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Clitoria ternatea in Ethanol Induced Anxiety

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ABSTRACT

The present study aimed to investigate the anxiolytic properties of *Clitoria ternatea.* The organic solvent (Petroleum ether, Ethyl acetate, and Methanol) extracts from the leaves of *Clitoria ternatea* (Papilionoideae) were tested against the ethanol-induced mice in open arm and closed arm. The results showed promising anxiolytic activity against ethanol-induced mice. Among these, methanol extract was found to possess a more potent inhibitory activity effect when compared to the other extracts (Petroleum ether and Ethyl acetate). The results of this study validate the use of methanol extract in the anxiolytic properties of *Clitoria ternatea*.

INTRODUCTION

Our brain is an electrically operated switch on a delicate balance of chemicals and processes. Alcohol is a depressant, which means it can disrupt the whole and sole balance, affecting our thoughts, feelings, and actions and sometimes our long-term mental health also damaged. The glass of wine after a hard day might be a help to relax, in the long run, it can contribute to feelings of depression and anxiety and make stress and it's very difficult to deal with them. This is because regular, heavy drinking interferes with neurotransmitters in our brains (1). Anxiety and alcohol use can both be characterized at two different levels, symptomatic and syndrome. A big relationship between feelings of anxiety and drinking behavior. Some researchers told that a higher level of anxiety would be related to a higher intake of or frequency of drinking behavior (2, 3). Physiologically, alcohol withdrawal can often produce anxiety symptoms such as shakiness (4) or increased startle, a common symptom of PTSD (5). Also, neural adaptation occurs with frequent and excessive alcohol use over time such that repeated alcohol withdrawals sensitize this withdrawal-induced anxiety (6). This has often been referred to as the ''kindling-stress hypothesis''; that is, repeated withdrawals from chronic heavy drinking are thought to worsen, or ''kindle'' withdrawal-induced anxiety A number of studies have also demonstrated increased norepinephrine activity as well as hyperexcitability of the central nervous system, especially of limbic structures, during alcohol withdrawal (4,7). These are the same neural systems that have been implicated in panic attacks and panic disorder, providing a possible physiological explanation for the link between panic disorder and alcohol use disorders (7). Extract of *Clitoria ternetea* is useful for alcohol-induced anxiety.

Anxiety and alcohol use can both be characterized at two different levels: symptomatic and syndromal. An association at the former level would entail a clear relationship, for example, between feelings of anxiety and drinking behavior. That is, one would expect that higher levels of anxiety would be related to higher quantities and/or frequency of drinking behavior.

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MATERIALS AND METHODS

Extraction

The aerial parts were collected from the local areas of Pune. The dried aerial parts were powdered. The powdered material was extracted with ethanol (95%) by the maceration extraction method. It was then filtered and concentrated by evaporation. *Clitoria ternatea* (CT, 5 % w/w) was concentrated under reduced pressure. The dried extract was dissolved in distilled water and administered orally.

Animals

Male Swiss albino mice (22-25g) were housed in groups of five under standard laboratory conditions of temperature, humidity, and light. Animals had free access to food and water. Each group consisted of six animals. All experiments were carried out during the light period (11-13 h). All the protocols were approved by the Institutional Animal Ethics Committee (Approval no. SCOP/IAEC/2015- 16/03/231) constituted for the control and supervision of experimental animals by the Ministry of Environment and Forests, Government of India, New Delhi, India.

Drug

Ethanolic extract of *Clitoria ternatea* (100, 200 and 400 mg/kg, p. o.) was dissolved in the suspension of gum acacia (2 % w/v) in distilled water. All other chemicals were of analytical grade. Absolute ethanol was given orally with a liquid diet in drinking water to make a different % w/v solution. Control animals received corresponding saline solution (0.9%) in all cases.

