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Original Article

Housing In An Enriched Environment Enhances The Neuroprotective Effect Of *Celastrus Paniculatus* And *Tribulus Terrestris In* An Animal Model Of Chronic Stress

Babiker Bashir Haroun Baraka[1,2], Bhagya Venkanna Rao[1]*, Tanuja Krishnamurthy[1], Ramya Vasudev[1]

ABSTRACT

Introduction: Prolonged exposure to stress can contribute to depressive episodes. Preclinical studies have shown that stimuli like environmental enrichment (EE) can produce beneficial effects against stress by positively modulating neuroplasticity, neurochemistry, and behaviour. Hence, we explored whether exposure to EE can augment the neuroprotective activities of *Celastrus paniculatus* (CP) and *Tribulus terrestris* (TT) in stressed conditions. **Materials and Methods**: Rats were placed in immobilisation bags and stressed for two hours a day for ten days. After that, these stressed rats were treated by CP or TT, alone or in combination with enriched housing. Behavioural analysis in elevated plus maze, open field, forced swim and sucrose preference tests. The novel object recognition test and the rewarded alteration test on the T-maze were used to assess working memory. The brain-derived neurotrophic factor, interleukin-6, and tumour necrotic factor-alpha were measured in the hippocampus and prefrontal cortical tissues following stress and herbal treatment combined with an enriched environment. **Results**: We found that *Celastrus paniculatus* and *Tribulus terrestris*, combined with an enriched environment, produced a synergistic neuroprotective effect. CP + EE and TT + EE improved working memory and recognition memory in CIS animals, but they also reduced anxiety and depressive-like behaviours. TNF-α and IL-6 levels were decreased while brain-derived neurotrophic factor levels were raised in the frontal cortex and hippocampus regions, respectively. **Conclusion**: Our results show that living in an enriched environment can improve CP and TT neuromodulatory activities, highlighting the potential of combining sensory-motor interventions with herbal remedies for psychiatric disorders.

Key words: Chronic Immobilization stress, Depression, Enriched Environment, Hippocampus, Neurotrophic factor, Pro-inflammatory cytokines.

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INTRODUCTION

As per the World Health Organization, depression exerts a significant negative influence on health, affecting over 300 million people worldwide and standing as a leading cause of disability.[1] Persistent stress has emerged as a primary risk factor for developing neuropsychiatric disorders such as anxiety and affective disorders.[2-6] Prolonged exposure to stress can result in neurodegeneration and cognitive impairments due to the release of pro-inflammatory markers and glucocorticoids.[5-7]

Persistent stress and heightened neuroinflammation are implicated in triggering the overproduction of inflammatory molecules like TNF-α, interleukin (IL)-6, and IL-1β, potentially contributing to major depressive disorder.[8-10] Inflammation is associated with significant changes in behaviour, including depressive symptoms such as fatigue, hopelessness, loss of pleasure, slowed movement, and social withdrawal.[11] The aetiology of severe depression involves factors related to brain-derived neurotrophic agents.[12-15]

Environmental enrichment (EE), namely, the exposure to sensory, motor, cognitive, and social stimulation higher than that received in standard housing conditions,[16-17] has been shown to decrease anxiety- and depression-like behaviours.[18-20] EE also improves learning and memory[21-22] and increases 5-HT levels in the hippocampus and the prefrontal cortex.[18-19]

Traditional medicine systems have always used herbal plants as the primary constituent of medicine. Many herbs and extracts have been studied for their neuroprotective properties due to flavonoids and other phenolic compounds. [23-24] Because of its ability to improve cognition, *Celastrus paniculatus* is also known as the "elixir of life" in traditional medicine.[25] Research has shown that CP oil protects neurons from glutamate-induced toxicity by modulating glutamate receptors.[26] It is beneficial in stress-induced cognitive dysfunction and exhibits dose-dependent anti-cholinesterase activity in the rat brain.[25] However, the effect of CP seed oil combined with an enriched environment on chronic stress-associated cognitive deficits has not yet been explored. Therefore, this study aims to assess the neuroprotective effects of CP seed oil in combination with EE using a chronic immobilization stress model.

Tribulus terrestris Linn is a traditional medicinal plant used for various health conditions. This plant exhibits many pharmacological activities, including anti-cancer, anti-diabetic, antispasmodic, aphrodisiac, cardiotonic, diuretic, nerve tonic, and immunomodulatory effects.[27-29] These studies highlight its therapeutic potential against multiple diseases, likely due to the presence of flavonoids and phenolic compounds.[29-30] Previous studies demonstrated the neuroprotective effects of TT in different animal models.

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Chauhdary et al. demonstrated the neuroprotective effect of TT extracts in aluminium chloride-induced Alzheimer's disease in rats. The antioxidant activity of the extract and the chelating properties of flavonoids were connected with biochemical and behavioural improvement. [31] The *Tribulus terrestris* saponins pre-treated cerebral ischemic injury group showed significantly reduced infarct volume, brain oedema and neurobehavioral abnormality, and reduced pathologic changes in the brain, as well as had lower levels of serum TNF-α and IL-1β, and higher levels of brain NF-κB.[32] TT has protective effects against rotenone-induced oxidative damage and dopamine neuron loss in the substantia nigra of mice; it can also improve motor dysfunction and inhibit DNA damage and oxidative stress in PD mice.[33]

Hence, the current study investigated the effects of social enrichment combined with herbal drugs *Celastrus paniculatus* and *Tribulus terrestris*on memory, neurotrophic support, and neuroinflammation in male Wistar rats exposed to immobilisation stress for 10 days.

METHODS

Animals

Adult male Wistar rats (body mean weight 180-200g), were kept in standard lab settings at room temperature (25± 2°C) with a 12 h light/dark cycle. Animals were provided with standard food and adequate water during the experiment. The KLE College of Pharmacy Institutional Animal Ethics Committee, Bengaluru approved all procedures of the current research study (02/BVR/2021. The study was carried out according to CPCSEA guidelines.

