

Hydroxycinnamates alleviate chronic unpredictable mild stress-induced depressive-like behavior and neuroinflammation in mice

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The polyphenolic compounds ferulic acid (FA) and p-coumaric acid (PCA) have been extensively studied for their free radical scavenging and anti-inflammatory properties. Both compounds are present in food and beverages commonly consumed globally. Our molecular modeling, *in-vitro*, and *in-vivo* studies suggest that the compounds may be neuroprotective by modulating the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome pathway. The current study explored the dose-dependent neuroprotective potential of FA and PCA in a chronic unpredictable mild stress (CUMS) mouse model. Male Swiss albino mice were divided into nine groups consisting of control (CON), CUMS, FA10 (10 mg/kg FA), FA40 (40 mg/kg FA), FA160 (160 mg/kg FA), PCA10 (10 mg/kg PCA), PCA40 (40 mg/kg PCA), PCA160 (160 mg/kg PCA), and FLX (10 mg/kg fluoxetine). All animals, except the CON group, received random mild stressors for 21 days, and from day 22–42, the treatments were administered alongside the stressors. Behavioral assessments were performed on day 42, followed by sample collection. Brain homogenates from CUMS-exposed animals expressed elevated levels of the pro-inflammatory cytokines interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α), and oxidative stress markers. Treatment with FA and PCA effectively reduced cytokine release and oxidative stress, alleviating the depressive-like behavior.

Key words: ferulic acid, p-coumaric acid, pro-inflammatory cytokines, NLRP3 inflammasome, depressive-like behavior, neuroinflammation, oxidative stress

INTRODUCTION

Depressive disorder, or major depressive disorder (MDD), is a common mental health condition that, along with anxiety, is ranked in the top 25 global health burden (GBD, 2020). Depressed mood and a lack of interest are two of the primary symptoms in MDD patients (American Psychiatric Association, 2013). The involvement of inflammation and inflammatory markers as modulatory factors in MDD has been investigated extensively in recent years (Das et al., 2024),

though the clinical correlation of this hypothesis remains inconclusive.

Animal models have played a pivotal role in our understating of MDD pathophysiology, including learned helplessness, early life stress, and social defeat (Krishnan & Nestler, 2011). Although these models are effective, they have disadvantages, with the most common being a lack of translational efficacy, as the disease outcomes do not align with MDD clinical symptoms. In animal models, a state of reduced exploratory behavior or increased immobility is considered analogous to the development of depressive-like behavior, and

decreased sucrose preference represents anhedonia (Wang et al., 2017).

Prolonged unpredictable stress is linked to the etiology of neurodegenerative and neuropsychological disorders (White et al., 2024), and the rodent model of chronic unpredictable mild stress (CUMS) has emerged as one of the most relevant to the human depressive state (Markov & Novosadova, 2022), and is commonly used to understand pathophysiology and the effects of test compounds (Antoniuk et al., 2019). Chronic stress scenarios consistently and reliably reproduce an increase in pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β , nuclear factor-kappa B (NF- κ B), and toll-like receptor 4 (TLR4) (White et al., 2024). Glial cells are the principal source of innate immune responses in the central nervous system and are responsible for producing these pro-inflammatory cytokines.

Polyphenolic compounds of hydroxylated cinnamic acid derivatives are abundantly distributed in nature and are commonly consumed in food and beverages (El-Seedi et al., 2012). Originating from the catecholamine precursors phenylalanine and tyrosine via the Shikimate pathway, ferulic acid (FA) and p-coumaric acid (PCA) have attracted significant attention in recent years due to being the most commonly consumed antioxidants (Alam et al., 2016). Various preclinical *in-vitro* studies and a few clinical investigations have highlighted the health benefits of these hydroxycinnamate derivatives, with most data suggesting that their beneficial effects are due to free radical scavenging (van Acker et al., 1996; Zhang et al., 2016; Taofiq et al., 2017). Although the neuromodulatory effects of FA and PCA remain mechanistically unproven, we have recently demonstrated their neuroprotective potential using *in-vitro* models and a model of lipopolysaccharide (LPS)-induced sickness behavior (Kinra et al., 2021; 2024). Other studies have shown FA to have anti-inflammatory effects through inducing autophagy and blocking nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome activation (Liu et al., 2022). Similarly, PCA reduced neuroinflammation by regulating mitogen-activated protein kinase (MAPK)/NF- κ B signaling pathways and modulating monoaminergic signaling (Cao et al., 2023; Oh et al., 2023).

