Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 12 [8] July 2023 : 185-203

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Ellagic Acid Ameliorates Behavioral Impairment and Brain Oxidative Stress Induced by Chronic Unpredictable Mild Stress in Mice

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ABSTRACT

Stressful circumstances, the body's response to stress, and the onset of clinical depression all have complicated relationships with one another. Such animal models can be used for the pre-clinical evaluation of antidepressants. Chronic unpredictable stressors can create a situation that is similar to clinical depression. Clinical studies found that depressed patients had altered neurotransmitters, elevated MAO activity, nitrosative stress, and inflammation. The aim of this study was to examine the impact of ellagic acid on the behavioral, biochemical, and neurochemical changes induced by unpredictable chronic stress in mice. During the study, animals were exposed to various stress-inducing situations for a period of 21 days, resulting in depressive-like behavior. The stressed mice exhibited significant changes in immobility period, locomotor activity, memory acquisition, and retention. These behavioral changes were linked to a decrease in biogenic amines such as dopamine, norepinephrine, and serotonin, as well as an increase in nitrosative stress (characterized by increased lipid peroxidation and nitrite levels and decreased levels of glutathione, superoxide dismutase, and catalase activities), increased MAO activity, and enhanced activity of inflammatory cytokines (TNF-α, TGF-β, and IL-1β). Chronic administration of ellagic acid demonstrated significant restoration of the behavioral deficits induced by unpredictable chronic stress, such as increased immobility period. Furthermore, it restored the biochemical deficits, such as decreased nitrodative stress, MAO, and inflammatory cytokine activity, as well as the neurochemical deficits in dopamine, norepinephrine, and serotonin levels in the stressed mice. The findings of the study suggest that ellagic acid has potential antidepressant effects in chronically stressed mice by regulating biogenic amines, MAO, nitrosative stress, and inflammation. These results imply that ellagic acid could be useful in the clinical treatment of stress-induced depression.

Keywords: Ellagic acid, Duloxetine, Chronic Unpredictable Mild Stress, Depression, etc.

Received 25.04.2023 Revised 15.06.2023 Accepted 23.07.2023

INTRODUCTION

Stress is a daily burden, which is sustained by living organisms for some time, but when it exceeds the adaptive capacity it is associated with numerous illnesses and diseases. Epidemiological data support a role for stress plays an important role in the development of depression [50, 4]. Stress is one of the most widespread public health issues, with a lifelong prevalence of about 15-20%. According to the World Health Organization (WHO), disease-induced disability will be the leading cause of death by 2030 [62].

Stress can be acute (lasting a few days to a few weeks) or chronic (usually lasting weeks to months). Acute stress is recognized as a threat, and the autonomic nervous system is activated. As a result, adrenaline and cortisol levels rise, increasing heart rate and other cardiac effects. [21]. An acute stressful event is generally adaptive, at least in the short term but may become maladaptive if continuously activated suggesting the paradoxical action of the key stress response mediators [25]. Chronic stress is a condition of ongoing physiological arousal. This condition occurs when the body experiences multiple stressors or a single stressor continuously and the body does not have the capability or chance to activate the relaxation response [35]. Poorly managed or ignorance of everyday stressors may lead to traumatic events [58]. It has numerous negative consequences such as heart attacks, strokes, immune system suppression, infertility, and hastening of the aging process, etc. [1].

The stress response and the resulting hypothalamic– pituitary–adrenal (HPA) axial hyperactivity are considered to be one of the most powerful triggers for depressive disorders [57]. Preclinical and clinical

studies have indicated that the stress response can result in depression-like behavior accompanied by hyperactivity of the HPA axis, [17, 18] as indicated by increased serum glucocorticoid concentrations and corticotrophin-releasing hormone (CRH) expression in the hypothalamus [63]. Chronic mild unpredictable stress (CUMS) has been used in animal depression research because it can simulate the representative symptoms of depression, including anhedonia and despairing behaviour [32]. Given the roles of the HPA axis in the pathophysiology of depression, plasma corticosterone concentration was evaluated in CUMS rats in this study.

Increasing evidence supports the view that depression is accompanied by the activation of the inflammatory response system, and the overproduction of pro-inflammatory cytokines may play a role in the pathophysiology of depressive disorders [34, 40]. It has been reported that the most robust evidence-based inflammatory markers associated with depressive disorder include interleukin-1β (IL-1 β), Transforming growth factor- β (TGF- β), and tumor necrosis factor-α (TNF-α) [31, 14]. In animals, it has been shown that depression-like behaviors are induced by cytokines [56] and cytokine inducers, including lipopolysaccharide administration [72] and chronic mild stress. Therefore, IL-1 β, TGF- β, and TNF-α levels were evaluated in CUMS mice with and without antidepressant administration in this study. Altered mitochondrial complexes activity are highly relevant to neurological disorders as many of these disorders are characterized by mitochondrial electron transport chain inhibition, energy metabolism impairment, and elevated levels of oxidative stressors [5]**.**

Polyphenols are phytochemicals derived from phenylalanine and contain an aromatic ring with a reactive hydroxyl group. According to their structure, polyphenols can be divided into different classes. The most important ones are phenolic acids, which include polymeric structures, such as hydrolyzable tannins, lignans, stilbenes, and flavonoids. Flavonoids are divided into different subclasses: flavones (eg, chrysin), flavonols (eg, quercetin, myricetin & rutin), flavanones (eg, naringenin), anthocyanidins (eg, malvidin), flavan-3-ols (eg, catechin, epicatechin and EGCG), isoflavones (eg, genistein), and chalcones (eg, xanthohumol) [8, 9, 61]. Ellagic acid is a naturally occurring polyphenolic compound found in various fruits and vegetables such as pomegranates, strawberries, raspberries, and walnuts. It has been widely studied for its potential health benefits, including anti-inflammatory, antioxidant, and anticancer properties [22].

MATERIAL AND METHODS

Animals

Healthy adult male healthy Swiss albino mice (32-38g) were used for the experiment. The animals were housed under standard laboratory conditions, maintained on a 12:12 h light: dark cycle, and had free access to food and water. Animals were acclimatized in standard animal house environmental conditions for five days before the start of the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee and performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India on animal experimentation.

Drugs & Treatment

Ellagic acid purchase from Yucca Enterprises, Mumbai, India, and duloxetine was obtained as gift sample from Torrent Pharmaceuticals Ltd., Ahmedabad, India. Tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β) and Interleukin 1β (IL-1β) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Minneapolis, MN, USA, while nuclear factor kappa light chain-enhancer of activated B cells (NF-kB) and caspase-3 ELISA kits were procured from Imegenex, San Diego, USA, and Biovision, USA, respectively. All other chemicals used for biochemical estimations were of analytical grade. Drug solution was freshly prepared and administered in a constant volume of 10 ml/kg body weight. Ellagic acid (25 mg/kg, 50 mg/kg and 100 mg/kg) [20] by oral gavage and duloxetine (20 mg/kg) [67] was administered by intraperitoneally daily for 21 days.

Experimental Design

Male Swiss mice were randomly divided into 6 different groups, consisting of eight mice in each group for the CUMS protocol as follow: **Group I** consisted of control unstressed mice; **Group II** comprised of mice subjected to series of different types of stressors for 21 days; **Group III, IV and V** comprised of stressed mice receiving ellagic acid (25, 50 and 100 mg/kg, oral gavage), respectively, daily for 21 days; **Group VI** consisted of stressed mice receiving duloxetine (20 mg/kg, i.p.) for 21 days. Mice were subjected to various stressors daily 30 minutes after drug administration.

