Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

Available online at www.ijpsdronline.com

Research Article

Anxiolytic Activity of Methanolic Extract of *Cardia dichotoma* Leaves in Rats

Shalaka S. Shendage[1], Sayali Jadhav[2], Kirankumar Dhawale[1], Sameer H. Sawant[3*]

[1]*Department of Pharmacology, Sinhgad College of Pharmacy, Vadgaon. Pune, Maharashtra, India.*
[2]*Department of Quality Assurance Technology, Sinhgad Institute of Pharmacy, Narhe, Pune, Maharashtra, India.*
[3]*Department of Pharmacology, Sinhgad Institute of Pharmacy, Narhe, Pune, Maharashtra, India.*

ARTICLE INFO

Article history:
Received: 25 June, 2023
Revised: 06 July, 2023
Accepted: 10 July, 2023
Published: 30 July, 2023
Keywords:
Cordia dichotoma, Anxiolytic, Antioxidant, Rats
DOI:
10.25004/IJPSDR.2023.150416

ABSTRACT

The present study aimed to evaluate methanolic extract of *Cordia dichotoma* (MECD) leaves (MECD) for anxiolytic activity in rats. Elevated plus maze test (EPM) and light and dark test used to evaluate the antianxiety property of MECD. Diazepam (2 mg/kg) and methanolic extract of *C. dichotoma* (50, 100, and 200 mg/Kg, p.o.) were administered daily for 21 days. EPM and light and dark test were assessed on the 7th, 14th, and 21st days. On the 21st day, animals were killed, and the Hippocampus was excised for the estimation of various biochemical parameters like malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) as well as histopathological study. In this study, the (MECD) demonstrated a significant and dose-dependent increase in time spent in the open arm of the elevated plus maze (EPM) test and the time spent in the light compartment in the light and dark test. It is also significantly decreased the MDA level and increased CAT, GSH, and SOD levels significantly ($p < 0.001$) in the MECD-treated group compared to the control group. Furthermore, MECD significantly restored histological abnormalities, revealing reduced stress-induced damage. It is suggested that MECD possessed an anxiolytic effect via modulation of endogenous enzymes in experimentally induced anxiety in rats.

Introduction

Anxiety is the most common mental disorder, affecting one-eighth of the population, and it has become a major focus of psychopharmacology research in the last decade.[1] Anxiety disorders are estimated to impact over 40 million American individuals aged 18 and up each year, causing them to be plagued with worry and uncertainty.[2] Anxiety is among the most prevalent psychological problems among school-aged children and adolescents around the world. Anxiety in children is linked to poor social and coping abilities, which often leads to social avoidance, loneliness, low self-esteem, fears of social rejection, and trouble.[3]

Because of the high frequency of anxiety disorders and the associated impairments, the etiology of anxiety disorders is becoming a prominent focus of research.[4] Anxiety disorders are not only painful for people, but they also cost a lot of money.[5] The genesis of anxiety disorders has been investigated using a variety of methods, including genetic and family investigations, to look into the disorder's familial aggregation.[6] Anxiety disorders commonly begin in childhood, if left untreated, they can last into adulthood.[7]

Anxiety disorder treatment varies and depends on the nature of the condition and the features of the particular patient.[8] The therapy generally entails medications, psychological treatment and alternative therapy. Medications will not cure anxiety problems but can help people manage them while they get psychotherapy.[9] Selective serotonin reuptake inhibitors (SSRIs) are often

***Corresponding Author:** Dr. Sameer H. Sawant

Address: Department of Pharmacology, Sinhgad Institute of Pharmacy, Narhe, Pune, Maharashtra, India.

Email ✉**:** sampharma@gmail.com

Tel.: +91-8275207398

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

recommended drugs for generalized social anxiety and may be the first line of treatment. These medications, including fluoxetine, sertraline, paroxetine, citalopram, and others, increase the amount of the neurotransmitter serotonin, among other things.[10] Herbal treatments have seen a growth in popularity in recent years, owing to their safety, effectiveness, and superior therapeutic effects, as well as their lower cost when compared to synthetic or allopathic drugs, which have several therapeutic difficulties.[11]

Cordia dichotoma is a medicinally significant deciduous plant that may be found throughout India. *C. dichotoma* is a plant that grows in tropical and subtropical climates. Many phytoconstituents were screened and identified from *C. dichotoma*, including flavonoids, terpenes, alkaloids, tannins, and glycerides, all of which have diverse pharmacological actions.[12]

The selection of this plant, *C. dichotoma* (Boraginaceae) was made on the basis of its high therapeutic value, and easy availability. We have selected *C. dichotoma* for the evaluation of its anxiolytic activity as a new hope for a promising therapeutic approach. *C. dichotoma* reported to have various pharmacological activities like antioxidant,[13] anticancer,[14] antimicrobial and antifungal[13] in different doses in various species. However, very few studies have reported about the anxiolytic potential of *C. dichotoma*. Therefore, this current study aimed to investigate the effect of MECD in various anxiety models in rats.

