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Evaluation of the anti-depressant potential of EGCG-loaded nanoparticles in unstressed and stressed mice

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Epigallocatechin-3-gallate (EGCG) is a key bio-active component of green tea and has demonstrated significant antidepressant activity in laboratory animals. Nano-particulate drug delivery offers great potential to overcome drawbacks associated with EGCG *i.e.* its low solubility and stability by transforming it into effective deliverable drugs. In the current study, nano-formulations of EGCG alone and with piperine were synthesized using antisolvent precipitation methodology followed by evaluation of their *in vivo* antidepressant effect in unstressed and stressed Swiss male albino mice. The mice were exposed to distinct stressors *i.e.* tail pinch, induction of immobilization, *etc*. throughout a span of three weeks. Zein, a protein nanocarrier, was nano-encapsulated with EGCG (25 mg) and EGCG + piperine (25 mg + 5 mg). For a continuous three weeks, the mice were administered EGCG-loaded nanosuspensions (25 mg kg^{-1}) and EGCG–piperine nanocomplexes (25 mg kg^{-1}). To determine the impact of various medication treatments on stressed and unstressed mice, the tail suspension test (TST) was employed as a behavioural paradigm. Mice exposed to various drug treatments were also evaluated for the effect on locomotor activity. The animals were euthanized followed by further estimation of plasma corticosterone, plasma nitrite, brain malondialdehyde, brain MAO-A, brain reduced glutathione, and brain catalase levels. The EGCG–piperine nanocomplex (25 mg kg^{-1}) and paroxetine HCl (10 mg kg^{-1}) *per se* significantly reduced immobility time in unstressed and stressed mice as compared to their respective control groups treated with a vehicle. However, in the case of locomotor activity, no significant effect was observed. EGCG loaded nanosuspension, EGCG–piperine nanocomplex and paroxetine HCl significantly decreased plasma nitrite, and brain MAO-A, brain malondialdehyde and brain catalase levels. However, these drug treatments significantly increased plasma corticosterone and brain reduced glutathione levels in unstressed and stressed mice as compared to their respective control groups treated with a vehicle. So, the intraperitoneal administration of nanoformulations synthesized using EGCG alone and along with piperine significantly improves the antidepressant-like behavior in mice.

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1. Introduction

Depression, a debilitating psychiatric illness, inflicts an extensive health hazard to humanity. According to WHO statistics, 3.8% of the population face depression, including 5% of the adult population (4% in males and 6% in females), and 5.7% of individuals aged 60 years and above. Approximately 280 million individuals globally experience depression.[1] Although depression causes disability both in males and females, females are more prone to depression than males. It is expected to become the single leading cause of sickness burden worldwide by 2030.[2] Symptomatically, it is characterized by anhedonia (loss of interest and pleasure), pervasive low mood, suicidal inclination, sleep disturbances, exhausted, feeling of worthlessness/guilt and diminished concentration.[3] The brain exhibits one of the most significant rates of oxygen consumption per unit mass in the body. Even slight disruptions in the balance of antioxidant defenses and oxidative stress can have harmful effects on neurons. Molecular pathways linked to oxidative stress may play a role in the development of depressive and anxiety disorders. A disparity between the production of reactive oxygen species (ROS) from internal

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or external sources and the body's ability to neutralize ROS through antioxidants has been identified in these types of disorders.[4] Carcinogenicity and erratic effectiveness are one of the assessed outcomes associated with antidepressant drugs as approved by the United States Food and Drug Administration (FDA).[5] Consequently, to develop a nontoxic and effective antidepressant drug, traditional herbs are becoming a new area of interest for researchers.

Epigallocatechin gallate (EGCG), a flavonoid compound in green tea, holds potential to protect the human body from numerous neuropsychiatric disorders such as anxiety,[6] depression[7] and other neurodegenerative disorders like Alzheimer's and Parkinson's disease.[8] Besides neuroprotection, EGCG also offers numerous health benefits like anticancer, antioxidant, anti-atherogenic, and anti-inflammatory effects *etc*.[9,10] Despite being efficient, features like poor bioavailability, degradation in the gastrointestinal tract, rapid metabolism, fast elimination and various bioconversions such as methylation, glucuronidation, and sulfation in the body restrict its therapeutic use.[11] There are two different approaches to improve its therapeutic potential *in vivo* by increasing its bioavailability which includes (a) the use of combinational therapy *i.e.*, the use of potential inhibitors along with the main drug (b) the use of nano-drug delivery systems. An increase in the bioavailability of EGCG was demonstrated by inhibiting the process of glucuronidation *via* co-administration of piperine with EGCG. Piperine, an alkaloid present in black pepper, acts by inhibiting the effect of modulations resulting in an increase in its bioavailability.[12] Piperine has also been used to enhance the bioavailability of other polyphenols (curcumin and resveratrol) by increasing their absorption.[13,14] Thus, a combination strategy is a very reliable approach that reduces the adverse side effects and increases the therapeutic potential of polyphenols. Another way to improve the bioavailability of polyphenols is the development of nano-drug delivery systems wherein herbal bioactive compounds are encapsulated into nanoparticles. This new approach offers benefits like target specific delivery, enhanced solubility, improved bioavailability, low dose, sustained release, reduced elimination, and metabolism of phytochemicals. The nano-drug delivery system not only increases the selectivity and efficacy of the phytonutrients but also protects them from thermal and photo-degradation by minimizing their side effects too.[15]

Keeping in view the above findings, the current study was intended to prepare nanoformulations of EGCG alone (EGCG loaded nanosuspension) and with piperine (EGCG–piperine nanocomplex) loaded into protein nanoparticles and evaluate their potential as an antidepressant agent on Swiss male albino mice. The effect of the synthesized nanoformulation(s) on depression was investigated by evaluating their effect on catecholaminergic and serotonergic/5-hydroxytryptamine (5-HT) systems, monoamine oxidase (MAO) system(s) enzymes such as glutathione oxidase (GSH) and catalase and lipid peroxidation by employing behavioral model (tail suspension test, TST), biochemical, and neurochemical approaches. This is the first report of the evaluation of the anti-depressant potential of a nanoformulation prepared using EGCG in unstressed and stressed mice.

2. Materials and methods

2.1. Drugs and chemicals

Paroxetine HCl hemihydrate was received as a gift sample from Jubilant Generics Limited, Noida (U.P.). Epigallocatechin gallate was procured from MP Biomedicals, India. Zein and tween-80 were procured from Sigma Aldrich, St Louis, USA and S D-fine-chem, Mumbai, respectively. Lecithin, piperine, disodium hydrogen phosphate, sodium dihydrogen phosphate, Tris, EDTA disodium (AR), sucrose, sulfanilamide, 5-hydroxytryptamine creatinine sulphate, *N*-(1-naphthyl) ethylenediamine dihydrochloride, and *meta*-phosphoric acid were obtained from HiMedia Lab. Pvt. Ltd, Mumbai, India. Total protein was estimated using a kit from Erba Chem, Mumbai, India.

