BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, March 2026. Vol. **23**(1), p. 305-317

Vitamin D3 and L-Theanine Co-Therapy Alleviates Behavioral and Biochemical Deficits in a Chronic Unpredictable Stress Rodent Model

Indu Ghooli, Archana Jorige* and Tejasri Talloju

Department of Pharmacology, R.B.V.R.R. Women's College of Pharmacy
(Affiliated Osmania University), Hyderabad, India.

http://dx.doi.org/10.13005/bbra/3499

(Received: 04 January 2026; accepted: 30 January 2026)

Stress, particularly chronic stress, affects physical and mental health and causes adverse outcomes. Mental disorders account for 14% of all diseases, with depression being the main cause. Chronic Unpredictable Stress (CUS) models are used to study stress-induced disorders and evaluate therapeutic agents. Vitamin D3(Vit D3), essential for mood regulation and neuroplasticity, L-theanine, an amino acid in tea with anxiolytic and neuroprotective qualities, have been connected to depression and stress susceptibility. This study used adult male Albino Wistar rats aged ten–fifteen-weeks weighing 250–350 g to investigate the combined effects of L-theanine and Vit D3in the CUS Model. This involves administering Vit D3 and L-theanine to rodents after exposure to random stressors for a predetermined duration. Behavioural tests including forced swim test(FST), sucrose preference test(SPT), open field test(OFT), splash test, and novel object recognition test(NORT) evaluated cognitive and anhedonia symptoms resembling depression. Biochemical assays measured glucose, total cholesterol, triglyceride, and cortisol levels in serum, and adrenal gland relative weight was measured. Results indicated that L-theanine and Vit D3 significantly mitigated CUS-induced behavioural changes, showing reduced immobility in forced swim test, increased exploratory behaviour in OFT, normalized SPT and increased cognition in NORT. Biochemical assessment showed triglycerides, cholesterol, glucose, and cortisol levels were lower in treatment groups than stress control group. The results demonstrate that co-administration of L-theanine and Vit D3confers superior protection against CUS-induced behavioural and neurochemical deficits compared with monotherapy, indicating a synergistic therapeutic interaction. This finding supports the potential of combination strategies in stress-related mood disorders. Further research is required to define the molecular pathways mediating this enhanced effect.

Keywords: Behavioural assessments; Biochemical deficits; Chronic unpredictable stress (CUS); Cortisol; L - Theanine; Vitamin D3.

Stress, particularly chronic stress, affects both mental and physical health, contributing to neuropsychiatric changes and cognitive impairments such as depression and dementia through hormonal imbalances, neuroinflammation, and neurodegeneration.[1] Chronic stress (When stress persists over an extended period), they can cause cumulative psychological and physiological effects, elevated risk of health problems involving anxiety, cardiovascular disease,depression.[2]Persistent stress might also alter immune cells, leading to gene transcription changes, posing higher susceptibility to infectious diseases and autoimmune disorders.[3]

*Corresponding author E-mail: archanacology@gmail.com

The HPA axis and sympathetic nervous system are activated in the pathophysiology of chronic stress, which results in elevated cortisol levels and immune response suppression. Stress hormones like glucocorticoids and adrenaline are released by the body in reaction to stress. Dysregulation of GABA and glutamate further disrupts the brain's excitatory–inhibitory balance, contributing to cognitive and emotional instability. On a broader level, reduced BDNF (brain-derived neurotrophic factor) impairs synaptic plasticity and resilience, while altered VEGF (vascular endothelial growth factor) signaling affects neurogenesis and vascular support in the brain. Together, these changes create a neurochemical environment that sustains depressive symptoms and hinders recovery. This can make people more susceptible to infections, delayed wound healing, and exacerbate inflammatory diseases.[2]

Chronic Unpredictable Stress (CUS) model is used to research the effects of chronic stress on behaviour and physiology, particularly for mood or anxiety disorders. It involves exposing animals to a variety of mild stressors in an unpredictable sequence over several weeks, inducing behavioural and physiological changes. The CUS model offers ecological validity, induces anhedonia and depressive symptoms, and allows for the development of chronic behavioural changes.[4]Nutraceuticals, derived from natural sources, may offer therapeutic effects on stress-related disorders with lesser side effects when compared with allopathic medicine. Supplements such as L-theanine and Vit D*f* , have gained attention due to their potential therapeutic effects on stress-related disorders. L-theanine, a compound predominantly found in green tea, demonstrates a range of physiological effects, including potential anti-anxiety and antidepressant properties.[5]Research has indicated its effectiveness in alleviating major depressive disorder. Similarly, vitamin D supplementation have been linked to improvements in depression and cognitive function.[6]Thus, this study uses the CUS model to investigate how L-theanine and Vit D_3 work together to affect depression and memory impairment.

MATERIALS AND METHODS

Animals and Drugs

A total of 36 animals utilized for the experimental period of 42 days. They have been kept in polypropylene cages in a room with a 12/12 hr night/day cycle, with a 24±2degree Celsius temperature and 45–64% humidity. The animals were given a commercially balanced meal and unlimited water during the study period. Every animal procedure had been performed in compliance with the CCSEA(Committee for Control and Supervision of Experiments on Animals) standard set of guidelines for use as well as care of experimental animals. The protocol received approval by RBVRR/1328/02/2024.

The doses of L-theanine (15 mg/kg, i.p.) and Vit D_3 (10 000 IU/kg, p.o.) were selected based on previously published evidence demonstrating antidepressant and neuroprotective efficacy in rodent models of chronic stress while maintaining a favourable safety profile. L-theanine within the 5–50 mg/kg range produces significant antidepressant-like and anxiolytic effects by modulating monoaminergic neurotransmission and reducing oxidative stress.[5,7,8] A dose of 15 mg/kg was therefore chosen as a sub-sedative yet pharmacologically active concentration suitable for chronic administration. Vit D_3at 10,000 IU/kg was adopted from studies showing restoration of 25-hydroxyvitamin D levels, enhancement of BDNF expression, and suppression of oxidative and inflammatory changes during unpredictable chronic mild stress [9,10]These doses were expected to provide complementary neurochemical and antioxidative protection, justifying their combined evaluation against CUS-induced behavioural and biochemical impairments.Everyday dose was given at 10'O clock and stressor was given after dosing. On the day of behavioural parameters assessment stressor was not given and tests were performed after giving dose. Blood collection was also done after giving dose on the last day.

The animals that have been split into the following groups at random.

Group 1-Vehicle control (V.C)-Distilled water (p.o.)

