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Matrine ameliorates anxiety and depression-like behaviour by targeting hyperammonemia-induced neuroinflammation and oxidative stress in CCl4 model of liver injury

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ABSTRACT

Acute or chronic liver injury is associated with hyperammonemia which induced neuroinflammation and oxidative stress in the brain. Neuroinflammation, oxidative stress, reduced neurogenesis, and apoptosis are critical factors for the development of anxiety and depression. The present study was aimed to evaluate the anxiolytic and antidepressant properties of matrine against acute liver injury in the rodent model. Acute liver injury in mice was induced by administration of the acute hepatotoxic dose of carbon tetrachloride (CCl4) (1 ml/kg, i.p.). Pretreatment of mice with matrine (50 mg/kg i.p.) remarkably ameliorated CCl4-induced anxiety and depression-like behavior as evident from the results of open field test (OFT), elevated plus maze test (EPM), light-dark box test (LDB), forced swimming test (FST), and tail suspension test (TST). Moreover, matrine significantly inhibited CCl4-induced neuroinflammation in mice by reducing pro-inflammatory cytokines such as interleukins (IL-1β, IL-6) and tumor necrosis factor-α (TNF-α) levels in the hippocampus (HC) and prefrontal cortex (PFC). CCl4-induced oxidative stress was reduced by matrine due to its potential to enhance the levels of reduced glutathione (GSH), catalase (CAT), glutathione-S-transferase (GST), and decreased the malondialdehyde (MDA), and nitrite level in the PFC and HC of mice brain. Matrine remarkably reduced the levels of corticosterone, ammonia, AST, ALT, and creatinine. Matrine pretreatment remarkably ameliorated CCl4-induced morphological liver injury. Acute pretreatment of matrine enhanced neurogenesis by increasing the number of GFAF (glial fibrillary acidic protein) positive astrocyte, BDNF (brain-derived neurotrophic factor), and VEGF (vascular endothelial growth factor) in the hippocampus of CCl4-treated mice. Pretreatment of matrine inhibited apoptosis and DNA damage in the hippocampus. The present data revealed that hyperammonemia produced due to liver injury induced oxidative stress, neuroinflammation, reduced neurogenesis and apoptosis in the hippocampus, thus, resulting in anxiety and depression. Taken together, the present results suggested that matrine has a significant antidepressant and anxiolytic effects through modulation of neuroinflammation, oxidative stress, reduced neurogenesis and apoptosis induced by CCl4 administration.

1. Introduction

Liver diseases have been widely associated with anxiety and depression (Golden et al., 2005; Huang et al., 2017). Hyperammonemia is a common event that occurs in acute liver failure or chronic liver diseases and described by an elevated level of ammonia. Additionally, hyperammonemia triggers several neurological complications by inducing neuroinflammation and oxidative stress in the brain (Heidari et al., 2016). It has been reported that blood ammonia could be toxic for the human brain and implicated in the development of anxiety and depression (Duan et al., 2015). Depression and anxiety are the neuropsychiatric disorders that frequently exist in comorbid state and usually described by intense physiological, psychological, and social impairment (Sulakhiya et al., 2016). Over the past several decades, one of the most widely accepted etiology of depression has grown to be the monoamine hypothesis (Nestler et al., 2002). Several classes of

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antidepressants are based on the monoamine hypothesis of depression and have been utilized to ameliorate the symptoms of depression (Berton and Nestler, 2006). However, these antidepressants have major shortcomings, such as the slow onset of action (mostly > 21 days) and lower rates of therapeutic efficacy (Zhang et al., 2016). Currently, the most commonly prescribed anxiolytic medications include benzodiazepine (BZD). However, numerous studies reported that clinical uses of BZD is associated with tolerance, addiction, and severe adverse effects, such as sedation, amnesia, psychomotor and cognitive impairment (Bahi et al., 2014). Therefore, development of the novel anxiolytic and antidepressants based on different pharmacological target is still challenging.

Accumulative evidence reported the involvement of neuroinflammation and oxidative stress in the pathogenesis of depression and anxiety (Mello et al., 2013). The observed results include the decreased levels of antioxidants (GSH, GST, and CAT) and elevated levels of MDA, nitric oxide and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in the PFC and HC of mice brain (Maes, 2008; Salim et al., 2010; Zhang et al., 2016). An elevated level of proinflammatory cytokine produces sickness like behaviors such as loss of interest in social activities, major alterations in sleep pattern, reduction of locomotor activity, reduction in exploration, anhedonia, change in appetite, and body weight loss (Huang et al., 2008). Furthermore, proinflammatory cytokines caused hyperactivity of the hypothalamic-pituitary-adrenal axis (HPA-axis) which lead to an elevated level of cortisol in plasma (hypercortisolemia) and triggers symptoms of depression (Furtado and Katzman, 2015).

Hippocampal neurogenesis has been hypothesized to play a critical role in the pathogenesis of depression (Lee and Son, 2009). According to neurogenic hypothesis of depression reduced hippocampal neurogenesis may underlie the etiopathogenesis of depression while antidepressants therapeutic efficacy depends on the upregulation of neurogenesis in the hippocampus (Jacobs et al., 2000). GFAP, BDNF and VEGF are vital proteins which play crucial role in hippocampal neurogenesis. Studies have been reported that GFAP, BDNF and VEGF are decreased in rodent models of depression while increased by antidepressant (Ding et al., 2017; Han et al., 2017). Apoptosis and DNA damage in the hippocampal neurons have been proposed to be another critical mechanism involved in the pathogenesis of depression (Kosten et al., 2008; Czarny et al., 2018). Carbon tetrachloride (CCl4), is a potent hepatotoxic agent and extensively utilized to induce hepatotoxicity in rodent models (Li et al., 2015). CCl4 exhibits hepatotoxicity by free radical $CCl_3^{\bullet}$ generation and induced oxidative stress along with the elevation of inflammatory cytokines (IL-1β, IL-6, and TNF-α) level in the liver and brain of the rodent models (Anand et al., 2011; Makni et al., 2012).

Currently, numerous studies revealed that bioactive compounds from natural herbs act as potential candidates for the management of anxiety and depression in the rodent model (Zhang et al., 2016). Matrine is the chief alkaloid from *Sophora flavescens* which belongs to the family of Leguminosae (Yin and Zhu, 2005). Studies have been reported that matrine exhibits a number of pharmacological activities such as anti-inflammatory, anticancer, antiallergic and neuroprotective effect (Xu et al., 2012). It has been reported that matrine exhibited neuroprotective effects through inhibition of neuroinflammation and oxidative stress in various neurological disorders (Xu et al., 2012; Meng et al., 2017). The anxiolytic and antidepressant activities of matrine have not been studied up till now in acute liver injury. Therefore, in the present study, we established the CCl4-induced liver injury model of mice to simulate liver disease and observed the anxiety and depression-like behaviors through the behavioral and biochemical methods. It was hypothesized that matrine possesses anxiolytic and antidepressant properties via inhibiting neuroinflammation, oxidative stress, and apoptosis while enhancing hippocampal neurogenesis in CCl4-induced liver injury model. Furthermore, it was also explored that hyperammonemia is implicated in the pathogenesis of anxiety and depression after liver injury.

2. Material and methods

2.1. Animals

The entire experimentations were performed in adult male mice weighing (24–35 g) obtained from the NIH, Islamabad, Pakistan. All animal experimentations were performed in compliance with the NIH guidelines (Council, 2010). The behaviour experiments were performed during the light phase. This study was approved by Bio-ethical Committee (Approval No: BEC-FBS-QAU2017-60) of Quaid-i-Azam University, Islamabad. Animals were housed in the state of controlled environmental conditions (23 ± 2 °C, 60 ± 10% relative humidity and 12 h' light/dark phase) and retained with free access to water along with standard laboratory diet.

2.2. Chemicals and reagents

Matrine was received from Prof. YS Kim, Seoul National University, Seoul, Korea, fluoxetine, diazepam (Lowitt pharmaceutical, Pakistan), CCl4, Griess reagent, Nessler's reagent, 1-chloro-2,4-dinitrobenzene (CDNB), GSH, 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), hydrogen peroxide (H_2O_2), formalin, olive oil, and Elisa kits of cytokines (TNF-α, IL-1β, IL-6), corticosterone (eBioscience, Inc., United States), anti-BDNF antibody, anti-VEGF antibody, and glial fibrillary acidic protein (GFAP) (Santa Cruz Biotechnology, Inc) and diaminobenzidine substrate (DAB, Sigma).

2.3. Preparation and selection of doses

Matrine, diazepam, and fluoxetine were dissolved in 0.9% saline. Whereas, CCl4 was dissolved in olive oil. Three different doses of matrine (5, 25, and 50 mg/kg) were evaluated against the CCl4-induced animal model. The dose of matrine (50 mg/kg i.p.) was selected for further experiments due to its significant response in OFT as a preliminary test. Whereas, the doses of diazepam (2.0 mg/kg), fluoxetine (10 mg/kg), and CCl4 (1 ml/kg) were selected based on previous reports (Löw et al., 2000; Papp et al., 2003; Makni et al., 2012). All drugs were administered at the dose volume of 300 μL/ mouse by intraperitoneal (i.p) route before induction of anxiety and depression. All drug solutions were prepared instantly prior to starting the experiments.