Chronic immobilisation stress (CIS)

Rats experienced 10 days of immobilisation stress 2 hours per day as described earlier without access to food or water. After stress protocol, rats were placed back in their home cages with food and water.[5-6, 34-35]

Enriched Environment

Enriched animals were housed in large cages in groups of 8 rats for 3h/day for 14-day periods with water and food inside the cage. The cage contained toys of different sizes and materials, climbing ladders, and tunnels. To maintain novelty, all objects were changed on alternative days.[3,5, 36-37]

Drug treatment

The fruit of the plant TT was gathered from Siliguri, West Bengal, Maity, and verified by botanist Dr. N. Dhatchanamoorthy, Assistant Professor, Plant Systematic and Nomenclature, Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, India. The voucher specimen was stored in FRLHT Coll. No. 4720. The ethanolic extraction was performed using the condenser technique and a Soxhlet extractor. The powder was protected from direct sunlight and stored in an airtight container. CP seed oil, batch number 003S, was supplied by SadvaidyasalaPvt. Ltd., an analytical laboratory located at the M.G.S. Road in Nanjangud, India. To dissolve the oil, 1% Tween 20 and 5% DMSO were utilised.

Grouping

Animals were randomly divided into 5groups; eachconsistedof 8 rats for behavioural tests and 5 rats for biochemical estimation. Different sets of animals were used for behavioural and biochemical tests. Normal control (NC): Without stress and were housed in a standard cage; chronic immobilisation stress (CIS):Rats were subjected to immobilisation stress for 2 hours per day for 10 days; CIS + EE: Stressed rats were exposed to EE for 3 hours per day for 14 days;CIS + EE + TT: Stressed rats were exposed to EE for 3 hours per day, followed by oral administration of TT (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: Stressed rats were exposed to EE for 3 hours per day, followed by intraperitoneal administration of CP oil dissolved in DMSO and Tween-20 (250 mg/kg, i.p,.) for 14 days. After the treatment protocol, one set of animals was tested for anxiety and depressive behaviour in an open field, elevated plus maze, sucrose preference, and forced swim tests, respectively. The second set of animals was subjected to novel object recognition and T-maze tests to assess memory. The third set was sacrificed, and hippocampal and frontal cortical tissues were harvested to estimate BDNF, TNF-α, and IL-6 levels using an ELISA reader.

Sucrose preference test (SPT)

Rats were provided with drinking water and a 1% w/v sucrose solution in their home cages over a two-day training period. During this time, the volume of water and sucrose solution consumed by each animal was recorded daily. After 18 hours of food and water restriction, the animals underwent a test session where they were given a choice between two bottles: one containing sucrose water and the other containing plain water, for two hours. Sucrose preference was calculated using the following formula: the amount of sucrose water consumed divided by the total liquid consumed, multiplied by 100.[5-6, 25, 36-37]

Forced swim test (FST)

On the first day, each rat was placed individually in a plastic cylinder (50 cm tall and 23 cm in diameter) filled with water at 25 ± 2 °C, reaching a depth of 30 cm. They were made to swim for 15 minutes. Afterwards, they were taken out, dried with towels, and kept warm under a lamp for 10 minutes. The water was emptied after each trial to prevent any olfactory cues. After 24 hours, the rats were tested again for five minutes under the same conditions, and the session was video-recorded for subsequent analysis. The coded videos were reviewed for the duration of immobility by an experimenter who was blind to the treatment conditions. Rats were considered immobile if they only moved to keep their heads above water.[5-6, 25, 36-37]

Open field Test (OFT)

The open field test, a recognised method for assessing anxiety, was conducted in a wooden arena measuring 100 x 100 x 40 cm, with a black floor divided into 25 squares (20 cm x 20 cm each). The rats were given 5 minutes to explore the open field freely, starting from the centre. During offline video analysis, we recorded the number of squares crossed (16 squares), the time spent in the periphery (9 squares), and the duration spent in the centre.[5-6, 25, 36-37]

Elevated plus Maze (EPM)

The Elevated plus maze (EPM) is one of the most popular tests for evaluating anxiety behaviours in rodents. A cross-shaped maze with two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) that was 50 cm above the floor was used for the test. Each rat was put in the centre, facing an open arm, and its movements were recorded for five minutes using a mounted camera for offline analysis. The rats were put back in their cages following the test. The assessed variables included the time spent in both open and closed arms, the number of entrances into each arm, the number of head dips in the open arms, and the number of rearings in the closed arms.[5-6, 25, 36-37]

Spatial learning and memory evaluation in T-maze rewarded alternation task

To evaluate the working memory capabilities, rats were tested in a T-maze using a rewarded alternation task as described in previous studies. The wooden T-maze consisted of two side arms and one start arm. Before training, the rats were familiarized with the maze for 10 minutes over two days, during which food choco pellets were scattered

throughout. After the acclimatization phase, the rats underwent training for 10 consecutive trials each day, alternating between the left and right arms to receive the reward, continuing for 10 days. During the 30-second inter-trial intervals, the rats were returned to the start box, and the arms were cleaned with 70% alcohol to eliminate any odour cues. A memory retention test was conducted two days after the training session, consisting of ten consecutive trials separated by 30-second intervals. The number of correct choices made during both acquisition and retention was recorded manually.[3,25]

Novel object recognition test (NORT)

The NORT procedure consists of three phases: habituation, familiarization, and the test phase. During the habituation phase, each animal is free to move around the open-field arena without any obstacles for 10 minutes. After this, the animal is removed and placed in the cage. In the familiarization phase, each rat is left in the open-field arena with two identical objects (A + A) for ten minutes. In the test phase, the animal is returned to the open-field arena after 24 h, this time with two objects: A (the familiar object) and B (the novel object). The objects are placed in opposite, symmetrical corners of the arena, ensuring that the placement of the novel and familiar objects is balanced. Typically, normal rats show a preference for exploring the novel object more during the first few minutes of the test phase. This preference suggests that the animal remembers the familiar object.[34-35]

Biomarkers Analysis

After the treatment protocol, rats were sacrificed, and the hippocampus and prefrontal cortex were collected and stored at -20°C until biochemical estimation. Using BSA as a reference protein, the total protein content was calculated. Hippocampus and prefrontal cortex tissues were homogenized in PBS and centrifuged at 5000 g for 5 min. In the tissue lysate supernatants, IL-6, TNF-α, and BDNF levels were determined using commercial ELISA kits (BD OptEIA™, Biosciences).[34-35]

Statistical Analysis

The data are presented as the mean ± SEM for each group of 8 rats. We used versions 5 of the statistical software GraphPad Prism for data analysis. To compare all groups, we employed a one-way ANOVA followed by Tukey's multiple comparisons test or a repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test. P values less than 0.05 were considered statistically significant.