The herbal gel formulation of *C. dichotoma* is highly effective in mouth ulcer treatment. Our study may be the first report of the antianxiety effects of MECD in the EPM and L and D test, widely used animal models of anxiety.

Materials and Methods

Acute Toxicity Study

The acute toxicity of the extract of *C. dichotoma* leaves was evaluated in rat using the up and down procedure (OECD, 2001). Rat received methanolic extract at various doses (175–5000 mg/kg) orally by gavage. The animals were continuously observed for toxic symptoms for the first 4 hours after dosing; survivors were noted after 24 hours and then observed for 14 days. The next dose was reduced if toxicity was observed or increased not observed by 3.2 factor.

Experimental Animals and Protocol Approval

The present study was performed on male wistar rats (200–250 g) obtained from Global Bioresearch solution Pvt. Ltd, Pune India and housed at the institute animal house in groups of six animals per cage at standard laboratory conditions with a temperature of 25 ± 1°C, relative humidity of 45–55% and 12:12 hours dark and light cycle. Animals had free access to a standard rodent diet and tap water. All experimental procedures in the protocol were approved by the Institutional Animal Ethics Committee (IAEC) (Approval no. SCOP/IAEC/2021-22/10/288) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision of experiment on animals, India (CPCSEA).

Drugs and Chemicals

C. dichotoma leaves were obtained from the vital herbs, New Delhi, India. Diazepam (Sigma Aldrich), formaldehyde, potassium dihydrogen phosphate, sodium chloride, disodium hydrogen phosphate, and sodium bicarbonate are of laboratory grade and obtained from a local supplier in Pune

Extraction of *C. dichotoma* Leaves

Procured leaves of *C. dichotoma* Linn was shade dried and powdered. 250 gm of the powdered material was subjected to continuous soxhlet extraction by methanol (2.5 L) to get the crude extract. The appearance of colorless methanol (after 12 h of continuous extraction) in the siphon tube was considered the termination of soxhlet extraction. Crude extract obtained was filtered and then concentrated to dryness in a rotary evaporator under controlled temperature and reduced pressure.[14]

Phytochemical Screening

A preliminary phytochemical study of the methanolic extract of *C. dichotoma*[15] was done. All the extracts were dissolved in respective solvents and screened for different phytoconstituents classes. Table 1 shows the results of the phytochemical screening of MECD extract.

Method of Stress Induction

The elevated plus maze test (EPM) was used for animals in the experimental groups in order to induce stress, 30 minutes after the administration of *C. dichotoma* at doses 50, 100, 200 mg/kg. Animals in the experimental groups received a daily dose of *C. dichotoma* for 21 days using oral gavage. Additionally, one group received daily oral dosing of diazepam. We used the EPM apparatus, which has been recognized as a standard tool for measuring anxiety in rats, to determine the degree of anxiety.[16]

Table 1: Evaluation of preliminary phytochemical studies of MECD

Test for Phytoconstituents	*Result*
Flavonoids	Present
Alkaloids	Present
Steroids	Present
Anthraquinone glycosides	Absent
Cardiac glycosides	Present
Flavonoids and phenolics	Present
Carbohydrates	Absent
Proteins and free amino acids	Present

Elevated Plus Maze Test (EPM)

The EPM test is the most important model in contrast to all other models. The maze in the form of plus arms was made of wood. The elevated plus-maze was made of two sets of runways, each 61 cm long and 5 cm wide constructed at 90º each other. The arms of one runway were open and the other were surrounded by walls 15 cm high. Rats were placed in the center of the elevated plus-maze. For 5 minutes, rat was examined. The evaluation was performed based on the latency to number of entries on an open arm and close arm of the plus-maze and the total time spent on the open arms and close arm was recorded.[17]

Light and Dark Test (L and D test)

The apparatus consisted of two rectangular boxes (45 × 27 × 27 cm), partitioned into two compartments: light compartment and dark compartment connected by a 7.5 × 7.5 cm opening in the wall between compartments. An animal was placed in the light compartment's center and observed for 5 minutes. The evaluation was performed based on the number of entries on a light and dark compartment and the total time spent on the light and dark compartment.[18]