2.4. Experimental design

At the beginning of the experimentation, adult male mice (BALB/c) were randomly divided into seven groups. Every group includes five mice. All drugs were administered 30 min prior to CCl4 administration.

Group I: Vehicle control (animals received normal saline)
Group II: CCl4- treated group (1 mL/kg, i.p)
Group III: Diazepam (2 mg/kg, i.p)
Group IV: Fluoxetine (10 mg/kg, i.p)
Group V: Matrine (5 mg/kg, i.p)
Group VI: Matrine (25 mg/kg, i.p)
Group VII: Matrine (50 mg/kg, i.p)

The time-point 24 h was considered for the evaluation of anxiety and depression-like behavior. Anxiety related behavior was evaluated by OFT, EPM test as well as LDB test. Whereas, depressive related behavior was evaluated by FST and TST. After completion of behaviors experiment, mice were anesthetized using chloroform. And then samples of blood were collected from the heart of mice. By means of centrifugation, serum was separated and then stored at (-80 °C) for biochemical analysis. Subsequent to serum collection, anesthetized mice were killed through cervical dislocation and the liver and brain tissue were rapidly dissected out. Brain and liver samples were collected and stored at (−80 °C) for biochemical and histological analysis. Furthermore, within different animals both behavioral and biochemical

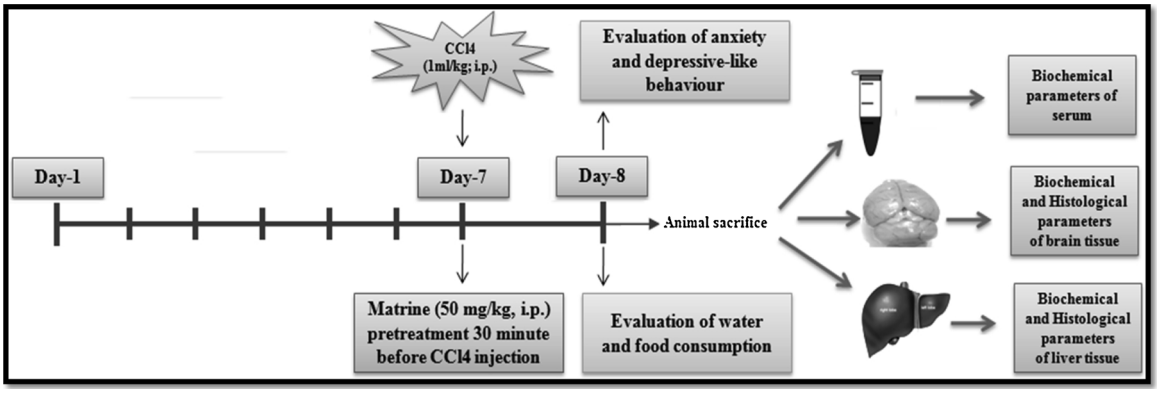

Fig. 1. A schematic overview of the experimental study plan.

tests were executed. The study plan is illustrated diagrammatically in (Fig. 1).

2.5. Evaluation of behavioral parameters

2.5.1. OFT

OFT was performed to assess locomotor activity as well as anxiety in rodents. Briefly, in the center square of the wooden box (50 L × 50 W × 40 H cm^3) each mouse was individually placed. The wooden box consisted of twenty-five equal squares on the base (10 × 10 cm^2). For every mouse, following parameters such as numbers of squares crossed as well as time spent in the center were recorded for 5 min (Jiang et al., 2015).

2.5.2. LDB test

LDB test was performed to assess the anxiety in rodents. The test apparatus was separated into 2 sections. A light chamber (42 × 30 × 20 cm^3; illuminated brightly with a bulb of 50 W) and a dark chamber (42 × 30 × 20 cm^3; black walls, with a black lid on top). The 2 chambers connected with each other with an open passageway (6 × 6 cm^2). Inside the dark chamber for 10 min individual mouse was placed and allowed freely to explore either compartment. For every mouse, the following parameters such as the number of light-dark transitions as well as time spent in the light compartment were recorded and evaluated (Jangra et al., 2016).

2.5.3. EPM test

EPM test was performed to evaluate the anxiety in mice. EPM test apparatus was composed of 2 open arms (35 × 5 cm^2) and 2 vertical closed arms (35 × 5 × 25 cm^2) connected by a small central podium (5 × 5 cm^2). At the center platform of EPM, each mouse was placed with the head facing in the direction of the open arm. For every mouse, the following parameters such as time spent in open arm and the total number of entries into the open arm were recorded and evaluated for 5 min (Jangra et al., 2014).

2.5.4. FST

The FST was performed to evaluate depression like-behaviour in mice. Briefly, the individual mouse was put into an open transparent plastic cylinder (10 W × 25 H cm), holding water at 23 ± 2 °C to a depth of 20 cm. For 6 min, each mouse was forced to swim. Immobility time was analyzed during the last 5 min. The absence of escape-oriented behavior was considered as an immobility time (Porsolt et al., 1977).

2.5.5. TST

The TST was also performed to evaluate depression like-behaviour in mice. Briefly, mice were individually suspended for 6 min on the rim of a rod 50 cm above the floor by using adhesive tape positioned about 1 cm from the tip of the tail. The immobility time duration was calculated in the last 5 min. Mice were considered to be immobile when they hung down inactively and were completely motionless (Steru et al., 1985).

2.5.6. Measurement of water and food consumption

100 ml of water and 50 g of food pellets were provided to the individual animal cage for the calculation of water and food consumption. 2 days prior to the experimentation, baseline water as well as food intake were determined by means of observing the animals in the individual cages.

2.6. Evaluation of biochemical parameters

2.6.1. Measurement of proinflammatory cytokines

Proinflammatory cytokines (TNF-α, IL1-β, as well as IL-6,) levels were measured in PFC and HC by using an ELISA (enzyme-linked immunosorbent assay) kits (eBioscience, Inc., United States). According to the protocol of manufacturer's, the cytokines levels were determined (Khan et al., 2013; Atiq et al., 2018).

2.6.2. GSH level

GSH levels in the HC and PFC were analyzed using a standard method reported by Ellman et al (Makni et al., 2012). The principle of this assay was based on the reaction of DTNB with free thiol groups of GSH to form a yellow chromophore. The resultant color was measured spectrophotometrically at 412 nm wavelength.

2.6.3. GST activity

GST activity in the HC and PFC was analyzed by using a method established by Warholm et al (Makni et al., 2012). The principle of this assay was based on measurement of the conjugation of CDNB with GSH. The change in the absorbance was monitored at 344 nm in the spectrophotometer.

2.6.4. CAT activity

CAT activity in the HC and PFC was evaluated by using a standard method reported by Aebi et al (Makni et al., 2012). The principle of this assay was based on the decomposition of H_2O_2 by catalase. The change in absorbance was measured at 240 nm in UV/visible spectrophotometer.

2.6.5. Nitric oxide (NO) levels

NO production was evaluated by the method reported elsewhere (Khan et al., 2013, 2014; Khan et al., 2015). The absorbance was measured at 560 nm by using a microplate reader. NO was calculated from a standard curve produced by NaNO2.

2.6.6. Lipid peroxidation assay

Lipid peroxidation was determined in the liver, HC, and PFC homogenate by measuring the malondialdehyde (MDA) level according

to Ohkawa et al with some modifications (Jangra et al., 2016). The absorbance was measured at 532 nm by using a microplate reader.

2.6.7. Determination of ammonia level in the liver, blood, and brain

Ammonia concentration in biological tissue was measured by the method reported by Gutierrez-de-Juan et al (Gutiérrez-de-Juan et al., 2017). This method was based on the Nessler's reagent (Potassium tetraiodomercurate (II)) utilization to detect ammonia accumulation in tissue. In the presence of ammonia, the pale yellow color of Nessler's reagent becomes darker yellow to a dark brown precipitate. By using UV/visible spectrophotometer, the absorbance was recorded at 425 nm wavelength. The content of ammonia was calculated from a standard curve produced by ammonium chloride.

2.6.8. Corticosterone (CORT) estimation

CORT level in the serum was measured by using the CORT ELISA (enzyme-linked immunosorbent assay) kits (eBioscience, Inc., United States). CORT level was determined according to the manufacturer's instruction (Ai et al., 2014).

2.6.9. Evaluation of ALT, AST and creatinine activities in plasma

The enzymatic biomarkers such as AST (aspartate aminotransferase), ALT (alanine aminotransferase), and creatinine were measured in the plasma for the assessment of hepatic and renal damage. In plasma ALT, AST, and creatinine levels were analyzed by enzymatic methods using commercially available kits (Abnova Corporation) (Khan et al., 2014; Khalid et al., 2018)

2.7. Histopathology, immunohistochemistry (IHC) and immunofluorescence

For histopathological examination, the whole brains and liver were kept in 4% paraformaldehyde (PFA) solution for fixing. The brain and liver tissues were fixed in PFA solution for 24 h. Subsequently, the tissues were sectioned at 5 µm by means of a rotary microtome and were stained with hematoxylin and eosin (H&E). The brain and liver tissues were examined under an optical microscope, and photos were taken according to Ai et al (Ai et al., 2014; Park et al., 2015).