Group 2 -Stress control(S.C)-Distilled water (p.o.) + Stress

Group 3-Standard group(Cit)-Citalopram (10 mg/kg) (i.p) + Stress
Group 4 -Test group1(L-The) - Theanine (15 mg/kg) (i.p)+ Stress
Group 5 -Test group 2(Vit D*f*)-Vit D*f* (10000 IU/Kg) (p.o.)+ Stress
Group 6 -Test group 3(L-The + Vit D*f*)-L-Theanine (15 mg/kg)(i.p)+ Vit D*f* (10000 IU/Kg) (p.o.) + Stress

Induction of stress

Stress was induced by stressors like Shaking ,Restraint, Tilted cage, Wet cage, Overnight illumination, Without sawdust and loud sounds, Foot shock, Social isolation, Tail pinching. Except for Vehicle control rats all other rats received CUS protocol. A total of nine stressors were used, and stressors are applied such that no animal received the same stressor for three days in a row to avoid predictability for the period of forty two days.[11,12]

Behavioural and Biochemical tests

Behavioural tests were assessed on 0week and completion of , 2nd, 4th and 6th week where Depression was assessed by the following tests. (1) SPT (Sucrose preference test),(2) OFT (Open field test),(3) Coat state test,(4) Splash test and (5) Forced Swimming test(FST). Memory was assessed by the tests (1)NORT (Novel object Recognition test) and EPM (Elevated plus maze test).[13-19]Biochemical parameters were conducted on the 6th week. Animals have been observed for the variations in their body weight, food intake (in gms), and water intake (in ml) throughout the 6 week study period. Sucrose preference in percentage and cognitive index was calculated using the below formulas.

$$\text{Sucrose preference percentage (\%)} = \frac{\text{Sucrose solution consumption (ml)}}{\text{sucrose solution consumption (ml)} + \text{water consumption (ml)}} \times 100$$

$$ci = (Tnew - Told)/ (Tnew + Told).$$

Assessment of relative weight of the adrenal glands was performed after removing the adipose tissue from the retroperitoneal connective tissue on the kidneys. The ratio of each rat's total body weight in grams to the relative weight of its adrenal glands (mg per pair of dry glands is divided by weight of each rat') was computed.[20]

Statistical analysis

Mean ± SEM (n = 6/group) was used to express all values. Graph pad prism 10 software has been utilized to analyze data by ANOVA(one-way analysis of variance) followed by Tukey test for repeated pairwise comparisons between the different treated groups. P less than 0.05 values have been considered as statistically significant.

RESULTS

Effect of L- theanine, Vitamin D*f* , and the Combination on Behavioural tests

Behavioural tests were assessed on 2nd week, 0 week, 4thweek and 6th week of the CUS protocol.

Effect of L- theanine, Vitamin D*f* , and the Combination on preference of consumption of Sucrose solution

The Figure 1 below represents sucrose preference by each group from 0 week to 6 week. Stress control rats showed significantly decreased preference towards sucrose over the period of Six weeks and the treatment groups showed significantly increased preference towards sucrose when compared with stress control group.

Effect of L- theanine, Vitamin D*f* , and the Combination on Time spent in central square and Total no.of Entries

As shown in Figure 2, Stress control group showed decreased inclination to enter the central square over the period of Six weeks, when compared with baseline values and vehicle control group. By sixth week, there were fewer entries in the central square of stress control than in the vehicle control group. By the end of Sixweek Standard group and Treatment groups L - Theanine, Vitamin D3 and L - Theanine + Vitamin D3 showed significant and progressive increase activity in central square demonstrating the effectiveness of the treatment in reducing the anxiety and enhancing exploratory behaviour at the Six week when compared to the stress control.

Effect of L- theanine, Vit D*f* , and the Combination on Coat State score

The Table1 below represents coat state score of all groups over the period of Six weeks. Rats exposed to the CUS protocol showed a markedly reduced self-care behaviour at the end of the 6-week CUS protocol, with unclean coats only being seen at weeks two, four and six in comparison to vehicle control group, as per coat state results.

Citalopram, L- The, Vitamin D3 and L- The + Vitamin D3 groups exhibited similar coat state as that of vehicle control group with clean coat.

Effect of L- theanine, Vit D*f* , and the Combination on Grooming latency

Figure 3 below displays average of grooming latency of all groups over the period of six weeks. Stress control group exhibited significantly high grooming latencies over the sixweek period owing to effectiveness of the protocol by confirming the presence of stress or depressive-like symptoms. Treatment groups L - Theanine, Vitamin D3 and L - Theanine + Vitamin D3 showed a marked and significant decrease in grooming latency over time when compared to stress control. The standard group showed a decrease latency by sixth week when compared with the baseline values, stress control.

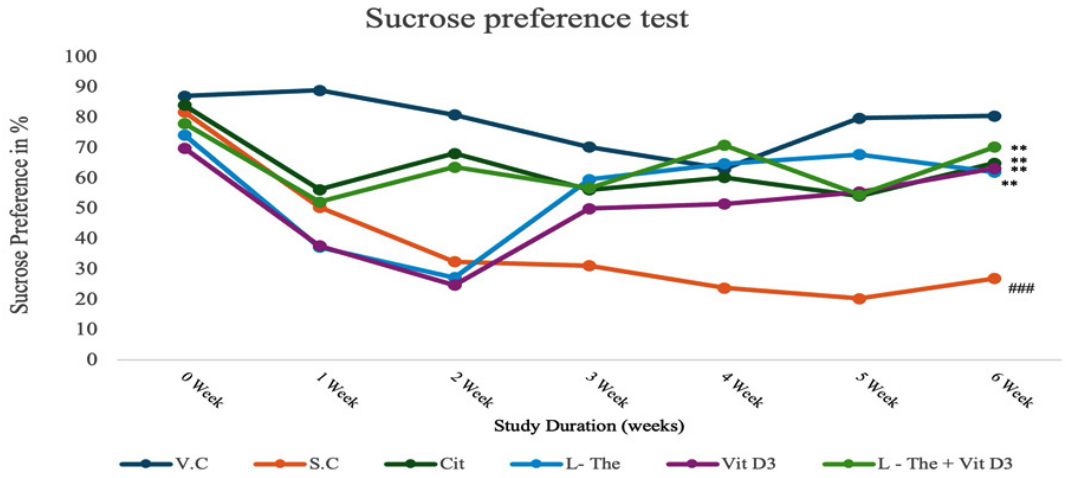

Fig. 1. Effect of L- theanine, Vit D*f* , and the Combination on Percentage preference of consumption of Sucrose solution

Values are "expressed as Mean ± SEM (n=6) #p<0.05, ## p<0.01, ###p<0.001,#### p<0.0001 compared with vehicle control, mean± SEM *p<0.05,** p<0.01, ***p<0.001,**** p<0.0001 compared to stress control