Evaluation of Endogenous Antioxidant Enzymes

Superoxide dismutase (SOD) activity in the brain

Brain tissue homogenate (supernatant) and distilled water were combined in equal parts. 0.25 mL ice-cold ethanol and 0.15 mL ice-cold chloroform were added to the mixture. The mixture was well mixed with a cyclomixer before being centrifuged at 600 g for 15 minutes at 4ºC. EDTA solution (0.5 mL) was added to this. The reaction was started using 0.4 mL of epinephrine, and the change in optical density/min was evaluated against a reagent blank at 480 nm.[19,20]

Glutathione (GSH) activity in the brain

A mixture of equal quantities of brain tissue homogenate (supernatant) and 20% TCA was used. After centrifuging the precipitated fraction at 600 g for 15 minutes at 4°C, 2 mL of DTNB reagent was added to 0.25 mL of supernatant. Phosphate buffer was used to increase the final amount to 3.0 mL. At 412 nm, the colour created was compared to a reagent blank.[19,20]

Catalase (CAT) levels in the brain

The capacity of CAT to produce the disappearance of hydrogen peroxide was used to test CAT activity, which was done according to Beers and Sizer's approach. One unit of CAT is the quantity of enzyme required to decompose 1-µmol of peroxide per minute. To 40 µL of the supernatant of brain tissue homogenate, 1960 µL of the substrate (Mixture containing 1800 µL of 10 mM H2O2 and 100 mL of Tris–HCl buffer and 60 mL of D/W) were added. At 25°C, the exponential disappearance of H_2O_2 at 240 nm was used to estimate activity.[20]

Malondialdehyde (MDA) levels in the brain

In 300 µL of 10% trichloroacetic acid (TCA), each sample was added to 150 µL and centrifuged at 1000 rpm for 10 minutes at 4°C. About 300 µL of the supernatant was incubated with 300 µL 0.67% thiobarbituric acid at 100°C for 25 minutes. The mixture was cooled for 5 minutes and the thiobarbituric acid reactive substances (TBARS) concentration as pink stains was read in a spectrophotometer at 535 nm.[19,21]

Histopathology

At the end of the investigation, animals were killed by cervical dislocation, and their brains were rapidly removed and preserved with 10% formalin. The brain samples were afterward processed for histological analysis.[22]

Statistics

All the values were expressed as Mean ± SEM. Analysis of data was performed using GraphPad prism 5.0 software. The data were analyzed by one-way ANOVA followed by Tukey's test and two-way ANOVA followed by Bonferroni *post hoc* test using GraphPad Prism 5. $p < 0.05$ were considered the minimum level of significance.

Results

Preliminary Phytochemical Studies of Methanolic Extract of *C. dichotoma*

The extract obtained was subjected to a qualitative phytochemical test to find out the active constituents. Evaluation of preliminary phytochemical Studies of MECD is given in Table 1.

Acute Oral Toxicity of Methanolic Extract of *C. dichotoma*

No mortality and any behavioural abnormality were observed after the oral administration of the MECD at doses 175, 560, 1792, 2000 and 5000 mg/kg according to the body weight. Thus, the results indicate that MECD is safe till 5000 mg/kg oral dose.

Antianxiety Activity of MECD (50, 100, 200 mg/kg) and Diazepam dose (2 mg/kg) in EPM Test in Rats

Effect of MECD on number of open arm entries

Number of entries of control group rats in open arm were found be 3.167 ± 0.60. MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) for 7 days showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$, respectively) increases in number of entries in open arm when compared with the control group. MECD 50 mg/kg showed no significant increase in entries in the open arm. Further 14th days of treatment, the number of entries in the open arm in the control group of rats were 3.00 ± 0.36. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) treatment showed significant ($p < 0.01$; $p < 0.001$ and $p <$

0.001, respectively) increases in number of entries in open arm when compared with the control group. However, MECD 50 mg/kg did not show any significant increase in number of entries in the open arm.