The sections of paraffin were also subjected to immunohistochemical studies for detection of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) as markers for neurogenesis and caspase-3 as a marker of apoptosis in the hippocampus using avidin-biotin-peroxidase complex (ABC) method (Noshy et al., 2018). Antibodies used in the IHC include an anti-BDNF antibody, anti-VEGF antibody, and anti-caspase 3 antibodies produced in rabbit (Santa Cruz Biotechnology, Inc). Briefly, brain sections were incubated with different antibodies mentioned above and the reagents required for the ABC method were added. Each marker expression was labeled with peroxidase and colored with DAB for detection of the antigen-antibody complex. The relative expression of BDNF, VEGF, and caspase-3 proteins were measured using ImageJ software.

Immunofluorescence staining was performed as previously described (Shah et al., 2018). Briefly, slides were treated with proteinase K (antigen retrieval step), washed with 0.1 M PBS, and incubated with 5% normal serum according to the source of the secondary antibody used. The slides were incubated with glial fibrillary acidic protein (GFAP) (Santa Cruz Biotechnology, Inc), overnight at 4 °C. The next morning, after being washed with PBS, the slides were incubated with fluorescently labeled secondary antibodies (Santa Cruz Biotechnology) for signal amplification in a dark chamber, then coverslipped in Ultra Cruz mounting medium (Santa Cruz Biotechnology, Inc). Immunofluorescence images (five images per slide) were captured using confocal scanning microscopes (Flouview FV 1000, Olympus, Japan). The relative expression of GFAP was measured using ImageJ software.

2.8. Evaluation of DNA damage in the neuron of the hippocampus

The comet assay (single-cell gel electrophoresis) was performed to measure DNA damage in the hippocampal neurons according to the method reported by (Nowsheen et al., 2012). The level of DNA damage was measured using CASP 1.2.3.b software.

2.9. Statistical analysis

Results were expressed as mean ± standard deviations (S.D.). Sigma plot version 10.0 (statistical software) was used for the statistical analysis. One-way ANOVA and unpaired Student's *t*-test were used to determine statistical significance between the groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of matrine in CCl4-induced anxiety-like behavior

3.1.1. OFT

Anxiety-like behavior was evaluated at 24 h after CCl4 administration. CCl4 administration was associated with anxiety as evident by remarkable reduction ($p < 0.001$) in the number of crossing and time spent in the center when compared to the vehicle control group in the OFT. For the selection of the most effective dose of matrine, different doses such as 5, 25, and 50 mg/kg were chosen for the study. The results demonstrated that 5 and 25 mg/kg doses of matrine didn't produce a remarkable anxiolytic effect in CCl4-treated mice. However, high concentration (50 mg/kg) of matrine exhibited very remarkable ($p < 0.001$) anxiolytic response against CCl4-induced anxiety. Therefore, matrine (50 mg/kg) was chosen for further study. Similar effects were also produced by the positive control such as diazepam (Fig. 2).

3.1.2. LDB test

Administration of CCl4 produced anxiety-like behaviour as evident by the significant reduction ($p < 0.001$) in time spent in the light compartment and the number of light-dark transitions as compared to vehicle control group in the LDB test. Diazepam and matrine demonstrated significant improvement ($p < 0.001$) in all the parameters of LDB test i.e. time spent in the light compartment and the number of light-dark transitions when compared to CCl4-challenged group (Fig. 3).

3.1.3. EPM test

A third behavioral model such as the EPM test was also performed for the assessment of CCl4-induced anxiety as well as an anxiolytic effect of matrine. CCl4 administration significantly reduced ($p < 0.001$) the number of entries in an open arm as well as time spent in an open arm. Matrine and diazepam showed remarkable increase ($p < 0.001$) in the number of entries in the open arm as well as time spent in the open arm while compared to CCl4-challenged group (Fig. 4).

3.2. Effect of matrine in CCl4-induced depressive-like behavior

3.2.1. FST and TST

Depression-like behavior was assessed after 24 h of CCl4 administration equally in FST and TST. CCl4-treated mice exhibited a striking increase ($p < 0.001$) in immobility time in both FST and TST when compared with vehicle control group. Matrine and fluoxetine significantly ameliorated ($p < 0.001$) the CCl4-induced depression as demonstrated by decreased immobility time in both FST and TST (Fig. 5).

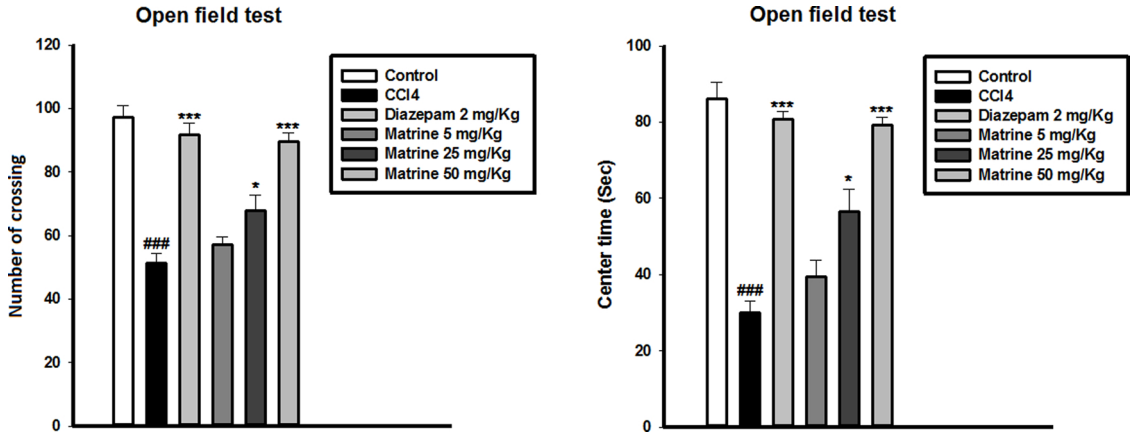

Fig. 2. Effect of matrine (5, 25, and 50 mg/kg), fluoxetine (10 mg/kg) and diazepam (2 mg/kg) on CCl4-induced anxiety in mice evaluated by open field test (A) locomotor activity (number of crossing) and (B) time spent in the center. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$ compared to control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to CCl4-treated group.

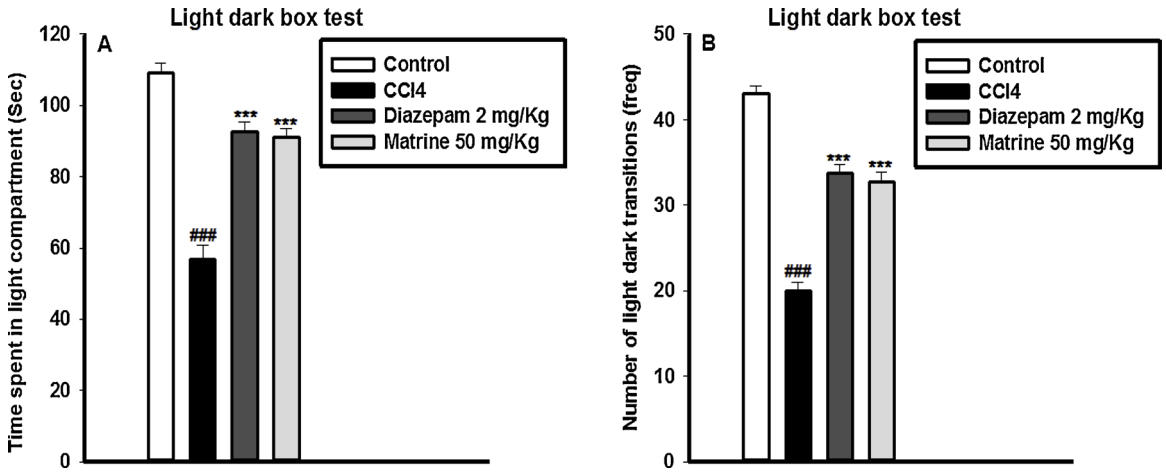

Fig. 3. Effect of matrine and diazepam on CCl4-induced anxiety-like behavior in mice evaluated by light-dark box test (A) time spent in light compartment and (B) the number of light-dark transitions in mice. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$ compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

3.2.2. Effect of matrine in the CCl4-induced alteration in food and water consumption

CCl4 administration significantly reduced ($p < 0.001$) water and food intake in mice at 24 h while compared to the vehicle control group. Matrine remarkably increased food and water intake in CCl4-treated mice (Table1).