2.2. Synthesis of the EGCG loaded nanosuspension and EGCG–piperine nanocomplex

Zein was used as a nano carrier in the anti-solvent precipitation process to prepare EGCG-loaded nanoparticles.[16,17] Zein-polymer and lecithin, which serve as stabilizers, were included in the organic phase and dispersed in 70% (v/v) ethanol before being filtered to remove contaminants. The antisolvent phase is made up of Tween-80 dissolved in ultrapure water. Blank nanoparticles are produced when the organic phase is added to the anti-solvent phase while being vigorously stirred at 1200 rpm (Fig. 1).

The same procedure utilized to prepare blank nanoparticles was also used to prepare drug-loaded nanoparticles. When EGCG (25 mg), EGCG + piperine (in 5 : 1 ratio) were combined in the organic phase, the formation of the EGCG-loaded nanosuspension and EGCG–piperine nanocomplex occurred, respectively. In order to increase the stability of the synthesized nanoformulations, the lyophilized nanosuspensions were cryoprotected with 1% (w/v) mannitol in a lab model freeze drier (Freezone 6-Plus Labconco, USA). Software with full-factorial (three levels three-factor 3^3) design expertise was used to optimise the nano-formulations. Particle size (PS), polydispersity index (PDI), and zeta potential (ZP) were among the physiochemical characterization methods used to evaluate the optimized nano-formulations.

2.3. *In vivo* study of the anti-depressant activity of the synthesised nanosuspensions

2.3.1. Experimental animals. Three month old Swiss male albino mice weighing between 25 and 30 g were purchased from Disease Free Small Animal House, LUVAS, Hisar, Haryana, India. Since female mice's estrogen hormone is thought to have antidepressant properties, female mice were not included in the study.[17] The animals were kept in regular environmental settings with an alternate 12-hour light/dark

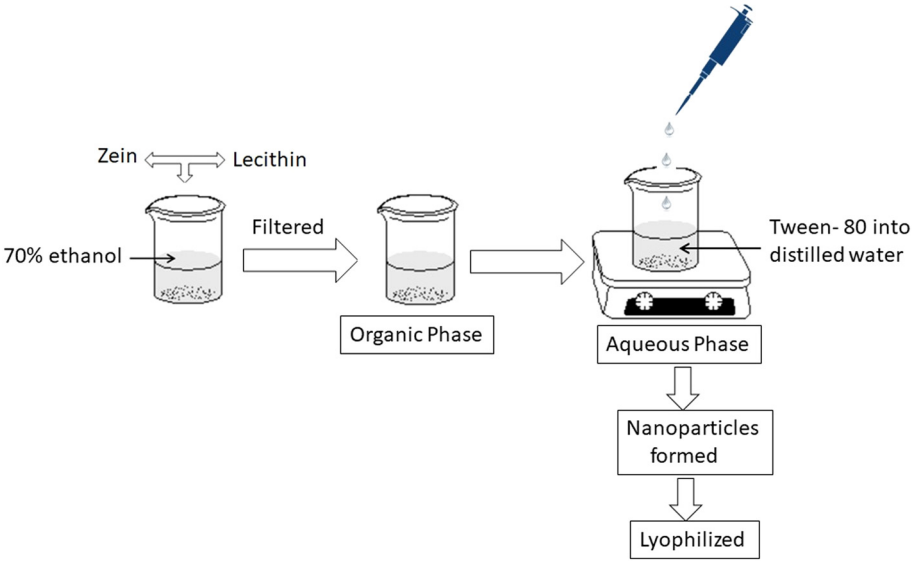

Fig. 1 Synthesis of zein encapsulated nanoparticles using the anti-solvent precipitation methodology.[17]

cycle, and 10 mice were housed in a polycarbonate cage (29 cm × 22 cm × 14 cm in size). During the whole trial, the animals had unrestricted access to food and water. Animals were fasted for two hours before and two hours post drug delivery to prevent food–drug interactions and improve drug absorption. The animals had a seven-day acclimatization period prior to experimental tests. To reduce circadian impacts, animal studies were conducted between 9:00 and 17:00 h. The Institutional Animals Ethics Committee (IAEC) approved the experimental protocol at its 27th meeting on December 17, 2014 (Endst. No. IAEC/2014/212-220, dated December 17, 2014). The treatment of animals was carried out in accordance with the regulations formed by the committee established by the Ministry of Environment and Forests, Government of India, New Delhi, for the purpose of regulating and supervising animal experimentation (Registration number. 0436/PO/Re/S/2001).

2.3.2. Vehicles. Paroxetine HCl and piperine were mixed in isotonic saline solution (0.9% NaCl). EGCG, blank nanosuspension, EGCG loaded-nanosuspension and EGCG–piperine nanocomplex were solubilized in deionized water. All solutions were prepared fresh every time before administration.

2.3.3. Chronic unpredictable mild stress (CUMS) procedure. The mice were exposed to distinct stressors throughout a span of three weeks as per the sequence reported earlier[18] and as shown in Table 1.

2.3.4. Dose selection and treatment schedule. The dosages of piperine (5 mg kg^{-1}), paroxetine HCl (10 mg kg^{-1}), and EGCG (25 mg kg^{-1}) were chosen based on the literature.[17,19,20] The dosage of the EGCG–piperine nanocomplex, EGCG-loaded nanosuspension, and blank nanosuspension was the same as that of EGCG in its purest form. Intraperitoneal (IP) administration of the medications was done every day for three consecutive weeks.

2.3.5. Experimental procedure. The animals were categorized into the following 14 groups (n = 10 each group):

Groups 1–7: Three weeks of intraperitoneal administration of the following substances: vehicle, paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (containing 25 mg kg^{-1} EGCG), EGCG–piperine nanocomplex (containing 25 mg kg^{-1} EGCG), blank nanosuspension (containing 25 mg kg^{-1} void drug particles) and piperine (5 mg kg^{-1}), respectively. The locomotor activity scores of mice were documented on the 21st day, 60 minutes after the vehicle or medication was administered. The immobility period in mice was then evaluated using the TST on the 22nd day after that.

Groups 8–14: For three weeks in sequence, the following medications were intraperitoneally administered: vehicle, paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (containing 25 mg kg^{-1} EGCG), EGCG–piperine nanocomplex (containing 25 mg kg^{-1} EGCG), blank nanosuspension (containing 25 mg kg^{-1}) and piperine (5 mg kg^{-1}). On the 21st day, scores of the locomotor activity of mice were taken post 60 minutes of inducing stress. Mice were used to perform a TST on day 22.