Chronic Stress Procedure

The CUMS procedure was performed as described by Taksande et al., [59], with slight modifications. This animal model of stress consists of chronic exposure to variable unpredictable stressors, none of which is sufficient alone to induce long-lasting effects. Briefly, CUMS consisted of exposure to a variety of

unpredictable stressors randomly scheduled over a 1-week period and repeated throughout the 3 weeks experiment (Table 1). Normal control mice were undisturbed except for necessary housekeeping procedures. The entire experimental protocol is depicted below.

Table 1: Schedule of stressors used in the 21 days of chronic unpredictable mild stress protocol (CUMS)

Stressor	Duration	Day Day
Food & Water Deprivation	24 h	Monday
Empty bottle Foreign Object	1 h 24h	Tuesday
Forced Swim cold water (12°C) Overnight illumination	6 min 12 h	Wednesday
Restraint Cage Tilted	2h 7h 7	Thursday
Food Deprivation & Soiled cage	24 h	Friday
Water Deprivation Overnight illumination	24 h 12 h	Saturday
Empty bottle Cage Tilt	1 h 7 h	Sunday

On the 21st day of CUMS protocol (60 min after drug administration), animals were subjected to various behavioral parameters viz learning and memory (Elevated Plus Maze), stress-induced depression (Tail Suspension Test), stress-induced pain (Thermal Hyperalgesia, Mechanical allodynia and Tail immersion), locomotor activity and muscle grip strength activity (Actophotometer, rota-rod), biochemical and neurochemical tests. The animals were given free access to food and water before administering drug on the last day. For biochemical and neurochemical investigations, mice were sacrificed by decapitation and the brain was isolated. Brain samples were then stored in phosphate buffer (0.1 M, pH 7.4) at −80 °C till further investigations.

Behavioral assessment

Assessment of immobility period

The animals were individually forced to swim in a 25×12×25 cm (L×B×H) filled with water (23±2°C) up to a height of 15 cm. After the initial 2-3 min of vigorous activity the animals showed period of immobility by floating with minimum movements. An animal is considered to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its nose above the water surface. The immobility period in last 4 min was recorded during the total 6 min test with the help of stop-watch [6].

Assessment of locomotor activity

In order to detect the association of immobility in the FST with changes in motor activity, the locomotor activity of mice was tested by a Digital Actophotometer. Each mouse was placed in the center of the actophotometer apparatus, and the locomotor activity was assessed one day before the FST. Total number of ambulatory movements was scored in the 5 min test period to evaluate locomotor activity [66].

Assessment of grip strength in Rota-rod test

Mice were subjected to motor function evaluation by placing them individually on rota rod, which was adjusted to the speed of 25 rpm. The fall-off time was recorded for each mouse and the longest period any animal was kept on the rod was 300 s [60].

Assessment of cognitive behavior using plus-maze test

Cognitive behavior was noted by using elevated plus-maze learning task [47]. Transfer latency (TL) that is the time taken by the animal to move from the open arm to enclosed arm, was considered as an index of learned task (memory process). The elevated plus maze consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) with an open roof. The maze was elevated to a height of 25 cm from the floor. The animal was placed individually at the end of either of the open arms and the initial transfer latency was noted on the first day. If the animal did not enter an enclosed arm within 90 s, it was gently pushed into the enclosed arm and the transfer latency was assigned as 90 s. To become acquainted with the maze, the animals were allowed to explore the plus maze for 20 s after reaching the closed arm and then returned to its home cage. Retention of the learned task was assessed 24 h after the 1st-day trial and expressed as a percentage of initial transfer latency.

Tail-suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method of Steru et al., [53]. Mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The total immobility was measured for a period of 6-min with the help of stop-watch. A mouse was considered immobile, when it hangs passively and completely motionless.

Assessment of Stress-induced hyperalgesia/ allodynia

Thermal hyperalgesia: Stress-induced hyperalgesia was assessed by tail immersion test. Each mouse was placed individually in restrainer leaving the tail hanging out freely. The terminal 1 cm part of the tail was immersed in a water bath maintained at 52.5±0.5^{0}C. The withdrawal latency was defined as the time for the animal to withdraw its tail from water. A cut-off time of 15 s was used to prevent damage to the tail [28].

2.4.6.2 Mechanical hyperalgesia: the nociceptive flexion reflex was quantified using the Randall Selitto paw pressure device (IITC, Woodland Hills, USA), which applies a linearly increasing mechanical force (in g) to the dorsum of the mouse's hind paw [2]**.**

2.4.6.3 Mechanical allodynia: mice were placed individually on an elevated mesh (1 cm^2 perforations) in a clear plastic cage and adapted to the testing environment for at least 15 min. von-Frey hairs (IITC, Woodland Hills, USA) with calibrated bending forces (in g) of different intensities were used to deliver punctuated mechanical stimuli of varying intensity. Starting with the lowest filament force, von-Frey hairs were applied from below the mesh floor to the plantar surface of the hind paw, with sufficient force to cause slight bending against the paw, and held for 1s. Each stimulation was applied 5 times with an inter-stimulus interval of 4-5s. Care was taken to stimulate random locations on the plantar surface. A positive response was noted if the paw was robustly and immediately withdrawn. Paw-withdrawal threshold was defined as the minimum pressure required to elicit a withdrawal reflex of the paw, at least one time on the five trials. Voluntary movement associated with locomotion was not considered as a withdrawal response. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to von-Frey hair application.

Biochemical Estimations

After behavioral assessment, mice were sacrificed under deep anaesthesia and brains were isolated and stored at 80°C for various biochemical estimations.

Measurement of Oxidative Stress

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills [65]. Tissue protein was estimated using the Biuret method. Reduced glutathione was assayed by the method of Jollow et al., [26]. Cytosolic superoxide dismutase activity was assayed by the method of Kono [30]. Catalase activity was assayed by the method of Claiborne [11].

Measurement of Nitrosative stress

Nitric oxide (nitrate- nitrite) by product in brain tissue was determined using the standard total nitric oxide assay kit (Assay Design, Inc. USA). Nitrate was reduced to nitrite by 3h incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Nitrite was converted to a deep purple azo compound by the addition of Griess reagent. Total nitrite/nitrate concentration was calculated using sodium nitrate as standard. Results were expressed as micromoles/mg protein.

Acetylcholinesterase activity

Cholinergic dysfunction was assessed by AChE activity. The assay mixture contained 0.01 ml of supernatant, 0.6 ml of 0.01 M sodium phosphate buffer (pH 8), 0.02 ml of acetylthiocholine iodide and 0.02 ml 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman reagent). The change in absorbance was measured at 412 nm for 5 min. Results were calculated using molar extinction coefficient of chromophore (1.36×104 M^{-1} cm^{-1}) and expressed as percentage of control group Ellman et al., [15].

Measurement of monoamine oxidase (MAO) enzyme Activity

The MAO activity was assessed spectrophotometrically method of Schurr & Livne [48]. The buffer-washed brain samples were homogenized in 10 volume of sodium phosphate buffer (0.1 M, pH 7.4) and centrifuged (Remi Instruments, Mumbai) at 15,000 g for 20 min. Pellets were discarded. Supernatant was pipetted out and used for the estimation of MAO-A and MAO-B activity. For estimating MAO-A activity, 2.75 ml Tris buffer (0.1 M, pH 7.4) and 100 µl of 4 mM 5-hydroxytryptamine were mixed in quartz cuvette which was then placed in double beam spectrophotometer (Perkin Elmer, USA). This was followed by the addition of 150 µl solution of brain homogenate to initiate the enzymatic reaction, and the change in absorbance was recorded at wavelength of 280 nm for 5 min against the blank. For estimating MAO-B activity, 2.75 ml Tris buffer (0.1 M, pH 7.4) and 100 µl of 0.1 M benzylamine were mixed in quartz cuvette which was then placed in double beam spectrophotometer. This was followed by the addition of 150 µl solution of brain homogenate to initiate the enzymatic reaction, and the change in absorbance was recorded at wavelength

of 249.5 nm for 5 min against the blank containing Tris buffer and 5-hydroxytryptamine. MAO activity was expressed as percent change in activity.