3.3. Effect of matrine in CCl4-induced proinflammatory cytokines levels

CCl4-induced neuroinflammation was determined by measuring proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in the PFC and HC regions of the brain. CCl4-treated mice showed remarkable elevation

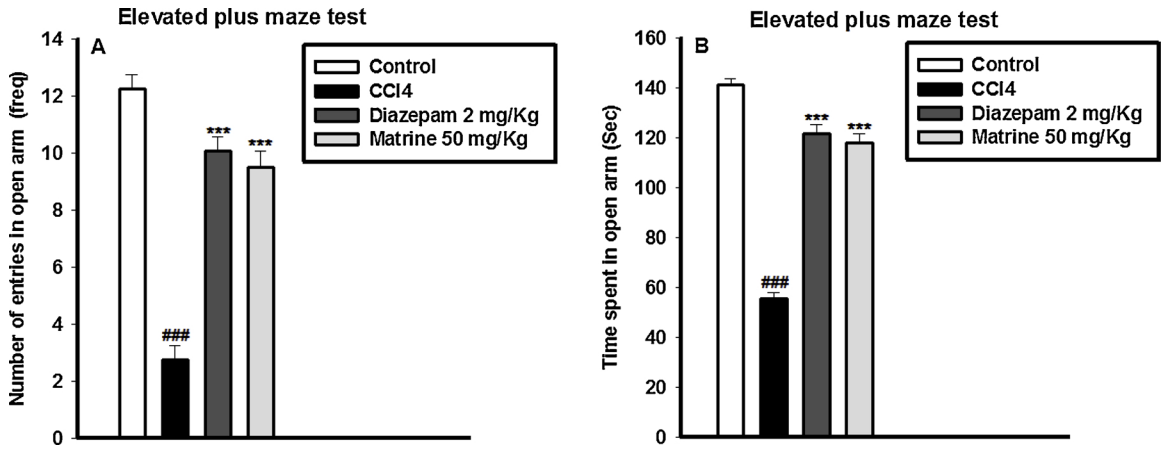

Fig. 4. Effect of matrine and diazepam on CCl4-induced anxiety in mice evaluated by an elevated plus-maze test (A) number of entries in open arm and (B) time spent in open arm. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$ compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

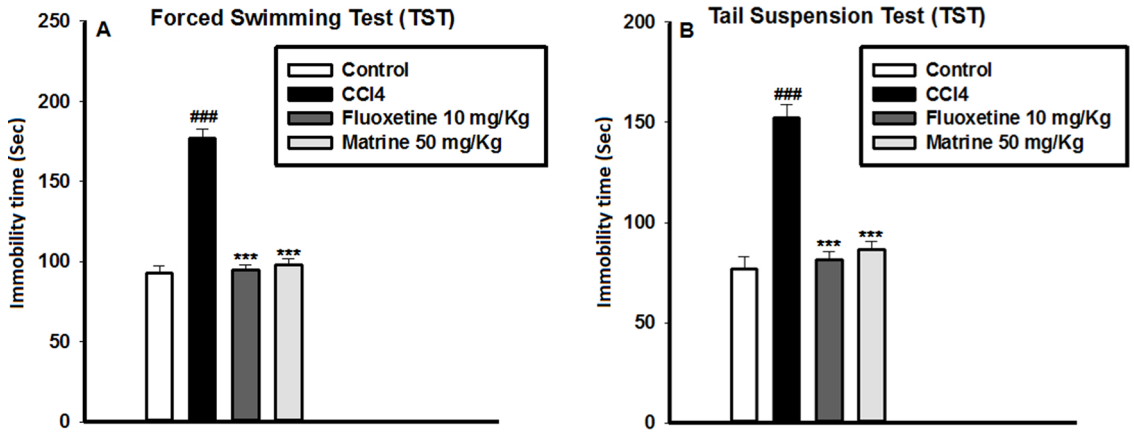

Fig. 5. Effect of matrine and fluoxetine on CCl4-induced depressive-like behavior in mice evaluated by (A) forced swim test and (B) tail suspension test. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$ compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

Table 1
Effect of matrine on CCl4-induced alteration in water and food consumption.

Parameters	Vehicle control	CCl4	Matrine
Water intake (ml/24 h)	5.03 ± 0.7	1.63 ± 0.45$^{###}$	4.4 ± 0.49***
Food intake (g/24 h)	4.93 ± 0.44	1.92 ± 0.21$^{###}$	3.94 ± 0.16**

All data were expressed as mean (n = 5) ± SD.
$^{###}$ $p < 0.001$ compared to control group.
** $p < 0.01$ and
*** $p < 0.001$ compared to CCl4-treated group.

($p < 0.001$) of IL-1β, IL-6, and TNF-α level in the brain (HC and PFC) as compared to the vehicle control group. Matrine significantly ameliorated ($p < 0.001$) the CCl4-induced proinflammatory cytokines level in the HC along with PFC regions of mice brain. Similar results were observed in diazepam and fluoxetine (Fig. 6).

3.4. Effect of matrine in CCl4-induced oxidative stress markers

CCl4-induced oxidative stress was determined by measuring antioxidants (GSH, GST, and catalase), nitrate, and MDA levels within the HC and PFC regions of the mice brain. It was noticed that CCl4 administration remarkably reduced ($p < 0.001$) GSH, GST and catalase levels in the PFC and HC of the mice brain as compared to vehicle control group. Matrine significantly elevated ($p < 0.001$) GSH, GST, and catalase levels in the PFC and HC region of the mice brain. Similar results were also observed by both fluoxetine and diazepam (Fig. 7). The nitrite level was significantly raised ($p < 0.001$) in mice both centrally (PFC and HC) and peripherally (serum) after 24 h of CCl4

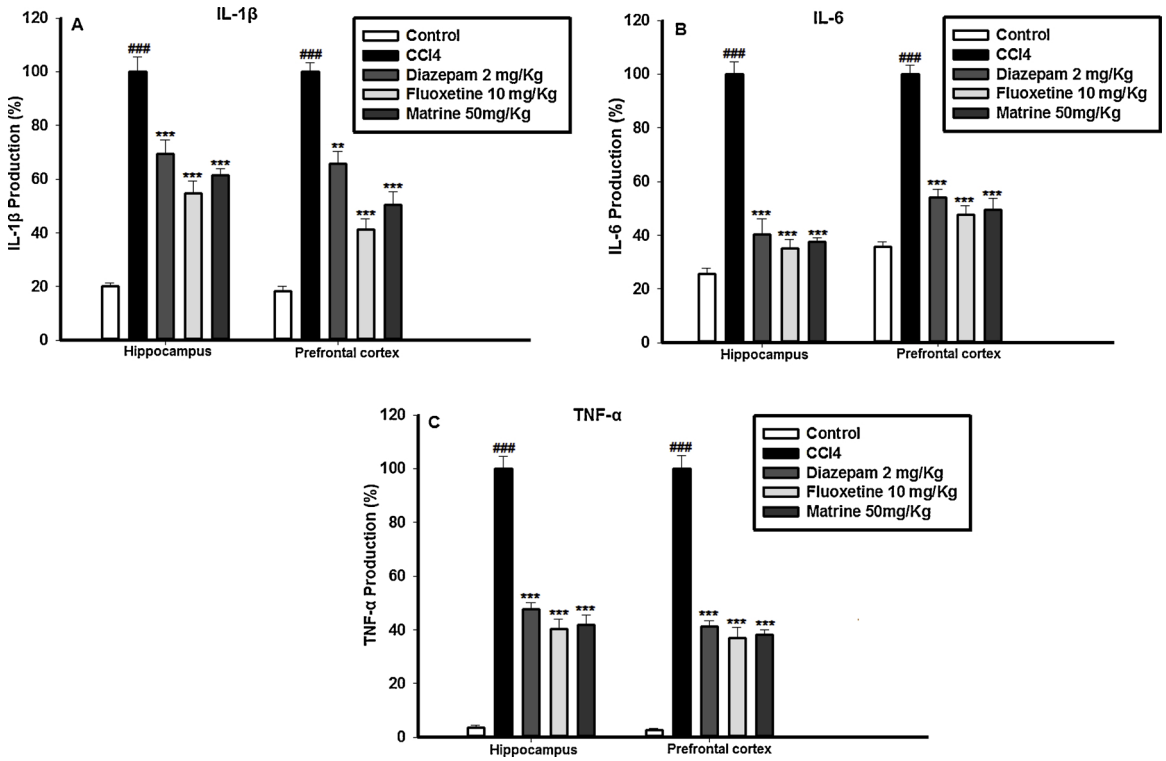

Fig. 6. Effect of matrine, diazepam, and fluoxetine on CCl4-induced proinflammatory cytokines (A) IL-1β, (B) IL-6), and (C) TNF-α level in the hippocampus and prefrontal cortex of mice brain. The results were expressed in percentage. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$ compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