The current study focused on the chronic administration of a broad FA and PCA dose range in a CUMS model of depressive-like behavior in mice. Compound administration commenced after establishing a depressive-like state through exposure to specific stressors, which was confirmed using the sucrose preference test (SPT).

METHODS

Animals

Male Swiss Albino mice weighing 25–30 g were selected for the study. The Institutional Animal Ethics Committee of Kasturba Medical College, Manipal, approved all experimental protocols (approval number IAEC/KMC/113/2020, dated November 7, 2020). Animals were housed in controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($50 \pm 5\%$) conditions, with a 12-hour light-dark cycle, and were provided with food and water *ad libitum*. All experiments were carried out following the guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Chemicals and reagents

All chemicals used in this study were analytical grade. FA, PCA, 2-thiobarbituric acid (TBA), and reduced L-glutathione (GSH) were purchased from Sigma-Aldrich Co. LLC (MO, USA). Carboxymethylcellulose, sodium dihydrogen phosphate anhydrous, disodium hydrogen phosphate anhydrous, and trichloroacetic acid were purchased from Merck Millipore Corporation (Darmstadt, Germany). Bicinchoninic acid (BCA) protein estimation and enzyme-linked immunosorbent (ELISA) kits were purchased from ThermoFisher Scientific (MA, USA).

Treatments and groupings

Animals were randomly divided into nine groups ($n=6$ per group), including a control group (CON), a CUMS group, three groups treated with FA at 10, 40, and 160 mg/kg (FA10, FA40, and FA160), three groups treated with PCA at 10, 40, and 160 mg/kg (PCA10, PCA40, and PCA160), and a 10 mg/kg fluoxetine (FLX) group, with all treatments administered via the oral route. CON and CUMS groups were administered a vehicle, carboxymethylcellulose (CMC, 0.25% w/v), at a dose of 10 ml/kg. FLX was used as a positive control because of its well-established efficacy in reducing depressive-like behaviors in animal models, which validates the CUMS paradigm and provides a positive control for evaluating the therapeutic potential of the test compounds. All groups, except CON, were exposed to randomized stressors, as described in Table 1. The stressors were randomly scheduled and changed every week.

Table 1. Randomized stressors for chronic unpredictable mild stress.

Food deprivation	24 h
Water deprivation	24 h
Cage tilting (45°C)	12 h
Wet cage	12 h
Overnight illumination	12 h
Swimming (4-6°C)	20 min
Restraint	6 h
Swimming (45°C)	20 min

The SPT was done only once on day 21, to randomize the animals into different groups. Before starting the test, the animals were habituated to a 1% w/v sucrose solution for 24 hours. After 24 h, a fresh solution of 1% w/v sucrose was prepared and weighed in the bottle, as was the weight of the bottle with fresh water. The mice were given a free choice of two bottles, one with 1% w/v sucrose solution and the other with water. The bottle positions were changed after 12 h to prevent potential side preference effects on drinking behavior. Twenty-four hours later, both bottles were removed and weighed, and sucrose preference was calculated (*sucrose intake (ml)/total liquid intake (ml) × 100*). The SPT also served as the exclusion criteria for CUMS-resistant mice. Test compounds were administered from day 21 to day 42, along with the continuation of CUMS stressors (Liu et al., 2017). Animal weight was monitored weekly from day 21 onwards. On day 42, behavioral tests were carried out, and the animals were sacrificed, with their brains collected

and stored at -80°C until further use (Fig. 1). Tissue samples were homogenized using ice-cold phosphate buffer (0.1 M, pH 7.4) for antioxidant and cytokine level estimations. The homogenate was centrifuged for 10 min at 6000 rpm at 4°C. Aliquots of the resultant supernatant were taken and stored at -80°C until further analysis.

Behavioral tests

All behavioral tests were performed using previously established protocols (Kinra et al., 2024; Mudgal et al., 2019; 2020). Spontaneous locomotor activity (LMA) and exploratory behavior were assessed using the open field test (OFT), while the forced swim test (FST) and tail suspension test (TST) were employed for behavioral despair quantification induced by the CUMS protocol. Briefly, LMA was assessed in a clean glass open field arena (30 × 30 × 60 cm), with the number of square crossings into nine virtual quadrants (10 × 10 cm each) counted over a five-minute period. FST was measured from the total immobility time over five minutes in a transparent plexiglass cylinder (30 × 20 cm), whereas TST was measured as immobility duration when the animals were hung 15 cm from the nearest surface for five minutes.