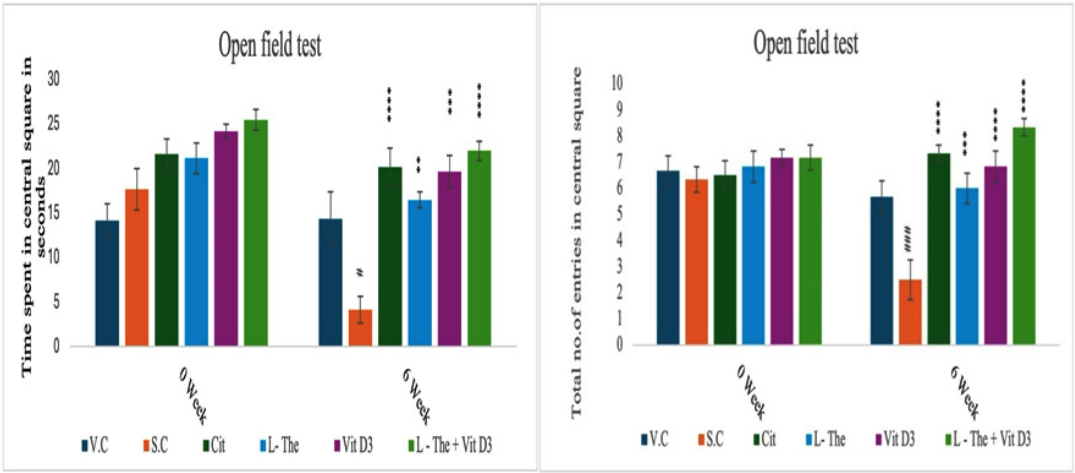

Fig. 2. Effect of L- theanine, Vit D*f* , and the Combination on time spent in central arm and Total no. of entries in central square

Values are "expressed as Mean ± SEM (n=6) #p<0.05, ## p<0.01, ###p<0.001,#### p<0.0001 compared with vehicle control, mean± SEM *p<0.05,** p<0.01, ***p<0.001,**** p<0.0001 compared to stress control.

Effect of L- theanine, Vitamin D*f* , and the Combination on Immobility time

The figure 4 below displays average immobility time of all groups over the period of six weeks in FST. Stress control group showed a marked increase in immobility time over sixweek period and increase in immobility time has been observed when associated to baseline value. When compared to the vehicle control group, the stress control group's immobility time increased significantly throughout the course of the sixweek period. Treatment groups L - Theanine, Vitamin D3 and L - Theanine + Vitamin D3 exhibited pronounced reduction in immobility time after treatment when associated with baseline values and stress control respectively. The combination group especially showed a substantial decrease(64.70%) in immobility time comparable with that of standard treatment(65.8%)

Effect of L- theanine, Vitamin D*f* , and the Combination oncognitive index

The figure 5 below displays average cognitive index of all groups over the period of six weeks. Stress control group showed decreased cognitive index for familiar over the time of six weeks whereas treatment groups exhibited an increase in the cognitive index. The L- Theanine + Vitamin D3 group showed a significant elevate

Table 1. Effect of L- theanine, VitD3 , and the Combination on Coat State score

Groups	Coat test Scores			
	0 Week	2 Week	4 Week	6 Week
Vehicle control	0.00	0.00	0.00	0.00
Stress control	0.00	0.17±0.17	1.17±0.48$^{\#\#}$	2.67±0.49$^{\#\#\#\#}$
Cit	0.00	0.00	0.00	0.00
L - The	0.00	0.00	0.00	0.00
Vit D*f*	0.00	0.00	0.00	0.00
L - The + Vit D*f*	0.00	0.00	0.00	0.00

Values are "expressed as Mean ± SEM (n=6) $^{\#}$p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$p<0.001,$^{\#\#\#\#}$ p<0.0001 compared with vehicle control, mean± SEM *p<0.05,** p<0.01, ***p<0.001,**** p<0.0001 compared to stress control".

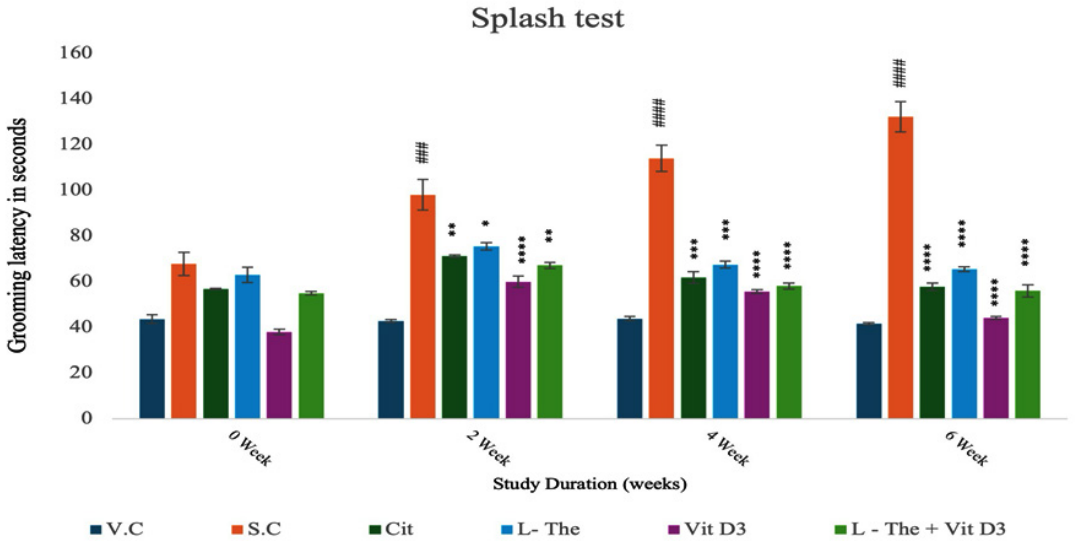

Fig. 3. Effect of L- theanine, Vit D3 and the Combination on Grooming latency

Values are "expressed as Mean ± SEM (n=6) $^{\#}$p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$p<0.001,$^{\#\#\#\#}$ p<0.0001 compared with vehicle control, mean± SEM *p<0.05,** p<0.01, ***p<0.001,**** p<0.0001 compared to stress control

in cognitive index from the second week over the sixweek period compared to any other treatment group.

Effect of L- theanine, Vitamin D*f* , and the Combination on Transfer latencies

The figure 6 below displays average transfer latency(TL) of all groups over the period of six weeks. The Stress control group showed decreased transfer latency on EPM over the period of six weeks. Before CUS induction all the groups showed decreased second day transfer latencies on EPM. After CUS protocol there is a significant increase in transfer latency validating the appropriate CUS induction and 6 weeks of treatment with stand and and test drugs significantly reduced the Transfer latency indicating their potential in inhibiting stress induced cognitive impairment.