After 21 days of treatment, the number of entries in the open arm in the control group of rats were found to be 3.167 ± 0.30. Treatment with MECD 100 and 200 mg/kg and diazepam (2 mg/kg) showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$) increases in number of entries in the open arm when compared with the control group. However, MECD 50 mg/kg ($p < 0.01$) showed no significant increase in the number of entries in the open arm. (Table 2)

Effect of Diazepam (2 mg/kg) and MECD on number entries in elevated plus maze test in rats

After 7 days of treatment, the number of entries in close arm in the control group of rats were found to be 12.50 ± 0.22. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$), respectively, decreases in number of entries in the close arm when compared with the control group. MECD 50 mg/kg did not show any significant decrease in number of entries in the close arm. Further, after 14 days of treatment, the number of entries in the close arm in the control group of animals were 12.66 ± 0.49. MECD rats (100 and 200 mg/kg) and diazepam (2 mg/kg) treated respectively showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$) decreases in number of entries in the close arm when compared with the control group. MECD 50 mg/kg did not show any significant decrease in number of entries in the close arm. Finally, after 21 days of treatment, the number of entries in the close arm in the control group of rats were 14.00 ± 0.51. MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) treatment respectively showed significant ($p < 0.001$ each) decreases in number of entries in the close arm when compared with the control group. MECD 50 mg/kg did not show any significant decrease in number of entries in the close arm (Table 3).

Effect of diazepam (2 mg/kg) and MECD (50, 100, 200 mg/kg) on time spent in open arm in elevated plus maze apparatus in rats

In this study we found that after 7 days of treatment with MECD (200 mg/kg), and diazepam (2 mg/kg) showed significant ($p < 0.05$ and $p < 0.01$) increases in the time spent in the open arm when compared with the control group. MECD 50 mg/kg did not show any significant increase in time spent in the open arm. After 14 days of treatment, the time spent in the open arm in the control group of animals was 98.50 ± 14.18. MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) treatment respectively showed significant ($p < 0.01$ and $p < 0.001$) increases in the time spent in the open arm when compared with the control group. MECD 50 mg/kg did not show any significant increase in time spent in the open arm. After 21 days of treatment, the time spent in the open arm in the control group of animals was 98.50 ± 14.18. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) treatment respectively showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$) increases in the time spent in the open arm when compared with the control group. MECD 50 mg/kg did not show any significant increase in time spent in the open arm (Table 4).

Effect of diazepam (2mg/kg) and MECD (50, 100, 200 mg/kg) on time spent in close arm in elevated plus maze apparatus in rats

We observed that the time spent in the close arm in the control group of animals was 195.66 ± 8.97. After 7 days of treatment with MECD (100 and 200 mg/kg), and diazepam (2 mg/kg) showed a significant ($p < 0.05$ and $p < 0.001$) decrease in the time spent in the close arm when compared with the control group. MECD 50 mg/kg did not show any significant decrease in time spent in the close arm. After 14 days of dosing, the time spent in the close arm in the control group of animals was 189.66 ± 4.44. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg)

Table 2: Effect of MECD on elevated plus-maze test on a number of open arm entries.

Days	*Control*	*MECD 50mg/kg*	*MECD 100mg/kg*	*MECD 200mg/kg*	*Diazepam*
7th	3.167 ± 0.60	3.833 ± 0.54	4.833 ± 0.30*	5.16 ± 0.47*	5.833 ± 0.30***
14th	3.00 ± 0.36	4.00 ± 0.44	5.00 ± 0.36**	5.667 ± 0.21***	6.167 ± 0.30***
21st	3.167 ± 0.30	4.66 ± 0.33*	5.667 ± 0.21***	6.33 ± 0.33***	6.50 ± 0.56***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

Table 3: Effect of MECD on elevated plus-maze test on a number of close arm entries

Days	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	12.50 ± 0.22	11.33 ± 0.49	10.50 ± 0.42**	10.16 ± 0.30**	6.16 ± 0.47***
14th	12.66 ± 0.49	11.16 ± 0.47	10.50 ± 0.22**	7.83 ± 0.54**	7.00 ± 0.68***
21st	14.00 ± 0.51	11.66 ± 0.55**	10.33 ± 0.42***	7.83 ± 0.477***	7.50 ± 0.42***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

Table 4: Effect of MECD on time spent in the open arm of an elevated plus-maze test

Days	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	104.33 ± 8.97	109.83 ± 8.38	132.83 ± 3.98	140.66 ± 16.23*	150.33 ± 16.03**
14th	98.50 ± 14.18	122.50 ± 3.72	146.50 ± 7.25**	163.00 ± 15.47***	172.00 ± 12.01***
21th	76.16 ± 10.97	128.66 ± 6.86**	164.50 ± 5.78***	181.833 ± 9.26***	186.00 ± 4.25***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p < 0.01, ***p < 0.001 as compared to control.

showed a significant (p < 0.05; p < 0.01 and p < 0.001) decrease in the time spent in the close arm when compared with the control group. MECD 50mg/kg did not show any significant decrease in time spent in the close arm. After 21 days of treatment, the time spent in the close arm in the control group of animals was 184.66 ± 15.13. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) showed significant (p < 0.01; p < 0.001 and p < 0.001) decrease in the time spent in the close arm when compared with the control group. However, MECD 50 mg/kg did not show any significant decrease in the time spent in the close arm (Table 5).