RESULTS

Combination treatment ameliorates anxiety-like behaviour in immobilised rats

The elevated plus maze (EPM) was utilised to assess anxiety-like behaviour in stressed rats, revealing significant differences between the stressed group and those receiving combination treatments. Data analysis from the EPM indicated that stressed animals spent considerably less time in the open arms (Figure 1A: $F_{4,35}$= 19.31; p<0.001) and made fewer entries into the open arms (Figure 1C: $F_{4,35}$ = 16.55; p<0.001) while spending significantly more time in the closed arms (Figure 1B: $F_{4,35}$ = 17.79; p<0.001) compared to the normal control group. Additionally, the number of head dips decreased in the stressed rats (Figure 1E: $F_{4,35}$ = 5.55; p<0.01), suggesting reduced exploratory behaviour and heightened anxiety levels.

In contrast, the combination treatment of EE and TT showed moderate anxiolytic effects, indicated by an increase in both the number of entries into the open arms (Figure 1C: $F_{4,35}$ = 16.55; p<0.001) and the number of head dips (Figure 1E: $F_{4,35}$ = 5.55; p<0.05). While there was a rise in time spent in the open arms and a reduction in the time spent in the closed arms, these changes were not statistically significant (p>0.05). Notably, the combination of EE and CP demonstrated superior anxiolytic activity, with significant increases in both the time spent in the open arms (Figure 1A: $F_{4,35}$ = 19.31; p<0.001) and the number of open arm entries (Figure 1C: $F_{4,35}$ = 16.55; p<0.001), along with a decrease in the time spent in the closed arms (Figure 1B: $F_{4,35}$ = 17.79; p<0.001). This treatment also enhanced exploratory behaviour, as shown bythe increased head dips (Figure 1E: $F_{4,35}$ = 5.55; p<0.01).

Effects of combining enriched environment with herbal drug treatment in restoration of exploratory behaviour in stressed rodents

The open field test (OFT) is a widely utilised experimental framework for evaluating anxiety-like behaviour in rodents. This test assesses exploratory behaviour, which is typically diminished in anxious animals. In the disease control group, both the number of squares crossed in the centre (Figure 2C: $F_{4,35}$ = 17.53; p<0.001) and the periphery (Figure 2D: $F_{4,35}$ = 20.94; p<0.01) were significantly reduced, along with a decrease in the time spent in the centre (Figure 2A: $F_{4,35}$ = 11.86; p<0.01) compared to the normal control group. These findings indicate that exploration was notably impaired in the stressed animals, suggesting the presence of anxiety-like behaviour.

However, anxiety-like behaviour induced by chronic immobilisation stress was effectively mitigated by herbal drugs when combined with an enriched environment. The combination of TT + EE and CP + EE led to a significant increase in the number of squares crossed in the centre (Figure 2C: $F_{4,35}$ = 17.53; p<0.001) and periphery (Figure 2D: $F_{4,35}$ = 20.94; p<0.001)and the time spent in the centre(Figure 2A: $F_{4,35}$ = 11.86; p<0.001). This enhancement in exploratory behaviour compared to the disease control group suggests a marked reduction in anxiety levels.

Combination of herbal drug treatment with EE restores sucrose preference in stressed animals

The sucrose preference test (SPT) is used to measure anhedonia, a primary symptom of depression. The current study shows that exposure to chronic immobilisation stress significantly decreased (Figure 3A: $F_{4,35}$ = 32.10; p<0.001) sucrose preference compared to healthy control animals, indicating anhedonia. However, the combination of treatment with TT and EE, CP with EE, successfully restored preference for the sucrose solution, showing a higher percentage of sucrose consumption (Figure 3A: $F_{4,35}$ = 32.10; p<0.001) than that observed in the stressed animals. Additionally, exposure to an enriched environment had an equal effect (p<0.001) as a combination treatment compared to normal control. Integrating herbal treatments with environmental enrichment enhances the effectiveness of reversing anhedonic behaviour.

Impact of combination treatments on behavioural despair in chronic stress

The forced swim test (FST), was used to evaluate behavioural despair by examining how animals cope with an uncontrollable and stressful environment. In this study, chronically stressed animals demonstrated a significant increase in immobility time (Figure 3B: $F_{4,35}$ = 33.59; p<0.001), a behaviour commonly linked to depressive-like states, compared to healthy controls. In contrast, the combination treatment and EE groupsnotably reduced immobility time (Figure 3B: $F_{4,35}$ = 33.59; p<0.001) relative to the disease control group.

The beneficial effect of combining EE with herbal drugs in mitigating chronic stress-induced learning and memory impairment

The T-maze alteration task was used to assess learning and memory in rodents. The present study indicates that chronic stress adversely affects