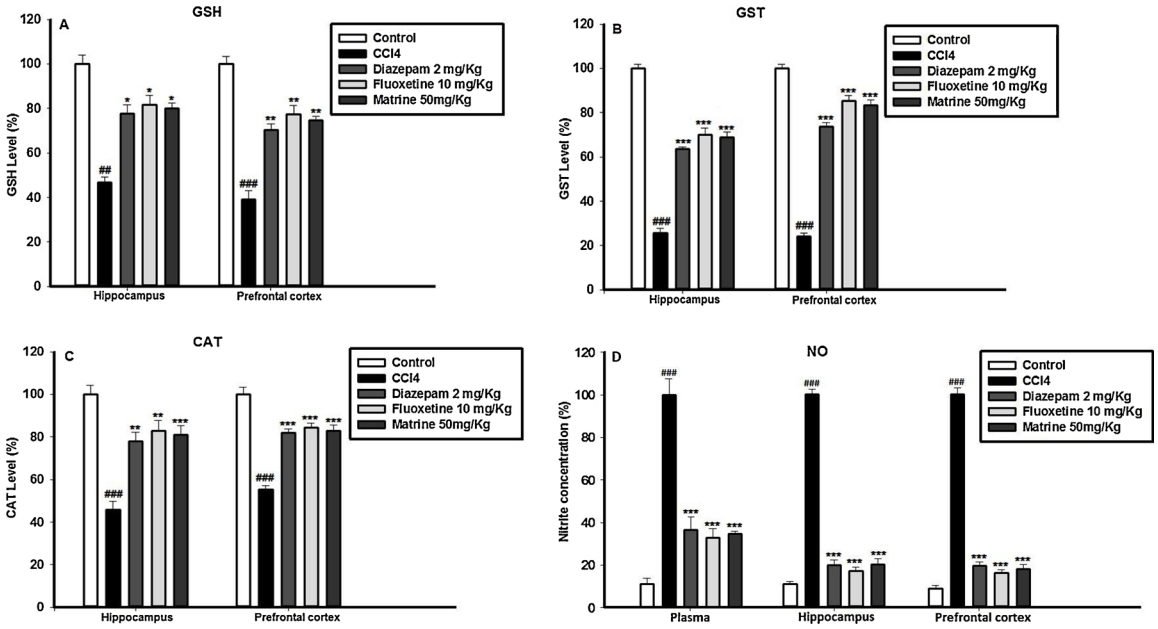

Fig. 7. Effect of matrine, diazepam, and fluoxetine on CCl4-induced oxidative stress which was evaluated by (A) GSH, (B) GST, and (C) catalase level in hippocampus and prefrontal cortex of mice brain (D) nitrite concentration in plasma and mice brain (hippocampus and prefrontal cortex). The results were shown in percentage. All data were expressed as mean (n = 5) ± SD. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to CCl4-treated group.

administration as compared to the vehicle control group. Matrine significantly reduced ($p < 0.001$) CCl4-induced elevated nitrite level both centrally (PFC and HC) and peripherally (serum). Similarly, fluoxetine and diazepam also suppressed NO level both centrally (PFC and HC) and peripherally (serum) (Fig. 7). Similarly, the MD level was remarkably raised ($p < 0.001$) in mice both centrally (PFC and HC) and peripherally (liver) after 24 h of CCl4 administration as compared to the vehicle control group. Matrine significantly reduced ($p < 0.001$) CCl4-induced elevated MDA level both centrally (PFC and HC) and peripherally (liver) (Fig. 8).

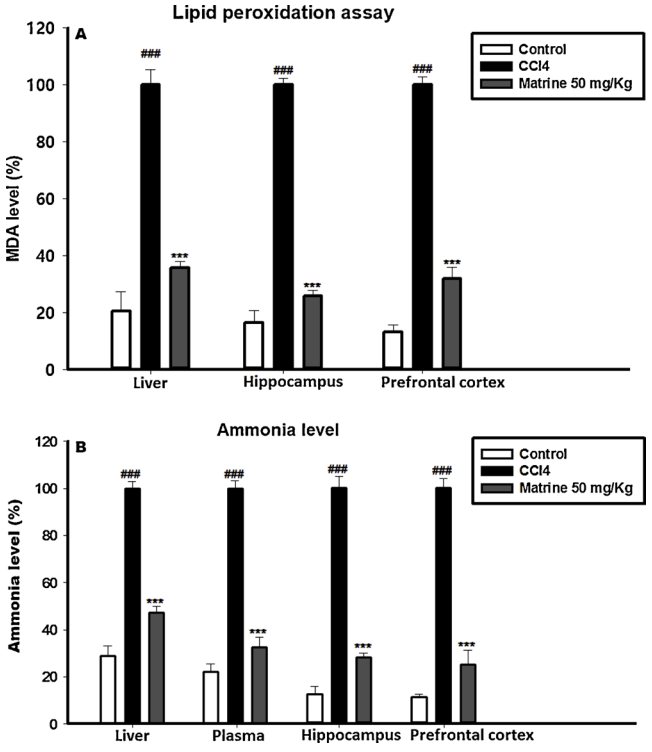

Fig. 8. Effect of matrine on CCl4-induced oxidative stress and ammonia concentration which was evaluated by (A) MDA level in the liver and mice brain (hippocampus, and prefrontal cortex) (B) Ammonia level in the liver, plasma, hippocampus, and prefrontal cortex. The results were shown in percentage. All data were expressed as mean (n = 5) ± SD. $^{\#\#\#}p < 0.001$compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

3.5. Effect of matrine in CCl4-induced ammonia level

The ammonia level was remarkably elevated ($p < 0.001$) in the mice liver, plasma, and brain (PFC and HC) after 24 h of CCl4 administration as compared to the vehicle control group. Matrine remarkably reduced ($p < 0.001$) CCl4-induced elevated ammonia level both centrally (PFC and HC) and peripherally (plasma and liver) (Fig. 8).

3.6. Effect of matrine in CCl4-induced serum corticosterone level

The corticosterone level was remarkably elevated ($p < 0.001$) in the serum of CCl4-treated mice. Matrine pretreatment significantly reduced ($p < 0.001$) the serum corticosterone level as compared to the CCl4-treated group (Fig. 9).

3.7. Effect of matrine on biochemical parameters of the hepatic and renal function

In order to confirm hepatotoxicity in CCl4-treated mice, hepatic biomarkers (ALT and AST) were evaluated in the plasma. It was observed that CCl4-treated mice showed enhanced ALT and AST activities ($p < 0.001$) when compared to the vehicle control group. Matrine remarkably reversed ($p < 0.001$) the elevated plasma ALT and AST activities in CCl4 treated mice. Additionally, CCl4 produced renal toxicity by enhancing plasma creatinine activities ($p < 0.001$) in the mice when compared to the vehicle control group. Matrine significantly reduced ($p < 0.001$) the plasma creatinine activities as compared to the CCl4-treated group (Table 2).

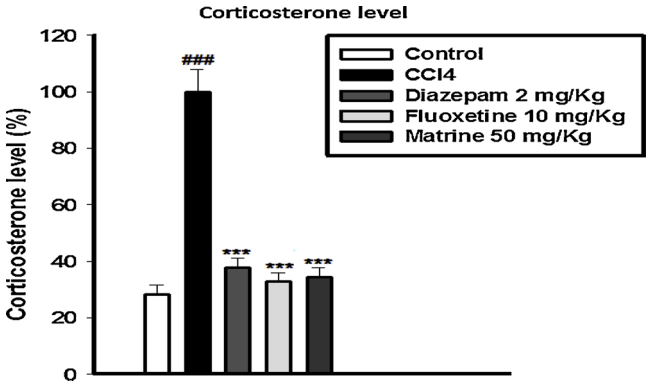

Fig. 9. Effect of matrine, diazepam, and fluoxetine on CCl4-induced changes in serum corticosterone levels. The results were shown in percentage. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$compared to control group; $^{***}p < 0.001$compared to CCl4-treated group.

Table 2
Effect of matrine on plasma ALT, AST, and creatinine enzyme activities in CCl4-treated mice.

Parameters	Vehicle control	CCl4	Matrine
ALT (IU/L)	41.33 ± 2.08	80.66 ± 2.30$^{###}$	54.66 ± 2.3***
AST (IU/L)	101.6 ± 2.51	200.33 ± 2.51$^{###}$	109.66 ± 3***
Creatinine (mg/dL)	0.51 ± 0.02	1.73 ± 0.03$^{###}$	0.71 ± 0.01***

All data were expressed as mean (n = 5) ± SD.
$^{###}$ $p < 0.001$compared to control group.
*** $p < 0.001$compared to CCl4-treated group.

3.8. Effect of matrine on histopathology of liver and hippocampus of mouse brain tissue

In histopathological studies of liver, photomicrography of vehicle control showed normal histology (Fig. 10A). While, photomicrography of the CCl4 treated mice demonstrated significant destruction, necrosis, and inflammation of the liver (Fig. 10B). Matrine significantly ameliorated the degeneration of liver tissue and nearly showed normal hepatocytes with minimal inflammatory infiltration (Fig. 10C). Additionally, the micrographs of mouse brain tissue sections are shown in (Fig. 11). A clear difference in the thickness of the granule cell layer of the dentate gyrus was noticed. The granule cell layer of the dentate gyrus in CCl4-treated mice was significantly thinner as compared to the vehicle control group. Matrine, fluoxetine, and diazepam significantly prevented this transformation (Fig. 11).

3.9. Effect of matrine on immunohistological markers of neurogenesis and apoptosis

Immunohistochemical staining of the hippocampus section revealed that CCl4-treated mice showed remarkable ($p < 0.001$) decreased in the expression of neurogenesis markers such as BDNF and VEGF. Matrine significantly increased ($p < 0.001$) the expression of BDNF and VEGF (Fig. 12). In order to further investigate the effect of matrine on hippocampal neurogenesis immunofluorescence staining of a true biomarker of neurogenesis such as GFAP was performed. Immunofluorescence analysis of hippocampus section showed remarkable reduction ($p < 0.001$) in GFAP cells in the hippocampus of the CCl4 group. Matrine treatment significantly ($p < 0.001$) increased the number of GFAP cells in the hippocampus (Fig. 13).

