Pharmacological Research - Modern Chinese Medicine 15 (2025) 100624

Contents lists available at ScienceDirect

Pharmacological Research - Modern Chinese Medicine

journal homepage: www.elsevier.com/locate/prmcm

Analysis of potential neuropharmacological activity and attenuating effect in chronic constriction induced neuropathic pain using Calotropis procera (Aiton) Dryand flower ethanol extract

Ashutosh Kumar [a], Brijesh Kumar [b,*], Rajesh Kumar [c], Vinod Tiwari [d], Pratistha Singh [e], Ajay Kumar [b], Manish Singh [b], Chandra Shekhar Azad [f], Ankit Uniyal [d]

[a] Department of Pharmacy, Ashoka Institute of Technology and Management, Varanasi, India
[b] Department of Pharmacology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India
[c] Department of Pharmacology, Maharaja Agrasen School of Pharmacy, Maharaja Agrasen University, Solan, 174103, India
[d] Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (BHU), Varanasi, India
[e] School of Biotechnology, Banaras Hindu University, Varanasi, India
[f] Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

ARTICLE INFO

Keywords:
Neuropharmacology
Antiepileptic
Cognition
Anxiety
Antinociceptive
GC–MS

ABSTRACT

Background: *Calotropis procera*, also known as "毒竹 du zhu" in Chainese, is used in several remedies to treat a variety of ailments, including inflammatory diseases, skin concerns, pain disorders, and respiratory issues. It has been observed that the various parts of the plant have been traditionally used for as anti-inflammatory and neuroprotective actions. The flower of this herb has not been investigated for these pharmacological properties.
Purpose: The aim of this research is to investigate the neuropharmacological profile and ameliorative potential of ethanolic extract of *C. procera* flower (EECP) in chronic constriction injury (CCI) induced neuropathic pain in rats.
Methods: GCMS analysis was performed to identify the active phytocmpounds of the plant. Neuropharmacological profile has been investigated by maximal electroshock seizure and pentylenetetrazole for antiepileptic, elevated maze plus and open field test for anxiety, tail suspension and forced swim test for depressant activity, and acetylcholinesterase and Morris water maze test for cognition. Anti-neuropathic pain was assessed via heat hyperalgesia and mechanical allodynia tests in rats after inducing CCI. Pro-inflammatory mediators (TNF-α, IL-1β, and IL-6) were determined by ELISA kits. SOD and nitrile level were measured for antioxidant activity. Sciatic nerve's histopathological changes for nerve deformity were evaluated by H &E staining.
Results: GCMS analysis revealed the presence of phytocompounds Lupeool, acetate, n-hexadecanoic acid, γ-sitosterol, hexadecanoic acid methyl ester, Octadecenoic acid (Z)-, methyl ester, β-Amyrin, phytol and other compounds. In neuropharmacological profile, EECP had a significant anticonvulsant effect, a decrease in locomotor activity, indicating a sedative effect but showed no anxiolytic effect. The immobility time decreased significantly in both the forced swim test and tail suspension test. The activity of acetylcholinesterase in the brain was decreased and Morris water test results revealed a shorter escape latency and greater time spent in the target quadrant. In anti-neuropathic pain assessment, the EECP reduced CCI-induced hyperalgesia and mechanical allodynia. TNF-α, IL-1β, and IL-6 levels were reduced while SOD levels increased and nitrite levels decreased in the sciatic nerve. Histological analysis revealed sciatic nerve deformity was reduced.
Conclusion: It is concluded that extract showed a potent antiepileptic, antidepressant, cognition enhancer and protective against nerve deformity and neuropathic pain. Phytocompounds identified via GCMS having neuroprotective, antioxidant, and anti-inflammatory properties, which may be correlated with the neuropharmacological and analgesic activities of the extract.

Abbreviations: AChE, Acetylcholinesterase; BW, Body weight; EECP, Ethanolic extract of *Calotropis procera*; MES, Maximal electroshock seizure; PTZ, Pentylenetetrazole.

* Corresponding author.
E-mail addresses: asthwal@rediffmail.com (B. Kumar), vtiwari.phe@iitbhu.ac.in (V. Tiwari), ankit.uniyal.rs.phe18@itbhu.ac.in (A. Uniyal).

https://doi.org/10.1016/j.prmcm.2025.100624
Received 9 February 2025; Received in revised form 3 May 2025; Accepted 3 May 2025
Available online 3 May 2025

1. Introduction

Mental health treatment is one of the most difficult areas in health care. Neurological illnesses are the leading cause of disability and the second leading cause of death worldwide [1]. The most common neurological and cognitive illnesses include epilepsy, Parkinson's disease, depression, anxiety, and Alzheimer's disease [2]. In some regions of the world, the majority of people continue to use traditional medicine to fulfill their basic health care needs. Plants that have traditionally been used as medicine contain a wide range of phytoconstituents that can treat a wide range of diseases [3].

C. procera is a plant in the Apocynaceae family that has been used traditionally to treat fever, asthma, rheumatism, digestion, colds, eczema, boils, elephantiasis, leprosy, cough, diarrhea, intestinal worms, and heal leucoderma [4].

The medicinal plant *C. procera* (Aiton) Dryand occur under its common name apple of Sodom or giant milkweed while existing as a member within the Apocynaceae family. Within traditional Chinese medicine (TCM) practitioners recognize *C. procera* as an effective therapeutic agent that treats inflammatory diseases together with skin conditions and pain disorders alongside respiratory complaints [5]. The plant exhibits a latex-rich material that includes active compounds including alkaloids together with flavonoids terpenoids glycosides and steroids responsible for its extensive therapeutic uses. The traditional Chinese medicine implements *C. procera* because of its dual effects on pain management and inflammation control [6]. The plant has medical applications for treating both joint pain and arthritis together with muscle spasms and rheumatism by improving blood circulation and decreasing inflammation. Both latex and root extracts from the plant get frequently used as poultice applications to treat swelling and sprains and various musculoskeletal disorders [7]. Traditional Chinese medical records show how practitioners used *C. procera* to remove wind and dampness elements from the body which matches TCM methods of rheumatic diseases treatment. Medical practitioners combine *C. procera* with additional plant substances to strengthen its capacity as an herbal medicine for reducing pain [8]. According to TCM practices the latex substance gets combined with various other natural ingredients to create ointments and pastes meant for external skin use. The substance requires careful use since it shows toxic properties yet healthcare providers tend to apply diluted latex solutions for treatment purposes [9].

Chinese medical practitioners employ *C. procera* to manage various respiratory problems including asthma as well as bronchitis and chronic coughs [10]. The roots and flowers of *C. procera* are used to create decoctions that medical practitioners frequently recommend for respiratory conditions involving mucus-related problems or breathing congestions. The practitioners who prescribe *C. procera* team it with *Glycyrrhiza uralensis* (licorice root) for stronger expectorant effect when treating respiratory obstructions [11]. The Traditional Chinese Medicine community values *C. procera* because it helps control digestive processes and treats stomach health problems [12]. People use latex along with root extracts from this plant because these substances effectively remove parasites and toxins from the body by acting as purgatives and anthelmintics. Administrative protocols for *C. procera* exist because its purgative effect can be potent [13]. The proper transformation methods and mixture preparation techniques enable the production of safe medications from this potentially toxic herb. The medicinal potential of *C. procera* in TCM remains a priority for scientific investigations because ongoing research proves its diversity of therapeutic uses in Chinese healing traditions [14].

Latex of the plant has been investigated for its potent antinociceptive activity. The goal of this research was to look into the neuropharmacological effects of an ethanolic extract of EECP flower in different animal models along with the identification of chemical compounds by GC–MS analysis [15–17].

One-sixth of the population is assumed to be affected by chronic pain. Chronic neuropathic pain is caused by nerve tissue damage, inflammation, or injury, and it has a high sensitivity to painful stimuli (hyperalgesia), which creates painful perception. It is frequently debilitating and treatment-resistant. Interleukin-1 (IL1β), tumour necrosis factor (TNF)-α, bradykinin, and nerve growth factor increase sodium and calcium currents at the nociceptor peripheral terminal, causing action potential discharge [18]. Peripheral immune cells and microglia in the spinal cord produce these same inflammatory mediators after neural damage, which contribute to neuropathic pain by activating nociceptive neurons [19]. Reactive oxygen species (ROS) have been linked to the development of chronic pain following nerve injury or an inflammatory offend. Interleukin (IL)-6 and other proinflammatory cytokines play important roles in neuronal response and inflammation [20–23].

The purpose of this study is to determine the analgesic and anti-inflammatory effects of EECP in CCI-induced neuropathic pain along with its applications in neuropharmacological activities.

2. Materials and methods

2.1. Preparation of extracts

The flowers of *C. procera* were collected from the from Central Nursery, Banaras Hindu University, Varanasi, India, in the month of April. Dr. Ashwini Kumar Kushwaha, Assistant Professor, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, authenticated the sample. Specimen with voucher number 2019–02 was deposited in the Herbarium at Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University (Barkachha) the flowers were shade dried and crushed into powder at room temperature. Percolation of milled power was made with 95 percent ethanol in a ratio of 1:7 w/v. The extract solvent was evaporated using a Buchi Rotary Evaporator R-210 Advanced, Switzerland. The yield percentage was found to be 11.2 percent w/w. Further study was conducted using a dried extract that had been prepared.

2.2. Drugs and chemicals

Silica gel (Merk Life Science Pvt. Ltd.), pentylenetetrazole (Sigma Laboratories), phenytoin, diazepam, scopolamine, imipramine, diclofenac, were purchased from Sigma-Aldrich, and organic solvents (ethanol, petroleum ether, and ethyl acetate) were purchased from Sisco Research Laboratories Pvt. Ltd. gabapentin (Neurontin) was purchased from Sigma-Aldrich, TNF-α, IL- 1β, and IL-6 assay ELISA kits were procured from Elabscience, Texas. Ethanol and other reagents were procured from Sisco Research Laboratories Pvt. Ltd.

2.3. Animals

Male Charles Foster rats weighing 200–280 g were provided by the Animal House, Institute of Medical Sciences, Banaras Hindu University. The rats were kept in the animal facility of the Department of Pharmaceutical Engineering at IIT, BHU, with standard food and water. The temperature in the house was maintained at a constant of 25 °C. All of the research was approved by the Central Animal Ethical Committee of Banaras Hindu University (Reg. No. 542/GO/Rebi/S/02/CPCSEA dated 26.5.2017) in accordance with CPCSEA guidelines. A limitation to the generalisability of the study is that it did not consider gender/sex issues.

2.4. GC–MS analysis

Gas Chromatograph Mass Spectrometer (GCMS) - Shimadzu QP-2010 Plus with Thermal Desorption System TD 20 was used for the analysis of EECP. Column oven temperature 100 °C, injection temperature 260 °C, column flow 1.21 mL/min, split ratio 10:0, ion source temperature 220 °C, interface temperature 270 °C, flow control mode-

linear velocity, pressure-90.4 kPa, solvent cut time 2.5 min, detector gain mode-relative, threshold-1000 were used for analysis. MS conditions start time 3.0 min, end time 39.98 min, ACQ mode-scan, and event time 0.20 *sec* were used. The parameters start *m/z* 40.00 and end *m/z* 650.00 were utilized [24].