Cytokine level changes in the brain

Proinflammatory cytokines, including TNF-α, IL-6, and IL-1β, were estimated using commercially available ELISA kits (Thermo Fisher Scientific, MA, USA) per manufacturer instructions.

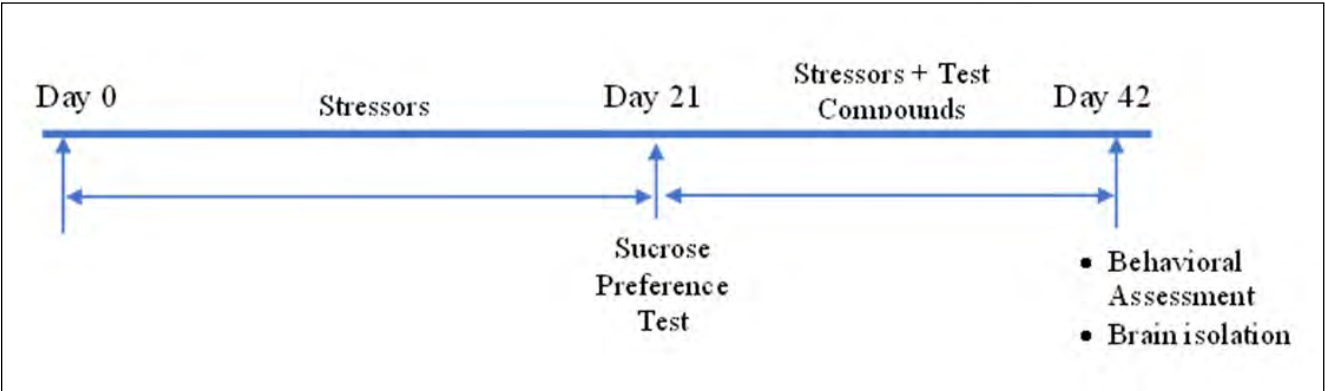


Fig. 1. The study timeline for assessing the effects of test compounds on chronic unpredictable mild stress-induced chronic depression in mice.

Estimation of oxidative stress in mouse brains

Lipid peroxidation (LPO) and GSH were quantified, as detailed earlier (Khan et al., 2013). Brain homogenates were incubated with equal volumes of TBA at 90°C for 10 minutes. Malondialdehyde (MDA) levels were measured spectrophotometrically at 532 nm. Similarly, GSH levels were measured by the absorbance of GSH and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) complex at 412 nm. Total protein estimation was carried out using a commercial kit (Thermo Fisher Scientific, MA, USA) per manufacturer instructions.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM) and analyzed using GraphPad Prism v10.0.0 (GraphPad Software, CA, USA). All the parameters were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, where $p < 0.05$ was considered statistically significant.

RESULTS

Open field test

Exposure of mice to the CUMS protocol significantly reduced the number of line crossings compared to the CON animals (24.33 ± 7.47 vs. 95.00 ± 18.83 s). Treatments with 40 mg/kg FA (61.50 ± 4.33 s), 160 mg/kg FA (82.33 ± 3.04 s), and all PCA doses (65.67 ± 2.93 s, 95.00 ± 2.38 s, and 95.67 ± 5.30 s) significantly alleviated CUMS-induced reductions in spontaneous activity ($p < 0.05$). A similar trend was observed with standard FLX treatment (84.50 ± 12.60 s). Interestingly, the low dose of FA (FA10) did not change OFT performance (49.33 ± 6.74 s) (Fig. 2A).

Forced swim test

Immobility time increased in the CUMS group (87.33 ± 7.83 s) compared to the CON (7.83 ± 1.30 s). All FA (41.33 ± 2.75 s, 33.17 ± 2.83 s, and 26.17 ± 2.61 s), PCA (57.50 ± 2.11 s, 48.83 ± 3.00 s, and 36.50 ± 6.53 s) and FLX doses (52.50 ± 3.17 s) significantly improved FST-induced immobility time (all $p < 0.05$; Fig. 2B).