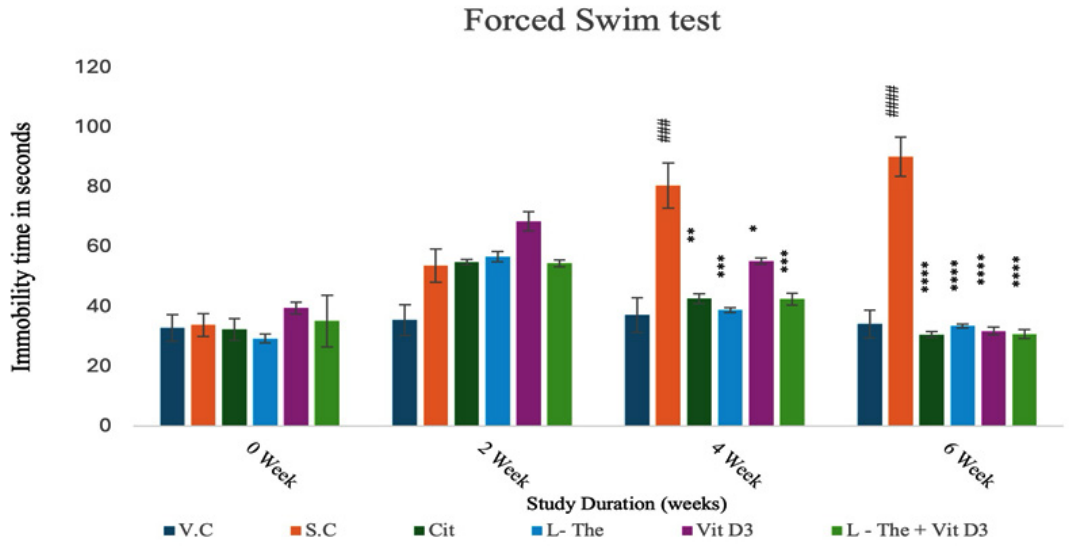

Fig. 4. Effect of L- theanine, Vit D3 and the Combination on Immobility time

Values are "expressed as Mean ± SEM (n=6) $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$,$^{\#\#\#\#}p<0.0001$ compared with vehicle control, mean± SEM $^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$,$^{****}p<0.0001$ compared to stress control"

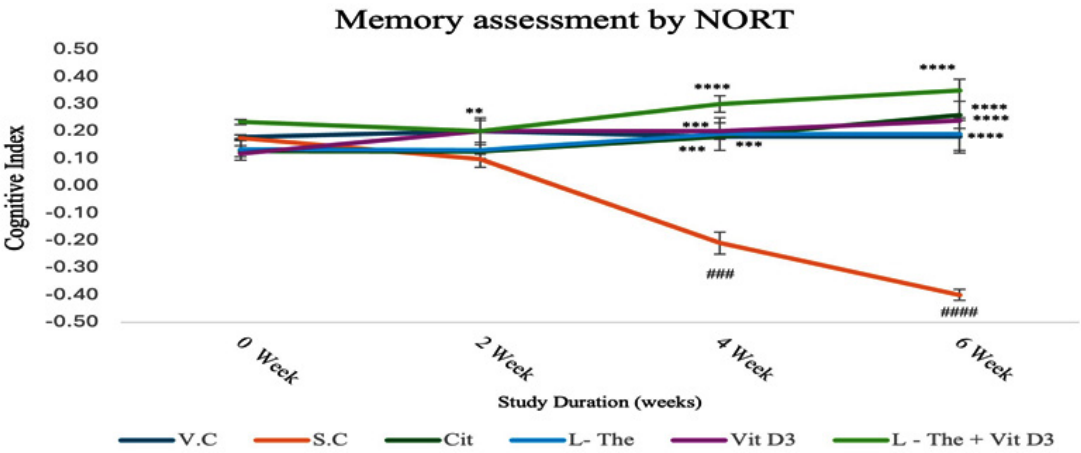

Fig. 5. Effect of L- theanine, Vit D3 and the Combination on Cognitive Index

Values are "expressed as Mean ± SEM (n=6) $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$,$^{\#\#\#\#}p<0.0001$ compared with vehicle control, mean± SEM $^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$,$^{****}p<0.0001$ compared to stress control

Effect of L- theanine, Vitamin D*f* , and the Combination on Biochemical parameters

Effect of L- theanine, Vitamin D*f* , and the Combination on Glucose levels, Total cholesterol and Triglyceride levels

Glucose levels were estimated on the 6thweek of the CUS protocol, and levels of glucose were raised in stress control group. But the elevation is statistically insignificant. The L-theanine + Vit D*f* treatment significantly reduced serum glucose levels. The triglycerides levels of stress control have been found to be significantly higher than vehicle control group. The citalopram treated standard group and treatment groups L - Theanine, Vit D*f* and L - Theanine + Vit D*f* triglyceride levels were found to be significantly lower when related to stress control group. It was discovered that the stress control group's Total Cholesterol levels were significantly greater than those of the vehicle control group. Total cholesterol levels were observed to be considerably lower in the L-theanine, Vit D*f* , and L-theanine + Vit D*f* and regular citalopram treatment groups than in the stress control group. Especially L - Theanine + Vit D*f* combination significantly reduced the cholesterol and LDL levels.

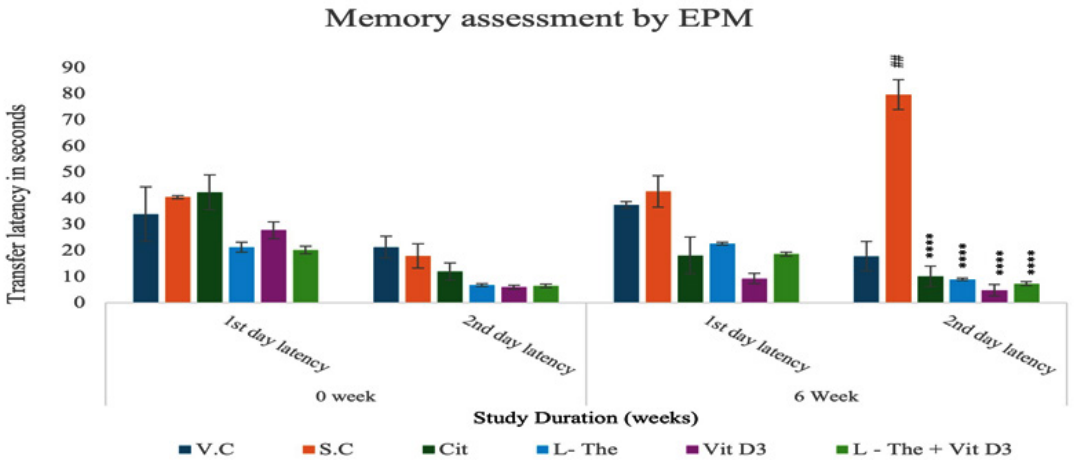

Fig. 6. Effect of L- theanine, Vit D3 and the Combination on Transfer latencies in sec
Values are "expressed as Mean ± SEM (n=6) $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$,$^{\#\#\#\#}p<0.0001$ compared with vehicle control, mean± SEM $^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$,$^{****}p<0.0001$ compared to stress control".