Induction of ethanol dependence

To develop dependence, mice were individually housed in small cages (28 ×14 × 14 cm) and provided with a nutritionally balanced control liquid diet on day 0 as their sole nutrient source (600 kcal/l). From day 1 to day 4, ethanol (1.8% v/v) was incorporated into the liquid diet, followed by 3.6% v/v of ethanol (from day 5 to 7) and 6.3% v/v of ethanol (from day 8 to10). On day 11, at 8.00 h, ethanol liquid diet was replaced with a nutritionally balanced control liquid diet. Mice from the control group were provided with a liquid diet without ethanol from day 0 to 10, which was isocaloric to ethanol liquid diet on a respective day.

Liquid diets were daily prepared and replaced at 8.00 h. At the end of this feeding pattern, no difference was observed in the weights of mice from the ethanol diet and control liquid diet groups (8).

Treatment schedule

Ethanol liquid diet was provided for days and on 11 days alcohol was withdrawn from the diet. *Clitoria ternatea* treatment was started on the first day of the study. Mice were randomly selected and divided into six groups of 5-6 animals each.

Group 1: Normal control (Vehicle)

Group 2: Ethanol control

Group 3: Ethanol + EECT (100 mg/kg)

Group 4: Ethanol + EECT (200 mg/kg)

Group 5: Ethanol + EECT (400mg/kg)

Symptoms

The ethanol dependence was assessed by scoring the withdrawal-induced physical signs of hyperexcitability, graded as per the scale. In brief, each mouse was lifted by the tail, spun gently through a 180o arc and held 30 cm away under an angle-poised lamp (60 W) for 3 sec; and ethanol withdrawal-induced physical signs were observed and graded at hourly (2,4,6,8 and 10 h) interval for 10 h to determine the time of peak withdrawal symptoms 11. Assessment of anxiety by elevated plus-maze test. The apparatus used for the elevated plus-maze test is in the configuration of a + and comprises two open arms (25 x 5 x 0.5 cm) across from each other and perpendicular to two closed arms (25 x 5 x 16 cm) with a center platform (5 x 5 x 0.5 cm). The open arms have a very small (0.5 cm) wall to decrease the number of falls, whereas the closed arms have a high (16 cm) wall to enclose the arm. The entire apparatus is 50 cm above the floor and is placed in an empty circular tank (100 cm diameter, 35 cm tall; normally used for the Morris water maze task) to protect the mice that fall or attempt to escape during the experiment. The apparatus is made of plastic materials. The platform is white and the walls are transparent. There is a variation in materials and colors of the apparatus of the elevated plus-maze. All the experimental mice are transferred to the

behavior testing room 30 min before beginning the first trial to habituate to the condition of the behavior testing room. The elevated plus-maze test is recorded using a video camera attached to a computer, which is controlled by a remote device. The number of entries (an entry is defined as the center of mass of the mouse enters the arm) into each arm and the time spent in the open arms is recorded and these measurements serve as an index of anxiety-like behavior. Mice are allowed to move freely about the maze for 10 min. Number of entries in open am and closed arm is measured. Later on, data was calculated and analyzed. The animal was considered immobile if it stops swimming and became motionless or showed a state of desperate where it halts the struggle to escape from the cylinder filled with water.

Bodyweight

The bodyweight of the animal was taken in the entire group before ethanol liquid diet consumption and after ethanol withdrawal and also measured daily for standardization of liquid diet (to confirm that intake of the liquid diet did not vary systematically over the days or between the groups).

Procedure

Bodyweight was measured gravimetrically using electronic balance every day and also % increase in body weight measured in a group before ethanol liquid diet consumption and after ethanol withdrawal.