2.5. Assessment of anticonvulsant activity

2.5.1. Maximal electroshock seizure (MES) test

A total of 25 rats divided into five groups. Group 1 (Control) received the vehicle, and Group 2 received phenytoin (25 mg/kg i. p.). EECP flower was given to groups 3, 4, and 5 at doses of 100, 200, and 300 mg/kg BW., respectively. All treatments were given to animals 45 min before the test, orally. The 150 mA of current through a corneal electrode (Electro- convulsometer, model no 100–3) was applied to induce generalized tonic-clonic- seizures in rats. The readings were taken as tonic flexion, tonic extensor phase, stupor, and recovery or death. In generalised tonic-clonic seizures, the reduction in the ratio of tonic extensor phase to flexion (E/F ratio) has been used as a measure of medication efficacy [25].

2.5.2. Pentyleneterazole (PTZ) induced convulsions

A total of 25 rats were divided into 5 groups. Group 1 (Control) received a vehicle while Group 2 received Diazepam 4 mg/kg i. p.) EECP flowers were given to groups 3, 4, and 5 at doses of 100, 200, and 300 mg/kg p. o. PTZ 50mg/kg i. p. To induce convulsions, PTZ 50mg/kg i. p. was given to the animals. The anticonvulsant activity was measured by delay in onset of a jerk, abolition of convulsion, and protection against PTZ-induced convulsion [26].

2.6. Assessment of anxiety activity

2.6.1. Elevated plus maze test

The anxiolytic and anxiogenic responses of the extract were investigated using the elevated plus maze test. It generates an approach-avoidance conflict which is stronger in the open arm compared to the enclosed arm. The anxious rat spent more time in the enclosed arm. The Elevated plus maze consists of two open arms (48.5 × 10 cm) and two close arms (48.5 × 10 × 35.5 cm) with an open roof. The apparatus is elevated to a height of 60.5 cm from the floor. Animals are divided into 5 groups (5 rats in each group). Group 1 (Control) received a vehicle, Group 2 received Diazepam 2 mg/kg i. p.). EECP flowers at doses of 100, 200, and 300 mg/kg p. o. were given to groups 3, 4, and 5. The experiment started 45 min after the rats were given the medication and ended five minutes later. Animals were placed in the central open square facing toward one of the close arms. The number of entries and time spent in closed arms, as well as the number of entries and time spent in open arms, were recorded during the 5-minute test. To be counted, all four paws must be entered into the arms [27].

2.6.2. Open field test

An open field test was carried out to determine the anxiolytic action of the extract on the rat. The floor of an open field measured as 60 × 60 cm. The floor is divided into white and black squares of diameter 15 × 15 cm. The height of the open field apparatus is 60 cm. The animal was placed in the central region and the activity of all grouped animals was noted for 5 min. The number of squares crossed, time spent in the central region, and central rearing time were noted during the experiment. If the animals spend more time in the central region after taking the medication, it is considered that the extract or medication has anxiolytic properties [28].

2.7. Assessment of depressant/ antidepressant activity

2.7.1. Forced swim test

Rats were forced to swim in an open cylindrical container (height 42 cm, diameter 16 cm) filled with fresh water at a temperature of 25 °C and a height of 15 cm. The treatment was given 45 min before the test. Individual rats were placed in containers and their immobility time was assessed during the 5-minute test. When the rat remained motionless, floating passively with its head above the water's surface, it was said to be immobile. The decrease in the immobility period is indicated by the antidepressant effect. Five groups of animals were used in the experiment. Group 1 was given a vehicle, and Group 2 was given imipramine (10 mg/kg p. o.). EECP flowers at 100, 200, and 300 mg/kg p. o. were given to groups 3, 4, and 5 [29].

2.7.2. Tail suspension test

A tail suspension test was used to identify the depressant action of the drug. Rats were suspended from a metal stand at a height of 57 cm. Adhesive tape was used to hang rat tails 1 cm from the distal end. The test was performed for 5 min. Rats were considered immobile when they hang passively. A decrease in immobility time is considered an antidepressant effect of the drug. For the test, the animals were separated into five groups. Group 1 was given a vehicle, while Group 2 was given imipramine (10 mg/kg p. o.). EECP flowers were given to groups 3, 4, and 5 at doses of 100, 200, and 300 mg/kg p. o., respectively) [30].

2.8. Assessment of effect on cognition

2.8.1. Morris water maze test

The water maze is a big circular metal pool that is filled with water (25 °$C \pm 1$ °C) and has an 85 cm circumference and a 30 cm height. The water-filled region is separated into four quadrants that are equal in size. A platform (10 cm diameter and 21 cm height) was placed slightly above the water surface, and the animals were left to find their way to the platform for training. The escape platform was then moved to the center of one quadrant, 1 cm below the water's surface, with swim starting locations in the other quadrant. The swim trials took place over four days, with different starting positions selected each day, but the site stayed the same on each day. From one of the starting points, each rat was placed in the water facing the pool's wall (N, E, S, W). The animals were permitted to swim for 90 s until they reached the platform. The time it took to get to the platform was used to calculate escape latency. If an animal was unable to reach the platform, the escape latency was recorded for 120 s. On the fifth day, a probe trial was conducted without the use of an escape platform. The time spent in the target quadrant is recorded as a spacial memory. The higher time spent in the target quadrant and the lower escape latency was interpreted as more cognition [31].

2.8.2. Inhibition of mouse brain acetylcholinesterase (AChE) (ex vivo ***experiments)***

The brain was removed and placed separately on an ice-cold plate after 4 hours of EECP and neostigmine treatment before being rinsed with iced buffer (0.5 M sodium phosphate, pH 7.4). The frontal cortex was taken right away, homogenised in 1.0 mL of buffer, and centrifuged at 9000 g for 5 min. As an AChE source, the supernatant was collected. AChE levels in brain homogenates (25 µL) were determined by mixing 100µ L Ellman's reagent with 190 µL propionylthiocholine iodide (75 mM). At 25 °C, the reaction mixture was incubated. The absorbance (412 nm) was measured after 60 min. The enzymatic reaction was stopped by adding 1.0 mL of quinidine sulphate (0.5 %). A blank reading was obtained without incubating the reaction mixtures. Each measurement of absorbance was double-checked [32].

2.9. Anti-inflammatory activity

2.9.1. Carrageenan induced rat paw edema

This test was carried out using the Plethysmometer. The paw volume was calculated using the mercury displacement caused by paw dipping. Carrageenan (0.1 % w/v in NS) solution 0.1 ml was injected beneath the

sub plantar tissue (right hind paw) to induce edema in all groups after 45 min of treatment drug (EECP 100 mg, 200 mg, and 300mg/Kg p.o.) and standard drug (Indomethacin 20 mg/kg s. c.). Animal paw volume was measured at 0, 30, 60, 120, and 180 min and expressed as paw volume increased (ml) [33].

2.9.2. Histamine induced rat paw edema

This test was carried out using the Plethysmometer. The mercury displacement caused by paw dipping was used to calculate paw volume. After 45 min of treatment drug (EECP 100 mg, 200 mg, 300mg/Kg p. o.) and standard drug (Indomethacin 20 mg/kg s.c.), histamine (0.2 % w/v) solution, 0.1 ml underneath the sub plantar tissue (right hind paw), was injected to induce edema in all groups. Animal paw volume was measured at 0, 30, 60, 120, and 180 min and an increase in paw volume (ml) was calculated [34].

2.10. Chronic constriction injury model

2.10.1. Animal groups

Sixty rats were randomly divided into six groups of ten rats each. The treatment started on the seventh day after surgery and was repeated once a day until the fourteenth day after the ligation. On days 0, 7, and 14, after surgery, the thermal withdrawal latency and paw withdrawal threshold (mechanical allodynia) were measured. On day 14 of the experiment, animals were sacrificed using di-ethyl-ether, the ipsilateral sciatic nerve and spinal cord were harvested for histological analysis, ELISA testing, and antioxidant measurement [35].

Group I (Normal control): No surgery was performed on rats Group II (CCI control): CCI+ Vehicle

Group III (Standard drug): CCI+gabapentin (Neurontin)

Group IV, V, and VI (Test drug): CCI+ (100, 200, and 300 mg/kg b. w. (p.o.) respectively) of EECP.

2.10.2. Induction of neuropathy by *CCI*

Neuropathic pain was induced by chronic constriction injury to the sciatic nerve. Ketamine (80 mg/kg i. p.) and xylazine (10 mg/kg i. p.) were used to anaesthetize the rats. The left sciatic nerve was exposed and the surrounding tissue was removed. Above the trifurcation, the sciatic nerve was ligated with chromic gut (silk4–0) at four points around the nerve, separated by 1 mm, and tightened until the ipsilateral hind limb twitched for the first time. After the ligation, the muscles and skin were sutured separately. During the surgery, sterile conditions were maintained. To prevent microbial infections, povidone-iodine was applied to the sutured area [36].

2.10.3. Behavioral *tests*

2.10.3.1. Mechanical allodynia (Von frey test). The paw withdrawal threshold (PWT) in gram was measured using Von Frey filaments to assess mechanical allodynia. Rats were placed in a transparent plexiglass box on a wire mesh platform. The filaments were gently bent perpendicular to the plantar region of the hind paw with increasing strength (ranging from 2 g of force to 15 g.). The forces were applied until the filament was slightly curved. Paw withdrawal and licking were considered allodynic or positive responses; otherwise, the filament was applied at a higher force strength. The mechanical allodynic threshold was defined as the force at which a response occurs. This test was performed on days 0, 3, 7, and 14 after the surgery, and readings from all the treatment groups were recorded [37].

2.10.3.2. Thermal hyperalgesia test. Hargreaves test was used to assess thermal hyperalgesia. The paw withdrawal latency to radiant heat stimulation was measured. The rats were placed on a glass floor in elevated Plexiglas cages and given an additional 15–25 min to acclimate to their surroundings before being tested. Under the glass floor beneath the hind paw, a radiant heat source was focused on the plantar surface of the ipsilateral side. To save the paw and avoid tissue damage, the cut-off time was reduced to 20 s. This test was performed on days 0, 3, 7, and 14 after the surgery, and readings from all the treatment groups were recorded. After three readings, the mean latency of the withdrawal responses for each foot was calculated [38].