2.4. Models employed for behavioral study

2.4.1. Tail suspension test (TST). Effects of various drugs on antidepressant behavior were evaluated in mice by performing the tail suspension test using a maze model as previously outlined[21,22] By using adhesive tape, each mouse was hung up at a height of 50 cm from the floor and tape was placed almost 1 cm away from tip of the tail. The assessment was conducted over a span of 6 minutes, during which the duration of immobility was measured. During the test, each animal was isolated both acoustically and visually from other animals. The mouse was considered to be in a state of immobility when no body movement was shown by it, hung passively and completely

Table 1 Order of stressors used for chronic unpredictable mild stress

Weeks	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1st week	I	E	F	O	T2	X	T1
2nd week	I	O	X	T2	E	T1	F
3rd week	O	F	T1	X	T2	I	E

I—induction of immobilization for a duration of 2 hours; E—exposure to empty water bottles for a period of 1 hour; F—subjected to foreign object exposure for 24 hours (such as a piece of plastic); O—continual illumination throughout the night; T2—application of tail pinch stimulus for 60 seconds; X—placement of cage at a 45-degree tilt for 7 hours; T1—application of tail pinch stimulus for 30 seconds.

still. To avoid any disturbances to animals, a test was performed in a silent room. The observer was blind to all the treatments.

2.4.2. Locomotor activity evaluation. The effect of different drug treatments on the locomotor activity was determined using a photo-actometer (INCO, Ambala, India).[23] The scores denoting the horizontal locomotor activity of mice in the control and test groups were recorded over a 5 minute period.

2.5. Biochemical estimation

On days 22 and 23, drug administration was also resumed. Animals were tested on the 22nd day. Mice were used in biochemical tests on the 23rd day, an hour after the medication was given. Blood samples (0.5–0.8 ml) from the retro-orbital plexus of mice were obtained, and then they were centrifuged for 10 minutes at 4 °C and 2500 rpm using a chilled centrifuge (C-30 Plus model, REMI, Mumbai, India) to separate the plasma. The corticosterone and nitrite levels were measured using separate plasma samples.

After the collection of blood samples, the animals were euthanized using cervical dislocation and their brains were isolated. Cold buffer containing 0.25 M sucrose, 0.1 M Tris, and 0.02 M EDTA (pH 7.4) was used to wash the isolated brain samples before weighing them. The brain sample was homogenised in cold buffer containing 0.25 M sucrose, 0.1 M Tris, and 0.02 M EDTA (pH 7.4), and then centrifuged twice using a refrigerator centrifuge at 2500 rpm for 10 min at 4°. The resulting pellet was removed, and the supernatant was centrifuged once more for 20 minutes at 12 000 rpm and 4 °C. The resulting supernatant was split into two portions:

Part I: The precipitates *i.e.*, the mitochondrial fraction was utilized to estimate the level of MAO-A.

Part II: The residual supernatant was used for evaluating the reduced glutathione, catalase, and lipid peroxidase activity.

2.5.1. Evaluation of the plasma nitrite level. Using the Green *et al.* approach,[24] the blood's plasma nitrite level was assessed. Sulfanilamide, 0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride, and 5% aqueous solution of *m*-phosphoric acid were combined and incubated for 60 minutes. Equal parts of the aforesaid combination and plasma were combined, and then the mixture was left at RT in the dark for 10 minutes. By using a UV-visible spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany), the absorbance was measured at 546 nm.

2.5.2. Evaluation of the plasma corticosterone level. The Bartos and Pesez, 1979 method[25] was used to determine the level of corticosterone in plasma. All the sample-containing tubes were placed in ice water for five minutes after the introduction of 1.0 ml of the sample in ethanol and 0.50 ml of a *p*-nitroso-*N*,*N*-dimethyl aniline solution in ethanol at a concentration of 0.10%. The mixture also included a 0.10 N; 0.50 ml solution of sodium hydroxide. All the tubes were sealed with cotton wool and stored at 0 °C for five hours while being shielded from light. The solution was mixed with 2.0 ml of buffer pH 9.8, 5.0 ml of phenol in ethanol solution at 0.10%, and 0.50 ml of potassium ferricyanide aqueous solution at 1.0%. The tubes were then submerged for 10 minutes in a water bath (20 ± 2 °C). The absorbance was measured at 650 nm using UV–visible spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany).

2.5.3. Estimation of brain MAO-A activity. Spectrophotometric analysis was performed to check the activity of MAO-A enzyme.[26,27] The brain mitochondrial fraction was suspended in a solution of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose at a ratio of 9 : 1) and mixed well for 20 min at 4 °C after being rinsed two times with sucrose-Tris-EDTA buffer (100 ml). After centrifugation, (15 000 rpm, 30 minutes, 0 °C), the obtained pellets were re-dissolved in ice-cold sodium phosphate buffer. A solution of 5-hydroxytryptamine (100 ml, 4 mM) and sodium phosphate buffer (2.75 ml, 100 mM, pH 7.4) was taken in a quartz cuvette and stored in a UV-visible spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany). 150 cc of mitochondrial fraction were added to begin the enzymatic process, and for 5 minutes, the alteration in absorbance at 280 nm wavelength was recorded. The 5-HT solution and sodium phosphate buffer were used as references.

2.5.4. Measurement of brain protein concentration. The complete protein concentration within the brain homogenate was assessed using a semi-autoanalyzer (CHEM-5 Plus, Erba, Mumbai, India), and then a total protein kit (Erba Chem, Mumbai, India).

2.5.5. Measurement of the brain lipid peroxidation level. Malondialdehyde concentration, an indicator of lipid peroxidation was examined using compounds reacting with thiobarbituric acid.[28] In a nutshell, 0.5 ml of post-mitochondrial supernatant and 0.5 ml of Tris-HCl were incubated at 37 °C for 2 h. 10% trichloroacetic acid (1 ml) was added after incubation, and then the sample was centrifuged for 10 min at

1000 rpm. After that, the test tubes were placed in boiling water for 10 minutes while combining supernatant (1 ml) and thiobarbituric acid (1 ml, 0.67% w/w). Redistilled water (1 ml) was added after chilling, and absorbance was recorded at 532 nm. By using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}, thiobarbituric acid-reactive compounds were computed and quantified in nanomoles of malondialdehyde per milligram of protein. The biuret method was used to assess tissue protein, and the amount of malondialdehyde in the brain was represented as nanomoles per milligram of protein.

2.5.6. Measurement of brain reduced glutathione. Brain reduced glutathione (GSH) was assayed be the method of Jollow *et al*. (1974).[29] In a nutshell, 10% post-mitochondrial supernatant (1 ml) and 4% sulfosalicylic acid (1 ml), respectively, were precipitated. The samples were stored at 4 °C for at least an hour before being centrifuged for 15 minutes at 1200 rpm. The test mixture has a final volume of 3.0 ml and is composed of the supernatant (0.1 ml), phosphate buffer (2.7 ml, 0.1 M, pH 7.4), and 5,5′-dithiobis-(2-nitrobenzoic acid) (0.2 ml, 0.1 mM, pH 8.0). The level of GSH was expressed using a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} and expressed as micromole per milligram protein as soon as the yellow color started to emerge at 412 nm.

2.5.7. Measurement of brain catalase activity. Catalase activity was assayed by the method of Claiborne (1985).[30] Phosphate buffer (1.95 ml, 0.05 M, pH 7.0), hydrogen peroxide (1.0 ml, 0.019 M), and 0.05 ml of post-mitochondrial supernatant (10%) were all included in the test mixture, which had a total volume of 3.0 ml. Absorbance changes were observed at 240 nm. Using the millimolar extinction value of H_2O_2 (0.07 mM), catalase activity was estimated and represented as micromoles of H_2O_2 decomposed per minute per milligram protein.