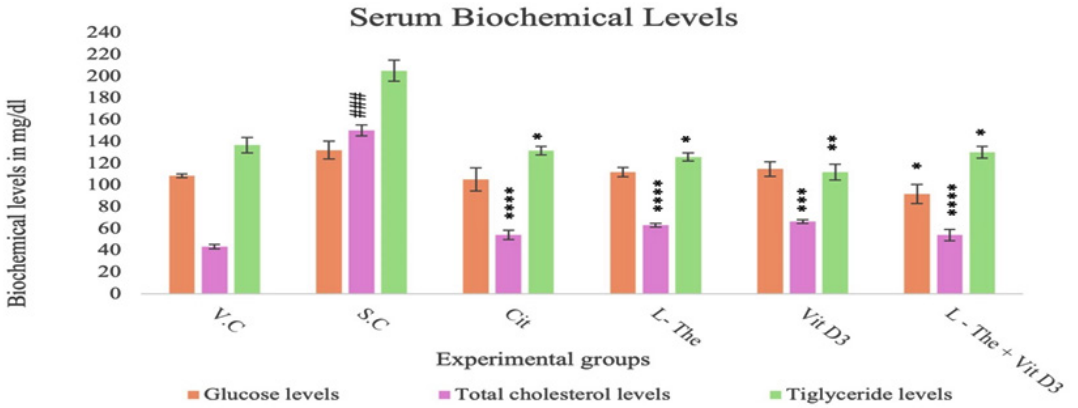

Fig. 7. Effect of L- theanine, Vit D3 and the Combination on Glucose levels, Total cholesterol and Triglyceride levels
Values are "expressed as Mean ± SEM (n=6) $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$,$^{\#\#\#\#}p<0.0001$ compared with vehicle control, mean± SEM $^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$,$^{****}p<0.0001$ compared to stress control.

Effect of L- theanine, Vitamin D*f* , and the Combination on Serum cortisol levels and Adrenal glands

The table 2 below shows serum cortisol levels of allgroups on the sixth week. The cortisol levels of stress control were found to be significantly higher compared to vehicle control group. Whereas the cortisol levels of the treatment groups are significantly lower than stress control group.

Effect of L- theanine, Vitamin D*f* , and the Combination on Relative weight of adrenal glands

The proportional weight of the adrenal glands after six weeks is shown in the Figure 8 below.The Stress control group showed increased weight of the adrenal glandwhen related with the vehicle control. By the end of sixth week, stress control showed significant increase in the adrenal gland weight when compared to the vehicle control group. All the treatment groups showed a reduction in Adrenal gland weight compared to stress control group. However L - Theanine and Vitamin D3 combination showed highest reduction in average adrenal gland weight compared to either of individual treatments

Table 2. Effect of L- theanine, Vit D3 and the Combination on Serum cortisol levels

Groups	Cortisol levels (ng/ml)
Vehicle control	1.83±0.03
Stress control	3.60±0.04$^{\#\#\#\#}$
Cit	2.90 ±0.03****
L - The	3.28±0.04****
Vit D3	3.12±0.06****
L - The + Vit D3	2.95±0.02****

Values are "expressed as Mean ± SEM (n=6) $^{\#}$p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$p<0.001,$^{\#\#\#\#}$ p<0.0001 compared with vehicle control, mean± SEM *p<0.05,** p<0.01, ***p<0.001,**** p<0.0001 compared to stress control.

DISCUSSION

The Sucrose Preference Test measures anhedonia, a depression core symptom characterized by a loss of interest in pleasurable activities. Rodents exposed to CUS typically show a reduced preference for sucrose solution compared to controls, indicating anhedonia. This reduction reflects the negative effect of chronic stress on reward processing systems in the brain, particularly involving dopaminergic pathways.[21]The increase in the sucrose preference of L- theanine can be due to the ability to increase the monoamine levels such as dopamine, serotonin and Treatment with Vit D 10,000 IU enhanced the sucrose preference [9,25]and the combination of both drugs could be the reason for improved performance of the combination group which was found to be consistent with earlier research.

Open Field Test evaluates anxiety-related behaviours as well as overall locomotor activity. In this test, rodents subjected to CUS often exhibit increased anxiety and depression, evidenced by decreased exploration of open field centre and elevated time spent in periphery. Neurotransmitter

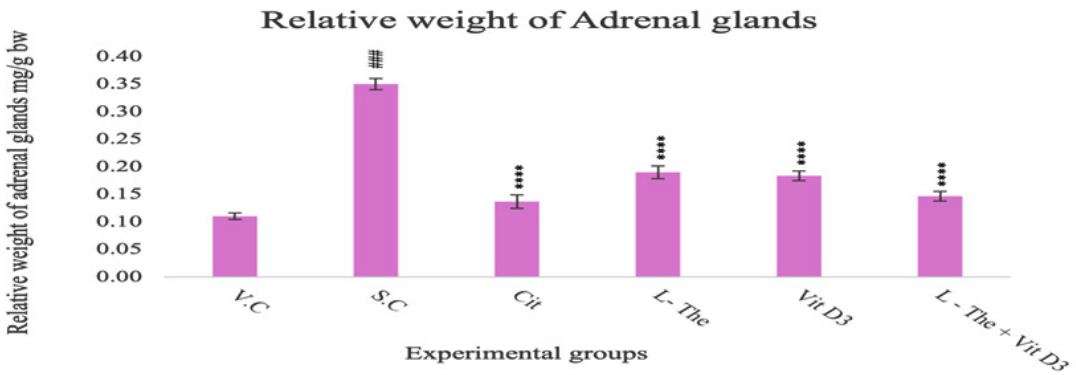

Fig. 8. Effect of L- theanine, Vit D3 and the Combination on Relative weight of adrenal glands
Values are "expressed as Mean ± SEM (n=6) $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001,$^{\#\#\#\#}$p<0.0001 compared with vehicle control, mean± SEM *p<0.05,** p<0.01, ***p<0.001,**** p<0.0001 compared to stress control".

imbalances and altered HPA(hypothalamic-pituitary-adrenal) axis function that were associated to these behavioural abnormalities.[26] Citalopram administered group showed significantly increase in time spent in central square activity in the model rats as previously reported.[27] L – theanine significantly increased the time in central square due to the antidepressant activity of L – theanine . Treatment with Vit D*f* enhanced the performance of rats in an OFT setting which can be due to antidepressant like effect of Vit D*f* which were found to be compatible with earlier research. [9,28]

Coat State Test examines the physical condition and grooming behaviour of rodents. A deterioration in coat condition, such as increased fur matting and poor grooming, is often seen in rodents exposed to CUS. This physical neglect reflects decreased self-care and an overall decline in physical and mental health, providing a visible marker of the depressive-like state induced by chronic stress. Groups administered with Citalopram, L-theanine, Vitamin D3 have a similar Coat state as that of the vehicle control group due to the antidepressant impact of the compounds and can be attributed to the serotonergic involvement.[5,29,30]