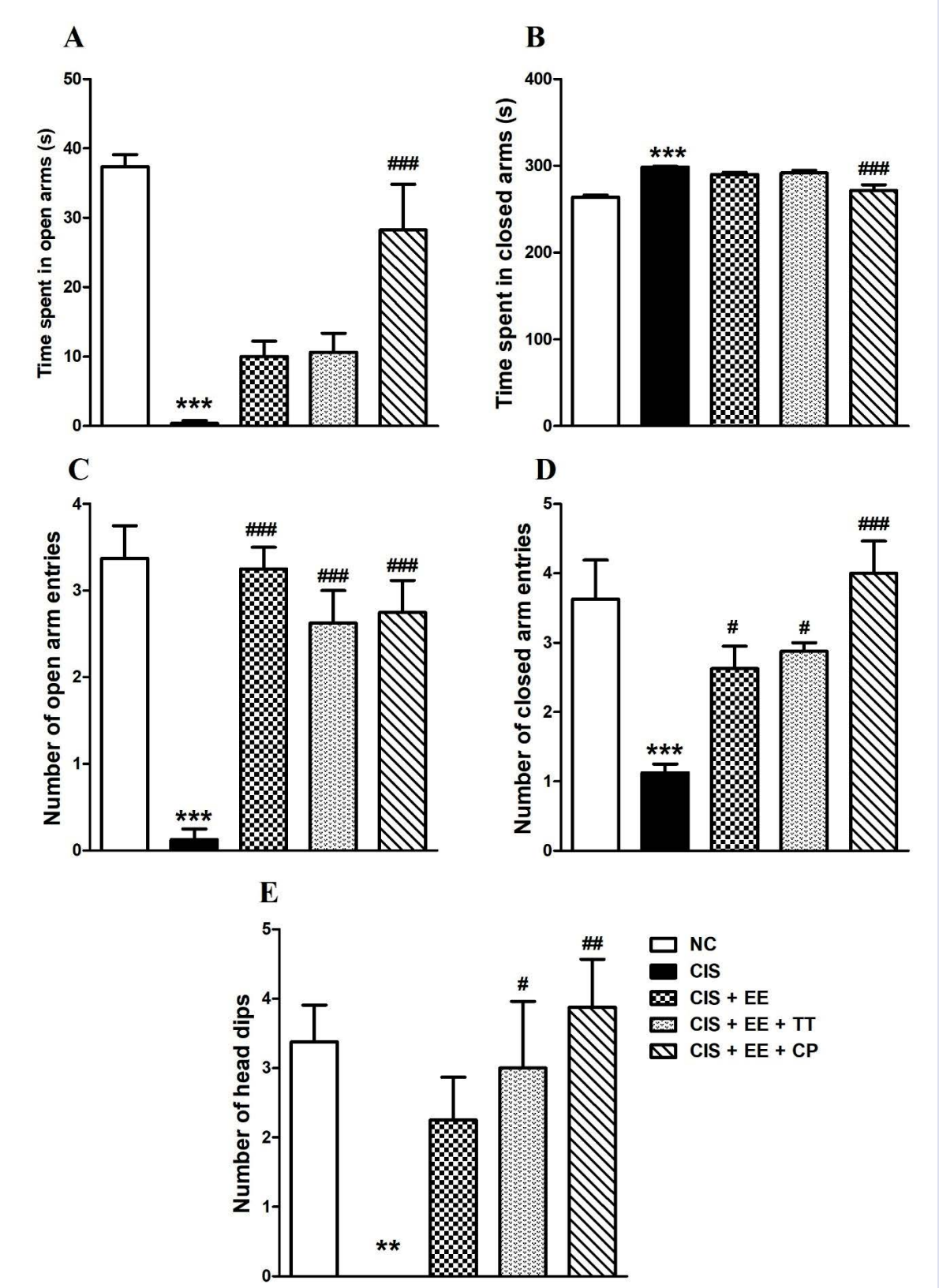

Figure 1: EE and herbal drug treated rats displayed an anti-anxiety effect in the elevated plus maze. (**A**) The time spent in the open arms. (**B**) The time spent in the closed arms. (**C**) The number of open-arms entries. (**D**) The number of closed-arms entries.(**E**) The number of head dips. Data is expressed as mean ± SEM (n = 8/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way ANOVA followed by Tukey's post-hoc test was done for analysis. $^{**}p<0.01$, $^{***}p<0.001$NC vs. CIS; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

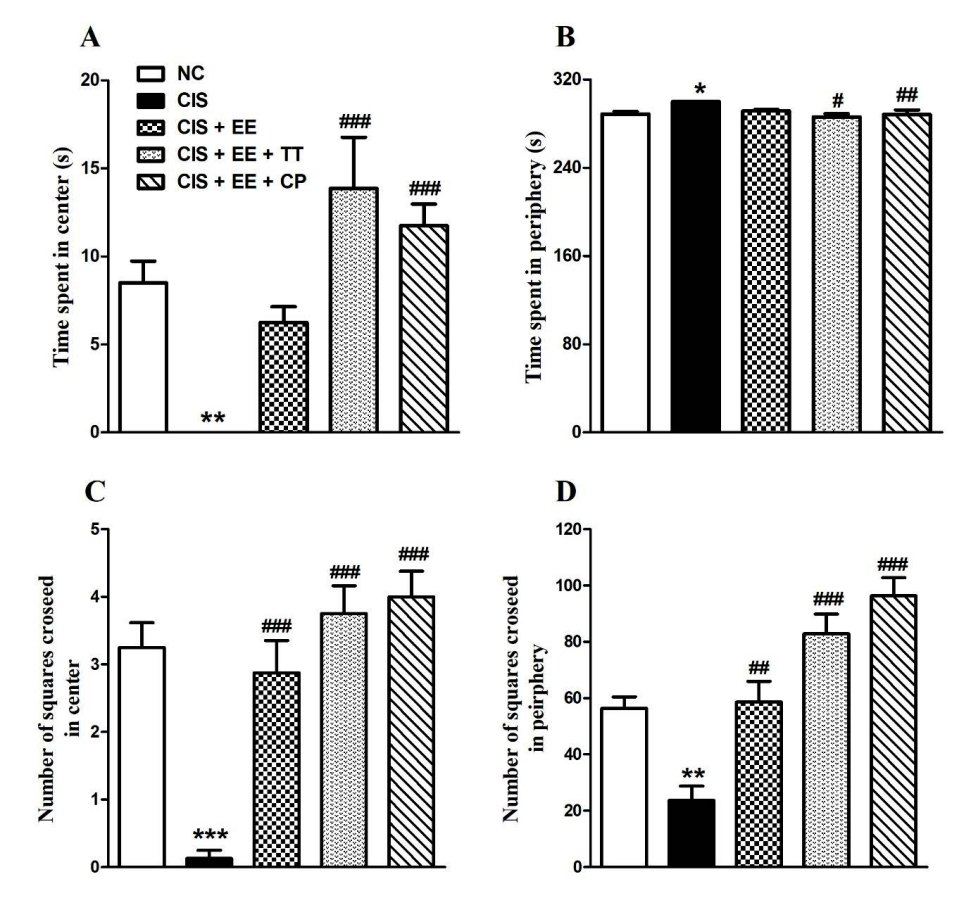

Figure 2: Combination treatment of EE and herbal drugs resulted in anxiolytic effects in the open field test. (**A**) Time spent in the centre of the arena with open arms. (**B**) Time spent in the periphery. (**C**) The number of squares crossed in the centre. (**D**) Number of squares crossed in the periphery. Data is expressed as mean ± SEM (n = 8/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way ANOVA followed by Tukey's post-hoc test was done for analysis. *p<0.05, **p<0.01, ***p<0.001NC vs. CIS;$^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