Assay of cytokines

Mouse TNF-α, IL-1β and TGF-β1 ELISA

The quantifications of TNF-α, IL-1β and TGF-β1 were done with the help of instructions provided by R&D Systems Quantikine mouse TNF-α, IL-1β and TGF-β1 immunoassay kit. The Quantikine mouse TNF-α, IL-1β and TGF-β1 immunoassay is a 4.5-h solid-phase ELISA designed to measure mousse TNF-α, IL-1β and TGF-β1 levels. The assay employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse TNF-α, IL-1β and TGF-β1 has been precoated in the microplate. Standards, control and samples were pipetted into the wells, and any mouse TNF-α, IL-1β or TGF-β1 present is bound by the immobilized antibody. After washing away any unbound substance, an enzyme-linked polyclonal antibody specific for mouse TNF-α, IL-1β and TGF-β1 is added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of mouse TNF-α, IL-1β and TGF-β1 bound in the initial steps. The sample values are then read off the standard curve. Values were expressed as mean±S.E.M.

Quantification of NF-kB p65 unit

The NF-kB/p65 ActivELISA kit (Imgenex, USA) was used to measure NF-kB-free p65 in the nuclear fraction of different brain regions. The nuclear levels of p65 may correlate positively with the activation of the NF-kB pathway. The NF-kB ActivELISA is a sandwich ELISA in which free p65 is captured by anti-p65 antibody-coated plates and the amount of bound p65 is detected by adding a second anti-p65 antibody followed by alkaline phosphatase conjugated secondary antibody using colorimetric detection in an ELISA plate reader at 405nm. The results were expressed as nanograms per milligram of protein.

Caspase-3 colorimetric assay

Caspase-3, also known as apopain, is an intracellular cysteine protease that exists as a proenzyme and becomes activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanaline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cytoplasmic fraction of different brain regions is directly proportional to the color reaction. The results were expressed as percentage of control.

Estimation of corticosterone

The quantifications of corticosterone levels were done with the help and instructions provided by R&D Systems kit.

Neurotransmitters estimation

Biogenic amines (dopamine, serotonin and norepinephrine) were estimated by HPLC with electrochemical detector. Waters standard system consisting of a high-pressure isocratic pump, a 20 ml sample injector valve, C18 reverse phase column and electrochemical detector were used. Data was recorded and analyzed with the help of empower software. Mobile phase consisting of sodium citrate buffer (pH 4.5)— acetonitrile (87:13, v/v). Sodium citrate buffer consist of 10 mM citric acid, 25 mM NaH_2HPO_4, 25 mM EDTA, and 2 mM of 1-heptane sulphonic acid [44]. Electrochemical conditions for the experiment were +0.75 V, sensitivity ranges from 5 to 50 nA. Separation was carried out at a flow rate of 0.8 ml/min. Samples (20 ml) were injected manually. On the day of experiment frozen brain samples were thawed and they were homogenized in homogenizing solution containing 0.2 M perchloric acid. After that samples were centrifuged at 12000 g for 5 min. The supernatant was further filtered through 0.22 mm nylon filters before injecting in the HPLC injection pump. Data was recorded and analyzed with the help of empower software.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was carried out by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparision test by using the software GraphPad Prism trial version 8.2 (GraphPad Software, Inc., La Jolla, CA, USA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of Effect of ellagic acid on Immobility Period in FST

Exposure to different stressors significantly increased the immobility period as compared to control mice. Chronic administration of ellagic acid (25, 50 and 100 mg/kg) significantly and dose-dependently reversed the increase in immobility period in stressed mice. The efficacy of ellagic acid (100 mg/kg) was comparable to that of duloxetine (20 mg/kg) (Figure 1).

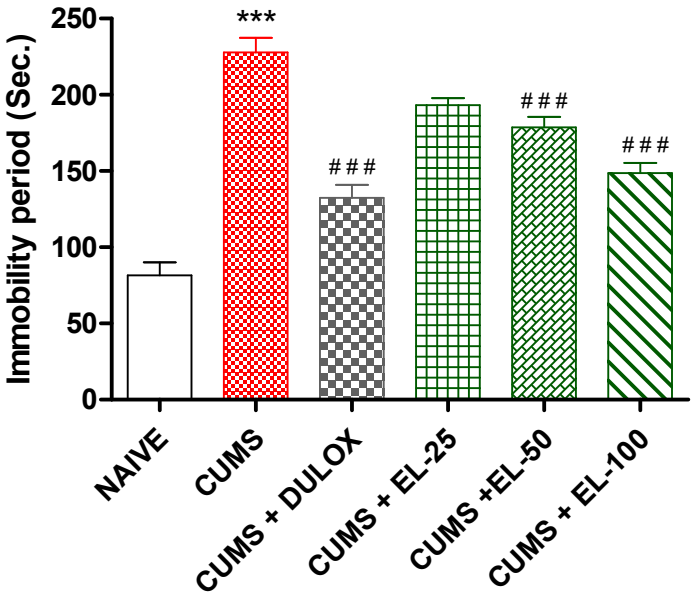


Figure 1 Effect of ellagic acid on immobility period in FST. Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

Effect of ellagic acid on locomotor activity

CUMS animal showed an increase in the locomotor activity compared to unstressed mice suggested that anxiety upsurge locomotion. However, chronic treatment with ellagic acid (25, 50 and 100 mg/kg) decreased the ambulatory scores as compare to CUMS group (Figure 2).

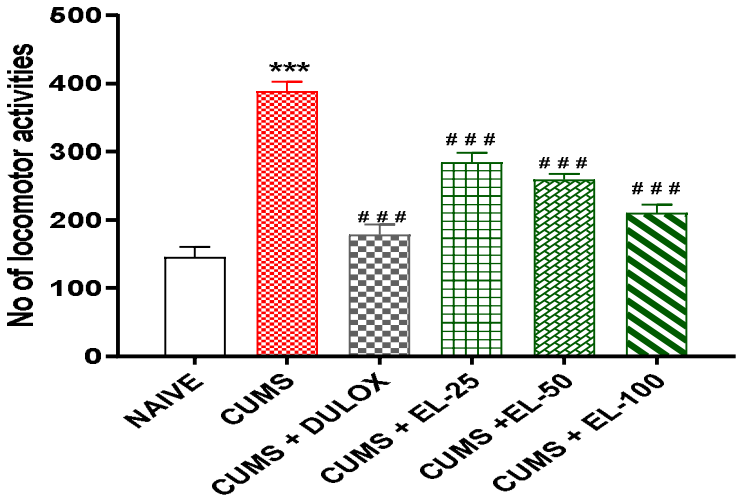


Figure 2 Effect of ellagic acid on locomotion. Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

Effect of ellagic acid on grip strength in Rota rod test

Chronically stressed mice showed a significant decrease in the fall-off time as compared to unstressed mice, thus displaying muscle in-coordination. Daily treatment with ellagic acid (25, 50 and 100 mg/kg) before the exposure increased the mean fall off time as compared to CUMS group (Figure 3).