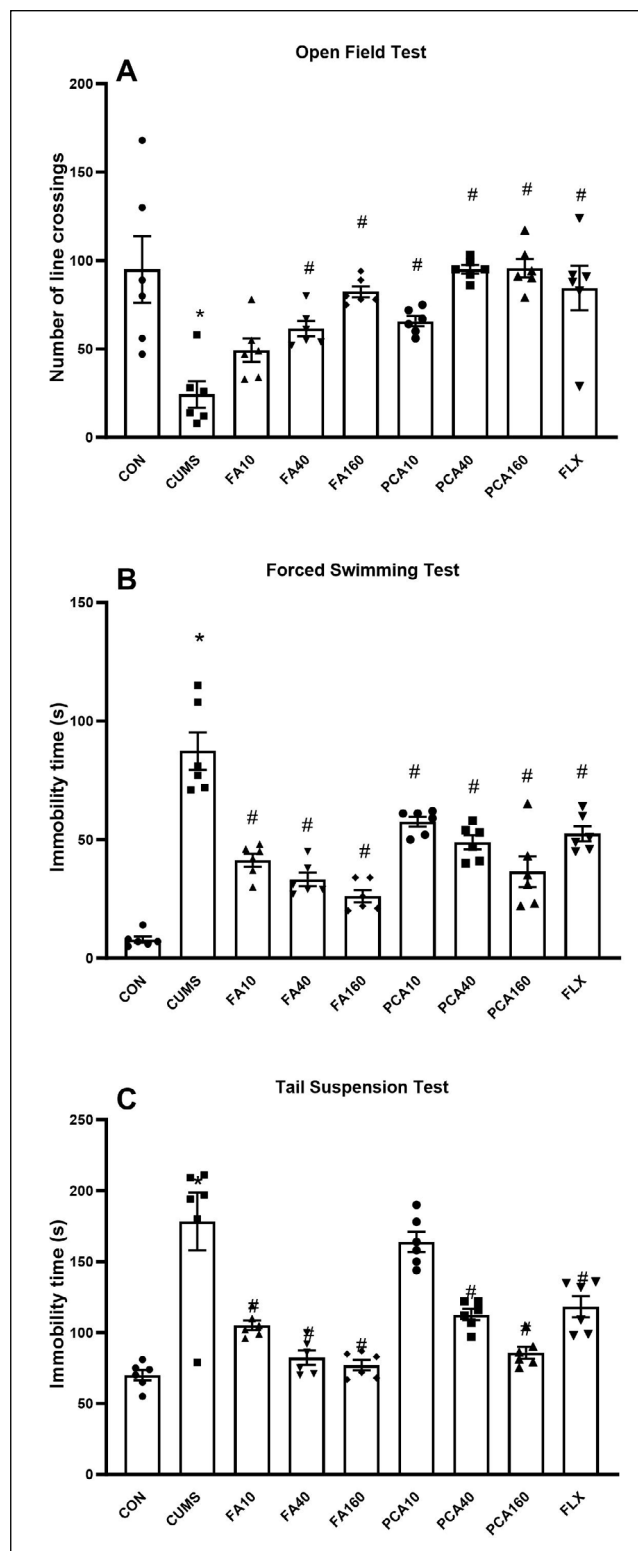


Fig. 2. The effect of test compounds on chronic unpredictable mild stress-induced behavioral changes in mice in the (A) open field test, (B) forced swimming test, and (C) tail suspension test. * represents significance at $p < 0.05$ compared to the control (CON), and # represents significance at $p < 0.05$ compared to the chronic unpredictable mild stress (CUMS) group. The data are mean $\pm$ standard error of the mean ($n=6$).

Tail suspension test

Similar to the immobility time in FST, exposure to CUMS significantly increased the immobility time in TST (178.30 ± 20.39 s [CUMS] vs. 70.00 ± 3.71 s [CON]). All FA groups (105.20 ± 3.40 s, 82.33 ± 5.01 s, and 77.00 ± 3.68 s), PCA40 and PCA160 (112.70 ± 3.93 s and 85.83 ± 4.22 s), and FLX (118.20 ± 7.42 s) improved TST-induced immobility time significantly (all $p < 0.05$; Fig. 2C). Interestingly, the lowest dose of PCA did not affect the immobility test (164.00 ± 7.08 s).

The effect of test compounds on chronic unpredictable mild stress-induced changes in cytokine levels in the mouse brain

The CUMS protocol significantly increased IL-1 β levels compared to the CON (194.50 ± 38.54 pg/mg vs. 15.41 ± 2.85 pg/mg). All FA (11.25 ± 2.51 , 15.09 ± 2.02 , and 11.49 ± 1.83 pg/mg), PCA (17.23 ± 3.38 , 18.52 ± 7.40 , and 13.68 ± 2.10 pg/mg), and FLX (8.88 ± 1.07 pg/mg) doses effectively reduced IL-1 β levels (all $p < 0.05$; Fig. 3A).