2.5.8. Statistical evaluation. One-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparison test were used to analyse the data using GraphPad Instat. All findings were presented as mean ± SEM. At a confidence level of 0.05, the statistical significance of the available experimental data was assessed.

3. Results

3.1. Synthesis and optimization of the EGCG loaded nanosuspension and EGCG–piperine nanocomplex

The anti-solvent precipitation method was employed to prepare EGCG-loaded nanosuspensions and EGCG–piperine nanocomplexes utilising protein nanocarriers. Full factorial design expert software (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN) was used for optimization.[31–33] The EGCG-loaded nanosuspension and EGCG–piperine nanocomplex were discovered to have particle sizes of 118.3 nm, 0.125, and −36.4 mV and 184.2 nm, 0.231, and −38.3 mV, respectively. In the EGCG–piperine nanocomplex, the increase in particle size was brought about by the encapsulation of two drug moieties into the polymeric matrix.

3.2. Behavioral models utilized for the assessment of the antidepressant effects of various drug treatments

3.2.1. Effect of immobility durations of mice in the tail suspension test (TST). In comparison with vehicle-treated, unstressed mice, chronic stress for three consecutive weeks significantly ($p < 0.001$) reduced the immobility duration of mice (Fig. 2). Piperine (5 mg kg^{-1}), blank nanosuspension (25 mg kg^{-1}), and EGCG (25 mg kg^{-1}) had no impact on the length of time that the mice remained immobile. But the administration of paroxetine HCl (10 mg kg^{-1}), EGCG loaded nanosuspension (comprising 25 mg kg^{-1} of EGCG) and EGCG–piperine nanocomplex (comprising 25 mg kg^{-1} of EGCG) *per se* over a span of three consecutive weeks significantly reduced immobility time of unstressed mice ($p < 0.001$, $p < 0.01$ and $p < 0.001$ respectively) and stressed mice ($p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively) compared to their corresponding vehicle-treated control groups. The outcome of the EGCG–piperine nanocomplex (25 mg kg^{-1}, IP) on the reduction of immobility period was better ($p < 0.001$) than EGCG loaded nanosuspension (25 mg kg^{-1}) in unstressed mice but both displayed a similar effect in the case of stressed mice. This suggests that piperine enhanced the efficacy of EGCG-loaded nanosuspension specifically in unstressed mice.

3.2.2. Effect on locomotor activity. As compared to the corresponding vehicle-treated controls, exposure of mice to CUMS did not significantly affect their locomotor activity. When compared to their corresponding vehicle-treated control

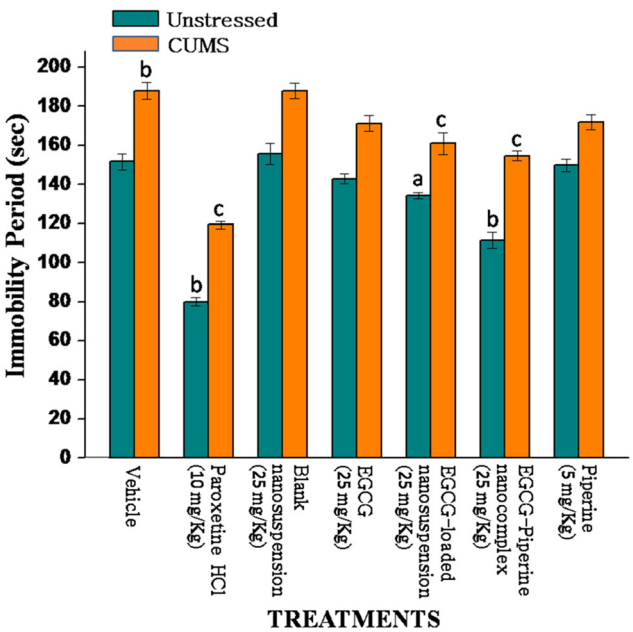

Fig. 2 Effect of various drug treatments on immobility periods of mice in TST. n = 10 in each group. Values are expressed as mean ± SEM. n = 10 in each group. Data were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparison test. F (13, 126) = 66.023; $p < 0.0001$. $^{a,b}p < 0.01$ and $p < 0.001$, respectively, as compared to vehicle-treated unstressed mice. $^{c}p < 0.001$ as compared to vehicle-treated stressed mice.

groups, various drug treatments, including EGCG, paroxetine HCl, blank nanosuspension, EGCG loaded nanosuspension, EGCG–piperine nanocomplex, and piperine had no noticeable ($P > 0.05$) impact on the locomotor activity scores observed in unstressed and stressed mice (Fig. 3).

3.2.3. Effect on plasma corticosterone levels. The unstressed mice did not show a significant effect on plasma corticosterone levels by paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG–piperine nanocomplex (25 mg kg^{-1} EGCG), or EGCG-loaded nanosuspension. Compared to stressed mice treated with a vehicle, animals subjected to persistent unpredictable mild stress had significantly higher plasma corticosterone levels ($p < 0.001$). Paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1} EGCG) and EGCG–piperine nanocomplex (25 mg kg^{-1} EGCG) *per se* administered for 3 subsequent weeks significantly ($p < 0.01$, $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively) lowered plasma corticosterone content of stressed mice in comparison with their corresponding vehicle-treated controls. Both piperine (5 mg kg^{-1}) and blank nanosuspension (25 mg kg^{-1}) had no noticeable impact on the plasma corticosterone levels of either stressed or unstressed mice. However, in the case of the EGCG–piperine nanocomplex (25 mg kg^{-1}), which contains piperine as a bioenhancer, the outcome of the EGCG loaded nanosuspension was potentiated, and the level of plasma corticosterone in stressed mice was significantly reduced ($p < 0.001$) (Fig. 4).

3.2.4. Effect on plasma nitrite levels. Animals exposed to CUMS had considerably ($p < 0.001$) higher plasma nitrite levels. Different drug treatments, such as EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (containing 25 mg kg^{-1} EGCG), blank nanosuspension (25 mg kg^{-1}) and piperine (5 mg kg^{-1}) were given to unstressed mice over the course of three weeks without showing any discernible effects on their plasma nitrite levels ($p < 0.05$). However, in comparison with the control group treated with the vehicle. Paroxetine HCl (10 mg kg^{-1}) and EGCG–piperine nanocomplex (comprising 25 mg kg^{-1} EGCG) both significantly ($p < 0.01$ and $p < 0.01$, respectively) reduced the plasma nitrite level. Paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1}) and EGCG–piperine nanocomplex (25 mg kg^{-1}) administered for 3 consecutive weeks resulted in a significant ($p < 0.001$) decrease in the plasma nitrite level in stressed mice in comparison with the control group treated with the vehicle. Piperine and blank nanosuspension had no discernible impact on the plasma nitrite level in stressed mice when compared to their corresponding vehicle-treated controls (Fig. 5).

