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Neuroprotective effects of nanophytosome-encapsulated citral on subcortical NGF gene expression and cortisol regulation in a mouse model of chronic unpredictable mild stress

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Abstract

Chronic unpredictable mild stress (CUMS) induces profound neuroendocrine and neurotrophic disturbances that contribute to depressive-like phenotypes, particularly through impaired subcortical plasticity and dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis. This study evaluated the neuroprotective effects of nanophytosome-encapsulated citral on subcortical nerve growth factor (NGF) gene expression and cortisol regulation in a CUMS mouse model. Twenty eight adult male mice were assigned to four experimental groups, including control, a CUMS (subjected to a six-week CUMS protocol), and two CUMS groups treated with citral-loaded nanophytosomes at doses of 20 and 50 mg/kg. All treatments were administered three times weekly during weeks 4–6. Behavioral assessments were complemented by molecular and biochemical analyses to quantify NGF mRNA levels assessed by Quantitative real-time PCR (qRT-PCR) in subcortical regions and circulating cortisol concentrations. CUMS exposure was associated with a significant downregulation of NGF expression, assessed by qRT-PCR, along with elevated cortisol levels measured by enzyme-linked immunosorbent assay (ELISA), indicating impaired neurotrophic support and HPA axis hyperactivation. Treatment with citral nanophytosomes produced robust antidepressant-like effects, characterized by partial restoration of NGF transcriptional activity and normalization of cortisol levels toward physiological ranges. These findings suggest that nanophytosome-based delivery substantially enhances the neuroprotective efficacy of citral, enabling simultaneous modulation of neurotrophic and endocrine pathways implicated in depression. By targeting both NGF-mediated neuronal resilience and stress-induced glucocorticoid imbalance, citral nanophytosomes represent a mechanistically integrated and translationally relevant strategy for developing next-generation antidepressant interventions.

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Introduction

Depression is a leading cause of global disability, affecting more than 300 million individuals worldwide and imposing a substantial socioeconomic burden on healthcare systems (Ferrari *et al.,* 2022). Despite extensive research, its pathophysiology remains complex and multifactorial, involving disruptions in neuroplasticity and endocrine regulation (McEwen and Akil, 2020). Core clinical features-including persistent low mood, anhedonia, and cognitive dysfunction-are closely associated with structural and functional alterations in subcortical brain regions, particularly the hippocampus, which plays a critical role in emotional regulation and memory processing (Lupien *et al.,* 2009). Notably, hippocampal atrophy and impaired neurogenesis have been consistently reported in both clinical and preclinical studies of depression (Cirulli and Alleva, 2009).

A growing body of evidence highlights the central role of neurotrophic factors, particularly nerve growth factor (NGF), in maintaining neuronal survival, synaptic plasticity, and adaptive stress responses. Reduced expression of NGF in hippocampal and other subcortical regions has been linked to depressive-like behaviors and impaired neuronal resilience (Maes *et al.,* 2011; Aloe *et al.,* 2012). In parallel, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis represents a hallmark of depression, leading to sustained elevations in glucocorticoids such as cortisol (Sapolsky, 2013). Chronic cortisol overexposure exerts neurotoxic effects within subcortical structures, where it disrupts neurotrophic signaling and accelerates neuronal dysfunction (McEwen and Akil, 2020).

Although conventional antidepressants such as selective serotonin reuptake inhibitors (SSRIs) remain the first-line treatment, their clinical utility is limited by delayed onset of action, incomplete response rates, and undesirable side effects (Cipriani *et al.,* 2018; Wittekind *et al.,* 2022). These limitations have prompted increasing interest in natural bioactive compounds with multi-target pharmacological actions. Citral, a bioactive monoterpene found in lemongrass and other medicinal plants, has attracted attention due to its reported neuroprotective and neuromodulatory properties (Ji *et al.,* 2023). However, its therapeutic application is restricted by poor aqueous solubility, chemical instability, and limited bioavailability.

To overcome these limitations, nanotechnology-based delivery systems have been developed to enhance the pharmacokinetic profile of phytochemicals. In this context, nanophytosome-based formulations have shown promising results in improving the stability and biological efficacy of citral, as previously demonstrated in our earlier work (Mashayekhpour *et al.,* 2025). Building upon these findings, the present study shifts the focus toward subcortical neurotrophic regulation and endocrine imbalance, providing a more targeted mechanistic perspective.