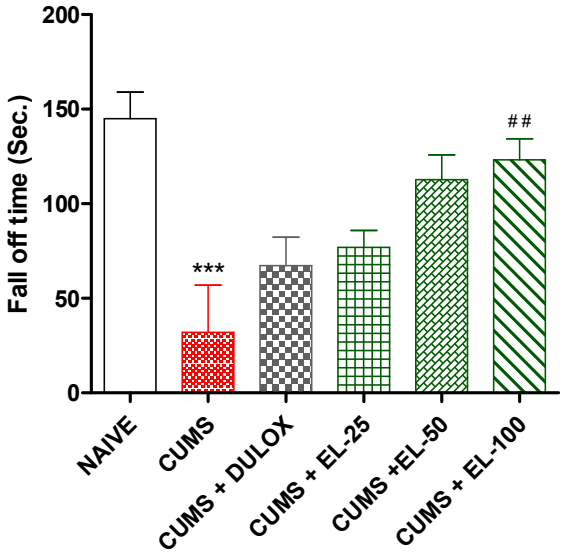


Figure 3 Effect of ellagic acid on muscle coordination. Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

Effect of ellagic acid on cognitive behavior using plus-maze test

Percent initial transfer latency (%ITL) was significantly increased in chronically stressed mice as compared to control mice. Treatment with ellagic acid (25, 50 and 100 mg/kg, p.o. for 21days) significantly and dose-dependently decreased percent initial transfer latency in mice. (Figure 4).

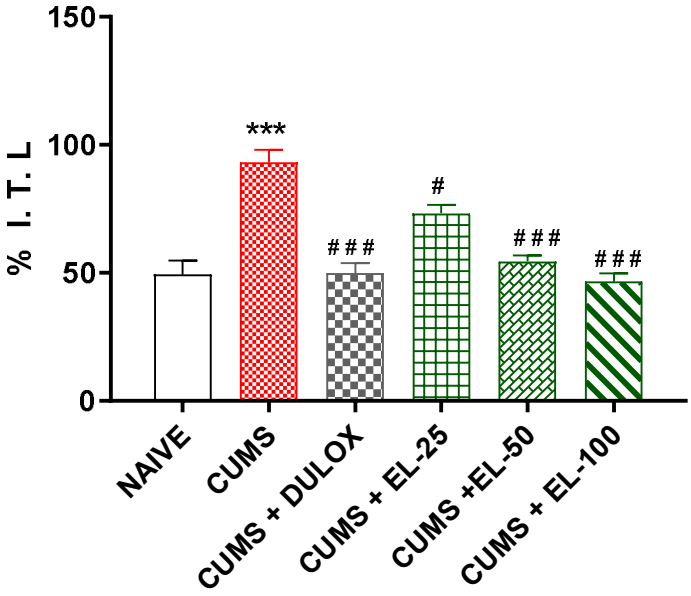


Figure 4. Effect of ellagic acid on cognitive behaviour. Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

Effect of ellagic acid on immobility period in the tail suspension test (TST)

The mice that were exposed to chronic unpredictable mild stress for 21 days showed a significant increase in the immobility period as compared to the unstressed mice. Treatment with ellagic acid refurbished these alterations by significantly reducing the immobility time in mice. (Figure 5).

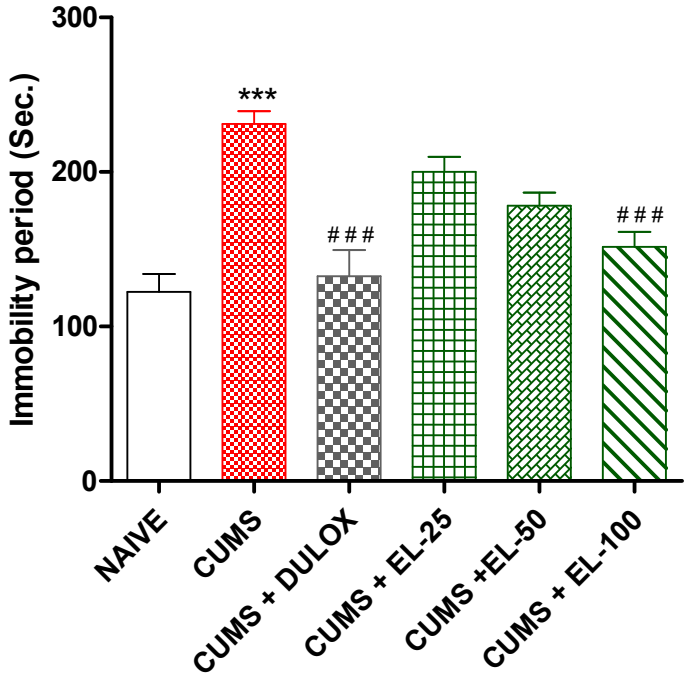


Figure 5. Effect of ellagic acid on immobility period in the tail suspension test (TST). Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

Effect of ellagic acid on stress-induced pain threshold

Thermal hyperalgesia

Animals chronically subjected to water immersion stress showed a significant decrease in tail withdrawal latency indicating hyperalgesia as compared to unstressed mice. Chronic treatment with ellagic acid (25, 50, 100 mg/kg, p.o. for 21 days) significantly attenuated the development of the hyperalgesia in chronically stressed animals (Figure 6A).

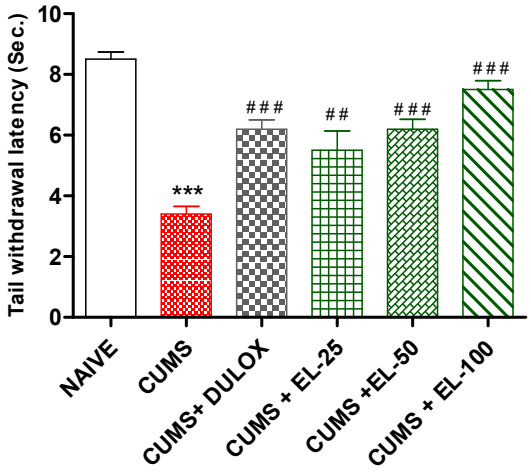
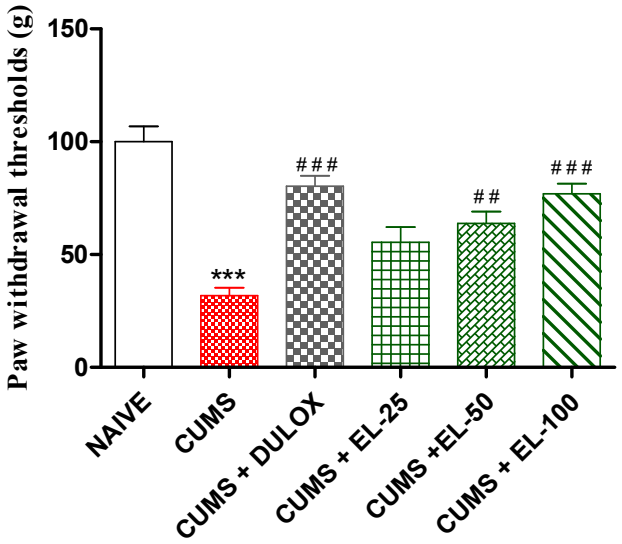


Figure 6A. Effect of ellagic acid on stress-induced pain threshold (Tail Immersion Test). Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

3.8.2 Mechanical hyperalgesia

CUMS produced a significant decrease in paw-withdrawal threshold in Randall Selitto paw pressure device as compared to unstressed group (Figure 6B). Ellagic acid (25, 50 and 100 mg/kg), significantly and dose-dependently increased the paw-withdrawal threshold in CUMS mice.