2.10.3.3. Preparation of tissue homogenate. On day 14 of the operation, the rats were euthanized using di-ethyl-ether. The sciatic nerve and spinal cord tissue were excised. The tissues were minced into small pieces and rinsed in ice- cold PBS (0.01 M, pH 7.4) to remove hemolyzed blood. Tissue pieces were weighed on an ice-cold surface and

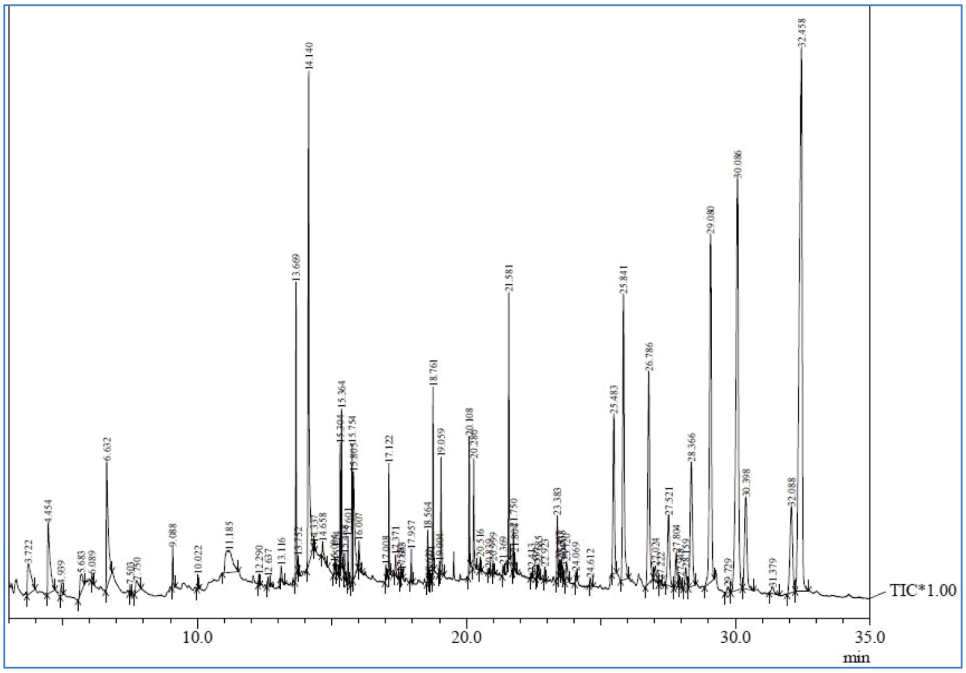

Fig. 1. GC–MS chromatogram of EECP flower.

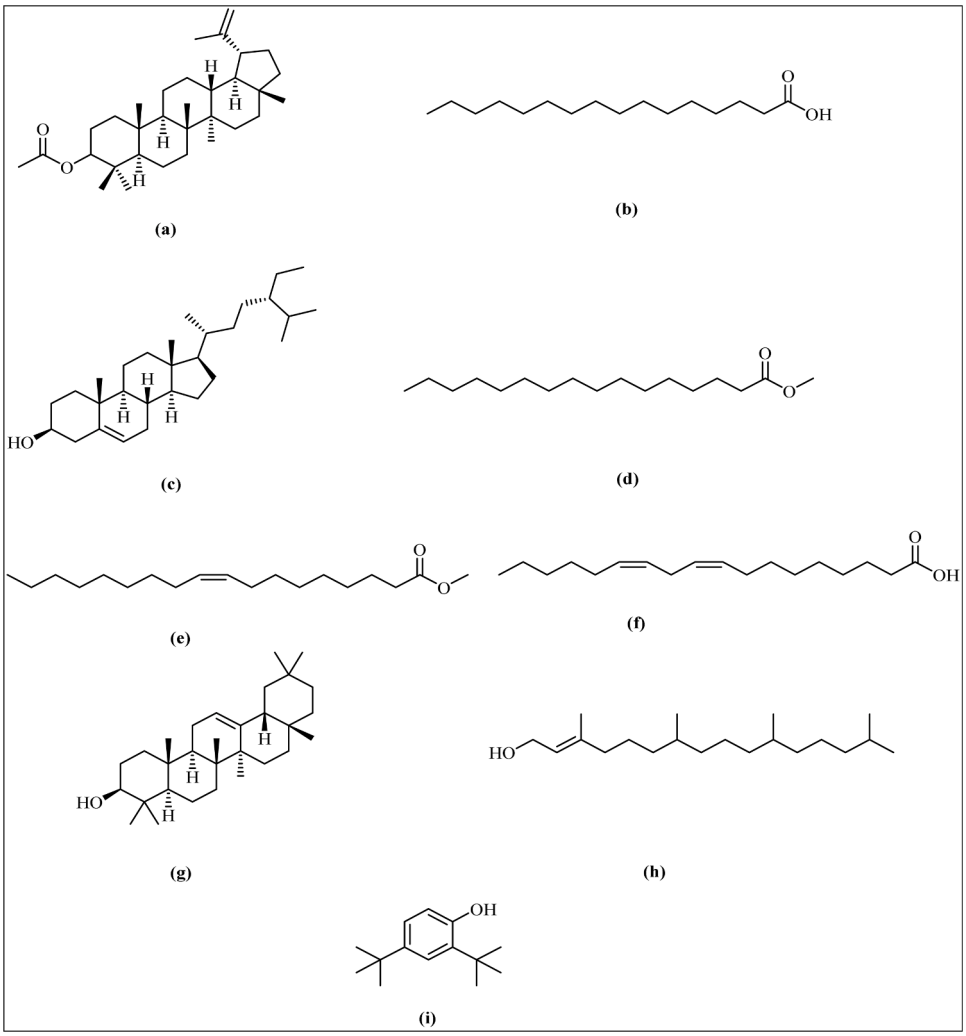

Fig. 2. Structures of various compounds identified through GC–MS analysis *(a) Lup-20(29)- en-3-ol, acetate (b) n-hexadecanoic acid (c) gamma.-Sitosterol (d) hexadecanoic acid methyl ester (e) 9-Octadecenoic acid (Z)-, methyl ester (f) 9,12-Octadecadienoic acid (Z,Z)- (g) beta.- Amyrin (1.70 %) (h) Phytol (i) 2, 4-Di-tert-butylphenol.*

homogenized in PBS with a glass homogenizer (tissue weight (g): PBS volume (mL) =1:9). The homogenate was then centrifuged for 5 min at 5000 g. The clear supernatant obtained was used for Elisa and other biochemical estimations [39].

2.10.3.4. Elisa test: TNF-α, IL-1β and IL-6 estimation. TNF-α, IL-1β, and f IL-6 levels were measured in the sciatic nerve and spinal cord using the R&D Systems Elabscience Rat TNF-α, IL-1β and f IL-6 Elisa kit. The experiment was carried out in accordance with the manufacturer's instructions [40].

2.10.3.5. Superoxide dismutase assay (SOD). In the test, 0.1 mM EDTA, 50 mM sodium carbonate, and 96 mM nitro blue tetrazolium were used. 2 mL of the above mixture, 0.05 mL of hydroxylamine, and 0.05 mL of the supernatant were added to the cuvette, and auto-oxidation of hydroxylamine was measured using a spectrophotometer at 560 nm for 2 min at a 30-second interval [41].

2.10.3.6. Nitrite estimation. The supernatant of the tissue homogenate was used for nitrite estimation using the Greiss reagent. A measure of 500 ml of Greiss reagent (1:1 solution of 1 % sulphanilamide in 5 % phosphoric acid and 0.1 % napthaylamine diamine dihydrochloric acid in water) was added to

100 ml of sciatic nerve supernatant and absorbance was measured at 546 nm using a spectrophotometer. Nitrite concentration was calculated using a standard curve for sodium nitrite and nitrite levels were expressed as mg/ml. Griess spectrophotometric analysis was used to estimate nitric oxide levels indirectly [42].

2.10.3.7. Histological evaluation. On the 14th day of the study, parts of sciatic nerve samples were collected and stored in a fixative solution (10 percent formalin). Dehydrated samples were embedded in paraffin. A microtome was used to section paraffin blocks at 4 μm transversely. Hematoxylin and eosin were used to stain the sectioned tissue, which was then observed under a high-powered light microscope [25].

2.11. Statistical analysis

The results of all the tests and biochemical parameters were analysed using one-way ANOVA followed by Dunnett's multiple comparisons tests, while the results of the escape latency (Morris water maze test), carrageenan-induced rat paw edema, and histamine-induced rat paw edema were analysed using two-way ANOVA followed by Dunnett's multiple comparisons tests. The data was presented in the form of a mean ± Standard error of the mean (SEM). The statistical analysis was carried out using the GraphPad Prism 8.0.2 software. In the comparison of outcomes, a p-value of <0.001 was considered significant.

Table 1
Major Chemical composition (%) of identified compounds in ethanolic extract of *Calotropis procera* flowers by GC–MS study and compared with NIST library.

S. No.	R. Time	Peak area	Area % age	Compounds Name
1.	20.839	84,677	0.03	n-Tetracosanol-1
2.	20.999	373,365	0.14	Eicosane
3.	21.369	378,925	0.14	Alpha.-TocospiroB
4.	21.581	5550,788	2.02	1-Eicosanol
5.	21.750	878,955	0.32	Tetracosane
6.	21.804	425,736	0.15	Eicosyl heptafluorobutyrate
7.	22.413	141,753	0.05	Eicosyl heptafluorobutyrate
8.	22.597	61,595	0.02	Hexatriacontane
9.	22.685	432,363	0.16	Cholesta-2,8-dien-6-ol, 14-methyl-, acetate, (5.alpha.,6.alph
10.	22.923	382,215	0.14	Stigmasta-4,7,22-trien-3.alpha.-ol
11.	23.383	1879,396	0.68	1-Eicosanol
12.	23.467	353,591	0.13	1-Hexacosanol
13.	23.538	402,472	0.15	Stigmasta-5,22-dien-3-ol, acetate, (3. beta.)-
14.	23.604	147,337	0.05	Hexatriacontane
15.	23.720	1115,605	0.41	1-Heptacosanol
16.	24.069	326,097	0.12	2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridec
17.	24.612	330,266	0.12	Ergosta-5,7-dien-3-ol, (3.beta.)-
18.	25.483	7825,026	2.84	Ergost-5-en-3-ol, (3.beta.,24r)-
19.	25.841	14,327,300	5.21	Stigmasterol
20.	26.786	12,496,149	4.54	Gamma.-Sitosterol
21.	27.024	917,613	0.33	Stigmasta-5,24(28)-dien-3-ol, (3. beta.)-
22.	27.222	226,366	0.08	Ergosta-8,24(28)-dien-3-ol, 14-methyl-, (3.beta.,5.alpha.)-
23.	27.521	4669,462	1.70	Beta.-Amyrin
24	27.804	1782,604	0.65	Lup-20(29)-en-3-one
25.	27.948	483,342	0.18	24(*S*)-Ethyl-3.alpha.,5.alpha.-cyclocholest-22(E)-en-6-one
26.	28.159	1102,145	0.40	9,19-Cyclolanost-24-en-3-ol, (3. beta.)-
27.	28.366	8695,060	3.16	24-Norursa-3,12-diene
28.	29.080	22,689,685	8.25	Olean-12-en-3-ol, acetate, (3.beta.)-
29.	29.729	365,818	0.13	Lup-20(29)-en-3-one
30.	30.086	33,430,866	12.15	Lup-20(29)-en-3-ol, acetate, (3. beta.)-
31.	30.398	7143,539	2.60	Lupeol
32.	31.379	625,338	0.23	9-Hexadecenoic acid, 9-octadecenyl ester, (Z,Z)-
33.	32.088	7335,944	2.67	A'-Neogammacer-22(29)-en-3-ol, acetate, (3.beta.,21.beta.)
34.	32.458	51,048,256	18.55	Lup-20(29)-en-3-ol, acetate, (3. beta.)-

R. Time: Retention Time (min), GC–MS: Gas chromatography-mass spectroscopy, NIST: National Institute of Standards and Technology.