Behavioral study

Elevated plus-maze test

Elevated plus-maze is the most significant model compared to all other models. The plus arms shaped maze was made of wood. It consists of two open arms (28.5×7 cm) (35×6 cm), and two enclosed (28.5×7×14 cm) (35×6×15 cm) with an open roof, arranged so that the two open arms are opposite to each other. The maze is elevated to a height of 50 cm. The plus-maze experiment was initiated by placing the mice in the center of the plus-maze facing an open arm. During this, the anxiety-like effect of ethanol withdrawal was measured time spent on the open arms and a total number of entries in open arm record for 5 min. in the plus-maze. An "arms entry" was recorded when the mice entered the arm with all four paws. The maze was carefully cleaned with tap water after each test session. Experiments were carried

out in a darkened and quiet room with a constant light of 15 W, located 80 cm above the maze and directed towards the apparatus. The light levels on the open- and enclosed arms were equal. The procedure was conducted preferably in a sound-attenuated room, with observations made from an adjacent room via a remote control TV camera (9).

RESULTS

Effect of EECT on ethanol-induced anxiety in mice (Elevated plus maze).

Effect of EECT on number of open arm entries

In the present study, we observed the effect of ethanolic extract of *Clitoria ternatea* (100, 200 and 400 mg/kg p.o. for 10 days). The development of ethanol withdrawal anxiety was assessed by counting the number of entries in open arm in elevated plus maze test on the 12th day. One way ANOVA revealed a significant decrease in open arm entries in ethanol withdrawal mice when compared with normal control ($P<0.001$). Tukey's post hoc test revealed that EECT significantly increased open arm entries in the treatment group when compared with ethanol control ($P<0.001$, $P<0.05$). EECT 400mg/kg shows more significant effect when compared with EECT 100 mg/kg ($P<0.01$).

Table No. 1: Effect of EECT on number of open arm entries

Treatment	No. of open arm entries
Normal Control	7.33±0.4472
Ethanol Control	3.167±0.3073*
EECT 100 mg/kg	5.167±0.3073$^{\wedge}$
EECT 200 mg/kg	6.000± 0.5574$^{\$}$
EECT 400 mg/kg	7.667± 0.4216$^{\$@}$

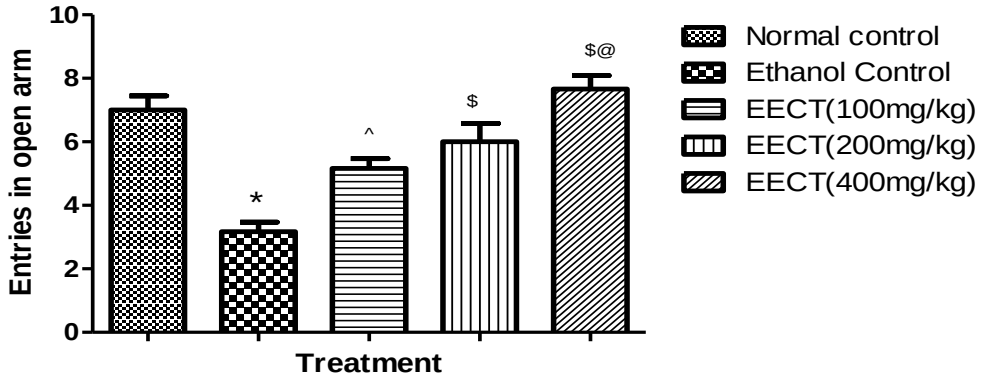


Figure No. 1: Effect on number of open arm entries.

n=6*P<0.001Vs. Normal control, $P<0.001, ^P<0.05Vs. Ethanol control, @P<0.01Vs. EECT 100mg/kg.

Effect of EECT on number of closed arm entries

After treatment with ethanolic extract of *Clitoria ternatea* (100, 200 and 400 mg/kg p.o.) for 10 days, the development of ethanol withdrawal anxiety was assessed by counting the number of entries in closed arm in elevated plus maze test on 12th day. One way ANOVA revealed a significant increase in closed arm entries in ethanol control when compared to normal control (P<0.001). Tukey's post hoc test revealed that the number of closed arm entries was significantly decreased in the treatment group (200 and 400 mg/kg oral) when compared with ethanol control (P<0.01, P<0.001 respectively). EECT 400 mg/kg was shown a more significant effect when compared with EECT 200 mg/kg (P<0.01).