In the present study, we investigated the antidepressant-like effects of citral nanophytosomes in a chronic unpredictable mild stress (CUMS) mouse model, a well-established paradigm that reproduces key features of human depression, including anhedonia and HPA axis dysregulation (Willner, 2017). We specifically evaluated NGF gene expression within subcortical regions as a marker of neurotrophic support, alongside circulating cortisol levels. By focusing on the interaction between neurotrophic signaling and endocrine function, this study aims to provide a mechanistically refined and translationally relevant framework for the development of novel antidepressant strategies.

Materials and Methods

Preparation of citral nanophytosomes

Citral-loaded nanophytosomes were prepared using a modified ethanol injection method as previously described (Lin *et al.,* 2024). Briefly, 6 g of citral was dissolved in 12 ml of ethanol at 55°C. Subsequently, 2.16 g of Tween 80 and 3 g of phosphatidylcholine were added to the solution and mixed thoroughly. The resulting mixture was then diluted with 120 ml of 96% ethanol. Thereafter, 0.01 M phosphate-buffered saline (PBS; 150 mM NaCl, pH 4.7) was added dropwise under continuous stirring at 55°C for 30 minutes using a water bath system. Following nanoparticle formation, ethanol was removed using a rotary evaporator at 55°C. The obtained aqueous suspension was further subjected to probe sonication for 30 minutes to enhance nanophytosome formation and stability. The morphology and size distribution of the nanoparticles were evaluated using scanning electron microscopy (SEM; KYKY-EM3200, China)

operated at an accelerating voltage of 26 kV.

Animals

A total of 50 adult male Balb/c mice (27 ± 3 g) were procured from the Animal Research Center of Mazandaran University. Animals were maintained under controlled laboratory conditions, including a temperature of 22–25°C and a 12-hour light/dark cycle, with free access to standard food and water. All experimental procedures were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals (revised 1996, Publication No. 80–23) and were approved by the Institutional Research Ethics Committee of UMZ (Approval Code: IR.UMZ.REC.1401.002).

CUMS protocol

After a one-week acclimatization period, mice in the experimental groups (excluding the control group) were exposed to a CUMS paradigm for a duration of 6 weeks, following established protocols (Liu *et al.,* 2015; Willner, 2017). The specific stressors and their schedule were summarized in Table 1. From the beginning of week 4, animals received oral administration of citral nanophytosomes at doses of 20, and 50 mg/kg via gavage, administered three times per week for the final 3 weeks of the experimental period.

Table 1. Detailed information about the chronic unpredictable mild stress (CUMS) procedure.

Time	Action or stressor
Monday 9:00 11:00	Closed light Food and water deprivation, 20 hours cold-wet cage (200 ml water (4°C) in 100 g sawdust bedding)
Tuesday 9:00 9:40	Replacing wet to dry cage, restoring food and water, and then case shaking (200 rpm for 40 minutes) Cease of case shaking, constant light for 24 hours
Wednesday 9:00 10:00	Closed light, monitoring animal weight 24 hours of 45-degree tilting of cage, and elimination of water
Thursday 9:00 15:00	Cease of 45-degree tilting of cage, restoring water, and changing to 5 mice/cage Transfer to single cage, elimination of food
Friday 9:00 9:40	Restoring food, case shaking (200 rpm for 40 minutes) Cease of case shaking and 20 hours hot–wet cage (200 ml water, 45°C, in 100 g sawdust bedding)
Saturday 9:00 10:00	Replacing wet to dry cage 24 hours of 45-degree tilting of cage and elimination of water
Sunday 9:00 15:00	Cease of 45-degree tilting of cage, restoring water, and changing to 5 mice/cage Transfer to single cage, constant light for 20 hours

Experimental design

Animals were randomly allocated into four experimental groups. The healthy control group (Control) consisted of mice not subjected to stress. The depressive control group (Vehicle) included mice exposed to CUMS and treated with the nanocarrier alone (three times per week during weeks 4–6). Three treatment groups received citral-loaded nanophytosomes at different doses: N-CIT20 (20 mg/kg), and N-CIT50 (50 mg/kg), all administered three times per week during weeks 4–6.

