protecting the brain by modulating intracellular signals and promoting cellular survival [45]. Steroid and terpenoid-derived phytochemicals are beneficial in alleviating neurodegeneration [46].

CONCLUSION

This study demonstrated that the methanolic leaf extracts of *Celosia cristata* and *Callistemon citrinus*, namely MCel, MCal, and MCel+MCal, exhibit promising learning and memory-enhancing activity as a neuroprotective and antiamnesic plant at various doses, specifically 200mg/kg and 400mg/kg. The extracts exhibited an antiamnesic effect by reversing scopolamine-induced learning and memory deficits and neurotoxicity, as evidenced by improvements in brain levels of SOD, GPx, TBARS, and AChE. Nonetheless, further research is needed to investigate the various other potential mechanisms of neuroprotection provided by both plants.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Vishwambhar Mishra designed the study, conducted the laboratory experiments, collected, analyzed, and interpreted the data, and drafted and revised the manuscript. Bhupendra Chauhan contributed to the design of the study, guided its execution, and drafted and revised the manuscript. Sanjiv Kumar Chaudhri guided the studies and helped to shape the final manuscript. Deepika Rani drafted and revised the manuscript. All the authors read, revised, and approved the submitted version of the manuscript.

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