Behavioural despair, a defining feature of depressive-like behaviour, is assessed using Forced Swim Test. In the FST, rats given CUS often show longer periods of immobility, indicating a sense of helplessness, which was also observed in this study. This behaviour correlates with decreased serotonergic and noradrenergic neurotransmission, mirroring the neurochemical changes observed in depression.[30] The combination of L Theanine and Vitamin D3 significantly reduced the immobility time, since L-theanine treatment can raise levels of NE, 5-HT, DA, it was highly effective in reducing depressive-like behaviour and greatly decreased immobility time in forced swim test.[5,7] Moreover addition of Vit D*f* treatment has enhanced the performance of rats in FST, which contributed to less floating time, highly effective in mitigating depressive like behaviour.[8,26]

The Novel Object Recognition Test evaluates recognition memory in particular. Rodents experiencing CUS typically show impairments in this test, as indicated by reduced exploration of a novel object compared to a familiar one. This cognitive decline is often linked to stress-induced alterations in hippocampal plasticity and neurotransmitter dysregulation, affecting learning and memory processes .[19,20] The increase of cognitive index of the groups administered with Vitamin D3 and L theanine administered group was found to be in accordance with earlier research can be attributed to increased BDNF levels in the brain by Vitamin D3 and L- Theanine.[9,21,22,23,24] A deficiency in the BDNF levels leads to dementia.[34]By modulating GABAergic neurotransmission L theanine improves memory.[35,36,37,50]

Rats' memory was assessed using the elevated plus-maze, which has two open and two enclosed arms. In animal models, the shortened TL has been proposed as a measure of how memory retention affects cognitive processes. The decreased latency of the treatment groups L - theanine and Vit D*f* can be due to increased levels of neurotransmitters [35,38] which were found by the earlier research and the shorter latencies of the combination group of L- theanine + Vitamin D3 can be due to the synergistic action.

Chronic stress conditions significantly influence sustained increases in cholesterol levels over time.[38]Taking Vitamin D could lower levels of total cholesterol, LDL and triglycerides in the blood.[39]According to the previous research that taking Vit D*f* could lower serum total cholesterol, LD Land triglyceride levels.[40] L- Theanine promotes lipolysis and inhibits fat formation.[41]

Cortisol rises in reaction to chronic stress. The adrenal cortex releases cortisol when HPA(hypothalamic-pituitary-adrenal) axis is activated during stress. Current CUS model shows how prolonged, unpredictable stress situations increase HPA axis activity and cortisol as the body copes with stress.[41] L-Theanine and Vitamin D3 synergistically reduced stress reactions, as shown by a substantial reduction from both stress control. L – theanine and Vit D*f* have the capacity to reduce cortisol levels, which might have been the reason in the reduction of cortisol levels, which are in line with earlier research.[43,44,45]Corticotropin-releasing hormone neurones in the hypothalamic paraventricular nucleus are involved in stress reactions on HPA axis. Paraventricular nucleus then transports peptide toanterior pituitary, where corticotrophs release adrenocorticotrophic hormone, increasing the production of glucocorticoids.[44] Memory impairment is closely associated with decreased

neurogenesis, the brain's process of producing new neurons.[46] Increased cortisol levels and inflammation have frequently been reported in patients with depression.[47] Rats under chronic stress typically show adrenal enlargement, suggesting that chronic stress also influences the periphery limb of the HPA axis.[48]Administration of L -Theanine is found to reduce psychosocial stress and might be the reason to reduce the adrenal gland weight as found in earlier research.[49,50] Combined supplementation of Vitamin D and L-theanine has demonstrated superior behavioural and neurochemical recovery in stress models compared with monotherapy, indicating multimodal and potentially synergistic mechanisms of action.[51] In addition to neurotransmitter modulation, the observed effects may also involve non-neurochemical mechanisms such as attenuation of neuroinflammation, oxidative stress, HPA axis dysregulation, and modulation of neurotrophic pathways associated with chronic stress.

CONCLUSION

The present study evaluated the effects of combined L-theanine and Vit D*f* supplementation in a chronic unpredictable stress (CUS) model and demonstrated significant protection against stress-induced behavioural and cognitive impairments. L-theanine and Vit D*f* combination effectively prevented anhedonia and depressive-like behaviours while improving anxiety-related responses and cognitive performance. These findings suggest a modulatory effect of the combination on stress-responsive neurobiological pathways beyond symptomatic relief. Collectively, the results indicate that L-theanine and Vit D*f* may represent a promising adjunctive approach for managing chronic stress–associated disorders. Future studies should elucidate underlying molecular mechanisms and validate these effects in clinical settings.

ACKNOWLEDGEMENTS

We are thankful to Principal and Management of RBVRR Women's College of Pharmacy for supporting and encouraging us to carry out this research.

Funding Sources

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Data Availability Statement

The manuscript incorporates all datasets produced or examined throughout this research study.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this article.

Ethics Statement

All experimental procedures were conducted in accordance with the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of Osmania University (Protocol No. RBVRR/1328/02/2024). Every effort was made to minimize animal suffering and to use only the number of animals necessary to obtain reliable scientific data.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Permission to reproduce material from other sources

Not Applicable

Authors' Contribution

Indu Ghooli: Methodology, Investigation, Data Curation, Formal Analysis, Writing – Original Draft, Visualization; Archana Jorige: Conceptualization, Supervision, Validation, Writing – Review & Editing; Tejasri Talloju : Data Collection, Writing – Review & Editing.

REFERENCES

1. Graybeal C, Kiselycznyk C, Holmes A. Stress-induced deficits in cognition and emotionality: a role for glutamate. In: Behavioral Neurogenetics. Springer; 2012:189–207.https://doi.org/10.1007/7854_2011_193
2. Chu B, Marwaha K, Sanvictores T, Awosika AO, Ayers D. Physiology, stress reaction. In: StatPearls [Internet]. StatPearls Publishing; 2024.https://www.ncbi.nlm.nih.gov/books/NBK541120/