Mechanical allodynia

In von-Frey hair test, CUMS mice showed significant increase in pain sensitivity to non-noxious stimulus as compared to unstressed mice (Figure 6C). Ellagic acid (25, 50 and 100 mg/kg), produced significant and dose-dependent increase in paw-withdrawal threshold in response to von- Frey hair stimulation.

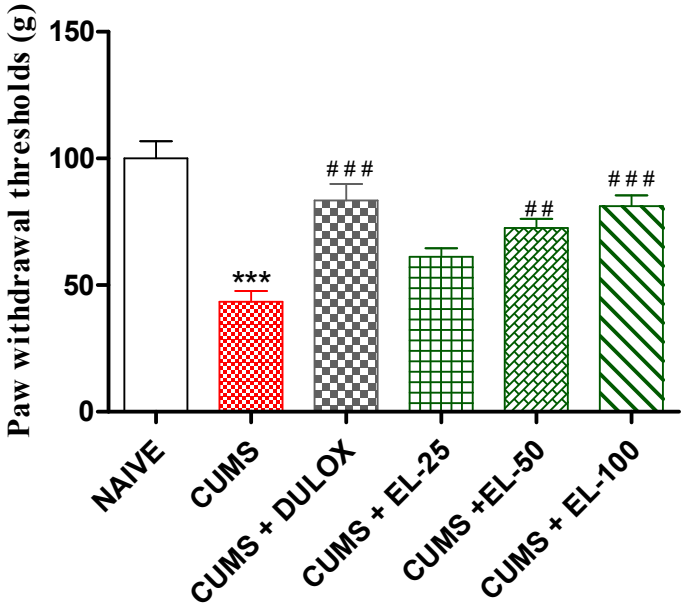


Figure.6C Effect of ellagic acid paw-withdrawal threshold (von- Frey hair stimulation). Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.)

Effect of ellagic acid treatment on CUMS-induced changes in biochemical alterations

Malondialdehyde (MDA) and nitrite levels were significantly increased in chronically stressed mice brain as compared to unstressed mice brain. Chronic treatment with ellagic acid produced significant ($p < 0.05$) and dose-dependent reduction in MDA and nitrite levels in chronic stressed mice brain. Reduced glutathione levels and enzyme activity of superoxide dismutase and catalase significantly decreased in the brains of chronic stressed mice as compared to unstressed group mice. This reduction was significantly and dose dependently augmented by the treatment with ellagic acid in the brain of CUMS group mice (Figure 7A-7E).

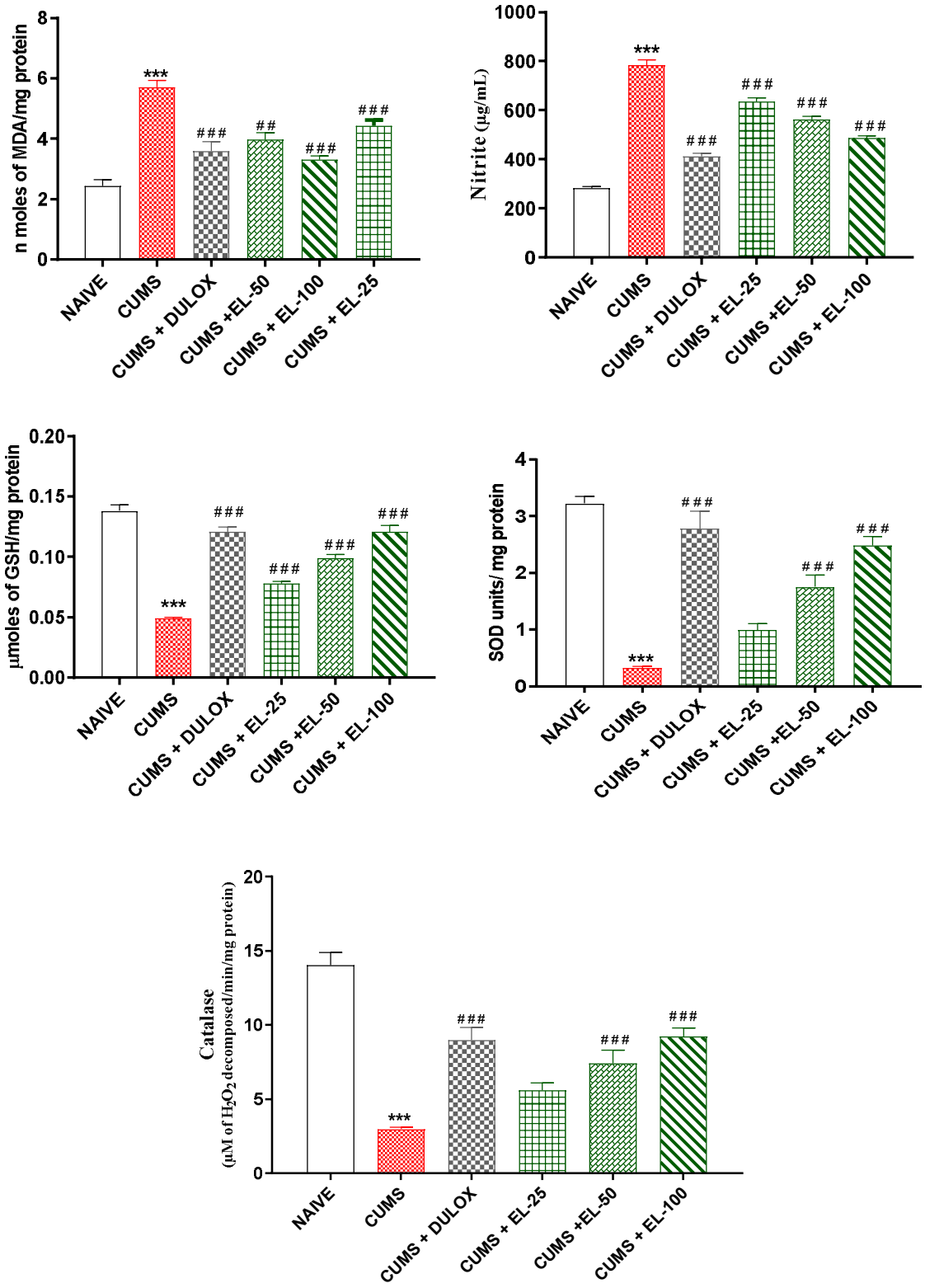


Figure 7A. Effect of ellagic acid on Malondialdehyde (MDA); 7B. Effect of ellagic acid on nitrite; 7C. Effect of ellagic acid on Glutathione (GSH); 7D. Effect of ellagic acid on Superoxide dismutase (SOD); 7E. Effect of ellagic acid on Catalase (CAT) (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

Effect of ellagic acid on monoamine oxidase (MAO) activity

Chronic stress procedure significant increase monoamine oxidase (MAO-A and MAO-B) enzymatic activity (Figure 8A and 8B). Chronic administration ellagic acid (25, 50 and 100 mg/kg) significantly reduced MAO-A and MAO-B enzymatic activity in fatigued mice as compared to control mice. Duloxetine (20 mg/kg) did not affect MAO activity in stressed mice.

