

## EVALUATION OF ETHANOLIC EXTRACT OF *CLITORIA TERNATEA* IN ETHANOL WITHDRAWAL INDUCED DEPRESSION

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### Abstract

The harmful and hazardous use of alcohol is a serious problem in the world. It results in serious health, social and economic harms, and is the third-leading risk factor for death and disability. The primary goal of ayurvedic medicine is to help people live long, healthy and balanced life with lesser side effects and higher efficacy. The present study evaluated the effect of ethanolic extract of *Clitoria ternatea* in ethanol withdrawal induced depression. Ethanol dependence was produced in mice to ethanol withdrawal signs; mice were individually housed in small cages and provided 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 in to 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 to 10). On day 11, at 8.00 h, ethanol liquid diet was replaced with nutritionally balanced control liquid. *Clitoria ternatea* extracts (100, 200 and 400 mg/kg) showed decrease in duration of immobility time than ethanol control mice. So it can be concluded that *Clitoria ternatea* may be beneficial in the treatment of ethanol withdrawal induced depression.

**Keywords:** Alcoholism, Ethanol withdrawal, EECT, Liquid diet.

## Introduction

Alcoholism is a serious public health problem that often results in medical, social and economic consequences throughout the world<sup>1</sup>. Alcohol affects our entire body, and daily excessive consumption of alcohol generally induces unpleasant physical symptoms such as headache, nausea and vomiting. World Health Organization defines alcoholism as the repeated (intermittent or continual) ingestion of alcohol leading to dependency, physical disease or other harm. Long-term consumption of alcohol in large quantities induces a number of disorders<sup>2</sup>. Alcohol withdrawal depends on signs and symptoms, including hyperactivity such as nausea, vomiting, sweating, shakiness, agitation, and anxiety, that develop when alcohol use is stopped after a period of heavy drinking. In other words, the ethanol withdrawal syndrome is a complex set of symptoms occurring in alcohol dependent patients after alcohol cessation. It involves a wide range of brain neurotransmitters implicated in the development of alcohol tolerance and reflects a homeostatic readjustment of the CNS<sup>3</sup>. Depression is a commonest temper disorder and at least has four symptoms such as changes in weight and appetite, changes in sleep and activity, feeling of crime, thought and decision problems and thought to self-murder<sup>4</sup>. According to the social and familial problems and also the risk of self-murder associated with depression, treatment programs gain more importance. Although using chemical drugs are among the most effective methods to deal with this disorder, but about one third of patients have no response to them, or shows several side effects after consuming these drugs. Thus finding new drugs with lower side effects is an important improvement on treatment of depression disorders<sup>5</sup>. At the other hand, medicinal plants introduce as an important source for finding new drugs with lower side effects. *Clitoria ternatea* (Family Fabaceae) is commonly known as "Butterfly pea". *Clitoria ternatea* are commonly used in the Ayurvedic system of medicine. The roots are bitter, refrigerant, laxative, intellect promoting, diuretic, anthelmintic and tonic and are useful in dementia<sup>6</sup>. *Clitoria ternatea* contains

antifungal proteins and has been shown to be homologous to plant defensin<sup>7</sup>. Rai et al., (2001) using passive avoidance test and spatial learning T-maze have shown that the aqueous root extract of *Clitoria ternatea* at dose 300 and 500 mg/kg doses enhances memory in rats<sup>8</sup>. The extract at 300 mg/kg dose produced significant memory retention, and the root parts were found to be more effective. We investigated the antidepressant activity of *Clitoria ternatea* by using forced swim test in mice at dose 100, 200 and 400 mg/kg.

## 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 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 a 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 purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

### Drug

Ethanol extract of *Clitoria ternatea* (100, 200 and 400 mg/kg, p.o.) was dissolved in suspension of gum acacia (2 % w/v) in distilled water. All other chemicals were of analytical grade. Absolute ethanol was given orally with 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

In order 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 in to 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 to 10). On day 11, at 8.00 h, ethanol liquid diet was replaced with nutritionally balanced control liquid diet. Mice from the control group were provided with liquid diet without ethanol from day 0 to 10, which was isocaloric to ethanol liquid diet on 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 ethanol diet and control liquid diet groups<sup>9</sup>.