Antianxiety Activity of MECD (50, 100 and 200 mg/kg) and Diazepam (2 mg/kg) on Light and Dark Test in Rats

Effect of MECD (50, 100 and 200 mg/kg) and diazepam (2 mg/kg) on the number of entries in light compartment

7 days treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) showed significant (p < 0.05; p < 0.001 and p < 0.001) increases in a number of entries in the light compartment when compared with the control group. Number of entries showed by control group were 5.50 ± 0.042. MECD 50 mg/kg showed no significant increase in entries in the light compartment. After 14 days of treatment, the number of entries in the light compartment in the control group of animals was 6.33 ± 0.42. Treatment with MECD (100 and 200 mg/kg) and diazepam(2 mg/kg) showed significant (p < 0.01; p < 0.001 and p < 0.001) increases in a number of entries in the light compartment when compared with the control group. After the 21 day of treatment, the number of entries in the light compartments in the control group of animals was 7.33 ± 0.42. Treatment with MCD (100 and 200 mg/kg) and diazepam (2 mg/kg) showed significant (p < 0.01; p < 0.001 and p < 0.001) increases in number of entries in the light compartment when compared with the control group. MECD 50 mg/kg did not show any significant increase in no. of entries in the light compartment (Table 6).

Effect of MECD (50, 100 and 200 mg/kg) and diazepam (2 mg/kg) on the number of entries in dark compartment

The number of entries in the dark compartment in the control group of animals was 8.00 ± 0.44. After MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) (p<0.001) treatment, respectively showed significant decreases in number of entries in the dark compartment when compared with the control group. MECD 50 mg/kg showed no significant decrease in entries in the dark compartment. After 14 days of treatment, the number of entries in the dark compartment in the control group of animals was 8.33 ± 0.49. MECD (100 and 200 mg/kg), and diazepam (2 mg/kg) respectively showed significant decreases in number of entries in the dark compartment when compared with the control group. MECD 50 mg/kg showed no significant decrease in entries in the dark compartment. After the 21 days of treatment, the number of entries in dark compartment in the control group of animals was 8.83 ± 0.40. Treatment with MECD (100 and 200 mg/kg)

Table 5: Effect of MECD on time spent in the close arm of an elevated plus-maze test

Days	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	195.66 ± 8.97	182.50 ± 6.83	167.16 ± 3.98	159.33 ± 16.2*	139.66 ± 15.35***
14th	189.66 ± 4.44	177.50 ± 3.72	152.33 ± 2.95*	137.00 ± 15.4**	128.00 ± 12.01***
21th	184.66 ± 15.13	171.33 ± 6.86	135.50 ± 5.78**	118.16 ± 9.26***	114.00 ± 4.25***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

Table 6: Effect of MECD leaves on a number of light compartment entries of light and dark test

Day	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	5.50 ± 0.42	6.83 ± 0.30	7.16 ± 0.40*	7.66 ± 0.55*	8.83 ± 0.54***
14th	6.33 ± 0.42	8.00 ± 0.25*	8.50 ± 0.42**	9.16 ± 0.30**	10.00 ± 0.36***
21st	7.33 ± 0.42	9.33 ± 0.21**	10.83 ± 0.60***	11.83 ± 0.54***	11.83 ± 0.30***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

and diazepam (2 mg/kg) treatment respectively showed significant ($p < 0.001$ each) decreases in number of entries in the dark compartment when compared with the control group. MECD 50 mg/kg did not show any significant decrease in number of entries in the dark compartment (Table 7).

Effect of MECD (50, 100 and 200 mg/kg) and diazepam (2 mg/kg) on time spent in light compartment treatment

After 7 days of treatment, the time spent in the light compartment in the control group of animals was 104.33 ± 8.97. Treatment with MECD (100 and 200 mg/kg), and diazepam (2 mg/kg), showed significant ($p < 0.05$; $p < 0.01$ and $p < 0.001$) increases in the time spent in the light compartment when compared with the control group. MECD 50 mg/kg did not show any significant increase in the time spent in the light compartment. After 14 days of treatment, the time spent in the light compartment in the control group of animals was 58.00 ± 5.48. MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) treated group showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$) increases in the time spent in the light compartment when compared with the control group. MECD 50 mg/kg did not show any significant increase in the time spent in the light compartment. After 21 days of treatment, the time spent in the light compartment in the control group of animals was 58.00 ± 5.48. Treatment with MECD (100 and 200 mg/kg)and diazepam (2 mg/kg) showed significant ($p < 0.001$ each) increases in the time spent in the light compartment when compared with the control group. MECD 50 mg/kg did not show any significant increase in the time spent in the light compartment (Table 8).