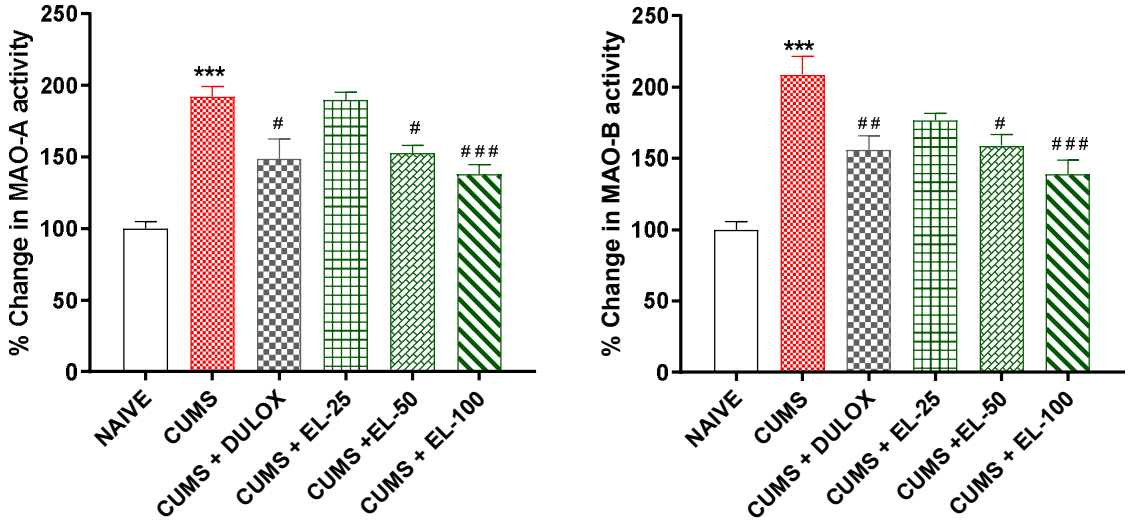
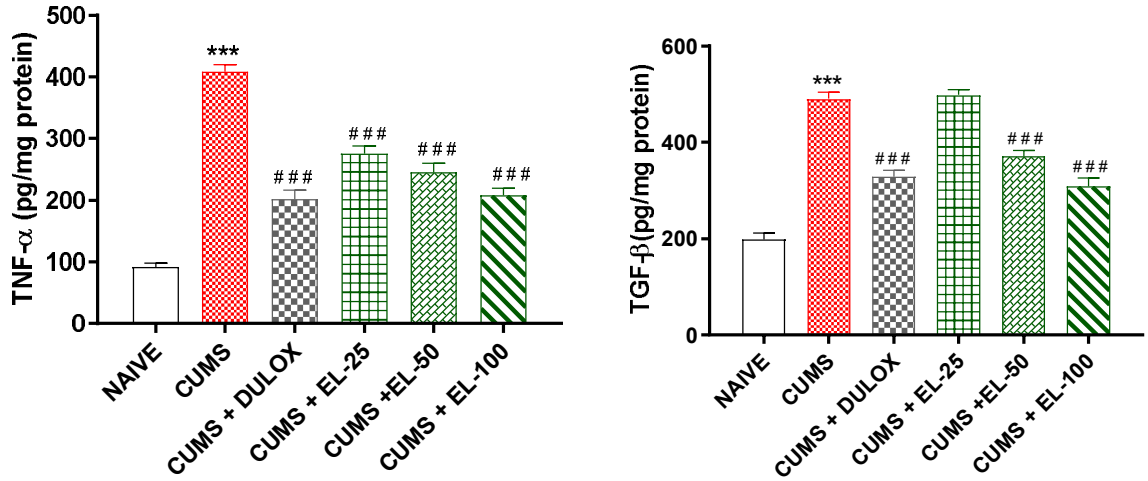


Figure 8A. Effect of ellagic acid on Monoamino oxidase-A (MAO-A); 8B: Effect of ellagic acid on Monoamino oxidase-B (MAO-B). (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

Effect of ellagic acid on TNF-α, TGF-β and IL-1β level

TNF-α, TGF-β and IL-1β levels were significantly increased in the brain of CUMS mice group suggesting involvement of neuroinflammation. Treatment with ellagic acid (25, 50 and 100 mg/kg) significantly and dose dependently inhibited these alterations (Figure 9A, 9B & 9C).



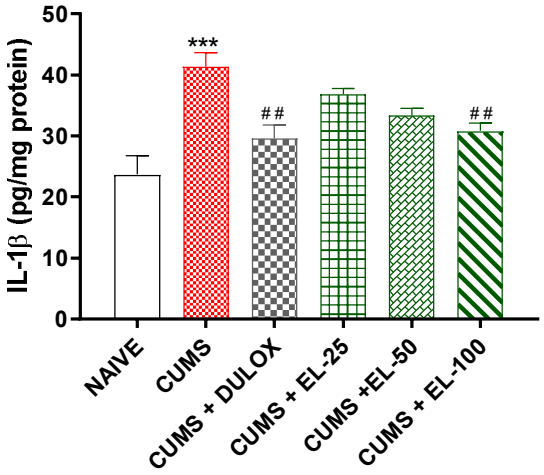


Figure 9A. Effect of ellagic acid on Tumor Necrosis Factor-α (TNF-α); 9B. Effect of ellagic acid on Transforming Growth Factor-β (TGF-β); 9C. Effect of ellagic acid on IL-1β (Interleukin-1β). (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

Effect of ellagic acid on serum corticosterone levels

Chronic stress significantly increased the serum corticosterone levels. Treatment with ellagic acid (25, 50 and 100 mg/kg) significant decrease in corticosterone levels compared to alone stress groups (Figure 10).

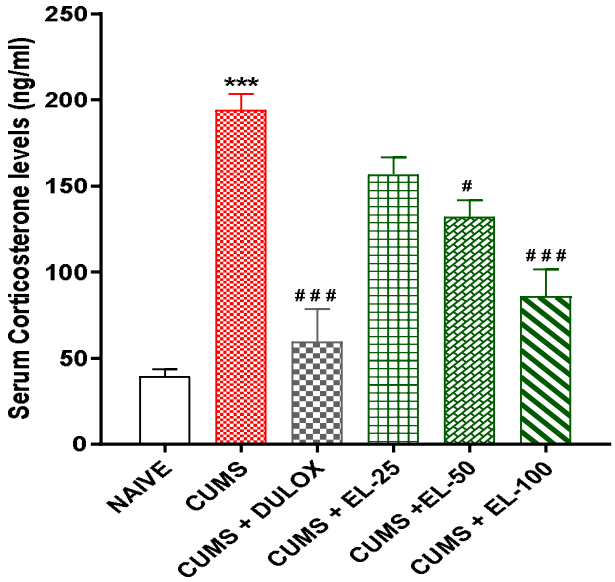


Figure 10. Effect of ellagic acid on serum corticosterone levels. (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

Effect of ellagic acid treatment on NFκβ and caspase-3 activity

NF-κβ p65 subunit and caspase-3 levels were significantly elevated in CUMS mice brain. ellagic acid (25, 50 and 100 mg/kg) treatment significantly inhibited enhanced NF-κβ p65 subunit expression in the nuclear fraction and caspase in dose-dependent manner (Figure 11A & 11B).