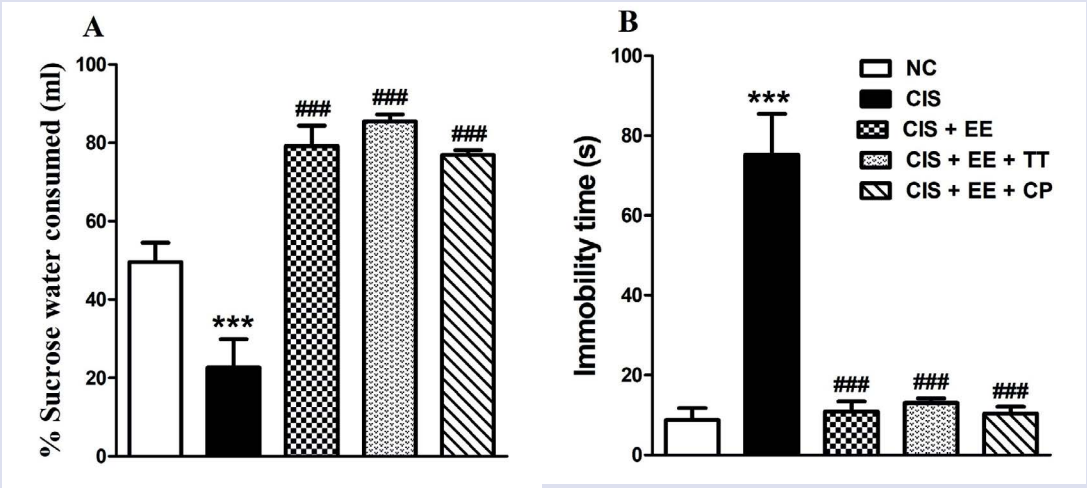

Figure 3: Exposure to an enriched environment with CP or TT showed antidepressant activity in an animal model of chronic immobilisation stress. (**A**) Sucrose preference test. (**B**) Forced swim test. Data is expressed as mean ± SEM (n = 8/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way ANOVA followed by Tukey's post-hoc test was done for analysis. ***p<0.001NC vs. CIS;$^{\#\#\#}p$<0.001 CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

these cognitive functions, as evidenced by the performance of animals in the CIS group. During the learning or acquisition phase, CIS animals exhibited significantly fewer correct choices (Figure 4A: $F_{4,35}$ = 23.85; p<0.001) compared to their non-stressed counterparts. Similarly, in the retention test, stressed animals again scored lower than normal animals (Figure 4C: $F_{4,35}$ = 34,48; p<0.001), highlighting deficits in learning and working memory, indicative of broader cognitive impairments.

The scores for CIS animals improved after exposure to an enriched environment, as seen on the 10th day of the acquisition phase (Figure 4B: $F_{4,35}$ = 23.85; p<0.01) and continued to show enhancements during the retention test (Figure 4C: $F_{4,35}$ = 34,48; p<0.001). When herbal drug treatments were combined with the enriched environment, significant reversal of learning and memory impairments occurred. Animals recalled their previous choices and adapted their behaviour to select the opposite arm. The combination of enriched environments with the herbal treatments (TT+EE and CP+EE) significantly boosted the scores of the CIS animals (Figure 4A: $F_{4,35}$ = 23.85; p<0.001).

Recovery of recognition memory in stressed rodents through enriched environment and herbal treatment

The novel object recognition test (NORT) was employed to evaluate recognition memory based on the natural tendency of animals to explore novel objects more than familiar ones. In the current study, diseased animals exhibited significantly greater exploration of the familiar object (Figure 5A: $F_{4,35}$ = 27.03; p<0.001) and less time spent with the novel object (Figure 5B: $F_{4,35}$ = 59.89; p<0.001). Both the recognition index (Figure 5C: $F_{4,35}$ = 87.99; p<0.001) and the discrimination index (Figure 5D: $F_{4,35}$ = 85.56; p<0.001) were significantly lower in these animals compared to the normal control group. The negative discrimination index indicates a clear preference for the familiar object, suggesting potential memory impairment.

In contrast, animals subjected to environmental enrichment (EE) and those in the combined treatment groups spent significantly more time exploring the novel object than the stressed animals (Figure 5B: $F_{4,35}$ = 59.89; p<0.001). The recognition index (Figure 5C: $F_{4,35}$ = 87.99; p<0.001) was substantially higher in these treatment groups, and the discrimination index was above zero (Figure 5D: $F_{4,35}$ = 85.56; p<0.001) compared to the CIS group. This finding suggests that the treated animals recognised and discriminated between the novel and familiar objects, indicating a recovery of impaired recognition memory.

Effect of chronic stress and enriched environment,Celastrus paniculatus, and Tribulus terrestrison BDNF, TNF-alpha, and IL-6 levels in the hippocampus and prefrontal cortex

In the hippocampus, stress decreased BDNF levels (Figure 6A: $F_{4,20}$ = 83.53; p<0.001)and increased TNF-alpha (Figure 6B: $F_{4,20}$ = 502.2; p<0.001)and IL-6 (Figure 6C: $F_{4,20}$ = 338.2; p<0.001)levels compared to the control group. When stressed rats were exposed to an enriched environment (EE), TNF-alpha and IL-6 levels were restored partially. However, EE did not restore BDNF levels in the hippocampal tissue. Notably, when EE was combined with CP or TT, the effects were even more pronounced, resulting in a greater restoration of BDNF levels