3. Rozanski A, Blumenthal JA, Davidson KW, Saab PG, Kubzansky L. The epidemiology, pathophysiology, and management of psychosocial risk factors in cardiac practice: the emerging field of behavioral cardiology. *J Am Coll Cardiol.* 2005;45(5):637–651. https://doi.org/10.1016/j.jacc.2004.12.005
4. Sequeira-Cordero A, Salas-Bastos A, Fornaguera J, Brenes JC. Behavioural characterisation of chronic unpredictable stress based on ethologically relevant paradigms in rats. *Sci Rep.* 2019;9(1):17403.https://doi.org/10.1038/s41598-019-53624-1
5. Shen M, Yang YI, Wu Y, et al. L-theanine ameliorates depressive-like behavior in a chronic unpredictable mild stress rat model via modulating monoamine levels in limbic–cortical–striatal–pallidal–thalamic circuit brain regions. *Phytother Res.* 2019;33(2):412–421. https://doi.org/10.1002/ptr.6237
6. Trivedi MK, Branton A, Trivedi D, Mondal S, Jana S. Vitamin D3 supplementation improves spatial memory, muscle function, pain score, and modulates physiological biomarkers in vitamin D3 deficiency diet-induced rats. *BMC Nutr.* 2023;9(1):108. https://doi.org/10.1186/s40795-023-00767-0
7. Bae D, Kim J, Oh DR, et al. Multifunctional antistress effects of standardized aqueous extracts from *Hippophae rhamnoides* L. *Anim Cells Syst.* 2016;20(6):369–383. https://doi.org/10.1080/19768354.2016.1250816.
8. Yin C, Gou L, Liu Y, et al. Antidepressant-like effects of L-theanine in the forced swim and tail suspension tests in mice. *Phytother Res.* 2011;25(11):1636–1639. https://doi.org/10.1002/ptr.3456
9. Bakhtiari-Dovvombaygi H, Izadi S, Zare M, et al. Vitamin D3 administration prevents memory deficit and alteration of biochemical parameters induced by unpredictable chronic mild stress in rats. *Sci Rep.* 2021;11(1):16271. https://doi.org/10.1038/s41598-021-95850-6
10. Bakhtiari-Dovvombaygi H, Izadi S, Zare Moghaddam M, et al. Beneficial effects of vitamin D on anxiety- and depression-like behaviors induced by unpredictable chronic mild stress by suppression of brain oxidative stress and neuroinflammation in rats. *Naunyn Schmiedebergs Arch Pharmacol.* 2021;394(4):655–667.https://doi.org/10.1007/s00210-020-02002-0
11. Burstein O, Doron R. The unpredictable chronic mild stress protocol for inducing anhedonia in mice. J Vis Exp. 2018;(140):e58184. doi:10.3791/58184.
12. Monteiro S, Roque S, de Sá-Calçada D, Sousa N, Correia-Neves M, Cerqueira JJ. An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice. Front Psychiatry. 2015 Feb 2;6:6. doi:10.3389/fpsyt.2015.00006. PMID:25698978; PMCID:PMC4313595.
13. Valenzuela RL, Velasco RI, Umerez DC, Urgena CR, Uy JA, Valdez MA, Villanueva-Uy LM, Vinasoy NA, Yu DI, Dasig DA. A comparative study on depression and anxiety of mice (Mus musculus) on a prebiotic-supplemented diet versus a standard diet during unpredictable chronic mild stress (UCMS). Acta Med Philipp. 2020.
14. Zhu H, Tao Y, Wang T, Zhou J, Yang Y, Cheng L, Zhu H, Zhang W, Huang F, Wu X. Long-term stability and characteristics of behavioral, biochemical, and molecular markers of three different rodent models for depression. Brain Behav. 2020 Feb;10(2):e01508. doi:10.1002/brb3.1508.
15. Hu C, Luo Y, Wang H, Kuang S, Liang G, Yang Y, Mai S, Yang J. Re-evaluation of the interrelationships among the behavioral tests in rats exposed to chronic unpredictable mild stress. PLoS One. 2017 Sep 20;12(9):e0185129. doi:10.1371/journal.pone.0185129.
16. Wu T, Li X, Li T, Cai M, Yu Z, Zhang J, Zhang Z, Zhang W, Xiang J, Cai D. Apocynum venetum leaf extract exerts antidepressant-like effects and inhibits hippocampal and cortical apoptosis of rats exposed to chronic unpredictable mild stress. Evid Based Complement Alternat Med. 2018 Jan 16;2018:5916451. doi:10.1155/2018/5916451. PMID:29576796; PMCID:PMC5822797.
17. Li F, Wang Y, Wang X, Zhao Y, Xie F, Qian LJ. Dynamic effects of chronic unpredictable mild stress on the hippocampal transcriptome in rats. Mol Med Rep. 2022 Apr;25(4):110.doi:10.3892/mmr.2022.12626.
18. Sharma AC, Kulkarni SK. Evaluation of learning and memory mechanisms employing elevated plus-maze in rats and mice. Prog Neuropsychopharmacol Biol Psychiatry. 1992;16(1):117-125. doi:10.1016/0278-5846(92)90014-6.
19. Bhuvanendran S, Kumari Y, Othman I, Shaikh MF. Amelioration of cognitive deficit by embelin in a scopolamine-induced Alzheimer's disease-like condition in a rat model. Front Pharmacol. 2018 Jun 25;9:665.doi:10.3389/fphar.2018.00665.
20. Blossom V, Gokul M, Kumar NA, et al. Chronic unpredictable stress-induced inflammation and quantitative analysis of neurons of distinct

brain regions in Wistar rat model of comorbid depression. Vet World. 2020;13(9):1870–1877. doi:10.14202/vetworld.2020.1870-1874