Table No. 2: Effect of EECT on number of closed arm entries

Treatment	No. of closed arm entries
Normal Control	9.000 ± 0.3651
Ethanol Control	14.000 ± 0.5164*
EECT 100 mg/kg	12.50 ± 0.4282
EECT 200 mg/kg	11.33 ± 0.4216&
EECT 400mg/kg	9.833± 0.4773$@

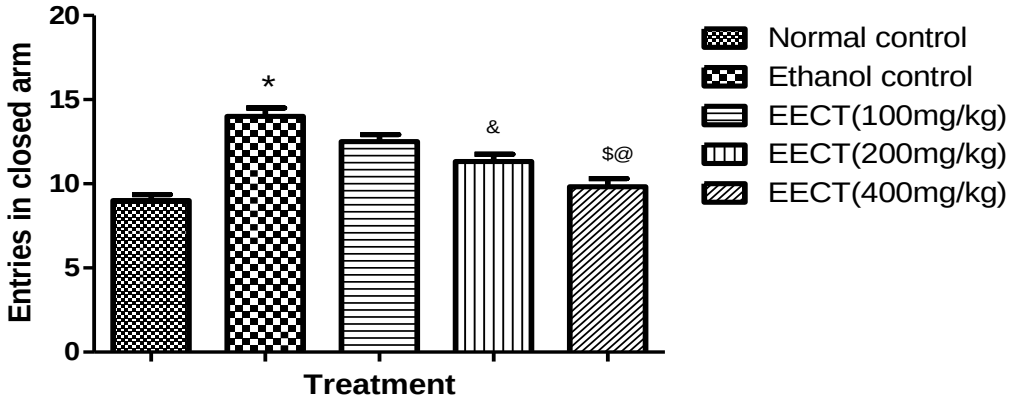


Figure No. 2: Effect on number of closed arm entries.

n=6 *P<0.001Vs. Normal control, $^{\&}$P<0.01, $^{\$}$P<0.001 Vs. Ethanol control, $^{@}$P<0.01 Vs. EECT 100mg/kg.

Effect of EECT on time spent in open arm

In the present investigation, the treatment of ethanolic extract of *Clitoria ternatea* (100, 200 and 400 mg/kg p.o.) for 10 days, the development of ethanol withdrawal anxiety was assessed by observing time spent in open arm in elevated plus maze test on 12th day. One way ANOVA revealed that time spent in the open arm was significantly decreased in the ethanol control group when compared to the normal control group (P<0.001). Tukey's post hoc test revealed that the treatment group significantly increased the time spent in the open arm when compared with ethanol control (P<0.001). EECT 400 mg/kg showed a more significant effect when compared with EECT 100 mg/kg (P<0.05).

Table No 3: Effect of EECT on time spent in open arm

Treatment	**Time spent in open arm**
Normal Control	73.67 ± 3.221
Ethanol Control	33.00 ± 2.129*
EECT 100 mg/kg	49.17 ± 1.276
EECT 200 mg/kg	47.00 ± 0.8433$^{\$}$
EECT 400 mg/kg	58.33 ± 1.838$^{\$\%}$

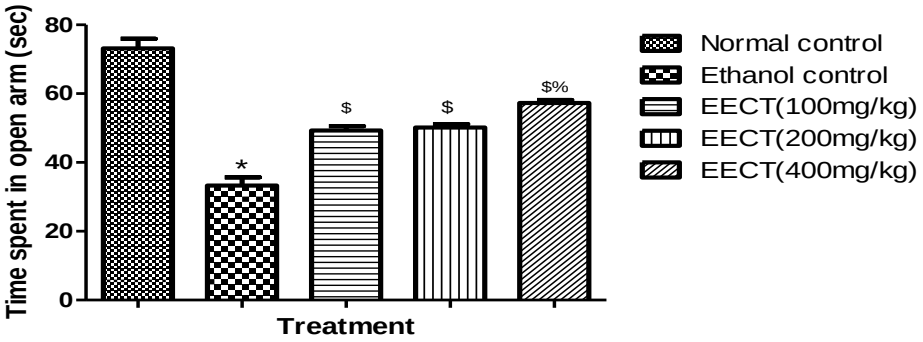


Figure no 3.Effect on time spent in open arm.