Effect of MECD (50, 100 and 200 mg/kg) and diazepam (2 mg/kg) on time spent in dark compartment

After 7 days of treatment the time spent in the dark compartment in the control group of animals was 236.16 ± 4.70. Treatment with MECD (100 and 200 mg/kg) showed a significant ($p < 0.05$; $p < 0.01$and $p < 0.001$) decrease in the time spent in the light compartment when compared with the control group. After 14 days of treatment, the time in the dark compartment in the control group of animals was 242.00 ± 5.48. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) showed a significant ($p < 0.01$; $p < 0.001$and $p < 0.001$) decrease in the time spent in the dark compartment when compared with the control group. MECD 50 mg/kg did not show any significant decrease in time spent in the dark compartment. After the 21 days of treatment, the time spent in the dark compartment in the control group of animals was 245.33 ± 3.98. Treatment with MECD (100 and 200 mg/kg) and diazepam (2 mg/kg) showed a significant ($p < 0.001$ each) decrease in the time spent in the dark compartment when compared with the control group. MECD 50 mg/kg did not show any significant decrease in the time spent in the dark compartment (Table 9).

Effect of MECD on Endogenous Antioxidant Enzymes in the Brain

Effect of MECD on superoxide dismutase (SOD) activity

In the present investigation, we found a significant decrease in the activity of SOD in the brain tissue of the control group. But the MDCD (50, 100 and 200 mg/kg) and Diazepam (2 mg/kg) treated group showed a significant increase the reduced level of SOD when compared with the control group. MECD 200 mg/kg showed a more significant effect when compared with MECD 50 mg/kg (Table 10).

Effect of MECD on catalase activity

At the end of the experiment, we found a significant decrease in the level of catalase (CAT) in the control group. However, MDCD (50, 100 and 200 mg/kg) and diazepam (2 mg/kg) treatment group showed significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$) increase the level of catalase when compared with the control group. MECD 200 mg/kg showed a maximum effect when compared with MECD 50 mg/kg (Table 10).

Table 7: Effect of MECD on light and dark test on the number of dark compartment entries

Day	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	8.00 ± 0.44	6.50 ± 0.61	5.83 ± 0.47*	5.50 ± 0.50**	4.00 ± 0.36***
14th	8.33 ± 0.49	6.83 ± 0.47	5.83 ± 0.65**	5.33 ± 0.88***	4.83 ± 0.70***
21st	8.83 ± 0.40	7.50 ± 0.22	5.83 ± 0.47***	5.16 ± 0.30***	4.83 ± 0.60***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control

Table 8: Effect of MECD on time spent in light compartment

Days	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	63.83 ± 4.70	74.50 ± 4.8	83.66 ± 8.01*	88.66 ± 4.44*	107.82 ± 2.59***
14th	58.00 ± 5.48	69.66 ± 11.79	84.83 ± 6.2**	105.00 ± 1.48**	121.83 ± 2.08***
21st	54.66 ± 3.98	80.50 ± 2.74$	120.16 ± 6.0***	140.66 ± 4.05***	143.33 ± 3.53***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

Table 9: Effect of MECD on time spent in the dark compartment

Days	*Control*	*MECD 50 mg/kg*	*MECD 100 mg/kg*	*MECD 200 mg/kg*	*Diazepam*
7th	236.16 ± 4.70	225.50 ± 4.86	217.83 ± 8.6*	215.50 ± 6.05**	183.00 ± 3.17***
14th	242.00 ± 5.48	230.33 ± 11.7	190.00 ± 4.0**	216.16 ± 2.75***	156.66 ± 3.50***
21st	245.33 ± 3.98	219.33 ± 2.74**	170.66 ± 5.2***	179.83 ± 6.03***	156.16 ± 3.67***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

Table 10: Effect of MECD on superoxide dismutase (SOD) activity, catalase activity, glutathione (GSH) activity and malondialdehyde (MDA) activity