To evaluate the effect of matrine pretreatment on CCl4-induced apoptosis, caspase-3 (apoptotic biomarker) was analyzed in the hippocampus. It was observed that caspase-3 was considerably increased ($p < 0.001$) in the hippocampus of CCl4-treated mice. While matrine pretreatment decreased caspase-3 expression in the hippocampus significantly (Fig. 14).

3.10. Effect of matrine on CCl4-induced hippocampal DNA damage

In order to explore further, DNA damage in the neuron of the hippocampus was assessed by comet assay. It was observed that CCl4-

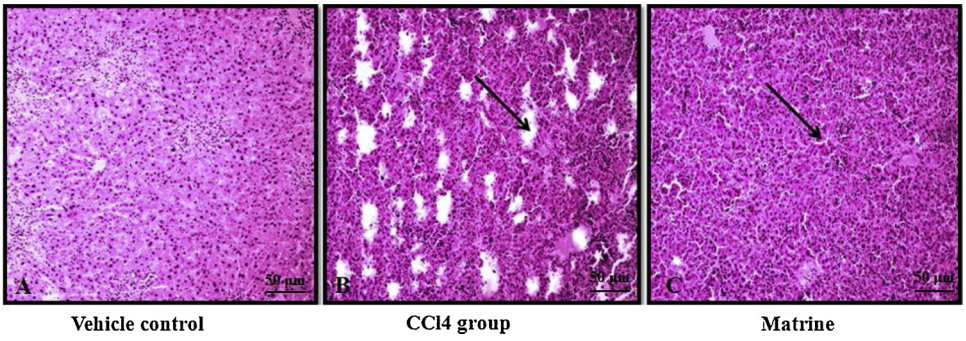

Fig. 10. Effect of matrine on histopathological changes in the liver of CCl4-treated mice. (H&E, × 200). (A) Liver of the vehicle control showing normal hepatocytes with no histopathological alteration (B) liver of the CCl4-treated mice showing disrupted hepatocytes and moderate inflammatory cellular infiltration (long arrow) (C) matrine group showing nearly normal hepatocytes with minimal inflammatory cellular infiltration (long arrow).

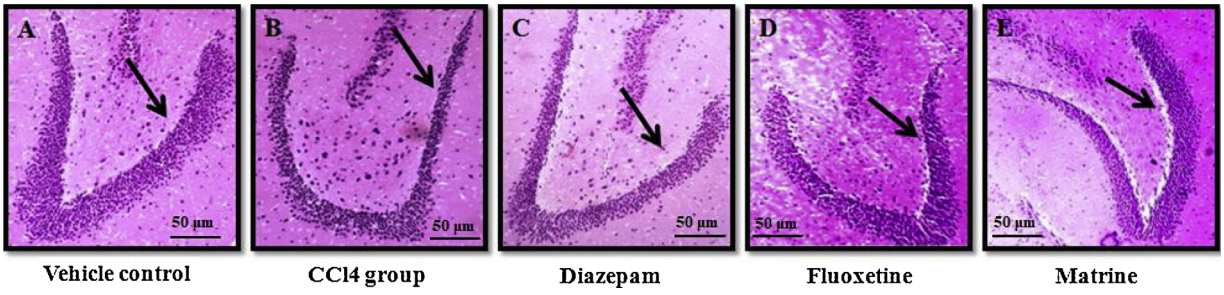

Fig. 11. Effect of matrine on the histopathology of the dentate gyrus of the hippocampus. The dentate gyrus of mice hippocampus subjected to CCl4 for 24 h is revealed in coronal sections stained with H&E at 200 × magnification. As shown by the arrow in (A and B), the granule cell layer of the dentate gyrus was thinner in the CCl4-treated group as compared to the control group. Whereas, as shown by the arrow in (C, D, and E) granule cell layer was thicker with fluoxetine, diazepam, and matrine treatments as compared to the CCl4-treated group.

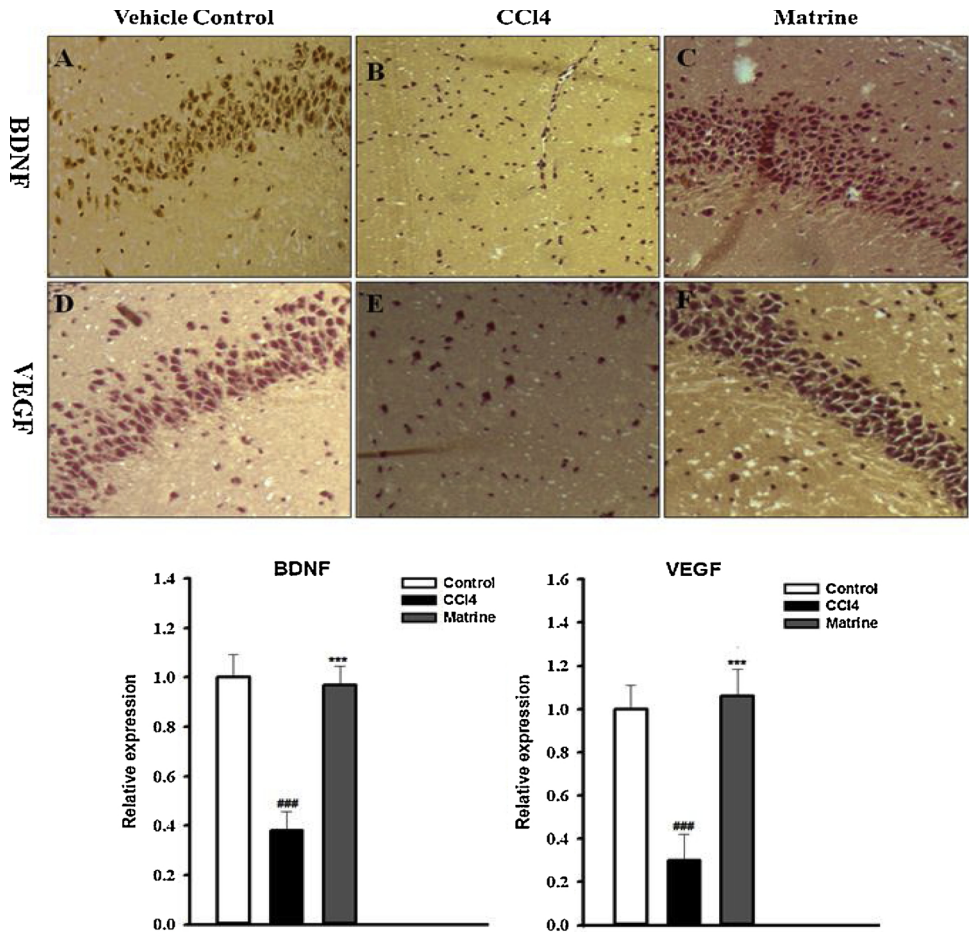

Fig. 12. Effect of matrine on the expression of BDNF and VEGF proteins in the dentate gyrus of mouse hippocampus. Representative micrographs of immunohistochemical staining for the BDNF protein (scale bar = 100 μm) (A) vehicle control (B), CCl4 and (C) matrine. Additionally, representative micrographs of immunohistochemical staining for the VEGF protein (scale bar = 100 μm) (D) vehicle control (E), CCl4 and (F) matrine. All data were expressed as mean (n = 5) ± SD. $^{###}p < 0.001$compared to control group; $^{***}p < 0.001$compared to CCl4-treated group.

treated mice showed significant enhancement ($p < 0.001$) in DNA damage by an increase in tail length and % DNA in the tail as compared to the vehicle control group. Matrine pretreatment significantly prevented ($p < 0.001$) DNA damage by a decrease in tail length and % DNA in the tail as compare to CCl4 group (Fig. 14).

4. Discussion

There are ample of evidence that several million people suffered from liver diseases (hepatitis and cirrhosis) exhibited psychiatric disorders such as anxiety and depression (Golden et al., 2005; Huang et al.,

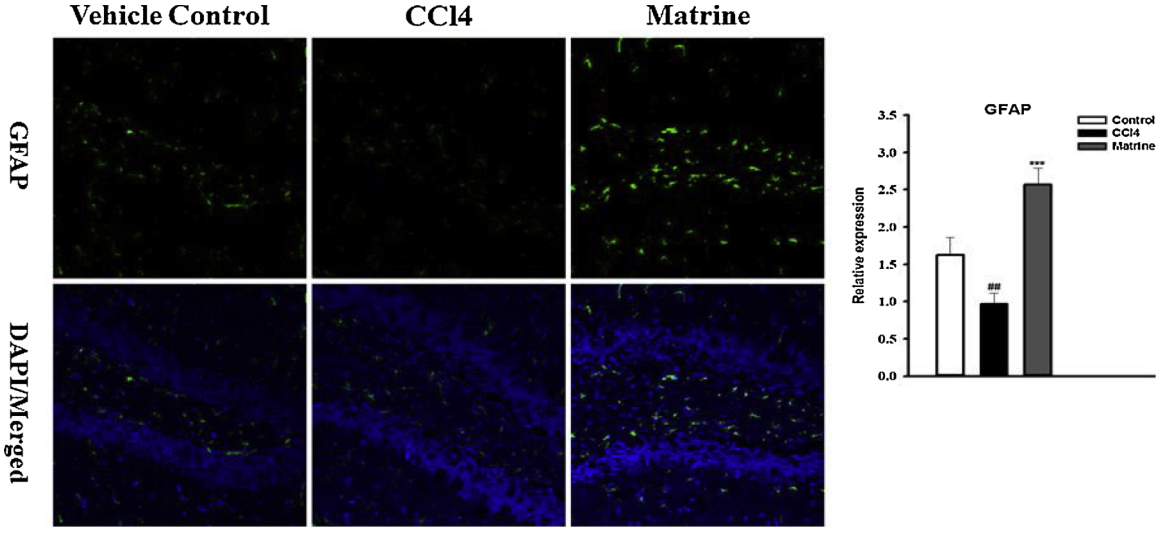

Fig. 13. Immunoreactivity of astrocytes (GFAP-positive cells) in the control, CCl4, and matrine group are shown. Scale bar = 40 μm. The GFAP-positive cells were visualized by FITC. Matrine increases the GFAP-positive cells in the hippocampus of CCl4 treated mice. Data presented was relative to control, while a number of experiment performed = 3, and (n = 5) ± SD. $^{###}p < 0.001$compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