All CUMS-exposed groups were subjected to the stress protocol for a total duration of six weeks. Drug and nanophytosome administrations were performed via oral gavage during the final three weeks of the experiment, starting from week 4.

Sucrose preference test (SPT)

Anhedonia, a core feature of major depressive disorder characterized by reduced sensitivity to rewarding stimuli, was evaluated using the SPT (Maes *et al.,* 2011; Willner, 2017). Prior to testing, mice were individually housed and subjected to 12 hours of water deprivation to standardize fluid intake behavior.

Subsequently, each animal was provided with two pre-weighed bottles: one containing a 1% (w/v) sucrose solution and the other containing tap water, for a 6-hour test period. To minimize

potential side preference, the positions of the bottles were alternated between the left and right sides during the experiment.

Fluid consumption from each bottle was determined gravimetrically. Sucrose preference (SP) was calculated as follows:

SP (%) = [Sucrose intake / (Sucrose intake + Water intake)] × 100

Assessment of serum cortisol levels

At the end of the CUMS protocol, animals were deeply anesthetized with a ketamine–xylazine combination according to the approved institutional animal-use protocol. Immediately after euthanasia, the brains were rapidly removed, and subcortical brain tissues were carefully dissected and collected. The tissue samples were immediately stored at −80°C until further analyses. Blood samples were centrifuged to obtain serum, which was subsequently collected for biochemical analysis. Serum cortisol levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's protocol.

Real-time PCR analysis

Subcortical tissues were rapidly dissected and homogenized in liquid nitrogen. Total RNA was extracted using TRIzol reagent according to standard procedures. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a cDNA synthesis (Tailing reaction) Kit via reverse transcription. Quantitative real-time PCR (qRT-PCR) was performed using QuantiFast™ SYBR Green PCR Master Mix (QIAGEN, Germany), with GAPDH used as the internal reference gene. The primer sequences utilized in this study were listed in Table 2.

Table 2. Sequences of primers used in qRT-PCR.

Gene	Primer	Sequence	Amplicon lengths (bp)
GAPDH	forward reverse	5′-ATCCTGCACCACCAACTGC-3′ 5′-ACGCCACAGCTTTCCAGAG-3′	129
NGF	forward reverse	5′-TGCGTTTTTGATCGGCGTA-3′ 5′-GTCTACAGTGATGTTGCGGG-3′	190

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (version 23.0; IBM Corporation, USA). Differences among multiple groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Characterization of citral nanophytosomes

The physicochemical properties of the citral nanophytosomes used in the present study were consistent with those reported in our previous work (Mashayekhpour *et al*., 2025). As shown in Fig. 1, scanning electron microscopy (SEM) images-derived from our earlier study-revealed that the nanophytosomes possessed a spherical morphology with a uniform and homogeneous distribution. The particle size ranged from 42 to 51 nm, confirming the successful formation of nanoscale vesicular structures.

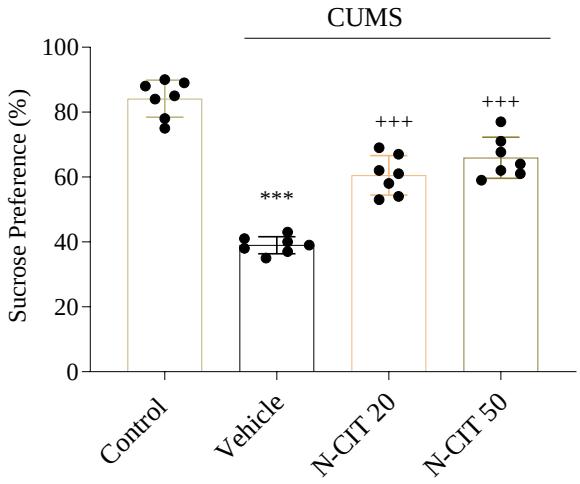


Fig. 1. Effects of citral nanophytosome on sucrose preference percent in the SPT. Data represented mean ± SD (n = 7). Control: saline, Vehicle: CUMS-induced depression, N-CIT20: citral nanophytosome (20 mg/kg), N-CIT50: citral nanophytosome (50 mg/kg). *** $p < 0.001$ vs. control group; +++ $p < 0.001$ vs. Vehicle group.