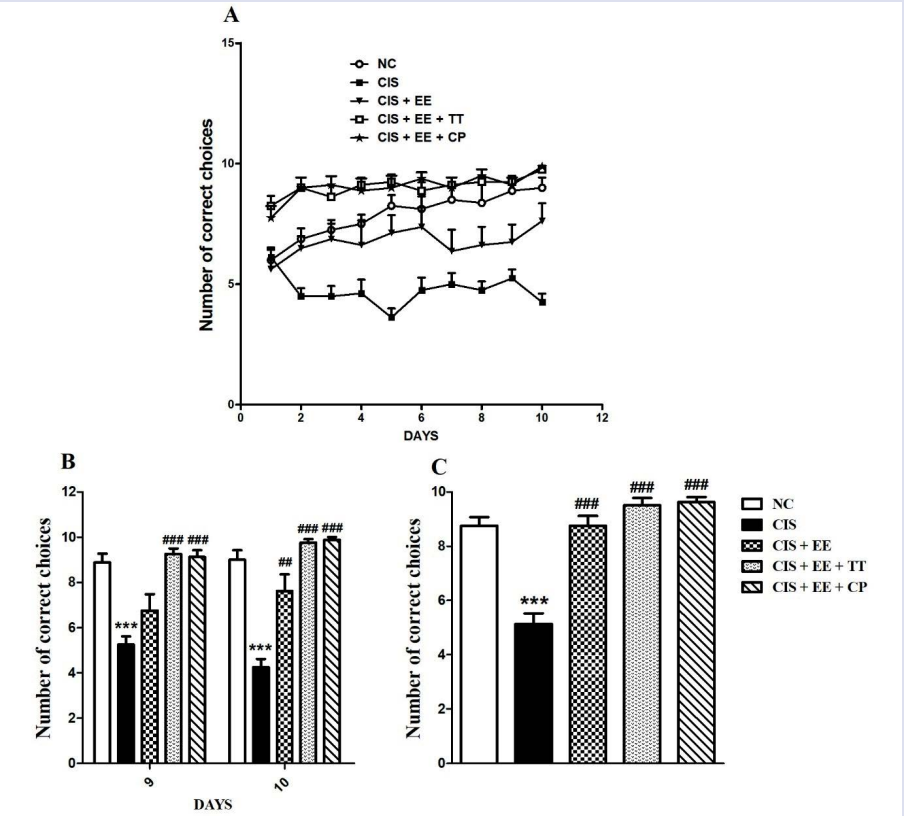

Figure 4: Enrichment and herbal drug combination improved spatial working memory deficits in the rewarded alternation task in the T-maze. (**A**) Learning curve during 10 days of training in the T-maze. (**B**) The number of correct choices on the 9th and 10th day of training. (**C**) The number of correct choices during the retention test. Data is expressed as mean ± SEM (n = 8/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way and Two-way ANOVA followed by Tukey's post-hoc test was done for analysis. ***p<0.001NC vs. CIS;$^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

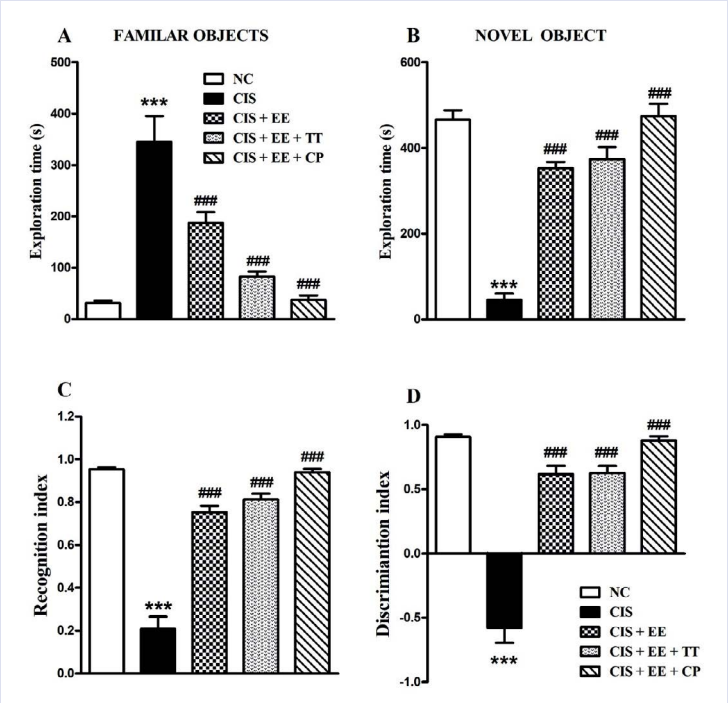

Figure 5: The social enrichment improved recognition memory in the novel object recognition test when combined with CP or TT. (**A**) Time spent exploring familiar objects. (**B**) Time spent exploring novel object. (**C**) Recognition index and (**D**) Discrimination index. Data is expressed as mean ± SEM (n = 8/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way ANOVA followed by Tukey's post-hoc test was done for analysis. ***p<0.001NC vs. CIS; ###p<0.001 CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

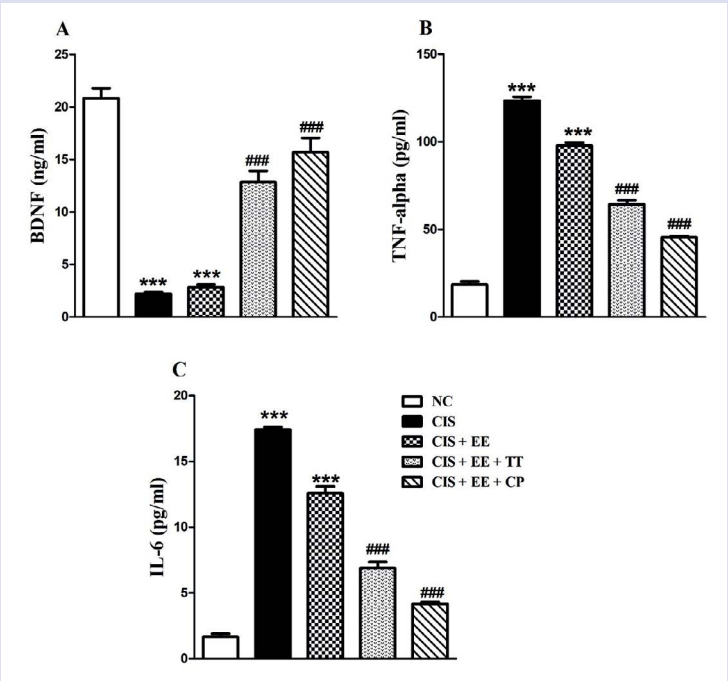

Figure 6: Exposure to two weeks of EE and herbal treatment completely restored neuroinflammation in the hippocampal tissue. (**A**) BDNF (**B**) TNF-α and (**C**) IL-6. Data is expressed as mean ± SEM (n = 6/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way ANOVA followed by Tukey's post-hoc test was done for analysis. ***p<0.001 NC vs. CIS; ###p<0.001 CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