3. Results

3.1. GC–MS analysis

The GC–MS spectra of the EECP are shown in Fig. 1. A total of 77 phytocompounds were analysed by GC–MS analysis and compared with the NIST library. The GCMS analysis confirmed the presence of Lup-20 (29)-en-3-ol, acetate, (3.beta.)- (18.5 %), n-hexadecanoic acid (6.38 %), gamma.-Sitosterol (4.54 %) hexadecanoic acid methyl ester (2.15 %), 9-Octadecenoic acid (Z)-, methyl ester (1.08 %), 9,12-Octadecadienoic acid (Z,Z)-, beta.-Amyrin (1.70 %), 2,4- Di-tert-butylphenol (0.34 %), and phytol (0.20 %), which are antioxidant and anti-inflammatory compounds (Fig. 1 and 2) (Table 1).

3.2. Assessment of anticonvulsant activity

3.2.1. MES test

EECP flowers decreased the duration of hind limb extension significantly in all groups of treatment compared to the control group. The duration of flexion was also significantly ($p < 0.001$–0.01) reduced in all treatment groups compared to the control group, whereas clonus and stupor phases were reduced in all treatment groups, but none was significant ($p > 0.05$) compared to the control group except for the phenytoin group ($p < 0.05$). Extensor and flexion were completely abolished in the groups of phenytoin, 200 and 300 mg/kg of EECP (Fig. 3).

3.2.2. PTZ induced convulsions

In all treatment groups, EECP flowers delayed the onset of PTZ -induced myoclonic jerks. The onset of convulsion was delayed by 180.80 s when a dose of 300 mg/kg BW. EECP was given, compared to 40 s in the control group. The duration of jerks in all the treatment groups decreased significantly ($p < 0.0001$) compared to the control group. The onset of clonus was delayed by 775±47 s in the 100 mg/kg group, compared to 153.83±16.64 s ($p < 0.001$) in the control group, whereas the other groups (phenytoin and EECP at doses of 200 mg/kg and 300 mg/kg) recovered and no clonus phase was detected. In all treatment groups except the control group, the onset of extensor was not observed. In all treatment groups, no incidence of mortality was recorded, whereas in the control group treated with a vehicle, 50 % mortality was recorded (Fig. 4).

3.3. Effect on anxiety

3.3.1. Elevated plus maze

The Elevated plus maze test showed a significant decrease in the number of entries in the open arm and close arm both by all the treatment groups. Time spent in the open arm by the EECP treated groups did not differ significantly from the control group, but time spent in the open arm by the diazepam treated group increased significantly (Fig. 5).

3.3.2. Open field test

The doses of EECP (100, 200, and 300 mg/Kg) and diazepam were given to separate groups of rats. The number of squares crossed by all treatment groups decreased significantly ($p < 0.001$–0.01) compared to the control groups. The number of central area rearing was increased in all groups but not significantly ($p > 0.05$) compared to the control group, except for the diazepam group ($p < 0.01$). Time spent in the central area did not change significantly ($p > 0.05$) in any of the treatment groups, while time spent in the central area increased significantly ($p < 0.001$) in the diazepam group compared to the control group (Fig. 6).

3.4. Effect on depressant/ antidepressant activity

3.4.1. Forced swim test

EECP flower at a dose of 300 mg/kg significantly decreased immobility time in the forced swim test compared to the control group ($p < 0.05$). (Fig. 7a).

3.4.2. Tail suspension test

The tail suspension test demonstrated that at a dose of 300 mg/Kg EECP flower-treated group had a shorter immobility time ($p < 0.05$) than the control group (Fig. 7b).

3.5. Effect on cognition

3.5.1. Morris water maze test

Rats were investigated for the effects of EECP flowers on scopolamine-induced amnesia. The extract's effects on escape latency and spatial memory were studied at doses of 100, 200, and 300 mg/Kg BW. The treatment with EECP flowers reduced escape latency significantly as compared to the scopolamine-induced vehicle-treated group. From day 1 to day 4 of the test, the control group considerably reduced the escape latency (Fig. 8a). The time spent in the target quadrant (Spatial memory) by the scopolamine group in the probe test on the fifth day was significantly lower than the control group ($p < 0.01$). When

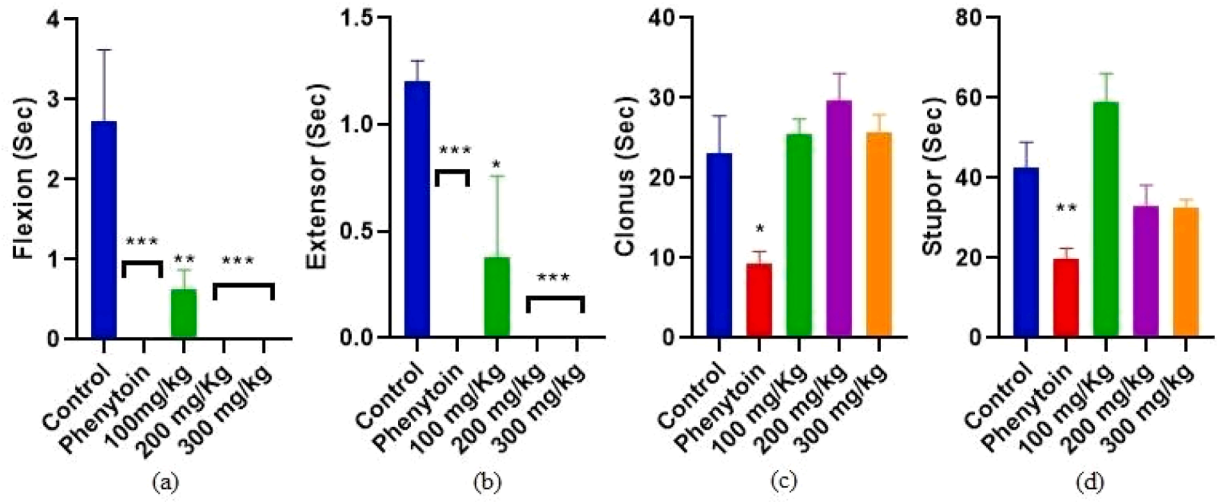

Fig. 3. EECP flower on maximal electroshock seizure: (a) Flexion was abolished by all doses of EECP compared to the control group (b) Extensor phage was also decreased at all doses of EECP (c) Clonus phase did not change significantly (d) stupor phase did not change significantly compared to control group. (***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$).

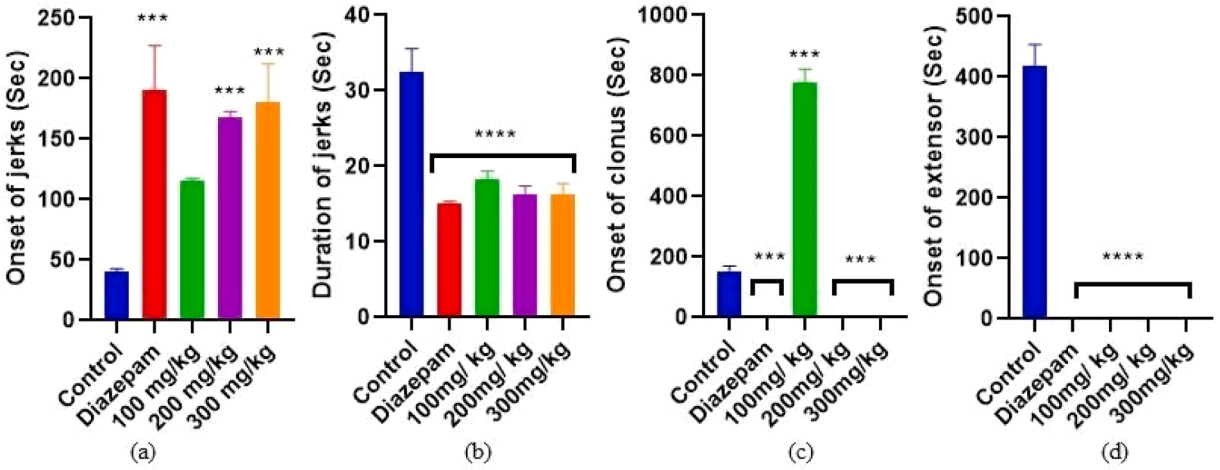

Fig. 4. Effect of EECP flower on PTZ induced convulsion: (a) Onset of jerk was delayed in EECP treated groups (b) Duration of jerk decreased in EECP groups (c) Onset of clonus was delayed in 100 mg/Kg group and did not occur in higher doses of EECP group. (d) Onset of extensor. (****= $p < 0.0001$, ***= $p < 0.001$).

compared to the scopolamine- induced vehicle-treated group, the donepezil ($p < 0.0001$) and EECP treated groups [100, 200, and 300 mg/Kg ($p < 0.05$–0.01)] spent more time in the target quadrant (Fig. 8b).

3.5.2. *Effect on ache activity*

In scopolamine-induced memory-impaired rats, the influence of EECP flowers on AChE activity was investigated. When compared to the control group, the scopolamine group showed a considerable rise in AChE activity. When compared to the scopolamine-induced vehicle-treated group, the EECP-treated groups significantly [100, 200, and 300 mg/Kg ($p < 0.05$- 0.001)] suppressed AChE activity. AChE activity was 72.87 %, 63.86 %, and 60.4 % at 100, 200, and 300 mg/kg, respectively (Fig. 8c).

3.6. *Anti-inflammatory activity*

The treatment of EECP at doses of 100, 200, and 300 mg/Kg significantly ($p < 0.05$) reduced the paw edema induced by carrageenan and histamine compared to the control group. The paw volume of animals was measured at 0, 30, 60, 120, and 180 min and expressed as an increase in paw volume (ml). (Fig. 9a, b).