21. Pepino MY, Eisenstein SA, Bischoff AN, et al. Sweet dopamine: sucrose preferences relate differentially to striatal D2 receptor binding and age in obesity. *Diabetes.* 2016;65(9):2618–2623. https://doi.org/10.2337/db16-0407
22. Elbaz EM, Ibrahim SM, Rashad E, Yasin NAE, Ghaiad HR, Mehana NA. Therapeutic role of L-theanine in mitigating cognitive dysfunction and neuropathology in scopolamine-treated mice. ACS Chem Neurosci. 2025;16(13):2528-2545. doi:10.1021/acschemneuro.5c00351.
23. Chandrasekhar BVS, Elango P, Maheswari SU, Rajukumar D. A focus on the effect of L-theanine on improving depression and cognition in C57BL/J male mice subjected for chronic stress induced neuroinflammation. Biomed Pharmacol J. 2017;10(2):1015-1027. doi:10.13005/bpj/1199.
24. Tian X, Sun L, Gou L, Ling X, Feng Y, Wang L, Yin X, Liu Y. Protective effect of L-theanine on chronic restraint stress-induced cognitive impairments in mice. Brain Res. 2013;1503:24-32. doi:10.1016/j.brainres.2013.01.048.
25. Al-Ramadhan FR, Abulmeaty MM, Alquraishi M, Razak S, Alhussain MH. Effect of vitamin D3 on depressive behaviors of rats exposed to chronic unpredictable mild stress. *Biomedicines.* 2023;11(8):2112. https://doi.org/10.3390/biomedicines11082112
26. Packard AE, Egan AE, Ulrich-Lai YM. HPA axis interaction with behavioral systems. *Compr Physiol*. 2016;6(4):1897–1934.https://doi.org/10.1002/j.2040-4603.2016.tb00722.x
27. Yang J, Pei Y, Pan YL, et al. Enhanced antidepressant-like effects of electroacupuncture combined with citalopram in a rat model of depression. *Evid Based Complement Alternat Med.* 2013;2013:107380. https://doi.org/10.1155/2013/107380
28. da Silva Souza SV, da Rosa PB, Neis VB, et al. Effects of cholecalciferol on behavior and production of reactive oxygen species in female mice subjected to a corticosterone-induced model of depression. *Naunyn Schmiedebergs Arch Pharmacol.* 2020;393:111–120.https://doi.org/10.1007/s00210-019-01714-2
29. Bezchlibnyk-Butler K, Aleksic I, Kennedy SH. Citalopram: a review of pharmacological and clinical effects. *J Psychiatry Neurosci.* 2000;25(3):241–254.
30. Neis VB, Werle I, Moretti M, et al. Involvement of serotonergic neurotransmission in the antidepressant-like effect elicited by cholecalciferol in the chronic unpredictable stress model in mice. *Metab Brain Dis*. 2022;37(5):1597–1608.https://doi.org/10.1007/s11011-022-00979-6
31. Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF. Factors influencing behavior in the forced swim test. *Physiol Behav.* 2013;118:227–239. https://doi.org/10.1016/j.physbeh.2013.05.012
32. Guzmán-Ramos K, Moreno-Castilla P, Castro-Cruz M, et al. Restoration of dopamine release deficits during object recognition memory acquisition attenuates cognitive impairment in a triple transgenic mouse model of Alzheimer disease. *Learn Mem.* 2012;19(10):453–460. doi:10.1101/lm.026070.112
33. Chang PK, Chu J, Tsai YT, et al. Dopamine D3 receptor and GSK3â signaling mediate deficits in novel object recognition memory within dopamine transporter knockdown mice. *J Biomed Sci*. 2020;27:1–12.https://doi.org/10.1186/s12929-019-0613-y
34. KuŸma E, Soni M, Littlejohns TJ, et al. Vitamin D and memory decline: two population-based prospective studies. J Alzheimers Dis. 2016;50(4):1099–1108. doi:10.3233/JAD-150811
35. Williams NT. The role of L-theanine in reducing stress and anxiety. Am J Lifestyle Med. 2016;10(6):439-447. doi:10.1177/1559827614561855.
36. Yamada T, Terashima T, Kawano S, et al. Theanine, ã-glutamylethylamide, modulates neurotransmitter concentrations in the brain striatum interstitium in conscious rats. *Amino Acids.* 2009;36:21–27.https://doi.org/10.1007/s00726-007-0020-7
37. Nguyen BT, Sharma N, Shin EJ, et al. Theanine attenuates memory impairments induced by klotho gene depletion in mice. *Food Funct.* 2019;10(1):325–332. DOI: 10.1039/C8FO01577E
38. Kasatkina LA, Tarasenko AS, Krupko OO, et al. Vitamin D deficiency induces excitation–inhibition brain imbalance and a proinflammatory shift. *Int J Biochem Cell Biol.* 2020;119:105665. https://doi.org/10.1016/j.biocel.2019.105665
39. Devaki M, Nirupama R, Yajurvedi HN. Chronic stress-induced oxidative damage and hyperlipidemia are accompanied by atherosclerotic development in rats. *Stress.* 2013;16(2):233–243. https://doi.org/10.3109/10253890.2012.719052
40. Dibaba DT. Effect of vitamin D supplementation on serum lipid profiles: a systematic review and meta-analysis. *Nutr Rev.* 2019;77(12):890–902. https://doi.org/10.1093/nutrit/nuz037

41. Dai H, Chen X, He J, et al. Dietary L-theanine supplementation improves antioxidant capacity and lipid metabolism in finishing pigs. *J Funct Foods*. 2023;110:105831. https://doi.org/10.1016/j.jff.2023.105831
42. Knezevic E, Nenic K, Milanovic V, Knezevic NN. The role of cortisol in chronic stress, neurodegenerative diseases, and psychological disorders. *Cells*. 2023;12(23):2726. https://doi.org/10.3390/cells12232726
43. Al-Dujaili EA, Munir N, Iniesta RR. Effect of vitamin D supplementation on cardiovascular disease risk factors and exercise performance in healthy participants. *Ther Adv Endocrinol Metab*. 2016;7(4):153–165. https://doi.org/10.1177/204201881665335
44. Herman JP, McKlveen JM, Ghosal S, et al. Regulation of the hypothalamic–pituitary–adrenocortical stress response. *Compr Physiol*. 2016;6(2):603–621. https://doi.org/10.1002/j.2040-4603.2016.tb00694.x
45. White DJ, De Klerk S, Woods W, et al. Anti-stress, behavioural and magnetoencephalography effects of an L-theanine-based nutrient drink: a randomized, double-blind, placebo-controlled, crossover trial. *Nutrients*. 2016;8(1):53. https://doi.org/10.3390/nu8010053
46. Geigenmüller JN, Tari AR, Wisloff U, Walker TL. The relationship between adult hippocampal neurogenesis and cognitive impairment in Alzheimer disease. *Alzheimers Dement*. 2024;20(10):7369–7383.https://doi.org/10.1002/alz.14179
47. Amasi-Hartoonian N, Sforzini L, Cattaneo A, Pariante CM. Cause or consequence? Understanding the role of cortisol in the increased inflammation observed in depression. *Curr Opin Endocr Metab Res*. 2022;24:100356.https://doi.org/10.1016/j.coemr.2022.100356
48. Ulrich-Lai YM, Figueiredo HF, Ostrander MM, et al. Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *Am J Physiol Endocrinol Metab*. 2006;291(5):E965–E973. https://doi.org/10.1152/ajpendo.00070.2006
49. Unno K, Hara A, Nakagawa A, et al. Anti-stress effects of drinking green tea with lowered caffeine and enriched theanine, epigallocatechin, and arginine on psychosocial stress-induced adrenal hypertrophy in mice. *Phytomedicine*. 2016;23(12):1365–1374. https://doi.org/10.1016/j.phymed.2016.07.006
50. Wang L, Brennan M, Li S, Zhao H, Lange KW, Brennan C. How does the tea L-theanine buffer stress and anxiety. Food Sci Hum Wellness. 2022 May 1;11(3):467–475. doi:10.1016/j.fshw.2021.12.004.
51. Hussain HK, Rasheed N, Rehman Z, Hanif S, Ashraf W, Anjum SMM, Mushtaq RMZ, Akhtar N, Alqahtani F, Imran I. Nutritional co-therapy with vitamin D and L-theanine reduces unpredictable mild chronic stress (UCMS) in aged mice by restoring alpha-oscillations, dopamine levels and behavioral improvement. Exp Gerontol. 2025 Oct 15;210:112881. doi: 10.1016/j.exger.2025.112881. Epub 2025 Sep 1. PMID: 40902457.