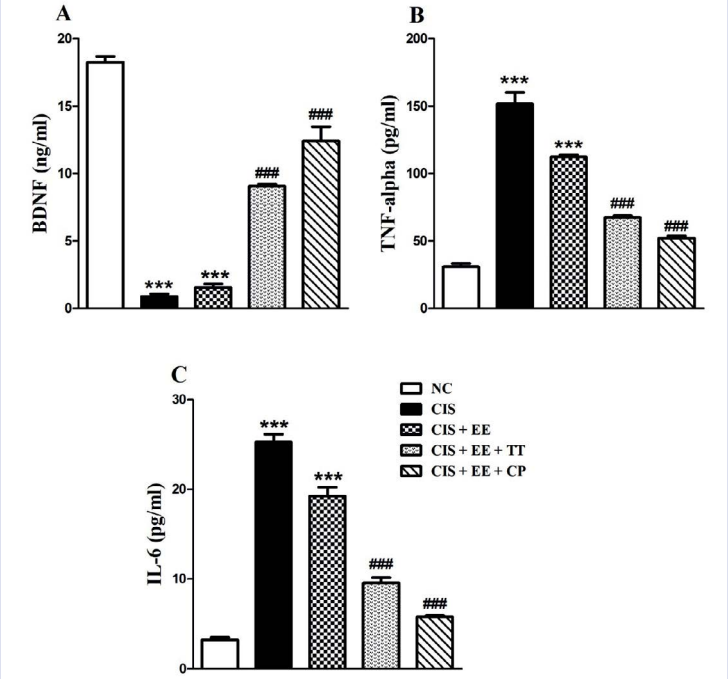

Figure 7: Chronic exposure to EE successfully reduced neuroinflammatory molecular markers in the prefrontal cortical tissue. (**A**) BDNF (**B**) TNF-α and (**C**) IL-6. Data is expressed as mean ± SEM (n = 6/group). NC: Control rats kept in a standard home cage; CIS: Rats exposed to chronic stress in immobilisation bags for 2h/day for 10 days; CIS + EE: CIS rats exposed to enriched environment (EE) for 3h/day 14 days; CIS + EE + TT: CIS rats exposed to EE for 3h/day followed by TT treatment (250 mg/kg, p.o,.) for 14 days; CIS + EE + CP: CIS rats exposed to EE for 3h/day followed by CP treatment (250 mg/kg, i.p,.) for 14 days; One-way ANOVA followed by Tukey's post-hoc test was done for analysis. ***p<0.001. NC vs. CIS; ###p<0.001 CIS vs. CIS + EE, CIS + EE + TT, CIS + EE + CP.

(Figure 6A: $F_{4,20}$ = 83.53; p<0.001)and further reductions in TNF-alpha (Figure 6B: $F_{4,20}$ = 502.2; p<0.001) and IL-6 levels (Figure 6C: $F_{4,20}$ = 338.2; p<0.001) in the hippocampus.

CIS significantly decreased BDNF levels (Figure 7A: $F_{4,20}$ = 187.6; p<0.001)in the frontal cortex, while TNF-alpha (Figure 7B: $F_{4,20}$ = 144.8; p<0.001)and IL-6 levels (Figure 7C: $F_{4,20}$ = 200.3; p<0.001) increased markedly compared to control animals (p<0.001). When stressed rats were placed in an enriched environment (EE), their BDNF levels did not recover (p<0.001). However, EE effectively reduced TNF-alpha and IL-6 levels in the prefrontal cortex of stressed animals (p<0.001). Combining EE with herbal drug treatment led to even more significant results, including greater restoration of BDNF levels (Figure 7A: $F_{4,20}$ = 187.6; p<0.001)and further decreases in TNF-alpha (Figure 7B: $F_{4,20}$ = 144.8; p<0.001)and IL-6 levels (Figure 7C: $F_{4,20}$ = 200.3; p<0.001). This suggests that integrating these interventions may enhance neuroprotective mechanisms and reduce inflammation associated with stress-induced cognitive impairment.

DISCUSSION

This study evaluated an enriched environment as a non-drug approach with *Celastrus paniculatus* (CP) and *Tribulus terrestris* (TT). Our goal was to restore behavioral depression and cognitive deficits caused by chronic immobilization stress in rats. Stress is a major factor in mental disorders, and social support can help improve depression, in addition to herbal treatment. In the current study, rats were immobilized for two hours each day for ten days. After this stress period, the rats received either CP or TT for two weeks while living in an enriched environment. Since rats are social animals, we believe that having "social support" could help lessen the negative effects of long-term stress.The current study assessed the impact of social housing conditions and the herbal treatments *Celastrus paniculatus* and *Tribulus terrestris* on neuroinflammation and cognitive deficits induced by chronic immobilization stress.

In animal models, stress is known to disrupt the negative feedback regulation of the HPA axis [35, 38-39], leading to increased depressive behaviors. [5-6, 36] Similar findings have been reported in several studies, where exposure to an enriched environment completely alleviated depressive symptoms, such as anhedonia and behavioral despair, in stressed animals.[5,8, 36,39] Previous studies have reported spatial learning and memory impairment in stressed and depressive-like conditions.[3,5-6, 25, 36-37] In the current investigation, we found that exposing stressed rats to enrichment for 3 hours completely restored spatial learning and memory impairment in the novel object recognition test and T-maze alteration task. Enrichment enhances learning while also rescuing maladaptive behaviours in social response, physical skills, and emotional reactivity.[41-42] Stress-induced elevated corticosteroid release and anxiety-like behaviour can be mitigated by an enriching environment.[5, 41,43]

Enrichment is essential for appropriate brain development because it stimulates sensory, motor, and cognitive stimuli, and it improves and modifies the neural circuitry.[44-45] EE significantly affects signalling molecules that support learning and memory through neural plasticity. Previous research revealed that stress inhibits LTP, a physiological mechanism that supports memory and learning.[3,46] Evidence shows that EE experience improves hippocampus LTP and spatial learning tasks in adult rats exposed to early life stress. [47-49]

The development of synapses, synaptic plasticity, and neuronal maturation in the brain are all influenced by BDNF.[50-51] Brain-derivedneurotropic factors play an important role in the pathophysiology of major depression.[12-15] Remarkably, earlier research found that an enriched environmentenhanced BDNF levels in stressed rats.[5,49,52-53] Further, we have not observed any improvement in the BDNF levels following short exposure to an enriched environment. This discrepancy may be because of the duration of the enriched environment and the method used to estimate the BDNF levels in

the brain regions. However, earlier studies were unable to show the correlation between the regulation of BDNF and an enriched environment.[54-57]