3.7. *Neuropathic pain*

3.7.1. *Effect on mechanical allodynia*

It shows that in the treatment groups, EECP affects mechanical allodynia stimulation. Oral daily administration of EECP (100, 200, and 300 mg/Kg) significantly increased the paw withdrawal threshold and eliminated mechanical allodynia during the 7 to 14-day treatment period when compared to the CCI group. gabapentin (Neurontin) showed the most effective followed by a 300 mg/Kg dose of EECP ($p < 0.001$) in the treatment. There was a significant decrease in increased paw withdrawal threshold when comparing the CCI group to the control group. Treatment was started on the seventh day of injury (day 0), when mechanical allodynia had already developed. (Fig. 10a)

3.7.2. *Effect on thermal hyperalgesia*

Oral daily administration of EECP (100, 200, and 300 mg/Kg) significantly increased paw withdrawal latency to radiant heat stimulation and eliminated hyperalgesia during the 7 to 14- day treatment period when compared to the CCI group. gabapentin (Neurontin) (Neurontin) showed the most effective followed by a 300 mg/Kg dose of EECP ($p < 0.001$) in the treatment. There was a significant decrease in

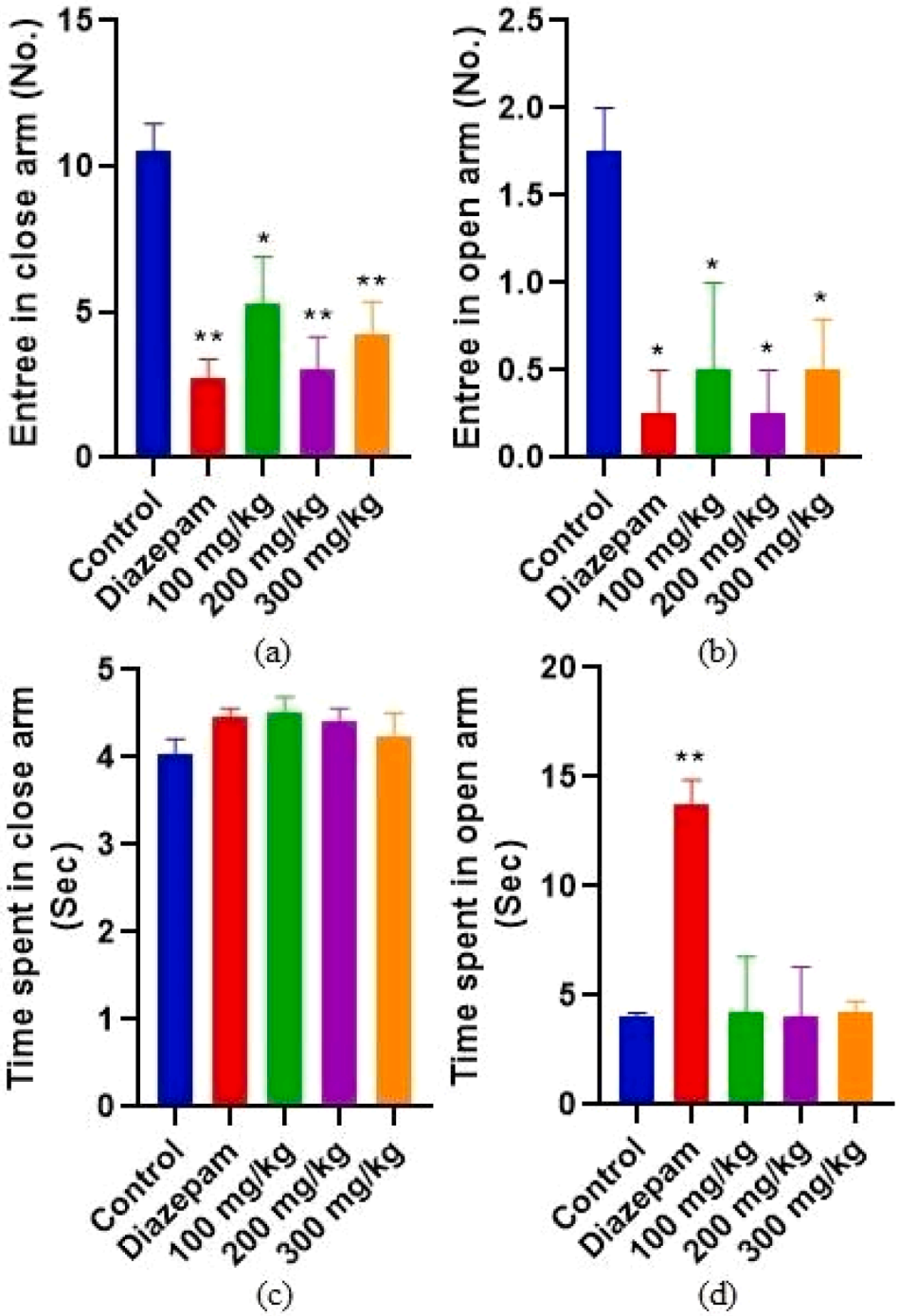

Fig. 5. Elevated plus maze Test: Time spent in open and closed both arm by all the treatment groups decreased (a&b) significantly (c) Time spent in close arm did not change significantly. (d) Time spent in open arm did not change significantly. Results were compared to control group. (**= $p < 0.01$, *= $p < 0.05$).

paw withdrawal latency when comparing the CCI group to the control group. Treatment was started on the seventh day of injury (day 0), when mechanical allodynia had already developed. (Fig. 10b)

3.7.3. Effect of treatment on TNF-α, IL-1β and IL-6 levels

3.7.3.1. Effect of treatment on TNF-α. Compared to the control group, the CCI group showed a significant increase in TNF-α levels in both the sciatic nerve and the spinal cord. Treatment with EECP at doses of 100, 200, and 300 mg/kg for 7 to 14 days significantly reduced TNF-α levels in these tissues compared to the CCI group. (Fig. 11a, 11b)

3.7.3.2. Effect of treatment on IL-1β. Compared to the control group, the CCI group showed a significant increase in IL-1β levels in both the sciatic nerve and spinal cord. Treatment with EECP at doses of 100, 200, and 300 mg/kg for 7 to 14 days significantly reduced IL-1β levels in the sciatic nerve and spinal cord compared to the CCI. (Fig. 11a, 11b)

3.7.3.3. Effect of treatment on IL-6 levels. Compared to the control group, IL-6 levels were significantly increased in the CCI group. However, treatment with EECP at doses of 100, 200, and 300 mg/kg significantly reduced the elevated IL-6 levels in both the sciatic nerve and spinal cord in the CCI + EECP groups, compared to the CCI group.

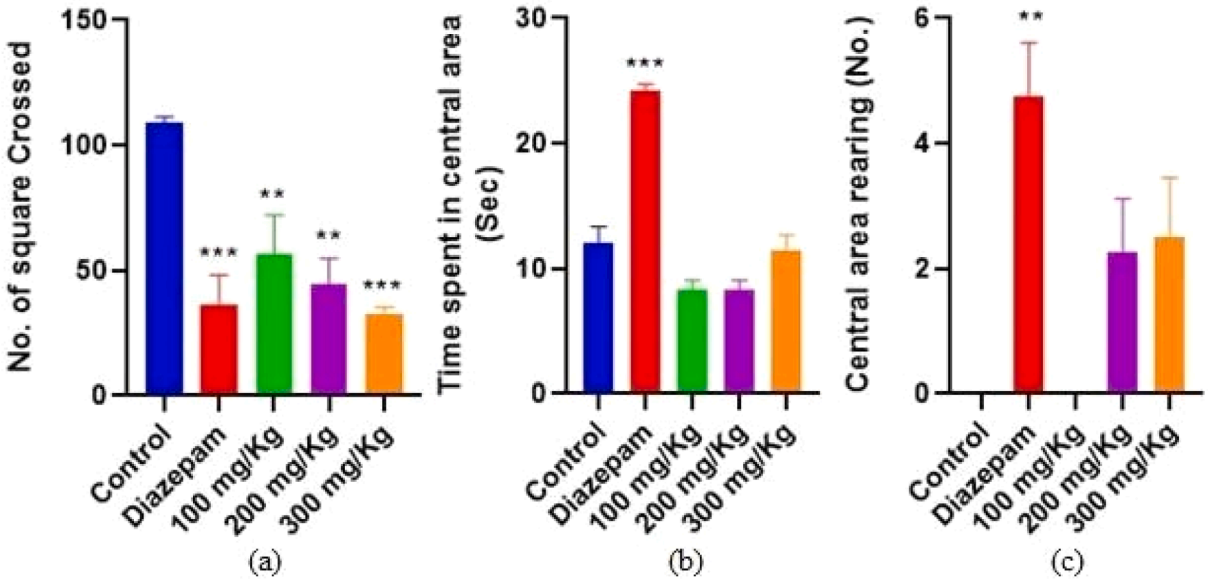

Fig. 6. Open field test: (a) The number of squares crossed by rats decreased significantly in all treatment groups compared to the control group. (b)Time spent in the central arm by the EECP group did not change significantly. (c) The number of central area rearing by the EECP group did not change significantly. (***= $p < 0.001$, **= $p < 0.01$).

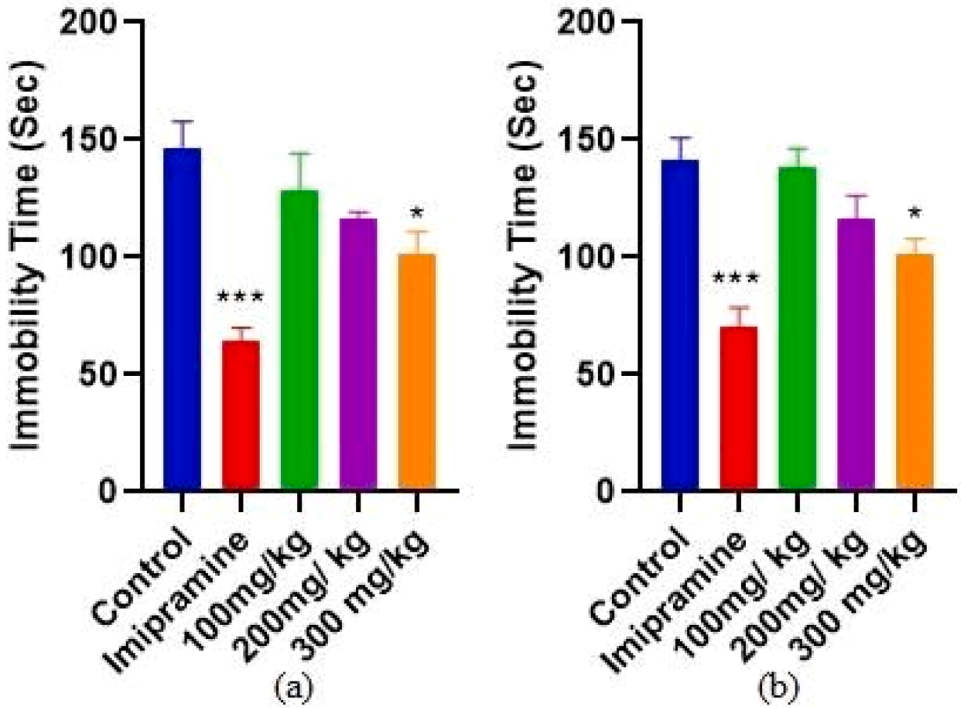

Fig. 7. (a) Forced swim test and (b) Tail suspension test. Immobility time decreased significantly at the dose of 300 mg/Kg BW. of EECP in forced swim test and tail suspension test both. (***= $p < 0.001$, *= $p < 0.05$).