#### Treatment schedule

Ethanol liquid diet was provided for days and on 11 days alcohol was withdrawn from diet. *Clitoria ternatea* treatment was started from first day of the study. Mice were randomly selected and divided in 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)

#### Assessment of blood ethanol concentration

Blood samples (40µl) were obtained from the retro-orbital sinus. Blood samples were precipitated in 3% perchloric acid (160 µl) and stored at 4°C until analysis. Ethanol was quantified by the alcohol dehydrogenase assay. Samples (60 µl) will be incubated in 3ml of 0.5mol/l Tris-Cl buffer (pH 8.8) containing 5.5 µg/ml of alcohol dehydrogenase and 1.5mmol/l β-nicotinamide adenine dinucleotide for 40 min at room temperature. Accumulation of β-NADH was measured by reading sample absorbance at 340 nm. The ethanol concentration was estimated using standard calibration curve<sup>10</sup>.

#### Assessment of ethanol withdrawal-induced symptoms

The ethanol dependence was assessed by scoring the withdrawal-induced physical signs of hyper excitability, graded as per the scale. In brief, each mouse was lifted by the tail, spun gently through a 180° 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<sup>11</sup>.

#### Assessment of depression forced swimming test (FST)

FST was done in line with our previously published reports. In short each individual animal was subjected to free swimming in a glass cylinder (10 × 25 cm) filled with water. Animals were allowed to swim for 6 min and the time of immobility was noticed for each animal for the last 4 min period. Later on, data was calculated and analysed. Animal was considered immobile if it stops swimming and became motionless or showed state of desperate where it halt the struggle to escape from the cylinder filled with water<sup>12</sup>.

#### Biochemical estimations

##### Lipid peroxidation

MDA is a secondary product of lipid peroxidation that reacts with thiobarbituric acid. The red pigment produced if extracted in n-butanol pyridine mixture gives colour was estimated by measuring the absorbance at 532 nm<sup>13</sup>.

##### Nitric oxide

Nitric oxide (NO) concentration was calculated using a standard curve for sodium nitrite and expressed as ng/mg of protein. An aliquot of crude homogenate was centrifuge at 21,000 g for 20 min at 4uC, and supernatant was used to analyse nitrite levels. Briefly, samples were incubated at room temperature for 20 min with Griess reagent [0.1% N-(1-naphthyl) ethylenediaminedihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1]. The absorbance was measured at 550 nm and compared to that of standard solutions of sodium nitrite<sup>14</sup>.

##### Reduced glutathione

Reduced glutathione (GSH) levels were estimated by the method of Ellman as described previously<sup>15</sup>. An equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5'-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexed and absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as mol/g tissue.

##### Superoxide dismutase

To 100 µl Supernatant, 1ml of sodium carbonate (1.06 gm in 100 ml water), 0.4 ml of

24 mM NBT and 0.2 ml of EDTA (37 mg in 100 ml water) was added and zero-minute reading was taken at 560 nm. Reaction was initiated by addition of 0.4 ml of 1mM Hydroxylamine hydrochloride, incubated at 25°C for 5 minutes and the reduction of NBT was measured at 560nm and expressed as  $\mu\text{g}/\text{mg}$  of protein<sup>16</sup>.

#### Histopathology study of brain

A brain were isolated and fix in 10% neural formalin solution, embedded in paraffin and cut into sections of 3-4  $\mu\text{m}$  thickness, which were stained with haematoxylin and eosin. Each sample was examined by light microscopy<sup>17</sup>.

#### Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) with post hoc tests for multiple comparisons. Effects were considered significant at  $p < 0.05$

### Results and discussion

*Clitoria ternatea* similarity with other anticonvulsant, antidepressant, anxiolytic and nootropic drugs with respect to its GABAergic and serotonergic properties further strengthens its potential<sup>18</sup>. *Clitoria ternatea* root extract was associated with increased levels of ACh and choline acetyltransferase (ChAT) in mice brain, but any relationship with inhibition of AChE activity was not established, and cortical AChE activity was actually found to be increased. An aqueous extract of the root also increased ACh levels in rat, hippocampus following oral administration, and it was hypothesized that this effect may be due to an increase in ACh synthetic enzymes.