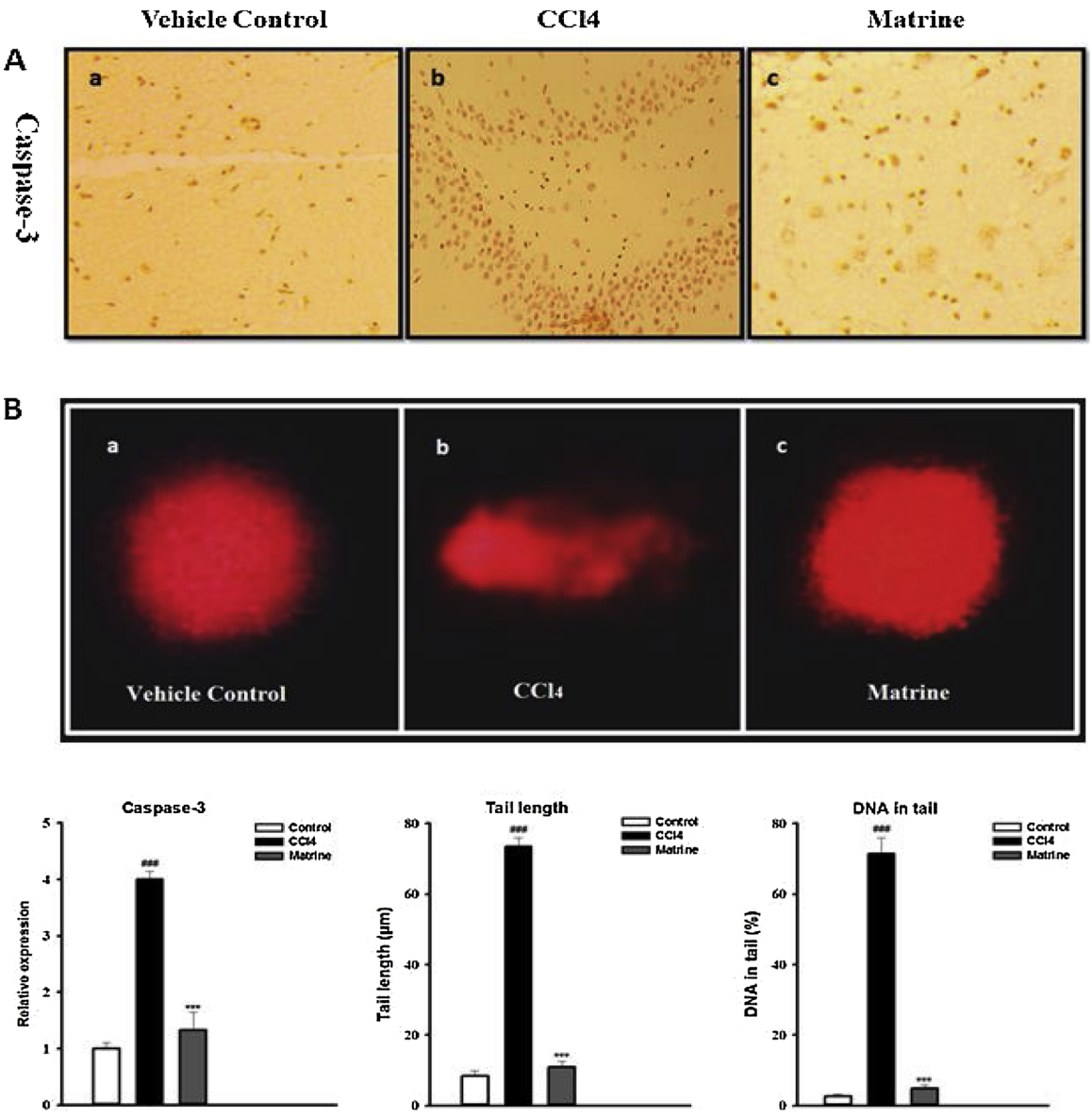

Fig. 14. Effect of matrine on the expression of caspase-3 proteins in the dentate gyrus of mouse hippocampus. (A) Representative micrographs of immunohistochemical staining of caspase-3 protein (scale bar = 100 μm) (a) vehicle control (b), CCl4 and (c) matrine. Additionally, the effect of matrine on the DNA damage of hippocampal neuron was investigated by performing a comet assay. (B) Representative fluorescence micrographs revealing the neuroprotective effect of matrine against CCl4-induced DNA damage of hippocampal neuron, (a) vehicle control (b), CCl4 and (c) matrine. The amount of DNA is measured by tail length and percent (%) of DNA in the tail. All data were expressed as mean ± SD. $^{###}p < 0.001$compared to control group; $^{***}p < 0.001$ compared to CCl4-treated group.

2017). Numerous studies revealed that liver failure or injury could lead to hyperammonemia which induced oxidative stress as well as neuroinflammation and triggers several neurological complications (Heidari et al., 2016). Studies have also been reported that hyperammonemia could act as a potential biomarker for anxiety and depression (Duan et al., 2015). Therefore, we established the CCl4-induced liver injury model of mice and observed the anxiety and depression-like behaviors through the behavioral and biochemical methods. Furthermore, the mechanism implicated in the development of anxiety and depression following liver injury was explored. CCl4 is a toxic lipophilic molecule, crosses cell membrane easily and well distributes in liver and brain tissues (Ritesh et al., 2015a). CCl4 elevate the oxidative stress and neuroinflammation in the liver as well as in various brain regions (cerebral cortex and hippocampus) of animal models (Makni et al., 2012; Ritesh et al., 2015b). CCl4 mediates hepatotoxicity after biotransformation by hepatic enzyme cytochrome P450 to generate trichloromethyl free radicals (CCl3•) which start attacks on lipids, membrane proteins, as well as thiols leading to necrosis of hepatocytes (Rahmat et al., 2014). Several studies reported that matrine exhibit neuroprotective properties through inhibition of neuroinflammation and oxidative stress in various rodent models (Kan et al., 2015; Meng et al., 2017). Based on this evidence, the current study explored the potential anxiolytic and antidepressant-like effects of matrine against CCl4-induced liver injury.

The behavioral results demonstrated that CCl4-treated mice exhibit anxiety as well as depressive-like behavior at 24 h post-CCl4 administration. In rodents, the behavioral models such as OFT, LDB test, and EPM test were performed for the evaluation of anxiety-like behavior. In the OFT, CCl4-treated mice demonstrated the remarkable reduction in the number of crossings and time spent in the center which clearly illustrated the anxiety-like behavior. In the same way, in the LDB test, CCl4-challenged mice illustrated anxiety-like behavior as indicated by

reduced time spent in the light compartment as well as the number of light-dark transitions. Similarly, in the EPM test, a remarkable reduction was observed in the number of open arm entries as well as time spent in opem arm which evidently signified the anxiety-like behavior by CCl4-challenged mice. In CCl4-treated mice, depressive-like behavior was indicated by a significant reduction in the immobility time in TST and FST when compared to the control group. Treatment with matrine reduced the anxiety and depressive-like behaviour by significantly improving the entire behavioral parameters related to anxiety and depression. Additionally, CCl4-challenged mice revealed a striking decrease in the water and food intake when compared to the control group. Reduced water and food intake by CCl4-treated mice may be due to hepatotoxicity (Al-Seeni et al., 2016). Matrine remarkably increased water intake and food consumption in CCl4- treated mice.

Mounting evidence reported that neuroinflammation plays a significant role in the pathophysiology of depression (Raison et al., 2006). Studies have been reported that proinflammatory cytokines (IL-1β, IL-6, and TNF-α) are elevated both centrally and peripherally in the patients of depression and anxiety, which signified immune dysregulation (Dowlati et al., 2010). Neuroinflammation produces an imbalance between oxidative stress and the antioxidant enzymes, which is also associated with depression (Kim et al., 2016). Biochemical results showed marked elevation in proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and nitrite contents in the brain (PFC and HC) of CCl4-treated mice. Matrine pretreatment remarkably reduced the levels of proinflammatory cytokines and nitrite contents in the brain (PFC and HC) of CCl4 treated mice. Additionally, it was also revealed that CCl4 induced oxidative stress by depleting antioxidants (GSH, GST, and CAT) and increasing MDA level in the brain (PFC, liver and HC) and liver of mice. Matrine diminished the oxidative stress by increased levels of antioxidants (GSH, GST, and CAT) and decreased level of MDA in PFC and HC of CCl4 treated mice.