n=6, *P<0.001Vs. Normal control, $P<0.001 Vs. Ethanol control, %P<0.05Vs. EECT 100mg/kg.

Effect on time spent in the closed arm

After treatment of ethanolic extract of *Clitoria ternatea* (100, 200 and 400 mg/kg oral) for 10 days, the development of ethanol withdrawal anxiety was assessed by time spent in open arm in elevated plus maze test on 12th day. One-way ANOVA revealed that time spent in the closed arm was significantly increased in the ethanol control group when compared to the normal control group (P<0.001). Tukey's post hoc test revealed that the EECT treatment group significantly decreased time spent in the closed arm when compared with ethanol control (P<0.001). EECT 400 mg/kg showed a more significant effect when compared with EECT 100 and 200 mg/kg (P<0.001, P<0.01 respectively).

Table no 4. Effect on time spent in the closed arm

Treatment	**Time spent in the closed arm**
Normal Control	209.0 ± 1.453
Ethanol Control	250.2 ± 1.962*
EECT 100 mg/kg	234.0 ± 1.483$
EECT 200 mg/kg	236.0 ± 1.147$
EECT 400mg/kg	225.0± 1.014$#@

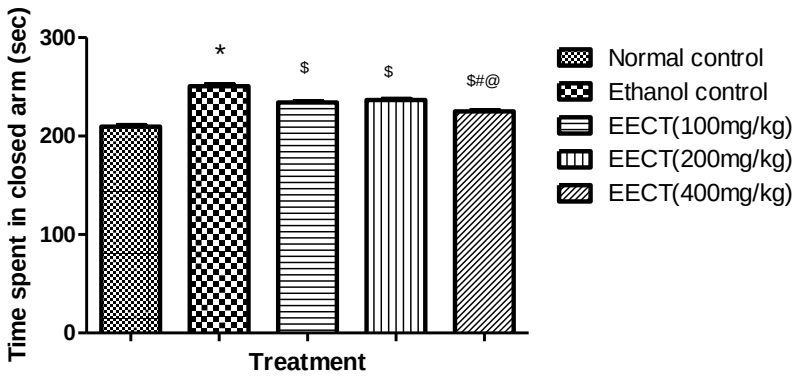


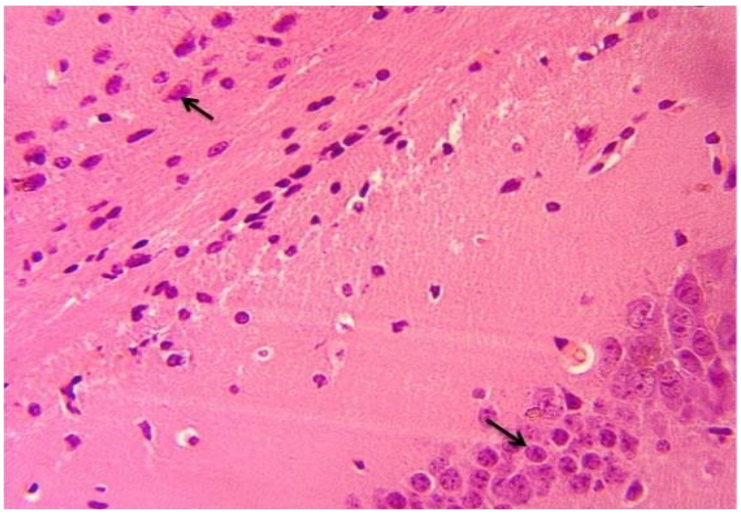
Figure No. 4: Effect of EECT on time spent in closed arm

n=6, *P<0.001Vs. Normal control, $P<0.001Vs. Ethanol control, @P<0.01, Vs. EECT 100mg/kg, #P<0.001, Vs. EECT 200mg/kg.