(Fig. 11a, 11b)

3.7.4. *Effect on biochemical parameters*

3.7.4.1. Effect on the enzymatic activity of superoxide dismutase. The CCI group showed a significant decrease in SOD activity as compared to the control group. When compared to the CCI group, the CCI+ EECP (100 mg/kg), CCI+ EECP (200 mg/kg), and CCI+ EECP (300 mg/kg), all demonstrated a significant increase in SOD activity. (Fig. 12)

3.7.4.2. Effect on nitrite level. CCI of the sciatic nerve in rats resulted in a substantial increase in nitrate levels as compared to the vehicle-treated control group. In CCI-induced rats, EECP (100, 200, and 300 mg/kg p. o.) and gabapentin (Neurontin) treatment significantly reduced nitrate levels in sciatic nerve homogenate. (Fig. 12)

3.7.5. *Effect on histological study*

Transverse nerve sections of a normal rat's sciatic nerve revealed normal structure and architecture, as well as no inflammation. The CCI

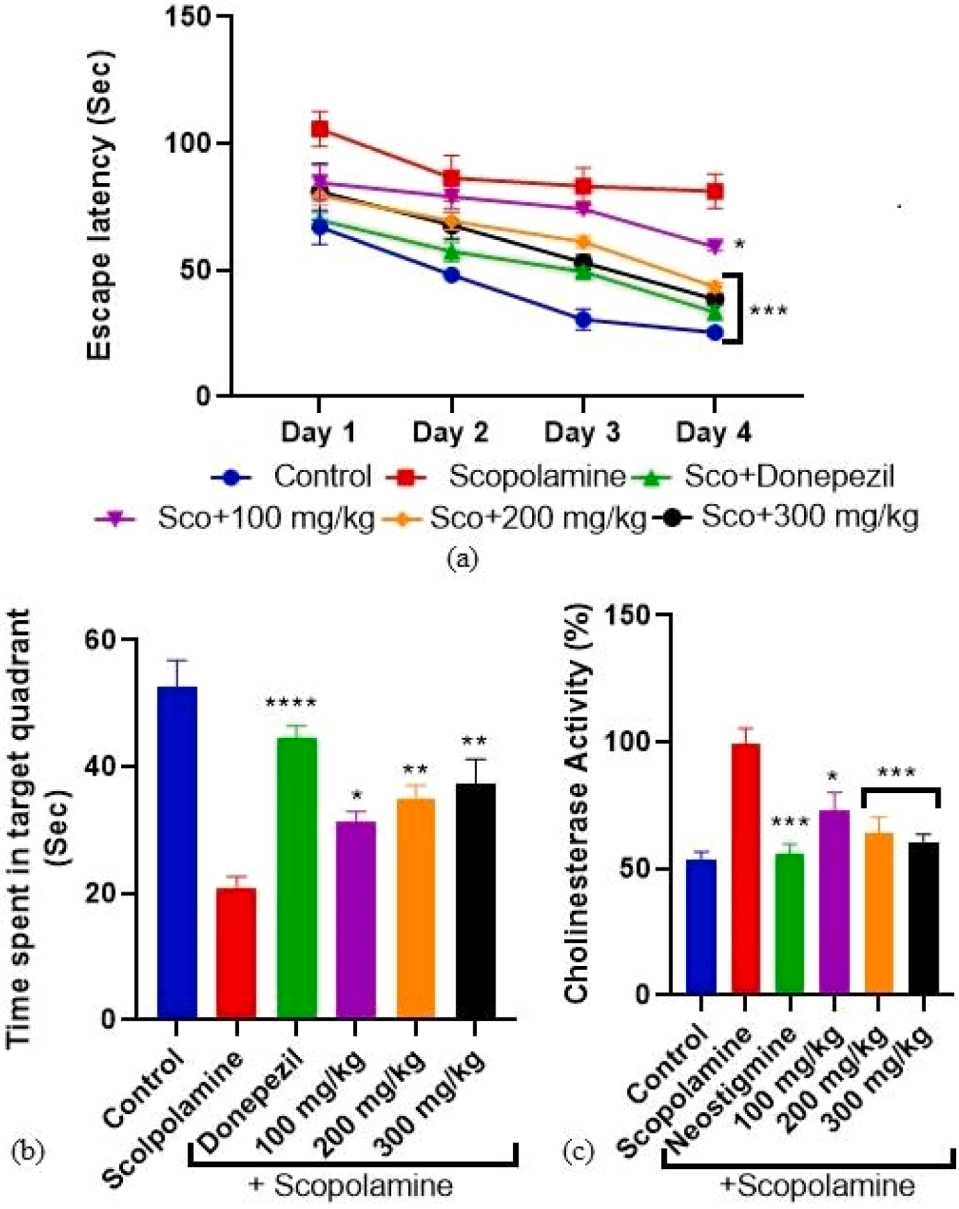

Fig. 8. (a). Morris water maze test: The escape latency was decreased in all the treatment groups compared to scopolamine group. **(b).** Morris water maze test: Time spent in the target quadrant during the probe test increased significantly at all doses of EECP flower compared to the scopolamine group. **(c)** Acetylcholinesterase activity decreased significantly by EECP treated groups compared to scopolamine treated group. (****= $p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$).

caused a noticeable histological change, as evidenced by axonal swelling, an increase in the number of Schwann satellite cells, and a change in nerve architecture. EECP 200 and 300 mg/kg/day/oral therapy for 7 to 14 days protected the sciatic nerve from CCI-induced structural damage and inflammatory alterations, whereas 100 mg/Kg EECP reduced inflammation but not as much as higher dosages. The CCI rats' derangement was normalised by gabapentin (Neurontin) therapy. (Fig. 13)

4. Discussion

In the study, EECP flower was tested for GC–MS and neuropsychopharmacological activities in rats, including antiepileptic, sedative, antidepressant, antianxiety, and cognitive effects [43–45]. The existence of phyto-compounds with antioxidants and other properties was found in the ethanol extract of *C. procera* flower [46–48]. Antioxidant phytocompounds are neuroprotective, and oxidative stress and inflammation must be reduced for brain health [49,50]. Natural antioxidants help to reduce memory loss and cognitive dysfunction, and they can also help patients with Parkinson's disease [51,52]. Some plant phytoconstituents' neuroprotective activities in the control of neurotransmitter metabolism, receptor function, and antioxidative defence have potential therapeutic uses in a variety of neurodegenerative illnesses [53]. GC–MS analysis revealed the presence of *n*- hexadecanoic acid, which has anti-inflammatory and anti-oxidative (6.38 %) activity [54,55]. According to an in-silico molecular docking study, gamma-Sitosterol (4.54 %) has neuropharmacological activity as an antidepressant. It has shown remarkable binding affinity against the human serotonin receptor (PDB: 5I6X). EECP also showed antidepressant action in the forced swim test and tail suspension test (Fig. 7) [56, 57]. Hexadecanoic acid methyl ester (2.15 %) (Jebastella and M 2015), 9-Octadecenoic acid (Z)-, methyl ester (1.08 %), 9,12-Octadecadienoic acid (Z,Z)-, beta.-Amyrin (1.70 %), phytol (0.20 %), 2,4-Di-tert-butylphenol (0.34 %), which are antioxidant and anti- inflammatory compounds [58]. In one study, phytol was found to reduce the incidence of pilocarpine (Salagen Tablet)-induced seizures and increase the survival

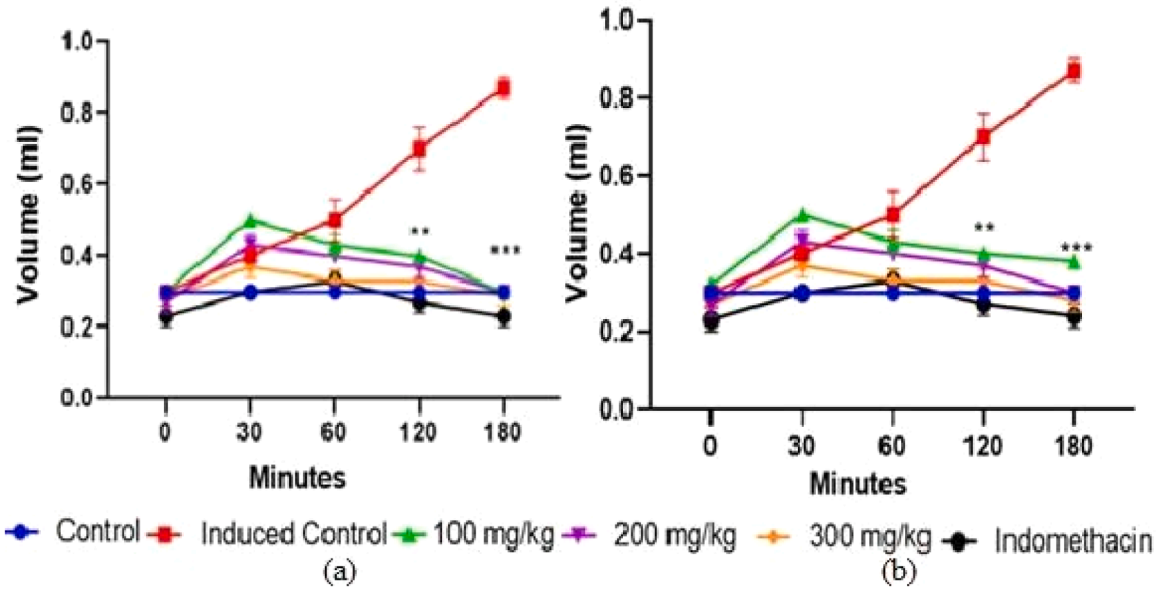

Fig. 9. **(a)** Effect of EECP on carrageenan induced paw oedema. **(b)** Effect of EECP on histamine induced paw oedema. Two-way ANOVA followed by Dunnette's multiple comparison test was performed to analyze the data. $P < 0.05$ was considered significant in comparison to induced control group. (****= $p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$).

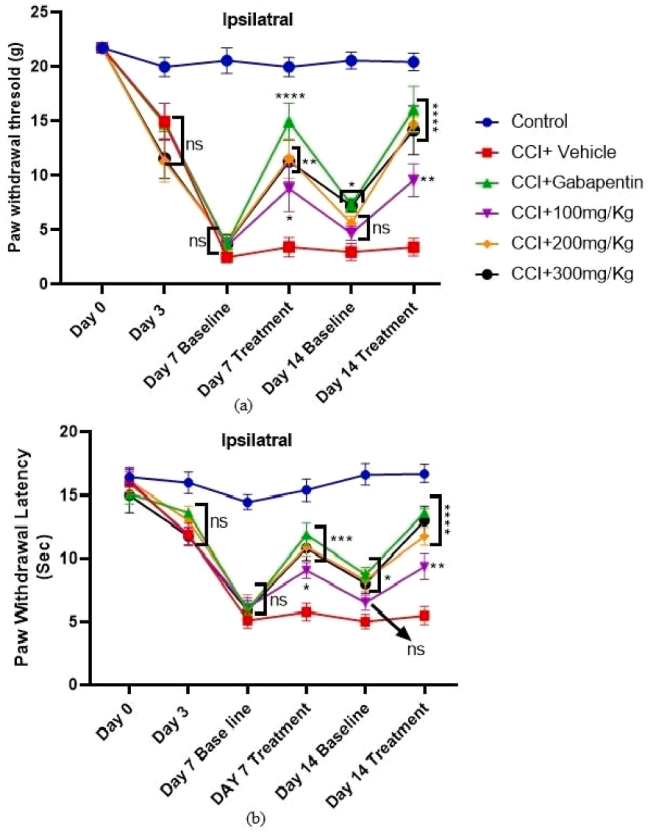

Fig. 10. In CCI induced neuropathy the effect of EECP on (a) mechanical allodynia (b) heat hyperalgesia was measured. Paw withdrawal threshold and paw withdrawal latency were increased from day 7 to day 14 days of the treatment. 300 mg/Kg dose of EECP reduced hyperalgesia most significantly but less than gabapentin (Neurontin). All the results were compared with CCI group. Two-way ANOVA followed by Dunnette's multiple comparison test was performed to analyse the data. Values are expressed as mean ± *S*.E.M. (****=$p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$), EECP: ethanolic extract of *C. procera* flower, CCI: chronic constriction injury.

rate [59,60]. These detected bioactive components may contribute to the neuroprotective or neuropharmacological activity of flower extract [61,62].