Interestingly, IL-6 levels were above the detectable limit in CUMS-exposed animals but not in the CON mice (>2000 pg/mg vs. 323.30 ± 49.26 pg/mg). All FA (305.10 ± 75.02 , 372.20 ± 56.21 , and 281.50 ± 44.40 pg/mg respectively), PCA (355.80 ± 33.26 , 474.00 ± 221.00 , and 304.30 ± 49.38 pg/mg respectively), and FLX (217.60 ± 31.93 pg/mg) doses significantly reduced IL-6 levels (all $p < 0.05$; Fig. 3B).

TNF- α levels were significantly elevated after CUMS exposure (245.80 ± 77.99 pg/mg) compared to the CON group, where the levels were below the detectable limit of the kit. Pretreatment with FA10, FA40, PCA40, and PCA160 reduced the CUMS-induced TNF- α levels to below its detectable limit, with only 1-2 animals in FA160 and PCA10 groups producing detectable concentrations (3.31 ± 3.31 pg/mg and 13.05 ± 10.75 pg/mg, respectively) (all $p < 0.05$; Fig. 3C).

The effect of test compounds on chronic unpredictable mild stress-induced changes in brain oxidative stress levels

The GSH levels indicated a significant reduction in the antioxidant defenses of the CUMS animals compared to the CON mice (69.83 ± 3.13 nmol/mg vs. 87.62 ± 3.89 nmol/mg). All FA doses effectively increased GSH levels (88.37 ± 3.71 , 84.45 ± 4.89 , and 85.68 ± 4.46 nmol/mg), as did PCA10 and PCA40 (87.07 ± 2.83 and 89.65 ± 4.01 nmol/mg, respectively). However, PCA160 and FLX did not markedly increase GSH levels

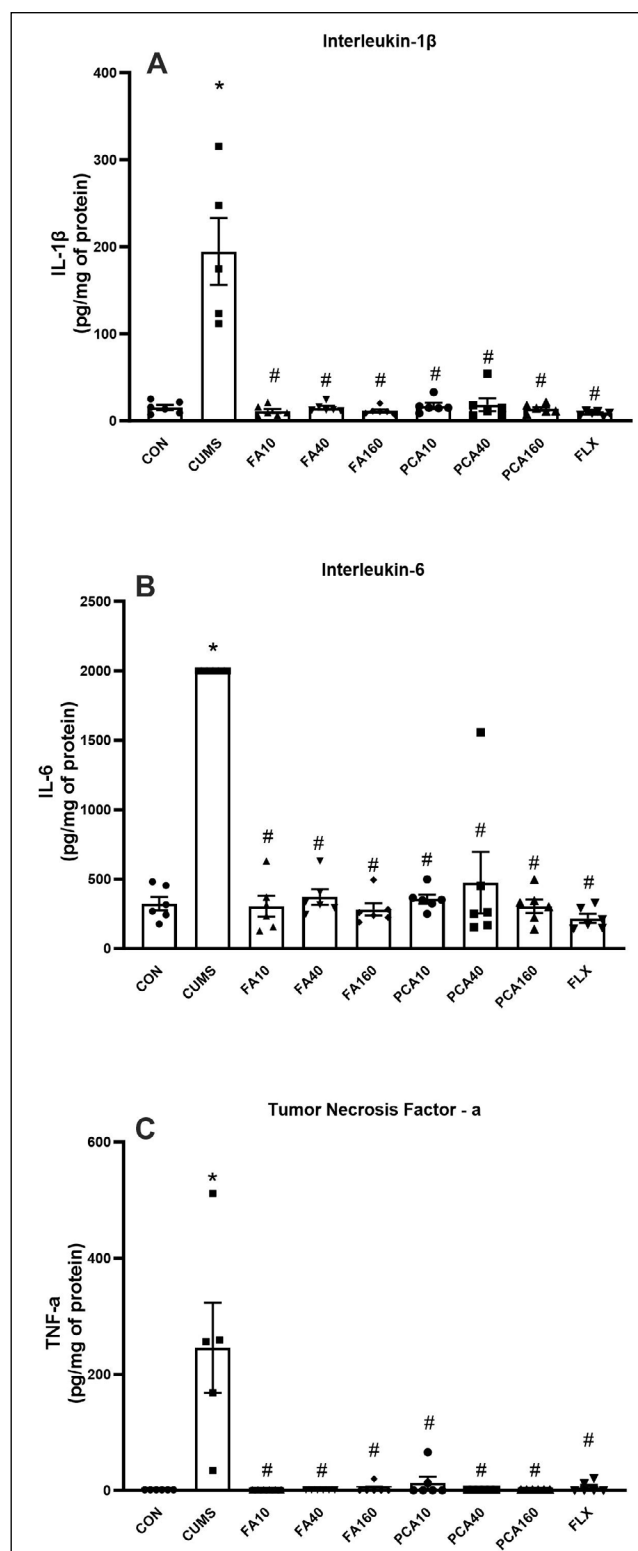


Fig. 3. The effect of test compounds on chronic unpredictable mild stress-induced changes in mouse (A) interleukin-1 β , (B) interleukin-6, and (C) tumor necrosis factor- α . * represents significance at $p < 0.05$ compared to the control (CON), and # represents significance at $p < 0.05$ compared to the chronic unpredictable mild stress (CUMS) group. The data are mean $\pm$ standard error of the mean ($n=6$).