Histopathological sections of mice brain

Ematoxylin and eosin stained histopathological sections of mice brain.

(A=Normal control, B- Ethanol control C- EECT 100mg/kg, D- EECT 200mg/kg, EECT-400mg/kg



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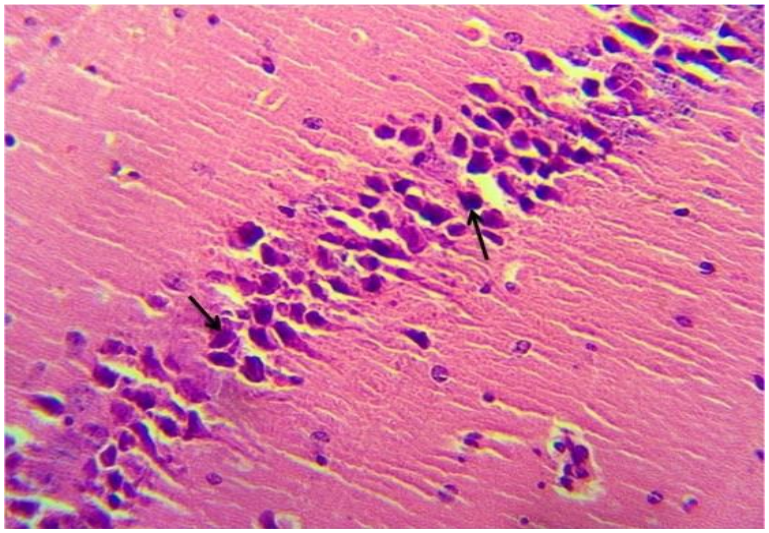

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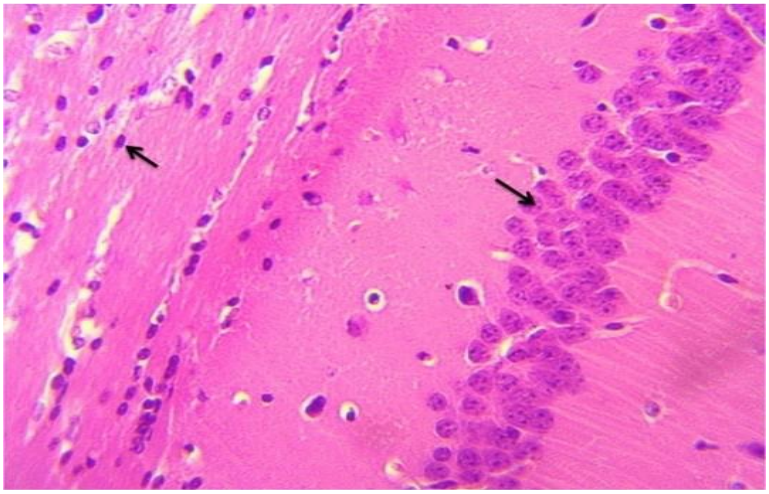