Both MES and PTZ-induced convulsions might be treated with EECP flowers. EECP flowers delayed the onset of convulsions in MES-induced epilepsy, and hind limb extensor and flexion were greatly reduced at 100 mg/Kg or entirely abolished at 200 and 300 mg/Kg EECP flower [63,64]. The extensor and flexion phases of convulsion were completely abolished whereas clonus and stupor phases were significantly decreased by positive control (phenytoin). The Extensor/Flexion ratio was used as the endpoint in the MES model to assess the antiepileptic potential of medicines efficacious in generalised tonic-clonic seizures [65]. Extensor/Flexion ratio significantly decreased in EECP treated groups. The MES-induced epilepsy model screens antiepileptic drugs which act by blocking voltage-dependent Na^+[66–69].

In comparison to the control group, EECP considerably delayed the onset of PTZ-induced convulsions in a dose-dependent manner, and the duration of jerk was significantly reduced in the treatment groups. At doses of 200 and 300 mg/kg BW of EECP, no clonus was seen, and the animals recovered. The control group had a 50 % mortality rate, while the EECP-treated groups had no mortality at all [70]. These findings suggest that the EECP flower was very efficient in abolishing PTZ-induced seizures. The effect of a medicine on animals with PTZ-induced epilepsy can be used to evaluate its impact on generalised absence seizures. Diazepam (benzodiazepines) is a GABA facilitator, whereas PTZ is a $GABA_A$ receptor antagonist. This suggests that the anticonvulsant effect of EECP against PTZ-induced seizures is mediated by the GABA-chloride channel [71,72].

The elevated plus maze test was based on the fear of balancing on a narrow space and the aversion to unexpected open spaces. As a result, avoiding open arms is regarded as anxious behaviour [73]. Anxiolytic medicines like diazepam, increase the time and number of entries into the open arms. The results of the elevated plus maze test in this study revealed that EECP-treated groups had fewer entrees in both the closed and open arms, which might be attributed to the extract's depressive impact [74,75]. The time spent in the open arm in EECP-treated groups was not significantly different from the control group, but diazepam-treated groups spent more time in the open arm. According to these findings, EECP flowers did not influence anxiety [76–79].

In an animal test model, an open field was used to examine anxiety

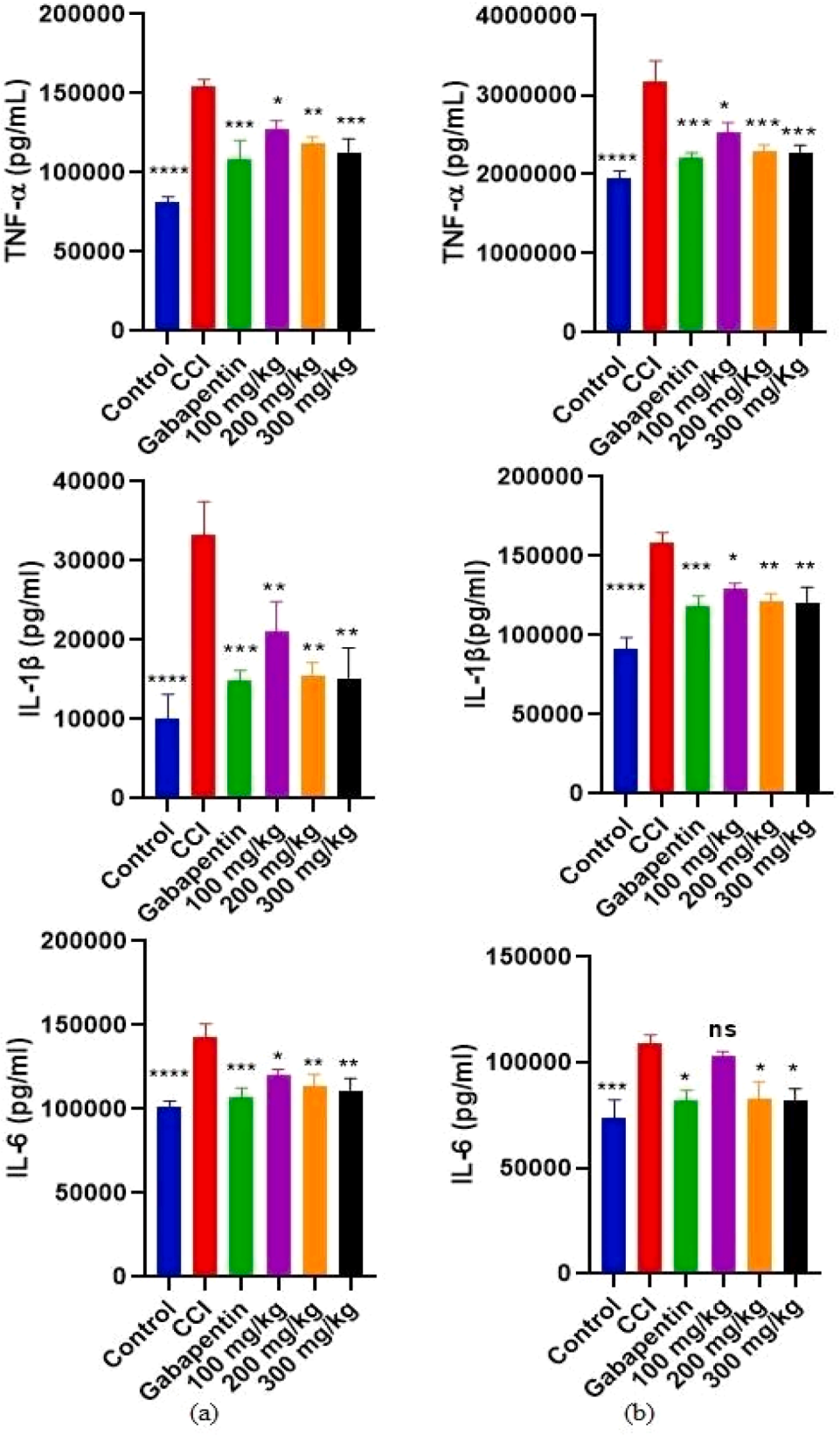

Fig. 11. TNF-α, IL-1β, IL-6 analysis by ELISA. CCI increased TNF-α, IL-1β and IL-6 levels in the sciatic nerve and spinal cord. TNF-α, IL-1β and IL-6 levels were significantly decreased in rats given EECP at the doses of 100, 200, and 300 mg/Kg in a dose-dependent manner orally for 7 to 14 consecutive days after CCI. All the results were compared with the CCI group. Values are expressed as mean ± *S*.E.M. (****=$p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$). ns (non-significant), EECP: ethanolic extract of *C. procera* flower, CCI: chronic constriction injury.

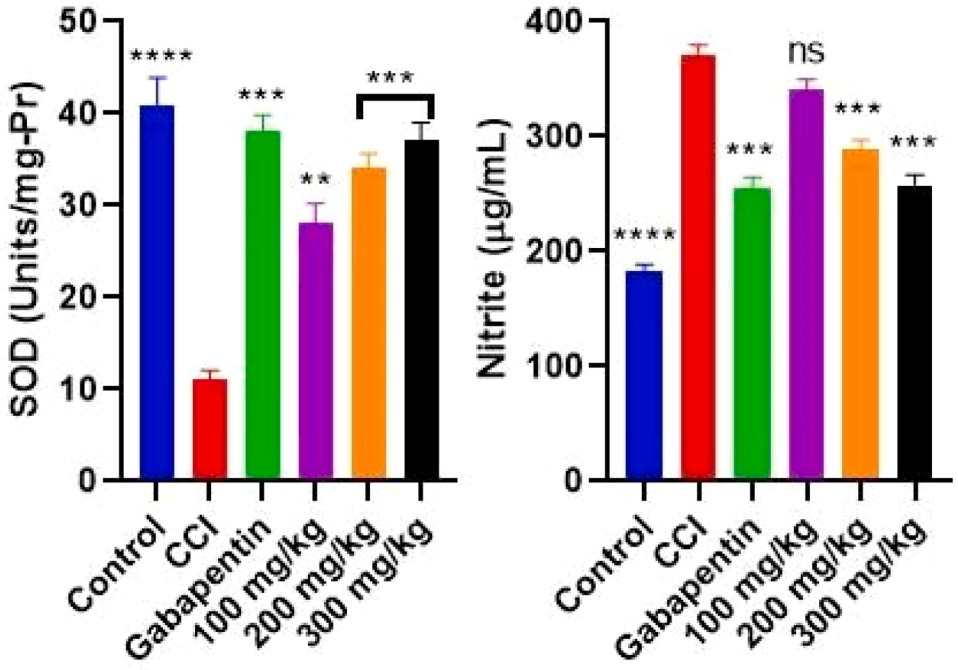

Fig. 12. CCI decreased super oxide dismutase (SOD) levels and increased nitrite levels in the sciatic nerve. SOD levels were significantly increased and nitrite levels decreased in rats given EECP orally for 7 to 14 consecutive days after CCI in a dose dependent manner. All the results were compared with the CCI group. Values are expressed as mean ± *S*.E.M. (****=$p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$). ns (non-significant), EECP: ethanolic extract of *C. procera* flower, CCI: chronic constriction injury.

behaviour. An open field test is used to objectively assess the emotionality of rats. The total number of square crossings was utilised to calculate the animals' locomotor activity throughout the experiment. The number of lines crossed by the animals in all of the EECP-treated groups, as well as the diazepam group, decreased significantly in this study [80,81]. The extract's sedative effect resulted in a decrease in locomotor activity. Time spent in the central region did not alter significantly in the EECP-treated groups, whereas time spent in the central area increased in the diazepam group as compared to the control group. These findings indicate that treatment with EECP-flower did not affect anxiety [82,83].

During the forced swim test, the animals' swimming behaviour is thought to be influenced by serotonergic activity in the brain. In the forced swim test, a decrease in the duration of immobility has an antidepressant effect. At a dose of 300 mg/kg BW, EECP significantly reduced immobility time ($p < 0.05$), whereas smaller doses of EECP flower were not effective [84]. Antidepressants lowered immobility time in the tail suspension test. When compared to the control group, EECP 300 mg/Kg BW considerably reduced immobility time during the tail suspension test. Thus, the EECP flower has an antidepressant effect in rats [85,86].