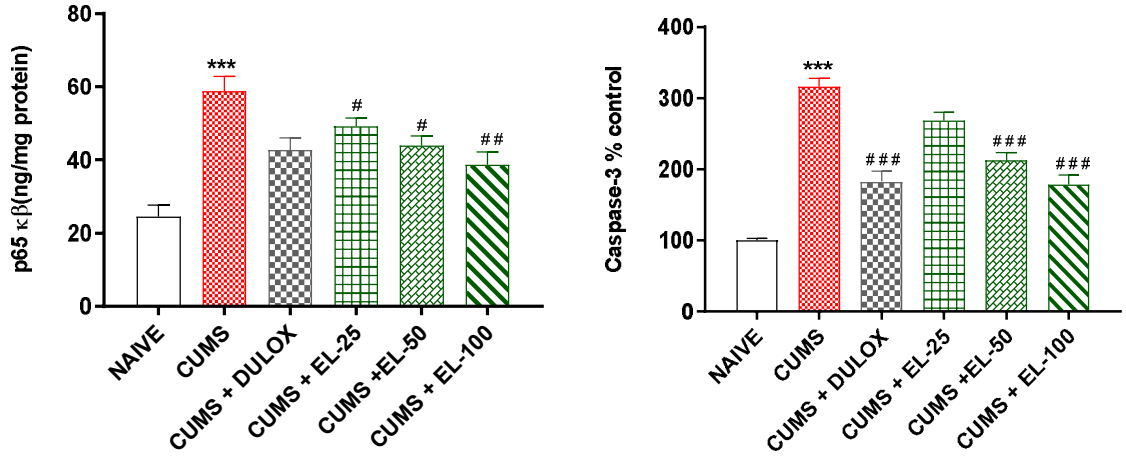


Figure 11A. Effect of ellagic acid on Nuclear Factor Kappa B (NF-κB); 11B. Effect of ellagic acid on Caspase-3 (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

Effect of ellagic acid on neurotransmitter levels

Chronic unpredictable mild stress resulted into decreased levels of dopamine (41%) (Figure 12A), norepinephrine (69%) (Figure 12B) and serotonin (61%) (Figure 12C) which was dose dependently replenished by ellagic acid (25, 50 and 100 mg/kg). ellagic acid 100 mg/kg produced a significant increase in the dopamine (56%), norepinephrine (121%) and serotonin (109%) in CUMS mice brain.

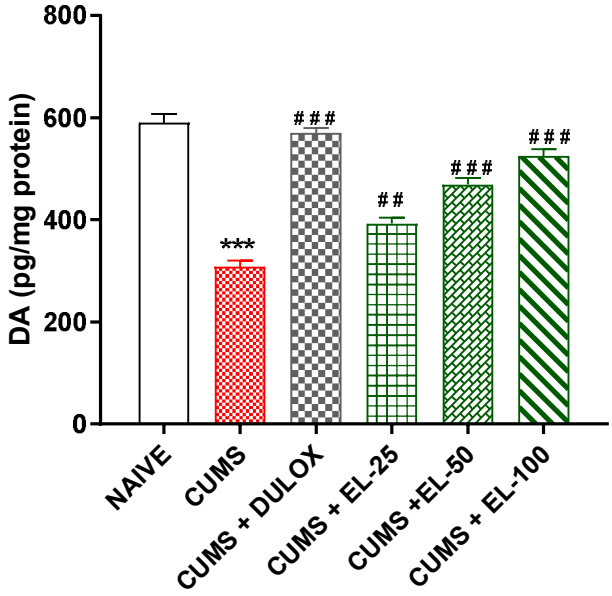


Figure 12A. Effect of ellagic acid on Dopamine level (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

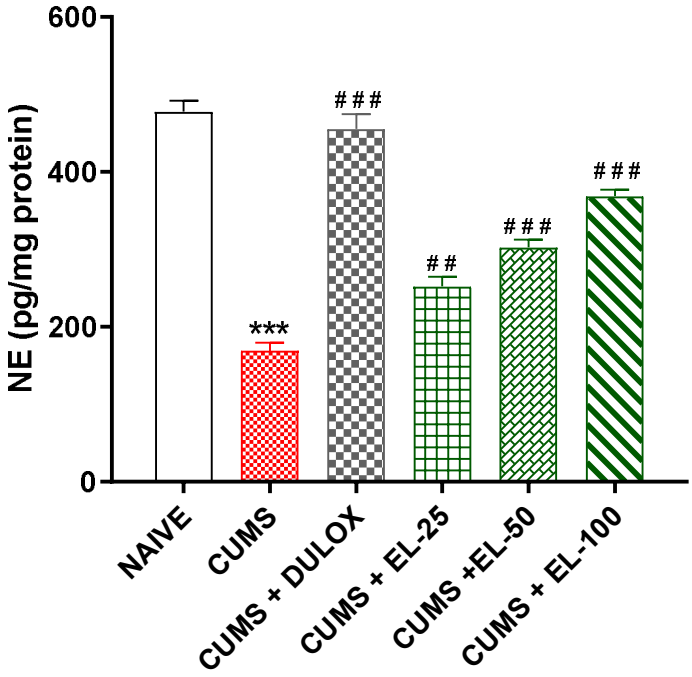


Figure 12B. Effect of ellagic acid on norepinephrine (NE) level (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p

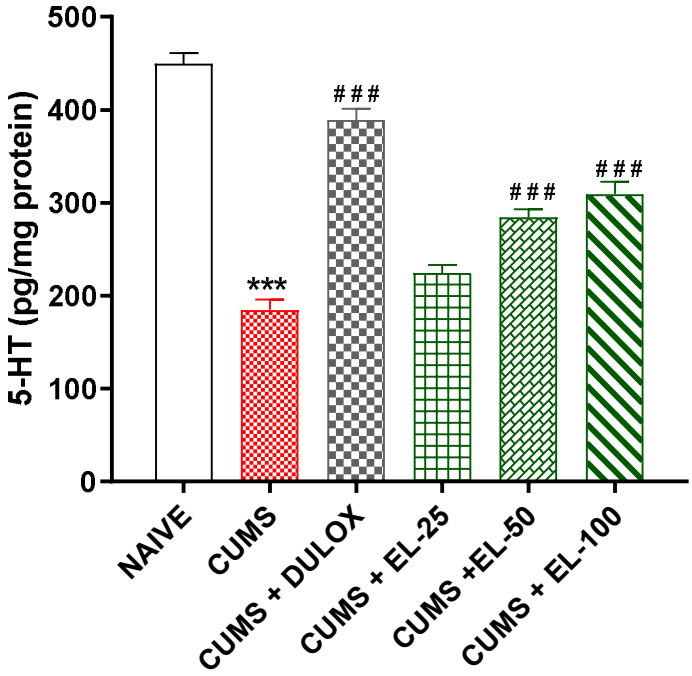


Figure 12C Effect of ellagic acid on 5-hydroxytryptamine (5-HT) level (Data are expressed as mean ± S.E.M. For statistical significance, ***p<0.05 as compared to **Control;** ### p<0.05 as compared to CUMS. **CUMS**: Chronic Unpredictable Mild Stress, **EL-25:** ellagic acid 25 mg/kg, p.o.; **EL-50:** ellagic acid 50 mg/kg, p.o.; **EL-100:** ellagic acid 50 mg/kg, p.o.; **DULOX(10):** duloxetine (20 mg/kg, i.p.).

DISCUSSION

CUMS, which is commonly employed in animals to simulate depression-like conditions, is considered to closely mimic the unpredictable stressors encountered in daily life [64, 71]. The induction of depressive-like behavior is significantly influenced by both the variability and unpredictability of the stress regimen. [51,42]. The underlying theory of this approach posits that depression results from an eventual incapacity to cope with a constant influx of dissimilar and unpleasant stimuli imposed by the environment. To replicate this effect in animals, stressors are employed to induce behavioral deficits that can later be ameliorated with antidepressant treatment [29].

Experimental investigations have revealed that CUMS activates two pathways that function to restore the disrupted processes in depressive disorders: the sympatho-adrenomedullary system and the hypothalamo-pituitary-adrenocortical (HPA) axis. Impairment of hippocampus-dependent functions is considered to be a hallmark of depressive disorders. The activation of the former results in a rapid increase in the release of adrenaline, which indirectly enhances the activity of noradrenergic neurons in the nucleus tractus solitarius and the locus coeruleus via vagal nerve activation. Consequently, the levels of noradrenaline (NA) in brain regions receiving projections from these nuclei are temporarily elevated, leading to functional alterations in neurons carrying NA receptors. The activation of the HPA system leads to a heightened release of corticosterone (the predominant hormone in most rodents, cortisol in humans) which readily penetrates the brain due to its lipophilic nature. The end products of these pathways, namely catecholamines and glucocorticoids, are frequently the mediators of stress-induced immunosuppression [55].