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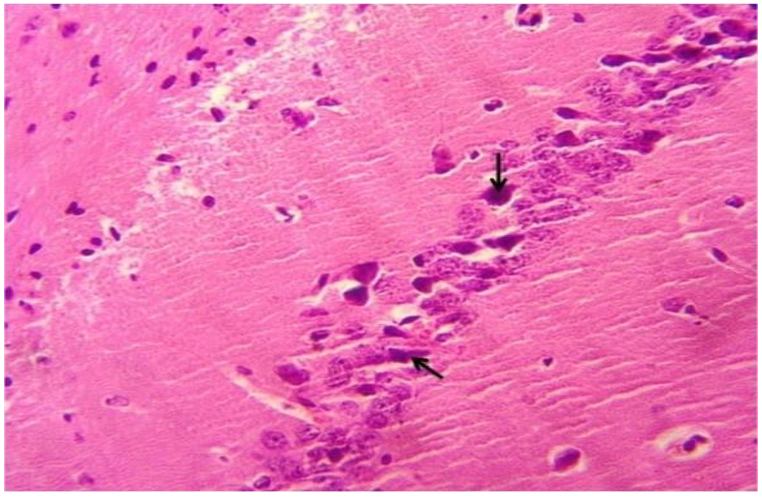
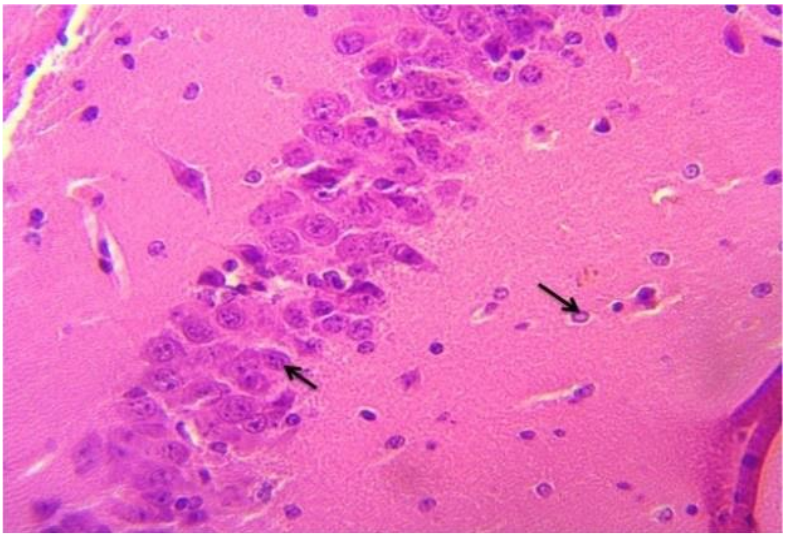


(D)



(E)

DISCUSSION

Alcoholism, also known as alcohol dependence, is a disease that includes the four symptoms i.e. Craving, a strong need or urge to drink. Loss of control meant not being able to stop drinking once drinking has begun (9) (Katzung, 2007). Tolerance is the need to drink greater amounts of alcohol to get high. Alcohol withdrawal is the change that the body goes through when a person suddenly stops drinking after chronic use. Cessation or reduction of alcohol consumption after periods of prolonged alcohol intake results in an alcohol withdrawal syndrome in humans and other animals (10).

Clitoria ternatea in ethanol-induced symptoms like depression, anxiety, and memory dysfunction. Ethanol dependence was induced in mice by chronic administration of ethanol liquid diet for 10 days as described in the earlier reported method. Bodyweight was measured a ^{1st}, 7^{th} and 11^{th} day. A significant increase in body weight up to day 7 when compared with control mice. But after day 7 the body weight was significantly decreased in the ethanol control group when compared with the normal control mice group. There is no significant effect on body weight between normal control and drug-treated groups.

Clitoria ternatea (100, 200 and 400 mg/kg) significantly increased the number of open arm entries whereas a decreased number of closed arm entries. The time spent in the open arm was increased whereas time spent in the closed arm was decreased in the elevated plus-maze test for anxiety. The exploration ratio was significantly increased in the treatment group (200 and 400mg/kg) when compared with ethanol control in novel object recognition test for

enhancement of memory dysfunction. The duration of immobility in the forced swim test for antidepressant activity was significantly decreased in the treatment group when compared against ethanol control.

Hence, it was observed that cognitive impairment in mice was mainly due to the degeneration of neurons in functional zones of the brain that controls memory and learning behaviour.

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