Effect of citral nanophytosome treatment on SP percentage

As illustrated in Fig. 1, the SP percentage was significantly decreased in the Vehicle compared to the control ($p < 0.001$). Treatment with citral nanophytosomes at the SP percentage was significantly elevated in both the N-CIT20 and N-CIT50 treatment groups compared to Vehicle group on days 2–4 ($p < 0.001$).

Effect of citral nanophytosome on serum cortisol levels

As shown in Fig. 2, serum cortisol concentrations were significantly elevated in the depression Vehicle compared to control ($p < 0.001$). Treatment with citral nanophytosome at the both N-CIT20 and N-CIT50 groups demonstrated more pronounced decreases in cortisol levels ($p < 0.001$ for both) compared to Vehicle animals.

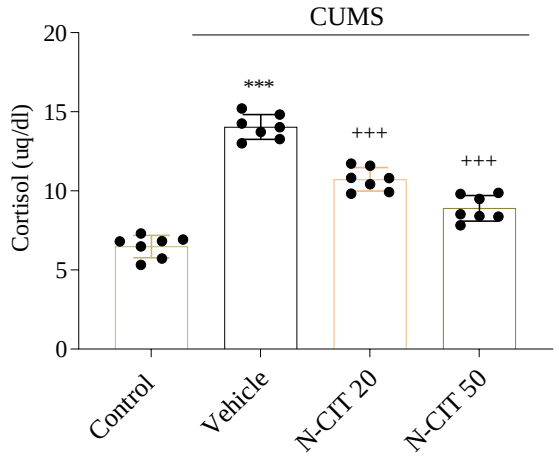


Fig. 2. Effects of citral nanophytosome on serum cortisol levels. Data represented mean ± SD (n = 7). Control: saline, Vehicle: CUMS-induced depression, N-CIT20: citral nanophytosome (20 mg/kg), N-CIT50: citral nanophytosome (50 mg/kg). *** $p < 0.001$ vs. control group; +++ $p < 0.001$ vs. Vehicle group.

Effect of citral nanophytosome on NGF mRNA expression

As shown in Fig. 3, NGF mRNA expression was significantly downregulated in Vehicle compared to control ($p < 0.001$). Three-week treatment with N-CIT20 and N-CIT50 significantly restored NGF mRNA levels relative to Vehicle animals ($p < 0.01$). These results demonstrated a clear dose-dependent neurotrophic effect of citral nanophytosome, with greater efficacy at higher concentrations.

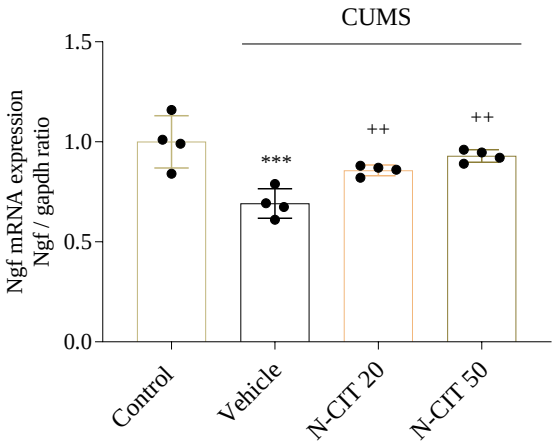


Fig. 3. Effects of citral nanophytosome on NGF in the brain hippocampus region. Data represented mean ± SD (n = 4). Control: saline, Vehicle: CUMS-induced depression, N-CIT20: citral nanophytosome (20 mg/kg), N-CIT50: citral nanophytosome (50 mg/kg). *** $p < 0.001$ vs. control group; + 5 $p < 0.05$ and ++ $p < 0.01$ vs. Vehicle group.

Discussion

The present study investigated the antidepressant-like and neuroprotective effects of citral-loaded nanophytosomes in a CUMS model, with a particular focus on subcortical dysfunction, HPA axis dysregulation, and NGF signaling. Subcortical regions, particularly the hippocampus, are highly susceptible to chronic stress and play a central role in emotional regulation and neuroplasticity, undergoing structural and functional alterations under prolonged stress exposure (Liu *et al.,* 2015; McEwen and Akil, 2020).