The current investigation demonstrated that mice subjected to stress exhibited symptoms resembling depression, characterized by an increased immobility period in the FST and reduced sucrose preference compared to the unstressed mice. These outcomes were in line with lower levels of 5-HT and DA in the central nervous system, rendering the animals more susceptible to displaying depressive symptoms under chronic stress [16, 36].

The current study observed a marked decrease in catecholamine levels (including serotonin, norepinephrine, and dopamine) in mice when subjected to Chronic Unpredictable Mild Stress. Additionally, CUMS significantly increased the activity of MAO-A in the brain [33]. The stressed animals also exhibited an increase in brain MAO-B activity, which may lead to the depletion of monoamine levels in the brain, including serotonin, dopamine, and norepinephrine. Catecholamines, which include serotonin, norepinephrine, and dopamine, play a role in regulating a wide range of behaviors and brain functions such as locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. Studies suggest that chronic unpredictable stress can impair nerve cells in the neural reward system, with damage linked to the serotonergic (5-HT) and dopaminergic (DA) systems. This damage can result in a loss of the capacity to experience happiness or pleasure [3, 27]. The current study found that ellagic acid was able to restore brain monoamine levels (including 5-HT, NE, and DA), resulting in improvements in immobility period, locomotor activity, and hedonic effects. The study discovered that CUMS led to a significant decrease in the depletion of dopamine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid, and also prevented the formation of 3-nitrotyrosine in the striatum in a dose-dependent manner. The researchers suggested that this neuroprotection could be linked to the anti-inflammatory responses triggered by statins, which include the reduction of the production of TNF-α, nitric oxide, and superoxide [49]. Duloxetine has been found to reverse hedonic deficits in animals subjected to unpredictable chronic mild stress [7]. The current study demonstrated that chronic administration of duloxetine significantly reversed the hedonic effects in mice that were exposed to chronic unpredictable mild stress.

Prior research indicates that the production of proinflammatory cytokines is elevated in humans in response to acute psychological stress [39, 52]. Studies conducted on animals have demonstrated that psychological stressors lead to an increase in cytokine levels, including IL-1β, IL-6, and TNF-α, both in the blood and various regions of the brain [43, 24] which may induce specific symptoms, labelled as the sickness behavior syndrome [46] such as anorexia, soporific effects, reduction of locomotor activity and exploration, anhedonia and cognitive disturbances, bear a strong similarity with those of depression [38, 68]. In the current study, exposure to chronic and varied unpredictable stressors resulted in an increase in inflammation and elevated TNF-α levels. However, administration of ellagic acid and duloxetine effectively reduced the levels of TNF-α in the stressed mice.

In this study, chronic stress exposure for 21 days was found to increase lipid peroxide and nitrite levels and decrease endogenous antioxidants, such as superoxide dismutase, catalase, and reduced glutathione. Previous studies have also reported oxidative modifications in the rat brain due to stressors. Moreover, intensive stress has been shown to alter the antioxidant defense system in rats. Stress-induced activation of the sympathetic-adrenal system may result in increased catecholamine metabolism, which can cause auto-oxidation and produce reactive oxygen species. This, in turn, can lead to oxidative stress, an imbalance

between oxidants and antioxidants, which can cause damage to various cell components, including lipid membranes. Lipid peroxidation has been regarded as an indirect measure of oxidative stress, which can lead to tissue injury. The production of free radicals during chronic stress might mediate cell damage and be responsible for neuronal disorders.

Exposure to chronic stress results in a decrease in antioxidant enzyme levels, both in the serum and hypothalamus. This reduction in the activity of superoxide dismutase and catalase enzymes may be attributed to their inactivation by interaction with oxygen radicals [70, 69]. Glutathione is a crucial endogenous antioxidant defense system that safeguards cells against singlet oxygen, hydroxyl radical and superoxide radical damage. GSH metabolism is tightly regulated and is involved in redox signaling, as well as providing protection against environmental oxidant-mediated injuries. The glutathione cycle involves oxidation of GSH to oxidized glutathione (GSSG), followed by regeneration of GSH from GSSG within cells catalyzed by the flavoenzyme glutathione reductase [41]. Therefore, a decreased level of GSH can lead to an imbalance in the redox status of cells, leading to oxidative stress. Changes in the ratio of the reduced and disulfide form (GSH/GSSG) can affect signaling pathways that participate in a broad array of physiological responses ranging from cell proliferation, autophagy, apoptosis, and gene expression.

The role of glutathione in cognitive function and synaptic plasticity processes as well as its involvement in neurotrophic and neurodegenerative events in rodents have been documented [12]. The tripeptide glutathione and its related enzymes participate in the maintenance of oxidant homeostasis in aerobic cells. Glutathione peroxidase is an extremely important antioxidant enzyme, with respect to cellular protection. Change in the concentration of glutathione peroxidase is one of the earliest signs of oxidative injury [37]. Increased free radical scavenging activity under stress condition might have caused the decrease in glutathione peroxidase levels in the hypothalamus. GSH has been shown to modulate glutamatergic activity and this may contribute to a dysfunction in glutamatergic pathways responsible for long-term potentiation required to encode memories [13]. Prediger et al. [45] had reported that GSH depletion causes disruption of spatial memory in the Morris water maze. In the present study, we also observed GSH depletion as well as memory impairment in mice induced by CUMS and restored by ellagic acid. Cognitive restoration by simvastatin may be related to its ability to block the production and activity of ROS thereby enhancing the activity of GSH [54]. Ellagic acid increases the levels of GSH and the high levels of GSH in neuronal cells might be able to cope with the toxicity of H_2O_2 and thus could be part of the mechanisms of neuroprotection. Ellagic acid has been demonstrated to play an important role as an antioxidant by up-regulating the gene expression of glutathione peroxidase (GSH-Px) [19] and may reverse the memory loss caused by CUMS by increasing GSH levels.

Increased level of hydroperoxides may arise from the reaction of superoxide with superoxide dismutase, the activity of several enzymes, or the oxidation of endogenous substances during stress [69,10]. Significant elevation of lipid hydroperoxide levels in the hypothalamus after stress confirms the previous researcher's observations, which are related to stress-induced lipid peroxidation [23]. Our study indicates that chronic stress might elevate the formation of $\cdot O_2^-$ and H_2O_2 and as a consequence, the main detoxifier of free radicals, the antioxidants (Cu, Zn, SOD, GPx) may work effectively to remove them. In the present study ellagic acid reverse the nitrodative stress in the hypothalamus caused by CUMS by restoring lipid peroxide, superoxide dismutase, catalase, GSH and nitrite levels.

CONCLUSION

Based on the aforementioned findings, it can be concluded ellagic acid ameliorate the neurobiological alterations in chronic unpredictable mild stress model by modulating neurotransmitter levels, nitrodative stress and inflammatory cytokine surge.

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CITATION OF THIS ARTICLE

Shilpi Sachdeva, Anand Kamal Sachdeva, Divya Yadav, Rakesh Yadav. Ellagic Acid Ameliorates Behavioral Impairment and Brain Oxidative Stress Induced by Chronic Unpredictable Mild Stress in Mice. Bull. Env.Pharmacol. Life Sci., Vol 12 [8] July2023: 185-203