The main purpose of the present study was to investigate the pharmacological effects of *Clitoria ternatea* in ethanol withdrawal symptoms like depression. Ethanol dependence was induced in mice by chronic administration of ethanol liquid diet for 10 days as described in the earlier reported method. Blood ethanol concentration was estimated before ethanol withdrawal. Treatment with EECT (100,200 and 400mg/kg) didn't affect the levels of blood ethanol concentration when compared against ethanol control group (Table 1 and fig. 1). Ethanol withdrawal on day 11 significantly increased scores of ethanol withdrawals sign in mice between 2 and 8 h with its peak at 6th h. Hence, this animal model was used to investigate the influence of *Clitoria ternatea* on ethanol withdrawal syndromes. It was

observed that administration of EECT (100,200 and 400mg/kg, p.o.), 10 min prior to the withdrawal of ethanol on day 11, significantly attenuated the scores of ethanol withdrawals, seen otherwise in vehicle treated group (Table 2 fig. 2). *Clitoria ternatea* (100,200 and 400 mg/kg) significantly decreased the duration of immobility in forced swim test in comparison with ethanol control (Table 3 and fig. 3).

In ethanol withdrawal mice there is excess elevation in oxidative stress which disturbs the level of the biochemicals in the brain. In present study, ethanol withdrawal mice showed elevated levels of oxidative stress markers (lipid peroxidation and nitric oxide) and reduced endogenous antioxidant (glutathione and superoxide dismutase) level in brain tissue. Treatment with *Clitoria ternatea* (200 and 400 mg/kg) significantly elevated the levels the glutathione and superoxide dismutase but reduced the level of lipid peroxidation and nitric oxide in the brain when compared with the ethanol control group (Table 4 fig. 4, 5, 6, 7).

The histopathological examinations of mice brain using hematoxylin and eosin stain were performed for all the groups which includes normal control group, ethanol control group, EECT treatment group(100, 200 and 400 mg/kg). Histopathological examination of stained sections of brain showed disturbed structural organisation and swelling and inflammation of neurons in ethanol control mice. In addition, itself ethanol impairs cellular antioxidant status because of the decreased brain levels of glutathione (GSH) and superoxide dismutase (SOD). EECT 100 mg/kg failed to restore the structural alteration of brain tissue. However, oral administration of EECT 200 and 400 mg/kg significantly restored the brain morphology and minimal degeneration of neurons with reduced swelling and inflammation (fig.8).

We can conclude that treatment with EECT is effective and further study can be revealed antidepressant action of this extract modifying the animal models in the ethanol withdrawal study. This will help the investigator in the area towards the exact mechanism that how this herbal plant acts towards ethanol withdrawal complications

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**Table 1:** Blood Ethanol Levels

Time (h)	Ethanol control	EECT (100 mg/kg)	EECT (200mg/kg)	EECT (400mg/kg)
0	169.000±9.259	152.333±2.418	147.5000±2.460	143.667±2.201
2	82.667±0.803	74.333±1.174	64.667±0.760	55.750±1.413
4	31.753±1.632	26.667±0.715	19.250±0.335	16.167±0.654
6	2.655±0.030	2.100±0.234	2.087±0.161	1.512±0.129

**Table 2:** Effect of EECT on Ethanol Withdrawals Induced Scores

Time (h)	Normal control	Ethanol control	EECT (100mg/kg)	EECT (200mg/kg)	EECT (400mg/kg)
2	1.000±0.168	6.333±0.422 <sup>*</sup>	4.167±0.401 <sup>§</sup>	2.833±0.167 <sup>§@</sup>	2.500±0.224 <sup>§#</sup>
4	1.333±0.2211	7.833±0.477 <sup>*</sup>	4.667±0.211 <sup>§</sup>	3.333±0.333 <sup>§@</sup>	1.500±0.223 <sup>§#</sup>
6	1.167±0.167	8.167±0.401 <sup>*</sup>	5.000±0.032 <sup>§</sup>	3.500±0.224 <sup>§#</sup>	2.400±0.221 <sup>§#</sup>
8	1.133±0.211	7.667±0.211 <sup>*</sup>	5.500±0.342 <sup>§</sup>	3.333±0.221 <sup>§#</sup>	2.333±0.212 <sup>§#</sup>
10	1.167±0.165	7.333±0.333 <sup>*</sup>	4.500±0.428 <sup>§</sup>	3.500±0.224 <sup>§%</sup>	1.667±0.227 <sup>§#</sup>

<sup>\*</sup>P<0.001 Vs. Normal control, <sup>§</sup>P<0.01 Vs. Ethanol Control, <sup>#</sup>P<0.001, <sup>@</sup>P<0.01, <sup>%</sup>P<0.05Vs. EECT 100mg/kg.