The elevated level of plasma CORT is a biomarker of HPA-axis activity in rodents (Zhang et al., 2016). Plasma CORT is secreted in excess amount in animal models of depression and could return to the normal level by antidepressants (Raone et al., 2007). In the current study, pretreatment of matrine remarkably decreased the CCl4-induced enhancement in plasma CORT, thus, recommending that matrine has the function of normalizing hyperactivity of HPA-axis. Additionally, CCl4-challenged mice revealed a significant increase in liver and kidney enzymes such as ALT, AST, as well as creatinine. An elevated level of liver enzymes in plasma signifies the induction of acute hepatotoxicity by CCl4 and might be attributed to breaking down of liver tissue, permitting the escape of intracellular enzymes from the cytoplasm into the bloodstream (Al-Seeni et al., 2016). This data is reliable with other studies which indicated that exposure to CCl4 leads to hepatic and renal damage in rodent models (Hismiogullari et al., 2015). The current findings also indicated that CCl4-treated mice showed a marked increase in ammonia level in the liver, blood, and brain. Studies have been reported that hyperammonemia is a causative factor in neurological disorders by inducing oxidative stress and neuroinflammation in the brain (Heidari et al., 2016). Matrine pretreatment remarkably reversed the elevated plasma ALT, AST, creatinine, and ammonia level induced by CCl4. Furthermore, histopathology of the liver was examined. Liver tissues demonstrated various histopathological changes as a consequence of CCl4-induced hepatotoxicity which were consistent with previous studies (Al-Seeni et al., 2016). Matrine significantly improved the liver tissues.

In order to explore further therapeutic effects of matrine, histopathology of the hippocampus was examined. It was revealed that the cellular layer of the dentate gyrus of the hippocampus in the CCl4-group was found to be evidently thinner compared to the control group. Matrine remarkably prevented this tendency. Hippocampal neurogenesis refers to a complex neurobiological process of generating new neurons from neural stem cells (NSCs) (Borsini et al., 2015). The process of hippocampal neurogenesis play a crucial role in depression i.e. reduced hippocampal neurogenesis is associated with the pathogenesis of depression while antidepressant drugs enhanced hippocampal neurogenesis (Malberg and Schechter, 2005; Kim et al., 2016). GFAP (glial fibrillary acidic protein) is a true molecular biomarker for hippocampal neurogenesis and neuronal survival expressed by astrocytes (Zhang and Jiao, 2015). Astrocytes play a critical role in survival, proliferation, and maturation of neurons. It has been reported that antidepressant stimulate astrocytes leading to increased expression of vital growth factors (BDNF and VEGF) which are involved in the therapeutic action of antidepressant (Lee and Son, 2009; Kajitani et al., 2012). Numerous studies reported that expression of GFAP positive astrocytes were significantly decreased in animal models of depression while antidepressant increased the expression of GFAP positive astrocytes (Ding et al., 2017). It has been reported that antidepressant (Fluoxetine) showed glioprotective effects by increasing the number of hippocampal GFAP positive astrocytes in chronic stress models (Czéh et al., 2006). The present study revealed that CCl4 administration remarkably decreased GFAP positive astrocytes, BDNF and VEGF expressions in the hippocampus region of the brain. It indicates that CCl4 administration negatively affected the granular cells proliferation in the dentate gyrus thus decreasing hippocampal neurogenesis. On the other hand, matrine significantly enhanced hippocampal neurogenesis by increasing GFAP positive astrocytes, BDNF and VEGF expressions. The present results were consistent with a previous study that also showed that matrine contribute to neuroprotection against cerebral ischemia by increased GFAP-positive cells (Xu et al., 2012).

In addition to neurogenesis, apoptosis has been proposed to be another crucial mechanism implicated in the pathogenesis of depression (Kosten et al., 2008). In the present study, apoptosis was detected in the hippocampus by measuring apoptotic biomarkers such as caspase-3. It was revealed that matrine significantly reduced CCl4-induced apoptosis in the hippocampus by reducing the expression of caspase-3. These results were consistent with the previous studies which suggested that antidepressant reduced apoptosis by decreasing caspase-3 expression in the hippocampus (Chen et al., 2007). Another vital pathological mechanisms involved in the pathogenesis depression is increased DNA damage in the hippocampal neuron which occurs due to oxidative stress (Czarny et al., 2018). The DNA damage in the hippocampal neuron was measured by means of comet assay. The present study explored that matrine pretreatment remarkably prevented CCl4-induced DNA damage in the hippocampal neuron.

5. Conclusion

Taken together, the present study provides convincing evidence that matrine, a naturally occurring alkaloid, produced significant anxiolytic and antidepressant effects in the CCl4-induced animal model. The anxiolytic and antidepressant activity of matrine was achieved by the suppression of neuroinflammation, oxidative stress, and apoptosis while enhancing hippocampal neurogenesis in the mouse brain tissue. The present study demonstrated that matrine could be a potential candidate for the treatment of anxiety and depression and associated disorders. The present study also exposed that hyperammonemia produced as a result of liver injury induced oxidative stress, neuroinflammation, apoptosis and decreased neurogenesis in the brain resulting in anxiety and depression (Fig. 15). This study reveals that liver injury induced by CCl4 affects brain physiology leading to psychiatric alteration. However, the direct or indirect neurotoxic effects of CCl4 on the brain is yet to be analyzed. In this regard, more in-depth molecular investigations are required to elucidate the direct or indirect neurotoxic effects of CCl4 on the brain. Furthermore, more in-depth investigations are needed to elucidate the detailed molecular mechanism of matrine.

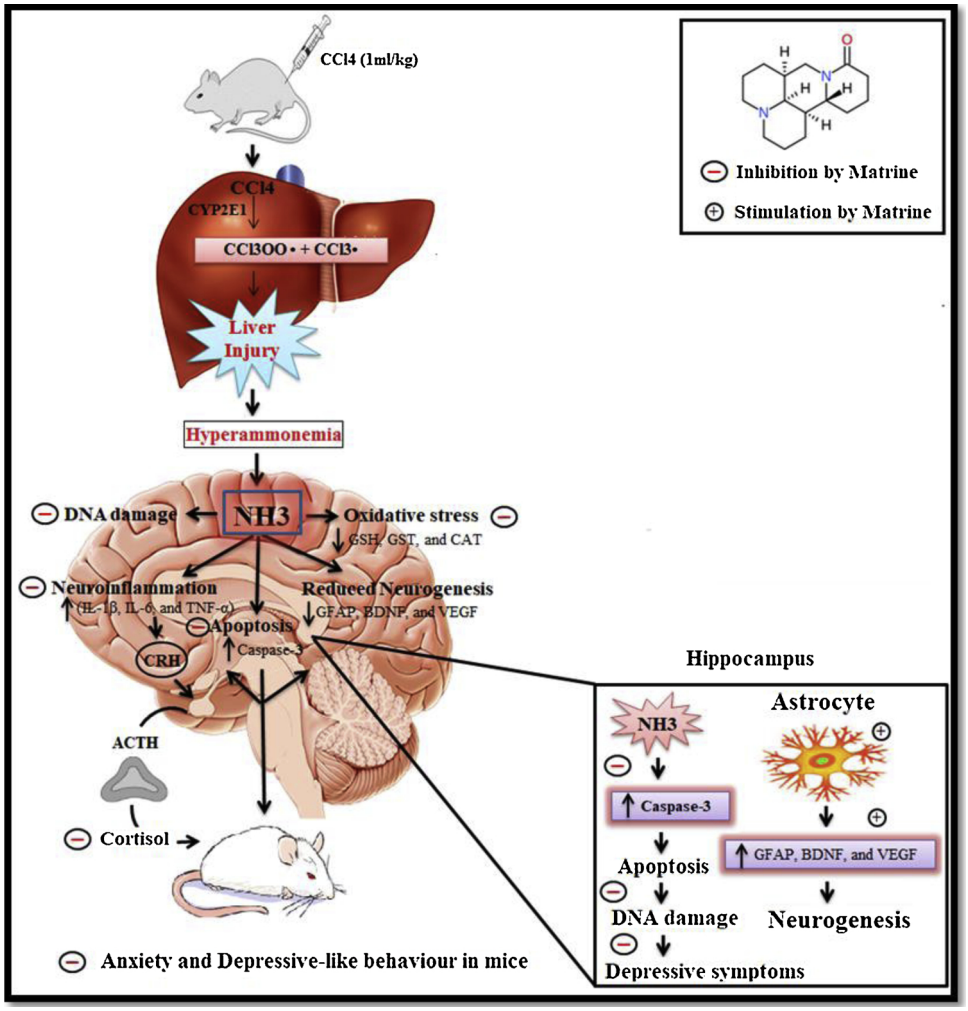

Fig. 15. Proposed model of the mechanism for the anxiolytic and antidepressant actions of matrine in CCl4-induced liver injury model of mice.

Acknowledgments

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