Groups	*SOD (nmol/mL)*	*Catalase (µmol/mL)*	*GSH (Mmol/g Protein)*	*MDA(µmol/mL)*
Control	1.46 ± 0.19	4.17 ± 0.25	17.79 ± 1.37	161.93 ± 4.38
MECD 50 mg/kg	2.48 ± 0.17*	5.76 ± 0.42	25.37 ± 1.11**	131.7 ± 3.4***
MECD 100 mg/kg	3.31 ± 0.14***	7.77 ± 0.34***	28.80 ± 0.85***	92.14 ± 6.06***
MECD 200 mg/kg	3.70 ± 0.30***	10.35 ± 0.38***	35.22 ± 1.61***	70.50 ± 4.40***
Diazepam 2 mg/kg	4.00 ± 0.25***	10.96 ± 0.47***	28.07 ± 228***	55.43 ± 3.11***

Data expressed as Mean ± SEM, n=6 per group. The data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

Effect of MECD on glutathione (GSH)

We found a significant decrease in the level of GSH in the control group. MDCD (50,100 and 200 mg/kg) and diazepam (2 mg/kg) were significant ($p < 0.01$; $p < 0.001$ and $p < 0.001$) increase the level of SOD when compared with the control group (Table 10).

Effect of MECD on malondialdehyde (MDA) activity

After 21 days, we observed a significant increase in the malondialdehyde (MDA) level in the control group. Treatment with MDCD (50, 100, 200 mg/kg) and diazepam 2 mg/kg showed a significant decrease in the elevated level of MDA compared with the control group (Table 10).

Histopathological Changes

Histopathology examination of the brain tissue in the control group showed changes in the hippocampus part. In the control group, there was seen more damage to neurons. MECD (50, 100, and 200 mg/kg) and diazepam (2 mg/kg) significantly reduced neuronal damage. The brain tissues of rats treated with MECD at 100, and 200 mg/kg revealed multifocal mild neuronal degeneration and prevented damage to the neurons in the brain, both groups show decrease in the number of darkened nuclei and damaged neurons (Fig. 1).

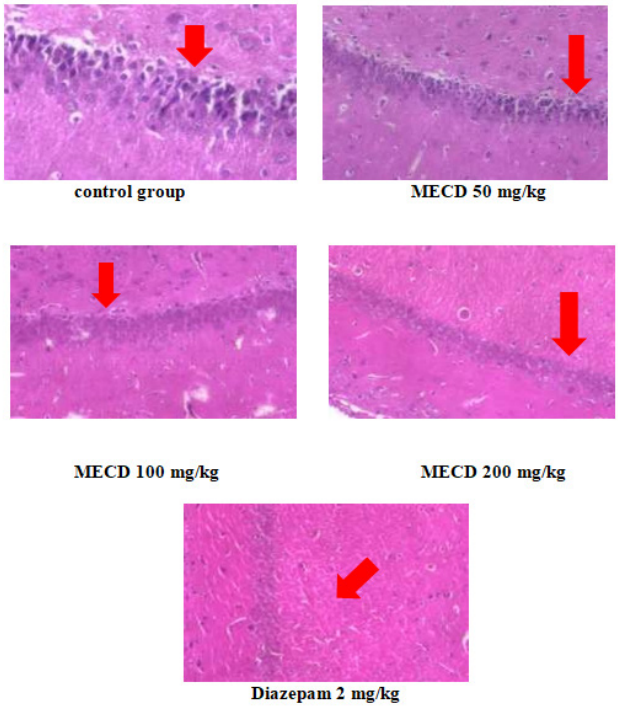

Fig. 1: Effect of MECD (50, 100, 200 mg/Kg) on histopathological changes

Discussion

It is reported that *C. dichotoma* and *C. myxa* are species of same genus and belong to family Boraginaceae.[23,14] Arora and Deswal, 2015 reported the antianxiety potential of *C. myxa* but there is no any report showed the antianxiety activity of its congener species *C. dichotoma* in rats.[24] However, other pharmacological activities like antimicrobial, antifungal, anti-inflammation and anticancer have already been reported in *C. dichotoma*.[1]

In present study the MECD was subsequently put through pharmacological testing utilizing several models. It is proved that methanolic extract showed the presence of steroid, carbohydrate, alkaloid, cardiac glycosides, and flavonoids.[25] In this study, we also investigated and confirmed the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, steroids, etc after performing the preliminary phytochemical test. These essential chemical constituents may be responsible for the anxiolytic activity in rats. Presence of flavonoids are

proven to possess antianxiety activity has been showed by researchers in their previous studies. However according to the previous reports, the antianxiety effect of flavonoids may be because of its potential to acts on central nervous system[10,11] and benzo diazepam receptors.[12,13] So, flavonoids in MECD may be responsible for antianxiety activity.