3.2.5. Effect on brain MAO-A levels. On comparing mice treated with the vehicle to unstressed mice, it was found that CUMS significantly ($p < 0.001$) enhanced brain MAO-A activity in the mice. Paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG–piperine nanocomplex (25 mg kg^{-1}) and piperine (5 mg kg^{-1}) *per se* administered for 3 consecutive weeks significantly ($p < 0.05$, $p < 0.05$, $p < 0.001$ and $p < 0.05$, respectively) decreased MAO-A activity of unstressed mice in comparison with the control group treated with the vehicle. However, in stressed mice, paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1}), and EGCG–piperine nanocomplex (25 mg kg^{-1}) significantly reduced

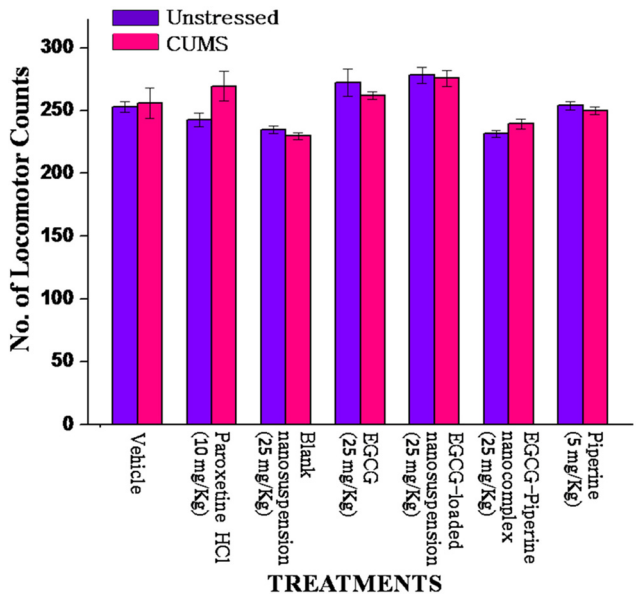

Fig. 3 Effect of various drug treatments on locomotor activity. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey–Kramer multiple comparison tests. CUMS – chronic unpredictable mild stress. F (13, 126) = 6.194. $p > 0.05$.

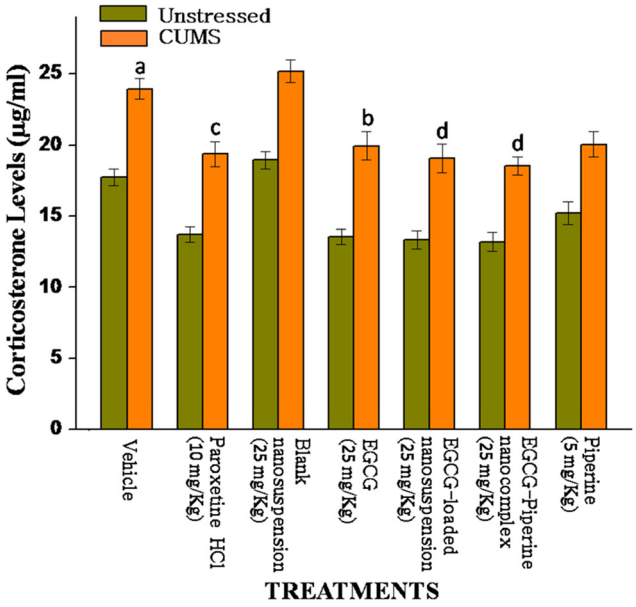

Fig. 4 Effect of various drug treatments on the plasma corticosterone levels. $n = 10$ in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparison test. CUMS – chronic unpredictable mild stress. F (13, 126) = 16.480; $p < 0.0001$. $^{a}p < 0.001$ as compared to vehicle-treated unstressed mice. $^{b,c,d}p < 0.05$; $p < 0.01$ and $p < 0.001$ as compared to vehicle-treated stressed mice.

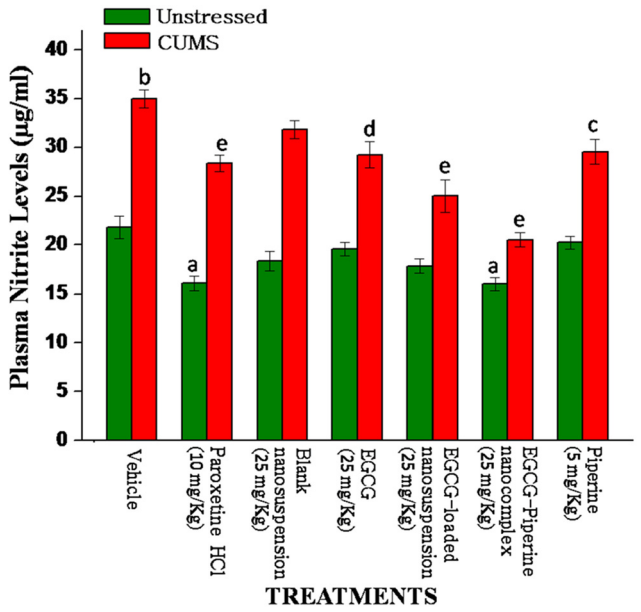

Fig. 5 Effect of various drug treatments on the plasma nitrite levels. n = 10 in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey–Kramer's *post hoc* test. CUMS – chronic unpredictable mild stress. F (13, 126) = 38.832; p < 0.0001. $^{a,b}p$ < 0.01 and p < 0.001 as compared to vehicle-treated unstressed mice. $^{c,d,e}p$ < 0.05; p < 0.01 and p < 0.001 compared to vehicle-treated stressed mice.

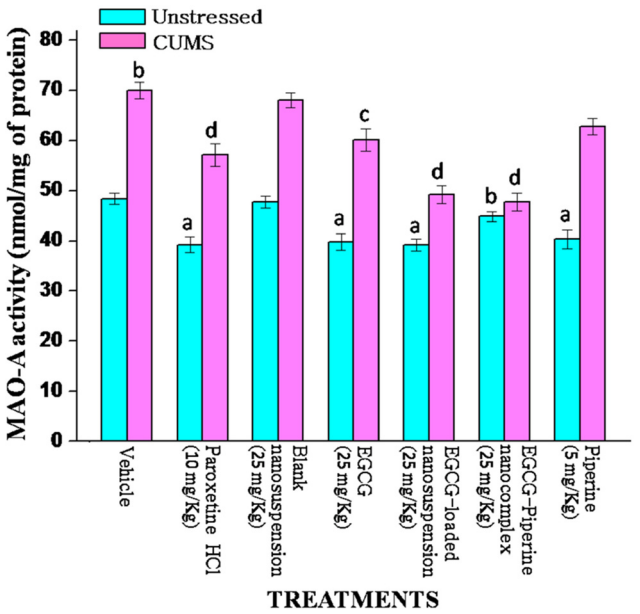

Fig. 6 Effect of various drug treatments on brain MAO-A activity. n = 10 in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparison test. CUMS – chronic unpredictable mild stress. F (13, 126) = 47. 037. p < 0.0001. $^{a,b}p$ < 0.05 and p < 0.001 compared to vehicle-treated unstressed mice. $^{c,d}p$ < 0.01 and p < 0.001 compared to vehicle-treated stressed mice.