(78.95 ± 3.33 and 80.08 ± 3.13 nmol/mg, respectively) (Fig. 4A).

In accordance with the GSH levels, LPO was significantly increased in the CUMS group compared to the CON (868.30 ± 36.98 nmol/mg vs. 309.20 ± 19.51 nmol/mg). All doses of FA (482.30 ± 16.04 , 688.80 ± 38.61 , and 631.80 ± 57.99 nmol/mg), PCA (601.70 ± 45.07 , 631.70 ± 60.91 , and 643.70 ± 57.62), and FLX (583.80 ± 33.47 nmol/mg) significantly reduced the CUMS-induced increase in LPO (all $p < 0.05$; Fig. 4B).

DISCUSSION

The CUMS model employs systematic and repeated exposure to variable, unpredictable, and uncontrollable stressors for a prolonged period, and this paradigm can recapitulate the major symptoms of human depression. Evidence suggests that depression is characterized by hypothalamic-pituitary-adrenal (HPA) axis hyperactivity and is accompanied by increased immune dysregulation in the brain, predominantly mediated by IL-1 β (Schiepers et al., 2004; Goshen et al., 2008).

In our previous study, we mechanistically explored the role of FA and PCA in LPS-induced sickness behavior and neuroinflammation (Kinra et al., 2024), with NLRP3 inflammasome pathway involvement established in the acute sickness behavior model. In the current study, the test compounds were administered daily for three weeks. Due to the chronic dosage regimen, the FA and PCA doses were reduced to 10, 40, and 160 mg/kg orally. The stressors (as detailed in the methods) were carefully selected based on various pilot studies and according to their feasibility, frequency, consistency, and intensity (Sequeira-Cordero et al., 2019). The induction of a depressive state in the animals was evaluated by the SPT on day 21.

The behavioral impact of the CUMS protocol was assessed by measuring spontaneous activity in the OFT and immobility time in the FST and TST. Reduced exploratory behavior in the open field and increased immobility in the forced swim and tail suspension tests indicate the development of a depressive-like state (Mallik et al., 2023; Kinra et al., 2024). The selected stressors were able to significantly reduce the LMA and increase the immobility time, suggesting that the animals developed a behavioral depressive-like state.

As indicated earlier, our selected dose range encompassed a low to moderately high dose of the test compounds administered orally and chronically and was based on our previous work and supporting published literature. Overall, there was a noticeable dose-dependent impact of the FA and PCA treatments, with an increase in spontaneous activity and a progressive de-