Chronic stress is the primary environmental risk factor for major depressive disorder (MDD), and there is compelling evidence that neuroinflammation is the major pathomechanism linking chronic stress to MDD. Rats exposed to chronic stress showed enhanced expressions of neuroinflammatory markers IL-6, and TNF-α.[58-59] Previous clinical research has demonstrated that patients with depression have higher concentrations of TNF-α and IL-6 levels.[60-61] Animal studiesshowed increased pro-inflammatory cytokines following prolonged immobilization stress.[62-63]

Interestingly, exposure to an enriched environment mitigated neuroinflammation.[58-59] Prior research has demonstrated the protective effects of an enriched environment against neuroinflammation. In mother rats going through a maternal separation, EE successfully reduces neuroinflammation, neuronal apoptosis, disruption to synaptic plasticity, and the ensuing depression-like behaviour.[64] By reducing anxiety-inducing behaviours, enrichment restored neuroinflammatory markers.[65] In the chronic unpredictable stress model, an enriched environment greatly reduced microglial activation and relieved depressive-like behaviour.[66]

Traditional medicine systems have long utilized herbal plants as a core component of therapeutic treatments. Many herbs and their extracts have been studied for their neuroprotective effects, particularly due to their content of flavonoids and other phenolic compounds. [23-24] *Celastrus paniculatus*, commonly known as the "elixir of life" in traditional medicine, is renowned for its cognitive-enhancing properties. [25] Research has shown that CP oil helps protect neurons from glutamate-induced toxicity by modulating glutamate receptors. [26] It has also been found to alleviate stress-induced cognitive impairments and exhibit dose-dependent anti-cholinesterase activity in the rat brain.[25] Previous studies have highlighted CP's positive effects on learning and memory.

Celastrus paniculatus is widely recognized for its neuroprotective effects, primarily attributed to its antioxidant properties[67-68], which may also account for the protective effects observed in this study. The seeds and aqueous extract of *C. paniculatus* have demonstrated neuroprotection against glutamate-induced neurotoxicity[26] and hydrogen peroxide.[69] The aqueous extract has been found to inhibit NMDA receptor activity and prevent the resulting increase in Ca^{2+} flux, thereby protecting neurons from glutamate-induced toxicity.[26] These findings are consistent with previous research, where various herbal remedies, including *C. paniculatus*, have exhibited antioxidant and neuroprotective effects in the context of neurodegenerative diseases. [26-25, 67]

The study shows that *Celastrus paniculatus* (CP) treatment reduces the levels of inflammatory cytokines. CP regulates Bax, Bcl-2, and caspase-3 expression in the brain tissue of mice with glutamate-induced brain injury.[70] Additionally, Jyothishamti oil increases nerve growth factor and decreases interleukin-6, nuclear factor-κB, and TNF-α levels. CP oil enhances synaptophysin immunoreactivity and reduces reactive gliosis, neuronal degeneration, and vascular proliferation. [71-72]

Zhu et al. reviewed the health benefits of TT, highlighting its various properties such as antioxidant, anti-inflammatory, antitumor, antibacterial, hepatoprotective, anthelmintic, larvicidal, anticaries, anti-aging, and memory enhancement activities.[73] Chaudary et al. demonstrated the neuroprotective effects of *Tribulus terrestris* extracts in a rat model of Alzheimer's disease induced by aluminum chloride.[31] They attributed these benefits to the extract's antioxidant activity and the chelating properties of its flavonoids, which contributed to improvements in both biochemical markers and behavioral outcomes. In another study, pre-treatment with TT saponins before cerebral ischemic injury resulted in a significantly reduced infarct volume, decreased brain edema, fewer neurobehavioral abnormalities, lower serum levels of TNF-α and IL-1β, and increased brain levels of NF-κB.[32] Additionally, *Tribulus terrestris* has shown protective effects against rotenone-induced oxidative damage and the loss of dopamine neurons in the substantia nigra of mice. It also improved motor function and reduced DNA damage and oxidative stress in a Parkinson's disease model.[33]

Tribulus terrestris (TT) has demonstrated significant antidepressant and anxiolytic effects against chronic mild stress-induced anhedonia and anxiety while also lowering elevated levels of corticotropin-releasing hormone (CRH) and corticosterone (CORT).[74] Behavioural experiments conducted by Bouabdallah *et al*., demonstrated that TT administration produces notable anxiolytic and antidepressant-like effects in scopolamine treated zebrafish.[75] Additionally, steroidal saponins isolated from *T. terrestris* exhibit promising bioactivity. Moreover, the ethanolic extract *of T. terrestris* (EETT) significantly reduced the expression of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), in RAW 264.7 cells stimulated by lipopolysaccharides, effectively lowering TNF-α levels and indicating EETT's potential for anxiolytic activity.[76]

This study aimed to assess the potential for integrating sensory-motor interventions with herbal treatments as a promising strategy for addressing stress-related psychiatric disorders, paving the way for future investigations into their combined effects on mental health.

CONCLUSION

This study aimed to examine the effects of a short period of environmental enrichment (EE) combined with *Celastrus paniculatus* (CP) and *Tribulus terrestris* (TT) on cognitive function in a chronic stress model, as well as the underlying molecular mechanisms. Our results showed that prolonged stress caused neuroinflammation in the hippocampus and cortex, increased IL-6 and TNF signaling, reduced BDNF levels, and impaired cognitive abilities. However, treatment with CP or TT along with EE helped reduce neuroinflammation and improved working memory, recognition memory, anxiety, and depressive behaviors. Short-term EE alone did not increase neurotrophic factor levels in the prefrontal cortex or hippocampus. Interestingly, when EE was combined with CP and TT, BDNF levels were restored in stressed rats. These findings suggest that combining EE with herbal treatments can improve cognitive function and offer insights for developing better rehabilitation strategies that use both environmental and pharmacological approaches.

The main limitation of this study is that the specific bioactive compounds responsible for the neuroprotective effect were not identified, isolated, or purified; instead, the entire plant extract was utilized. Another limitation is that neuronal morphology has not been asssesed. Further investigation is needed to determine which metabolites from *C. paniculatus* and *T.terrestris* protect neuronal tissue from chronic stress-induced toxicity, whether it is a single metabolite or the synergistic action of multiple components present in *C. paniculatus* seeds or *Tribulus terrestris* fruits.

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AUTHORS CONTRIBUTIONS

The final manuscript was prepared with input from all authors.

CONFLICTS OF INTEREST

We thus state that we have no competing interests.

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