MAO-A activity as compared to the corresponding vehicle-treated controls (p < 0.001, p < 0.01, p < 0.001, and p < 0.001, respectively) (Fig. 6).

3.2.6. Effect on brain malondialdehyde levels. As compared to the control, unstressed animals, the level of brain malondialdehyde was found to be higher in stressed animals. The levels of malondialdehyde in the brains of stressed mice were significantly reduced (p < 0.001, p < 0.01, p < 0.001, p < 0.001 and p < 0.01, respectively) following chronic treatment with paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG–piperine nanocomplex (25 mg kg^{-1}) and piperine (5 mg kg^{-1}) alone. Malondialdehyde levels in unstressed mice were not substantially lower in the presence of EGCG (25 mg kg^{-1}), blank nanosuspension (25 mg kg^{-1}), or piperine (5 mg kg^{-1}) compared to the control group. However, paroxetine HCl (10 mg kg^{-1}), EGCG-loaded nanosuspension (25 mg kg^{-1}) and EGCG–piperine nanocomplex (25 mg kg^{-1}) significantly (p < 0.01, p < 0.05, and p < 0.01, respectively) reduced malondialdehyde levels in unstressed mice in contrast to their corresponding vehicle treated controls (Fig. 7).

3.2.7. Effect on brain reduced glutathione levels. In the brains of stressed mice, a significant reduction was observed in the reduced glutathione level in comparison with vehicle-treated unstressed mice. Administration of paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1}) and EGCG–piperine nanocomplex (25 mg kg^{-1}) and piperine (5 mg kg^{-1}) *per se* for a duration of 3 weeks resulted in a significant (p < 0.05, p < 0.01, p < 0.01, p < 0.001 and p < 0.05 respectively) increase in GSH levels of unstressed

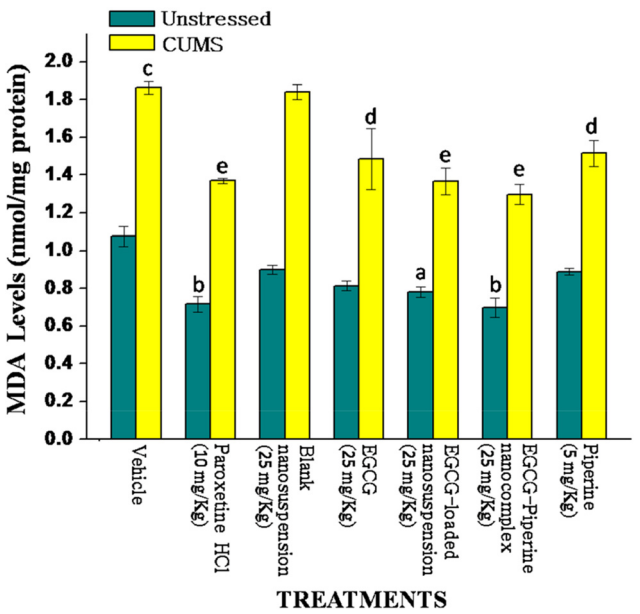

Fig. 7 Effect of various drug treatments on the brain malondialdehyde (MDA) levels. n = 10 in each group. Values are expressed as the mean ± SEM. Data were analyzed by one way ANOVA followed by the Tukey–Kramer multiple comparison test. F (13, 126) = 44.236; p < 0.0001. $^{a,b,c}p$ < 0.05, p < 0.01 and p < 0.001 compared to vehicle-treated unstressed mice. $^{c,d}p$ < 0.001 and p < 0.01 compared to vehicle-treated stressed mice.

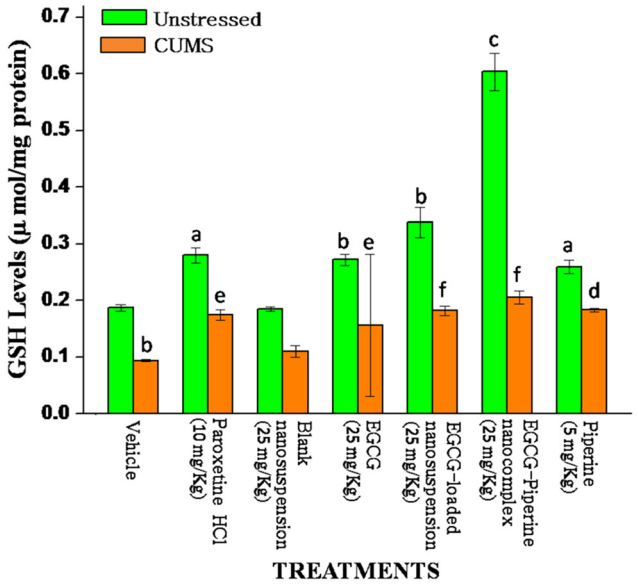

Fig. 8 Effect of various drug treatments on the brain reduced glutathione (GSH) levels. n = 10 in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparison test. CUMS – chronic unpredictable mild stress. F (13, 126) = 74.283; $p < 0.0001$. $^{a,b,c}p < 0.05$, $p < 0.01$ and $p < 0.001$ compared to vehicle-treated unstressed mice. $^{d,e,f}p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to vehicle-treated stressed mice.

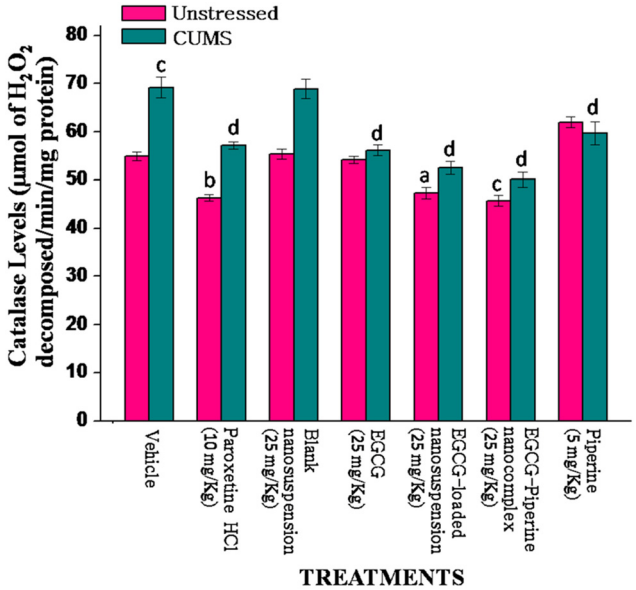

Fig. 9 Effect of various drug treatments on brain catalase levels. n = 10 in each group. Values are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey–Kramer multiple comparison tests. CUMS – chronic unpredictable mild stress. F (13, 126) = 28.500; $p < 0.0001$. $^{a,b,c}p < 0.001$, $p < 0.01$ and $p < 0.05$ compared to vehicle-treated unstressed mice. $^{d}p < 0.001$ compared to vehicle-treated stressed mice.

mice in comparison to the control group treated with the vehicle. However, paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1}), EGCG–piperine nanocomplex (25 mg kg^{-1}) and piperine (5 mg kg^{-1}) significantly ($p < 0.01$, $p < 0.01$, $p < 0.001$, $p < 0.001$, and $p < 0.05$, respectively) increased GSH levels of stressed mice in comparison to the control (Fig. 8).