In the present study, CUMS exposure induced a significant reduction in sucrose preference, reflecting anhedonia. This finding is consistent with previous studies validating the CUMS model as a robust paradigm for inducing depressive-like behaviors (Willner, 1997; Liu *et al*., 2015).

A key finding of the present study is the modulation of cortisol levels. Chronic stress induces HPA axis dysregulation, leading to sustained glucocorticoid elevation, which is a hallmark of major depressive disorder (Pariante

and Lightman, 2008). Elevated cortisol levels impair subcortical neuroplasticity and disrupt neuronal homeostasis (Sapolsky, 2013; Gulyaeva, 2023). Consistent with these findings, our results demonstrated that CUMS significantly increased cortisol levels, whereas citral nanophytosome treatment normalized these levels, suggesting restoration of HPA axis balance.

Another central finding of this study was the evaluation of NGF expression in subcortical regions. Neurotrophic factors are critical regulators of neuronal survival, synaptic plasticity, and adaptive stress responses (Aloe *et al.,* 2012; Capossela *et al.,* 2024). Emerging evidence indicates that chronic stress downregulates NGF expression and contributes to impaired neuroplasticity and depressive-like behaviors (Maes *et al.,* 2011; Ahmad *et al.,* 2021). In agreement with these reports, our study showed that NGF expression was significantly reduced following CUMS exposure and was effectively restored after treatment with citral nanophytosomes.

Mechanistically, glucocorticoid excess is known to negatively regulate neurotrophic signaling pathways. Sustained elevation of cortisol suppresses neurotrophin expression and impairs neuronal survival mechanisms, particularly in subcortical structures such as the hippocampus (Sapolsky, 2013; Gulyaeva, 2023). Therefore, the restoration of NGF expression observed in this study may be attributed, at least in part, to the normalization of HPA axis activity following treatment.

From a pharmacological perspective, the enhanced efficacy of citral in its nanophytosome form can be explained by improved bioavailability and brain delivery. Nanocarrier-based systems have been shown to enhance the transport of bioactive compounds across the blood–brain barrier and improve their therapeutic performance in neurological disorders (McEwen and Akil, 2020; Naim *et al.,* 2026). Given the inherent limitations of free citral, its nanoformulation likely plays a crucial role in achieving the observed therapeutic effects (Zhang *et al.,* 2024).

Taken together, the present findings demonstrated that citral nanophytosomes exert antidepressant-like effects through modulation of HPA axis activity and restoration of subcortical NGF expression. Compared to our previous cortex-based study, the present subcortical-focused investigation provided more precise mechanistic insight into neurotrophic regulation under chronic stress conditions (Shalikar *et al.,* 2026).

However, some limitations should be acknowledged. The present study assessed NGF at the gene expression level without evaluating protein expression. Additionally, downstream signaling pathways were not explored. Future studies should investigate NGF-related signaling cascades and evaluate the long-term translational potential of citral nanophytosomes.

In conclusion, the present study provided compelling evidence that citral-loaded nanophytosomes exert significant antidepressant-like and neuroprotective effects in a CUMS model, with a distinct mechanistic focus on subcortical regions. By normalizing HPA axis hyperactivity (cortisol) and upregulating subcortical NGF gene expression, this formulation demonstrated a coordinated, multi-target mode of action aligned with current neurobiological models of depression.

Importantly, this study advanced the field by shifting the focus from cortical outcomes to subcortical neuroplasticity, thereby offering more targeted insight into stress-induced neuronal dysfunction. The incorporation of nanophytosome technology enhanced the translational relevance of these findings by improving the pharmacokinetic limitations of citral and facilitating effective brain delivery.

Collectively, these results position citral nanophytosomes as a promising, mechanistically grounded therapeutic strategy for depression, particularly in the context of HPA axis–mediated and neurotrophic dysfunction. While further studies are required to validate protein-level changes and downstream pathways, the present work provides a strong foundation for future translational and clinical investigations.

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Conflict of Interest

The authors declare no conflicts of interest.

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