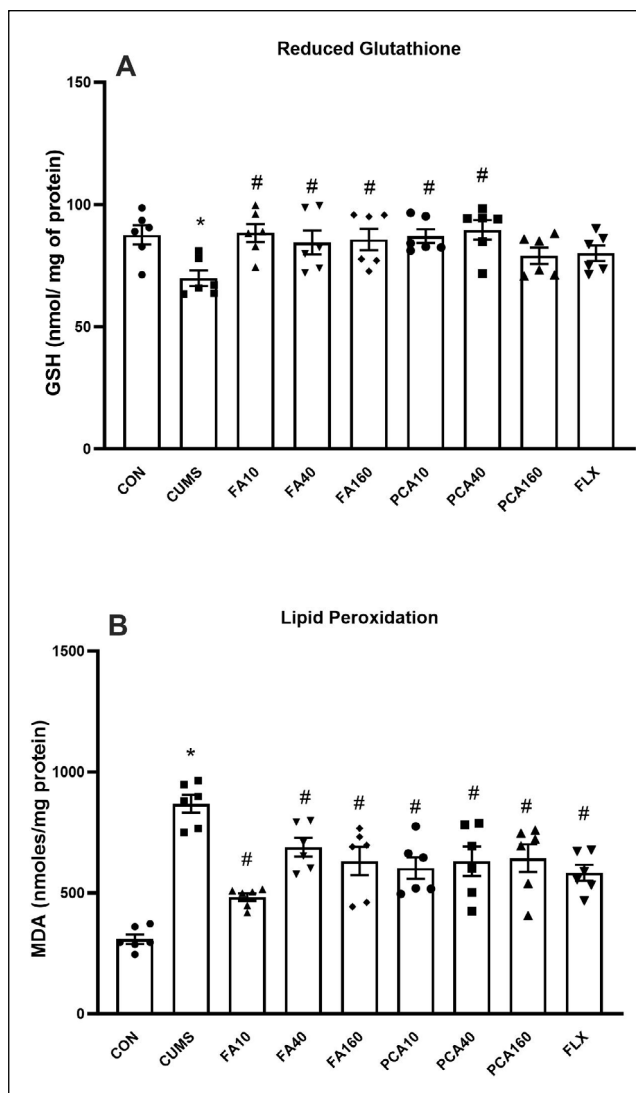


Fig. 4. The effect of test compounds on chronic unpredictable mild stress-induced changes in oxidative stress in the mouse brain with respect to (A) reduced glutathione (GSH) and (B) malondialdehyde (MDA). * represents significance at $p < 0.05$ compared to the control (CON), and # represents significance at $p < 0.05$ compared to the chronic unpredictable mild stress (CUMS) group. The data are mean $\pm$ standard error of the mean ($n = 6$).

crease in immobile behavior in the behavioral despair tests with an increasing dose. The standard drug, FLX, also produced a comparable behavioral change, though the effect was closest to the FA40 treatment group.

The CUMS model of chronic depression is known to have an underlying inflammatory component in the brain, which is responsible for behavioral changes (Wang et al., 2021). In order to establish CUMS-induced neuroinflammation, biochemical estimations of changes in the levels of IL-1 β , IL-6, and TNF- α in whole-brain homogenates were undertaken. Since this study aimed

to evaluate the effects of phenylpropanoic acid derivatives on neuroinflammation in a chronic model of depression, we opted for whole-brain homogenates rather than specific anatomical areas of interest. The CUMS protocol significantly increased IL-1 β levels in all the animals, and IL-1 β receptor knockout studies showed that this cytokine is linked with stress-induced behavioral changes (Goshen et al., 2008). Furthermore, some clinical studies have observed a positive correlation between MDD symptoms and pro-inflammatory cytokines (Cassano et al., 2017; Das et al., 2021). In our previous study, we found that LPS caused NLRP3 inflammasome assembly and upregulated IL-1 β (Kinra et al., 2024). IL-1 β is also the first cytokine known to enhance HPA axis activity during an immune response (Bernton et al., 1987; Sapolsky et al., 1987). All the treatment doses of FA, PCA, and FLX significantly reduced the CUMS-induced increase in IL-1 β .

CUMS activates the stress response system, particularly the HPA axis, leading to prolonged release of cortisol, the primary stress hormone. Persistent cortisol elevation disrupts homeostasis, initiating a cascade of inflammatory processes. As stress persists, systemic inflammation increases, partly due to the activation of glial cells in the brain, which release pro-inflammatory cytokines. These inflammatory markers can compromise the blood-brain barrier (BBB), making it more permeable and allowing immune cells to infiltrate the central nervous system. This disruption amplifies neuroinflammation, perpetuating a cycle of stress, cortisol release, and inflammation that sustains the systemic inflammatory response (Almutabagani et al., 2023).

A similar trend was observed in IL-6 levels, which were significantly elevated by CUMS in all groups. In the CUMS group, the levels were beyond the maximum detectable limit. Chronic stress increases IL-6 in various brain and blood components, and IL-6 knockout has been shown to reduce the development of depressive-like behavior in models of behavioral despair (Chourbaji et al., 2006). Both FA and PCA treatment groups showed a significant reduction in IL-6 compared to the CUMS group. Similarly, TNF- α was below detectable limits in most treatment groups.

Recent research demonstrated that stress represses the expression of tight junction proteins like claudin-5, which disrupts BBB integrity and causes neuroinflammation and various psychiatric disorders. Furthermore, the severity of depressive-like behavior is correlated with the extent of BBB integrity loss (Sun et al., 2024). Moreover, rodent positron emitting tomography (PET) shows that chronic stress decreases P-glycoprotein function at the BBB and affects its integrity (de Klerk et al., 2010).