3.2.8. Effect on brain catalase activity. In the brains of stressed mice, catalase levels were significantly ($p < 0.001$) increased in comparison to the control group treated with the vehicle. Administration of paroxetine HCl (10 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1}), EGCG–piperine nanocomplex (25 mg kg^{-1}), and piperine (5 mg kg^{-1}) *per se* for a duration of three consecutive weeks produced a significant ($p < 0.01$, $p < 0.05$, $p < 0.001$ and $p < 0.05$, respectively) decrease in catalase levels of unstressed mice as compared to the respective vehicle treated control. On the other hand, paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (25 mg kg^{-1}), EGCG–piperine nanocomplex (25 mg kg^{-1}) and piperine (5 mg kg^{-1}) *per se* showed significant ($p < 0.001$) reduction in catalase levels of stressed mice in comparison to the control group treated with the vehicle (Fig. 9).

4. Discussion

Epigallocatechin gallate, a polyphenol present in green tea, possesses numerous therapeutic benefits that provide protection against life-threatening conditions such as cancer and cardiovascular diseases and neurodegenerative disorders[30,34] and also in the prevention of psychiatric ailments by attenuating oxidative stress.[35] Besides all these benefits, a number of drawbacks like short shelf life, gastro-intestinal degradation, and inefficient permeability limit its therapeutic efficacy.[30] In addition, a significant variation in the bioavailability of catechins (EC, ECG, EGC, and EGCG) has been reported, with EGCG being the least bioavailable.[36] One of the reasons behind the low bioavailability of catechins is their large size.[37] The other reason for its low bioavailability is severe bioconversions during absorption, comprising methylation, sulfonation, and glucuronidation.[11]

There are two alternative approaches to combat the poor bioavailability of phytochemicals. One is the use of potential inhibitors that hinders the process of bioconversions and the other is the encapsulation of herbal bioactive into nanoparticles. According to a study, co-administration of EGCG with an inhibitor (piperine) slows down the process of elimination and prevents glucuronidation.[12] However, research has shown that encapsulating EGCG in chitosan nanoparticles greatly increased its bioavailability.[38,39] Additionally, mice who received an oral EGCG-loaded nanosuspension had plasma EGCG concentrations that were 1.5 times higher than those of free EGCG.[40] In rats, EGCG's oral bioavailability was more than doubled when it was enclosed in nanolipidic particles[41] compared to free EGCG.

Depression is becoming a new threat to the modern society. In order to decrease its prevalence and to antagonize the effects linked to detrimental psychiatric conditions, there is a

need to explore new agents that also overcome the side effects associated with antidepressant agents. The combination strategy for treating depression has gained popularity due to a range of potential benefits, including reduced demoralization from psychiatric effects in depressed patients experiencing therapeutic failure, diminished withdrawal syndrome, and the potential for faster and more effective clinical responses.[42,43] To estimate the stress pathology in animals at the pre-clinical level, chronic unpredictable mild stress (CUMS) is generally considered as the most reliable and valuable experimental model.[44] After CUMS simulation, the behavioral and biochemical changes were observed in mice such as an increase in immobility time, a decline in activity of antioxidant enzymes (GSH, SOD and CAT) and variations in the activity of monoaminergic responses confirming their depressive state of mind. Similar depressive symptoms were noticed when animals were exposed to multiple stressors for a very long time.[45,46] The present study suggests that intraperitoneal administration of nanoformulations synthesized using EGCG significantly improves the antidepressant-like behavior in mice subjected to chronic unpredictable mild stress (CUMS) for 3 successive weeks and the results far exceeded when a combination of EGCG and piperine were administered to mice than by either compound when used alone.[47–49]

One of the most popular behavioural models used to assess the effectiveness of antidepressants is the tail suspension test (TST).[50] The current study revealed that when the CUMS technique was used on the mice, their immobility duration increased considerably compared to controls, indicating their depressive-like behaviour. In comparison to vehicle-treated controls, the intraperitoneal administration of paroxetine HCl (10 mg kg^{-1}), EGCG-loaded nanosuspension (comprising 25 mg kg^{-1} EGCG), and EGCG–piperine nanocomplex (comprising 25 mg kg^{-1} EGCG) for three consecutive weeks significantly reduced the immobility time in both unstressed and stressed mice. Additionally, the administration of paroxetine HCl (10 mg kg^{-1}), EGCG (25 mg kg^{-1}), EGCG loaded nanosuspension (comprising 25 mg kg^{-1} EGCG) and EGCG–piperine nanocomplex (comprising 25 mg kg^{-1} EGCG) did not exhibit any effect on the locomotor activity of unstressed and stressed mice; hence this confirmed their CNS stimulant activity and indicated that the anti-depressant effect of EGCG in unstressed and stressed mice is specific. These results are in line with earlier studies.[51]

Besides behavioral abnormalities, chronic unpredictable mild stress induction also activates the hypothalamic–pituitary–adrenal (HPA) axis and is responsible for the synthesis and secretion of glucocorticoids from the adrenal cortex. The hyper-secretion of glucocorticoids in blood such as corticosterone in rodents or cortisol in primates disturbs the hormonal balance and ultimately leads to depression.[52] The studies denoted that antidepressant drugs produce therapeutic effect in depression patients by suppressing the activity of the HPA axis[53] and thus by restoring the functioning of the HPA axis to normal can be used as a treatment for depression.[52] In the present study, induction of the CUMS procedure resulted in an increase in the corticosterone level compared to the naïve animals and other observations also support this finding.[54] The administration of EGCG loaded nanosuspension significantly reduced the plasma corticosterone level in stressed mice. No significant effect was observed in the case of unstressed mice signifying that overactivity of HPA axis only occurred in stressed conditions.

These findings align with previous studies that the administration of EGCG prevents the increase in the corticosterone level occurring due to the induction of CUMS.[51,55]

Additionally, persistent unpredictable mild stress is thought to encourage oxidative stress and undermine the brain's antioxidant defense system.[56] Reactive oxygen species are produced as a result of oxidative stress, which causes oxidative damage to macromolecules like lipids, proteins, and DNA. This damage eventually results in neural malfunction and depression.[57] Since their levels reverted to normal post antidepressant treatment, antioxidant enzymes and lipid peroxidation can be viewed as important depression markers.[58] Chronic unpredictable mild stress that was induced caused significant oxidative damage, such as a rise in plasma nitrite levels and lipid peroxidation, as well as a decrease in the functioning of the antioxidant enzymes GSH, SOD, and CAT that are present in the brain tissue.[59] The current findings suggest that mice exposed to various stressors for three weeks in a row increased the levels of lipid peroxidation and nitrite while decreasing the activity of an endogenous antioxidant enzyme. Chronic administration of EGCG (25 mg kg^{-1}), EGCG–piperine nanocomplex (comprising 25 mg kg^{-1} EGCG), and EGCG-loaded nanosuspension reversed these parameters, resulting in a significant reduction in lipid peroxidation levels in stressed mice, an increase in GSH in unstressed mice, and a decrease in catalase levels in the brains of both unstressed and stressed mice. As a result, EGCG demonstrated considerable antioxidant activity in mice, and the findings support earlier research.[60,61] When compared to their naive animals, plasma nitrite levels of stressed and unstressed mice showed a significant decline after treatment with EGCG loaded nanosuspension (25 mg kg^{-1}) and EGCG–piperine nanocomplex. Therefore, EGCG demonstrated a strong protective effect on mice against oxidative stress brought on by CUMS. It has also been noted that mice exposed to various stressful conditions have higher plasma nitrite levels.[20]