The Morris water maze test and AChE activity were used to assess the effect of EECP flower on scopolamine-induced amnesia in rats. Morris water is used to evaluate spatial memory and learning ability. In the current study, the Morris water maze test demonstrated that EECP alleviated scopolamine-induced amnesia [87]. In the Morris water maze test, the time spent in the target quadrant in the probe test and the escape latency of repeated trials tests for 4 days were assessed. In this study, escape latency was decreased in 4 days of experiments, and time spent in the target quadrant was increased significantly compared to the scopolamine- treated group [88]. Acetylcholine is hydrolyzed and inactivated by AChE. Increased AChE activity diminished the availability of acetylcholine which leads to impaired memory, as shown in Alzheimer's disease patients' brains. Drugs, which are effective in the inhibition of the AChE enzyme may be beneficial in the treatment of Alzheimer's disease [89]. In the present study, EECP decreased the AChE activity in the brain of rats which indicates its diminishing effect on scopolamine-induced impaired memory in rats. Thus, EECP treatment reversed cognition impairment [90].

In the current study, we have also explored the effect of EECP flower on neuropathic pain. Neuropathic pain was induced by chronic constriction injury of rat's sciatic nerve. The CCI model of persistent neuropathic pain is a commonly used peripheral nerve injury model. The effect of EECP on behavioural, pharmacological, and histopathological changes in CCI- induced neuropathic pain was analysed [91].

In the rat model of CCI-induced neuropathic nociception, EECP oral treatment for 14 days significantly improved behavioural changes in the induced CCI model, including radiant heat hyperalgesia and mechanical allodynia [92].

Activated macrophages produce proinflammatory cytokines, which are involved in the up- regulation of inflammatory reactions. Certain pro-inflammatory cytokines, such as IL-1β, IL- 6, and TNF-, have been associated with the pathogenesis of pathological pain. Proinflammatory cytokines IL-1β upregulated after an injury to a peripheral nerve and after distress in microglia and astrocytes in the CNS. Following a peripheral nerve injury, IL-6 has been shown to play a key role in the neuronal response to nerve injury and contributes to the development of neuropathic pain behaviour [93]. Both inflammatory and neuropathic hyperalgesia has been linked to TNF- α. TNF-α intra-plantar injection causes mechanical and thermal hyperalgesia. In our study, we found that neuropathic pain increases the levels of inflammatory markers such as IL-1β, IL-6, and TNF-α after CCI. Treatment with EECP at doses of 100, 200, and 300 mg/kg reduced CCI-induced upregulation of inflammatory cytokines IL-1β, IL-6, and TNF-α in a dose-dependent manner in both the sciatic nerve and the spinal cord [94].

Catalase and superoxide dismutase, antioxidant defence enzymes, are deficient in nervous tissue followed by injury. Endoneural oxidative stress changes cause nerve dysfunction in rats with CCI. In the current study, superoxide dismutase levels were found to be significantly higher in the sciatic nerves of CCI-induced rats [95]. Reactive oxygen species

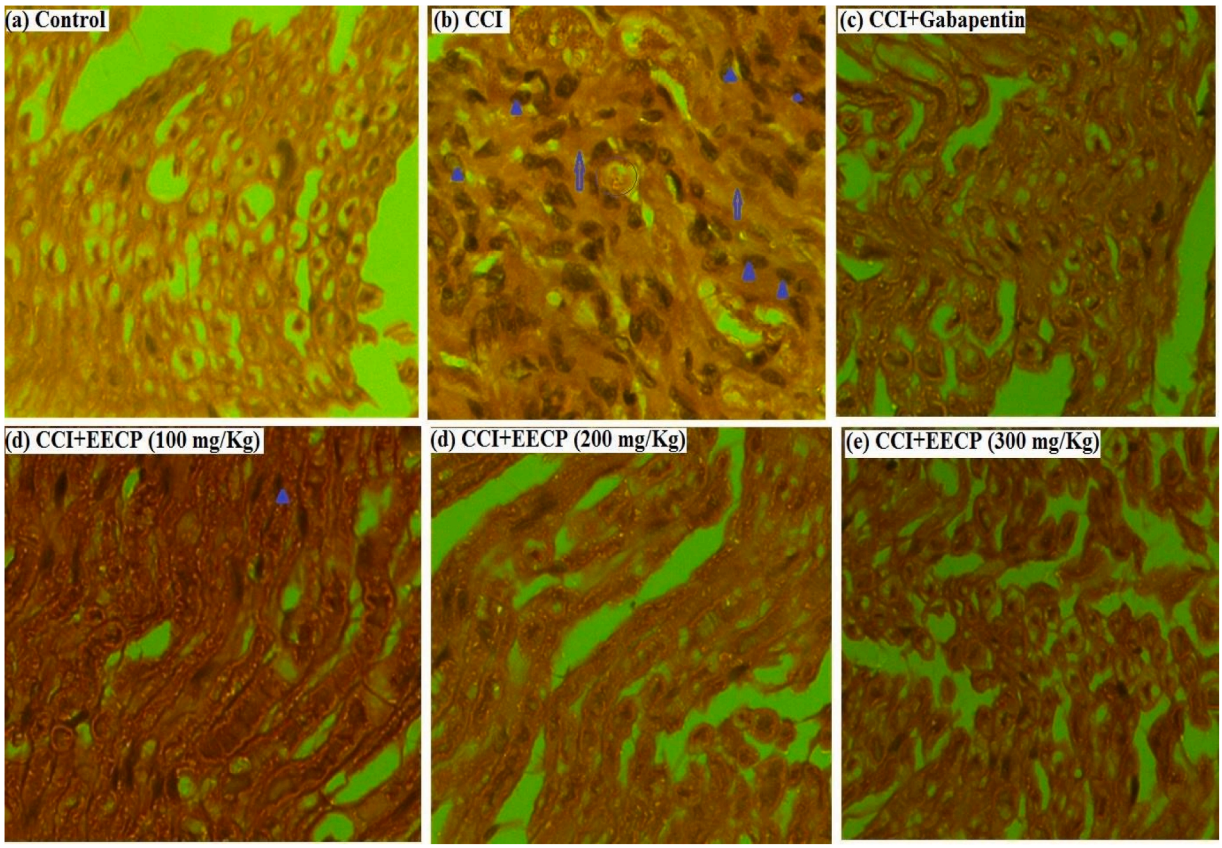

Fig. 13. Histological studies in sciatic nerve at 14 days after CCI induction (400x). Transverse nerve sections of a normal rat's sciatic nerve revealed normal structure and architecture, as well as no inflammation. The CCI caused an increase in the number of Schwann satellite cells (shown by arrow tip), and a change in nerve architecture (shown by arrow). EECP 200 and 300 mg/kg/day/oral therapy for 14 days protected the sciatic nerve from CCI-induced structural damage and inflammatory alterations, whereas 100 mg/Kg EECP reduced inflammation but not as much as higher dosages. The CCI rats' derangement was also normalised by gabapentin (Neurontin) therapy, EECP: Ethanolic extract of *C. procera* flower, CCI: chronic constriction injury.

(ROS) can harm neuronal function in a variety of ways. Superoxide is normally produced by mitochondria as part of the oxidative phosphorylation process, and superoxide dismutase easily converts superoxide to hydrogen peroxide [96]. NO is a key signalling molecule that affects pain at both the central and peripheral levels [97,98]. The presence of elevated nitrite levels in the sciatic nerve and spinal cord in CCI rats with hyperalgesia suggests that neuropathic pain may be mediated by elevated NO levels. In our study, EECP reduced oxidative stress by decreasing Nitrite levels and increasing superoxide dismutase activity in the sciatic nerve at all doses (100, 200, and 300 mg/Kg) [99].

Furthermore, when compared to CCI+vehicle treated group, histological features of sciatic nerve sections from the EECP treated groups showed a reduction in degenerated bodies and preservation of sciatic nerve architecture. CCI group has shown proliferated Schwan cells whereas EECP treated and gabapentin (Neurontin) treated groups showed fewer proliferations [100].

There are several phytocompounds in EECP, including flavonoids and saponins, which are powerful antioxidants. Major phytoconstituents detected in a GC–MS study included -sitosterol, stigmasterol, 9, 12-octadecadienoic acid (Z, Z)-, methyl ester, campesterol, acetate, -amyrin, hexadecanoic acid, methyl ester, 11-octadecenoic acid, methyl ester, 2-methoxy-4-vinylphenol. These compounds are powerful antioxidants, which may be responsible for the neuroprotective properties of the extract [101].

5. Limitations

The present research reveals important findings about how *C. procera* may become useful for treating neuropathic pain. Several important drawbacks need to be evaluated. The main experimental method used animal subjects because they prove insufficient for precise human neuropathic pain research. Additional human clinical trials are necessary to translate recorded results into knowledge about human physiological effects. The study investigates *C. procera* flower extract through ethanol extraction yet it fails to assess potential neuropharmacological benefits from other plant sections including leaves, roots and latex [102]. The research fails to provide a comprehensive examination of the molecular mechanisms which allow the extract to work as an analgesic and neuroprotective agent. Extensive investigation about receptor relationships might enhance the research results along with studies concerning inflammatory factors and neural transmitter regulation mechanisms [103]. The long-term safety aspects and potential toxic effects of *C. procera* extracts need to be investigated since they could pose risks to users who extend their treatment duration. Extract reproducibility and consistency face limitations due to variations in plant composition which result from environmental conditions together with seasonal changes. The study fails to compare its findings with standard treatments for neuropathic pain because it lacks analysis of existing protocols making it challenging to measure *C. procera* extract effectiveness against standard medications. Future clinical validation of Calotropis procera therapy for neuropathic pain requires the resolution of identified shortcomings in research [104].

6. Conclusion

GC–MS study of EECP found that it contains antioxidant and neuroprotective compounds. Some phytocompounds, such as phytol and stigmasterol, exhibit direct neuropharmacological actions. Animal

models demonstrated that the extract had anti-inflammatory activity. The neuropsychopharmacological effects of EECP flowers were studied for the first time in this work. This study also revealed that treatment with EECP decreased inflammatory markers (TNF-α, IL-1β, and IL-6) and oxidative stress. Our findings support the anti-inflammatory and anti-oxidant effects of EECP in the CCI-induced neuropathic pain model. Sciatic nerve deformity was also reduced after EECP treatment. The extract possesses antiepileptic, sedative, cognition enhancer, and anti-depressant properties, but no anti-anxiety activity. The ethanolic extract of *C. procera* flower was also effective in reducing neuropathic pain induced by chronic constriction injury of the sciatic nerve.

Compliance with ethical standards

The Central Animal Ethical Committee of Banaras Hindu University approved all of the experiments according to CPCSEA guidelines (Reg. No. 542/GO/Rebi/S/02/CPCSEA dated 26.5.2017).

CRediT authorship contribution statement

Ashutosh Kumar: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Brijesh Kumar:** Writing – review & editing, Visualization, Supervision, Methodology, Data curation, Conceptualization. **Rajesh Kumar:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Vinod Tiwari:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Pratistha Singh:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Ajay Kumar:** Writing – review & editing, Visualization, Formal analysis. **Manish Singh:** Writing – review & editing, Resources, Formal analysis. **Chandra Shekhar Azad:** Validation, Data curation. **Ankit Uniyal:** Software, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All the authors declares that there is no competing interests among the authors.

Acknowledgment

The authors acknowledge the Department of Pharmaceutical Engineering and Technology, IIT, BHU for their help and assistance in learning operative skills.

Data availability

The data used to support the findings of this study are included within the article.

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