In the current study, we have selected two most validated models, EPM and light and dark tests, which are well-accepted experimental animal models typically used to test the efficacy of antianxiety drugs.[26-28]

The EPM test is based on an idea where the entries to an open arm of EPM produce an approach-avoidance struggle that is stronger than that induced by the entries to an enclosed arm.[29] The reduction in dislike to the open arm is the result of an anxiolytic effect, stated by a rise in time spent and entries into the open arm. The primary index is spatiotemporal in nature; anxiolytic drugs reduce it and can be increased by anxiogenic compounds.[30]

Strong dislike to new open spaces and fear of balancing on narrow raised platforms can induce anxiety which is similar to human in rodents.[28] Therefore, avoidance to go to open arm is considered as anxiogenic behavior.[27,31]

In this study MECD increased the exploration of the open arms (time and entries into open arms) showed its anxiolytic effect.

The light and dark model used to predict the antianxiety activity of drug in rodents.[32] This model is very quick and easy to use without prior training of animal and food, and water withdrawal. In this model natural stimuli used anxiolytic drug specifically increase number of entries and time spent in light area in light and dark models. This behavioural activity of rats is the basis to use this model as screening model to evaluate antianxiety drug. In present study MECD (100 and 200 mg/kg) increased number of entries and time spent in light area explained its anxiolytic effect.

Increased reactive oxygen species (ROS) cause oxidative stress, which weakens the brain's defensive system. To prevent oxidative stress, antioxidants are necessary. All living cells contain GSH, a significant antioxidant. Converting hydrogen peroxide to oxygen and water shields the cell against superoxide, hydroxyl, and free oxygen radicals. Reduced glutathione levels cause mitochondrial damage, which exacerbates the neurological damage that anxiety sufferers experience. The catalase enzyme converts hydrogen peroxide into water and oxygen, preventing oxidative stress on the cell. A lipid peroxidation marker known as MDA is an aldehyde. Indicators of mitochondrial dysfunction brought on by oxidative stress include elevated MDA levels in the brain.[33]

In our study, oxidative stress was measured by brain antioxidant and lipid peroxidation levels. In the control group decreases in antioxidant levels such as SOD, CAT, and GSH and an increase in lipid peroxidation levels, ultimately leading to an increase in oxidative stress. These findings, which align with other research in the literature, demonstrate that oxidative stress plays a significant role in the development of anxiety.[34] The rats are pre-treated with MECD at 50, 100, and 200 mg/kg p.o doses. Significantly increasing the SOD, CAT, GSH level, and decrease in malondialdehyde (MDA) level confirmed its antioxidant activity and effective role in anxiolytic activity.

Histopathological examination was done to evaluate the possible protective effect of methanolic extract of *C. dichotoma* on the cellular level. The control group and the MECD treatment groups (50, 100, and 200 mg/kg) all had their brains histopathological examined with hematoxylin and eosin stain. In the control group results, neuronal cell damage in the hippocampus area. The brain tissue's structural alterations and loss of neuronal cells were shown to be not repaired by MECD 50 mg/kg. However, oral administration of MECD at doses of 100 and 200 mg/kg and in standard group diazepam shows considerably improved brain shape and reduced swelling and inflammation, with minimal loss of neurons. These findings support our postulation that *C. dichotoma* reduced the structural alterations and loss of neuronal cells and played an important role in anxiolytic activity.

It may be concluded that MECD in high dose (100 and 200 mg/kg) restored oxidative injuries associated with anxiety disease. This indicated that MECD has potent antioxidant properties. MECD also exhibits antianxiety activity in rats using EPM and light and dark test models of anxiety. The antianxiety effect was further revealed by histopathological examination of brain tissues of rat. Based on this observation, we concluded that MECD possessed an anxiolytic activity. In addition, a further detailed investigation is required to find out the phytoconstituent responsible for the anxiolytic effect of MECD and to explain its anxiolytic mechanism.

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HOW TO CITE THIS ARTICLE: Shendage SS, Jadhav S, Dhawale K, Sawant SH. Anxiolytic Activity of Methanolic Extract of *Cardia dichotoma* Leaves in Rats. Int. J. Pharm. Sci. Drug Res. 2023;15(4):519-527. **DOI:** 10.25004/IJPSDR.2023.150416