Monoamine oxidase (MAO) is a flavin enzyme situated within the outer mitochondrial membrane of cells throughout the body. MAO plays a crucial role in the metabolic breakdown of biogenic and xenobiotic amines within both the central nervous system and peripheral tissues. It is further characterized into two different forms – MAO-A and MAO-B. MAO-A exhibits a substrate preference for serotonin, epinephrine, and norepinephrine, while MAO-B shows a substrate preference for dopamine. During major depression, activity of MAO-A in the brain was elevated in the brain resulting in a decrease in the monoamine level.[62] Therefore, in the present study only the MAO-A level in the brain was measured. An increase in the activity of MAO-A was observed due to the exposure to different

Table 2 The relative order of effect on behavioral and biochemical parameters investigated on comparative basis in the current study

S. no.	Parameters	Order of significance in unstressed mice based on the 'q' value	Order of significance in stressed mice based on the 'q' value
1	Tail suspension test	Paroxetine HCl (19.343) > EGCG–piperine nanocomplex (11.384) > EGCG loaded nanosuspension (5.584)	Paroxetine HCl (18.533) > EGCG–piperine nanocomplex (9.064) > EGCG loaded nanosuspension (7.338)
2	Plasma nitrite	EGCG–piperine nanocomplex (5.810) > paroxetine HCl (5.757)	EGCG–piperine nanocomplex (14.452) > EGCG loaded nanosuspension (9.950) > paroxetine HCl (6.619) > EGCG (5.739) > piperine (5.428)
3	Plasma corticosterone	No effect was observed	EGCG–piperine nanocomplex (6.726) > EGCG loaded nanosuspension (6.058) > paroxetine HCl (5.674) > EGCG (4.973)
4	Brain MAO-A activity	EGCG–piperine nanocomplex (6.752) > EGCG loaded nanosuspension (5.539) > paroxetine HCl (5.531) > EGCG (5.189) > piperine (4.886)	EGCG–piperine nanocomplex (15.226) > EGCG loaded nanosuspension (12.551) > paroxetine HCl (7.752) > EGCG (5.935)
5	Brain lipid peroxidation	EGCG–piperine nanocomplex (6.275) > paroxetine HCl (5.945) > EGCG loaded nanosuspension (4.905)	EGCG–piperine nanocomplex (9.363) > EGCG loaded nanosuspension (8.191) > paroxetine HCl (8.158) > EGCG (6.275) > piperine (5.747)
6	Brain reduced glutathione	EGCG–piperine nanocomplex (28.457) > EGCG loaded nanosuspension (10.301) > paroxetine HCl (6.366) > EGCG (5.874)	EGCG–piperine nanocomplex (7.644) > EGCG loaded nanosuspension (6.127) > paroxetine HCl (6.045) > EGCG (5.533)
7	Brain catalase	EGCG–piperine nanocomplex (6.608) > paroxetine HCl (6.177) > EGCG loaded nanosuspension (5.459)	EGCG–piperine nanocomplex (13.719) > EGCG loaded nanosuspension (11.995) > paroxetine HCl (8.619) > EGCG (9.337) > piperine (6.823)

stressors. A significant inhibition in the brain MAO-A activity was observed in both unstressed and stressed mice upon administration of the EGCG loaded nanosuspension (25 mg kg^{-1}) and EGCG–piperine nanocomplex (25 mg kg^{-1}) for 3 successive weeks. The findings are in concurrence with the previous research demonstrating that EGCG exhibits monoamine oxidase inhibiting activity.[63] The present findings indicate that more inhibition of MAO-A activity was observed when the combination of EGCG and piperine was administered and this is also in alignment with a previous study where a combination of ferulic acid and piperine displayed a better inhibitory effect on MAO-A than the treatment with single drug.[64] The relative order of effects of various drug treatments (paroxetine HCl, blank nanosuspension, EGCG, EGCG-loaded nanosuspension, EGCG–piperine nanocomplex and piperine on behavioral model (TST) and biochemical parameters (plasma nitrite, plasma corticosterone, brain MAO-A, brain lipid peroxidation, brain GSH and brain catalase) is shown in Table 2.

In conclusion, the administration of synthesized nanoformulations shows a significant anti-depressant effect in unstressed and stressed mice possibly by decreasing the plasma nitrite level; inhibiting the activity of the MAO-A enzyme; and increasing the activity of the antioxidant enzyme in the brain. Antidepressant-like activity of the synthesized nanoformulation using EGCG was found comparable to paroxetine HCl, a standard antidepressant drug used in the current study. Indeed, the encapsulation of drug moieties into nanoparticles increases the bioavailability by improving the solubility and stability of phytochemicals[65,66] and it was also concluded from present findings that nano-encapsulation enhances the efficacy of a herbal drug (EGCG) and these effects were more pronounced when the combinational strategy (EGCG along with piperine) was used. Piperine (5 mg kg^{-1}) also displayed an anti-depressant effect, but it was less significant due to its low concentration. However, the anti-depressant effect was increased when it was combined with EGCG. It is concluded that the simultaneous administration of EGCG and piperine has the potential to produce a synergistic effect on depression-like behavior in mice. This combination could serve as a natural alternative for mitigating psychiatric disorders, offering improved efficacy and minimal side effects.

5. Conclusion

The bioavailability of polyphenols is very poor which restricts their therapeutic potential, and improving their systemic absorption is the real challenge. The use of bioavailability enhancers and their encapsulation into nanoparticles are the two approaches that can overcome the bioavailability issue. In this current investigation, EGCG was loaded into nanoparticles alone and with piperine and their anti-depressant potential in mice was evaluated. The antisolvent precipitation methodology was employed for the synthesis of nanoparticles loaded with EGCG and EGCG–piperine nanocomplex using zein as a nanocarrier. These findings show that the encapsulation of EGCG into nanoparticles produces a significant antidepressant effect on Swiss male albino mice and the results obtained are comparable to those with the standard antidepressant drug, paroxetine HCl. The antidepressant effect of EGCG is due to its antioxidant nature and these results are further amplified when it is combined with piperine. Moreover, the carrier *i.e.* zein used for the encapsulation of EGCG is biocompatible, biodegradable, non-toxic, FDA approved and safe for human use.

Conflicts of interest

The authors report no conflicts of interest in this work.

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