The inflammatory milieu induced by chronic stressors causes oxidative stress in the brain. Furthermore, mitochondrial oxidative stress adds to the inflammatory cascade, leading to the excessive generation of reactive oxygen species during the inflammatory phase. This process initiates cellular damage through the oxidation of cellular membrane lipids and proteins, causing increased membrane permeability and elevating lipid peroxidative adducts like MDA (Juszczyk et al., 2021). We quantified oxidative stress by measuring the levels of antioxidant defense (GSH) and increased LPO (MDA) markers. Exposure to CUMS stressors significantly reduced GSH and increased MDA levels. All treatment groups showed a decrease in overall LPO, with lower doses being more effective. Interestingly, with the exception of PCA160 and FLX, all treatments effectively increased antioxidant defenses.

CONCLUSIONS

This study highlights the importance of polyphenolic dietary compounds in reducing stress-related neurobehavioral changes in mice, with FA and PCA significantly improving spontaneous locomotion and reducing behavioral despair parameters. Furthermore, neuroinflammatory cytokine generation and oxidative stress were effectively reduced.

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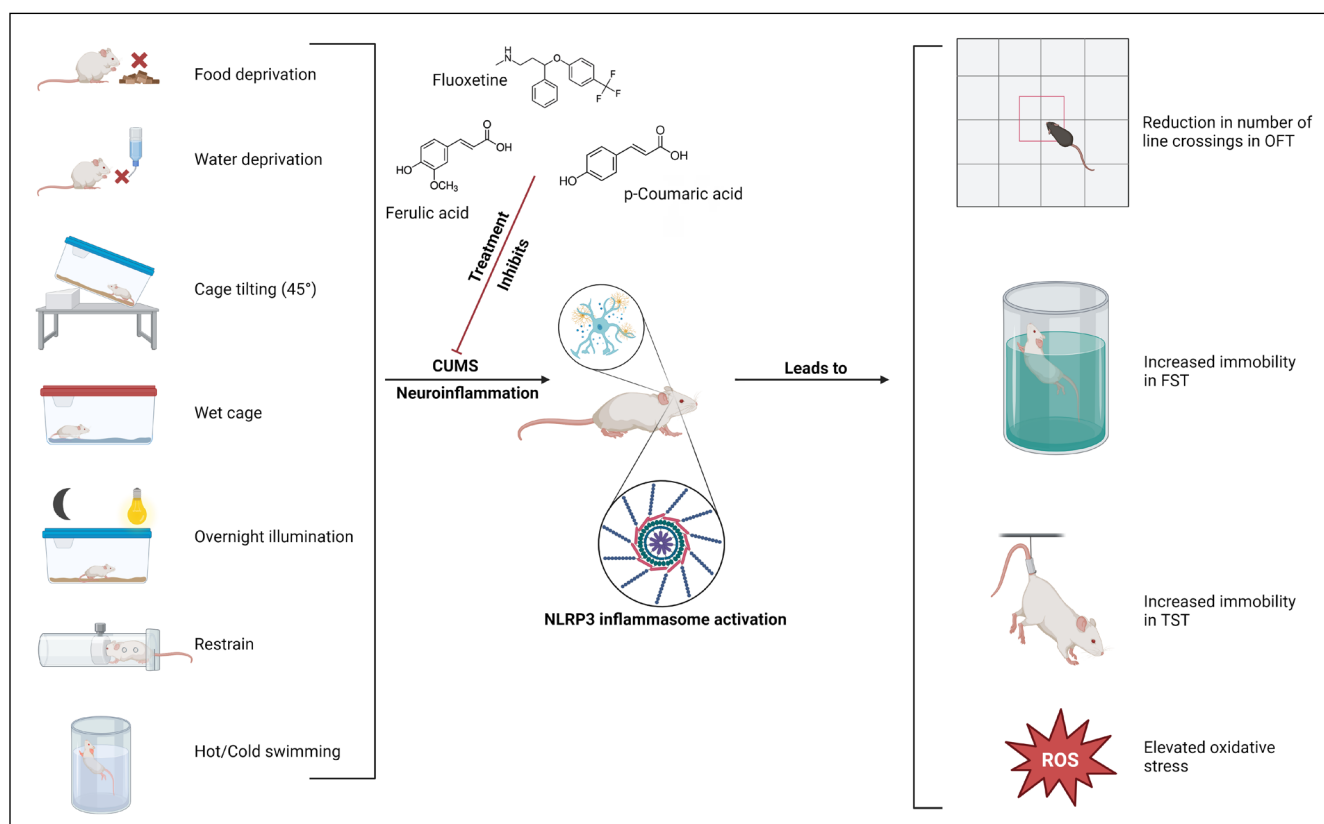
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SUPPLEMENTARY MATERIALS



CUMS graphical abstract