**Table 3:** Mobility Time in Sec

Treatment	Mobility time (s)
	Mean $\pm$ SEM
Normal Control	125.0 $\pm$ 3.950
Ethanol Control	89.67 $\pm$ 2.512 <sup>*</sup>
EECT 100 mg/kg	123.0 $\pm$ 2.530 <sup>§</sup>
EECT 200 mg/kg	132.2 $\pm$ 1.880 <sup>§</sup>
EECT 400 mg/kg	145.5 $\pm$ 3.510 <sup>§##</sup>

<sup>\*</sup>P<0.001Vs. Normal control, <sup>§</sup>P<0.001Vs. Ethanol control, <sup>#</sup>P<0.001, <sup>®</sup>P<0.05 Vs. EECT 100mg/kg.

**Table 4:** Effect of EECT on Oxidative Stress Parameters MDA and NO

Groups	MDA (nmol/mg)	NO (ng/mg)	SOD (%activity)	GSH (ng/mg)
Control	6.00 $\pm$ 1.155	146.5 $\pm$ 4.696	4.357 $\pm$ 0.5266	56.33 $\pm$ 1.764
Ethanol Control	35.00 $\pm$ 8.505 <sup>a</sup>	326.2 $\pm$ 8.514 <sup>*</sup>	1.297 $\pm$ 0.1761 <sup>*</sup>	22.67 $\pm$ 1.453 <sup>*</sup>
EECT 100mg/kg	24.33 $\pm$ 1.453	283.7 $\pm$ 11.68 <sup>^</sup>	2.217 $\pm$ 0.2002	29.00 $\pm$ 1.155
EECT 200mg/kg	21.00 $\pm$ 2.082	219.2 $\pm$ 4.629 <sup>§</sup>	2.797 $\pm$ 0.1934 <sup>^</sup>	34.00 $\pm$ 1.155 <sup>&amp;</sup>
EECT 400mg/kg	10.00 $\pm$ 0.5774 <sup>^</sup>	157.5 $\pm$ 5.421 <sup>§#</sup>	3.363 $\pm$ 0.0375 <sup>&amp;</sup>	48.67 $\pm$ 2.848 <sup>§#@</sup>

<sup>a</sup>P<0.01Vs Normal control, <sup>^</sup>P<0.05 Vs. Ethanol control

Figure 1: Levels of Blood Ethanol in Ethanol Withdrawal

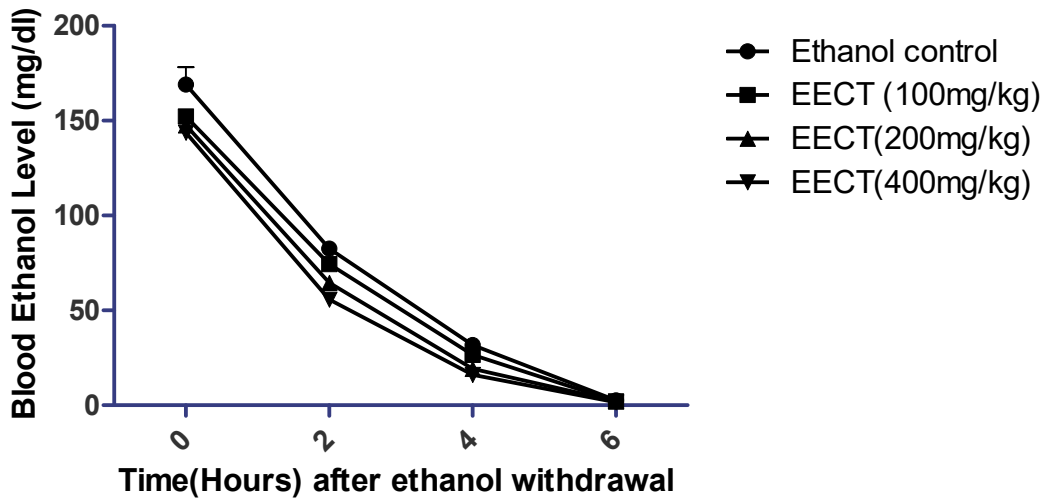
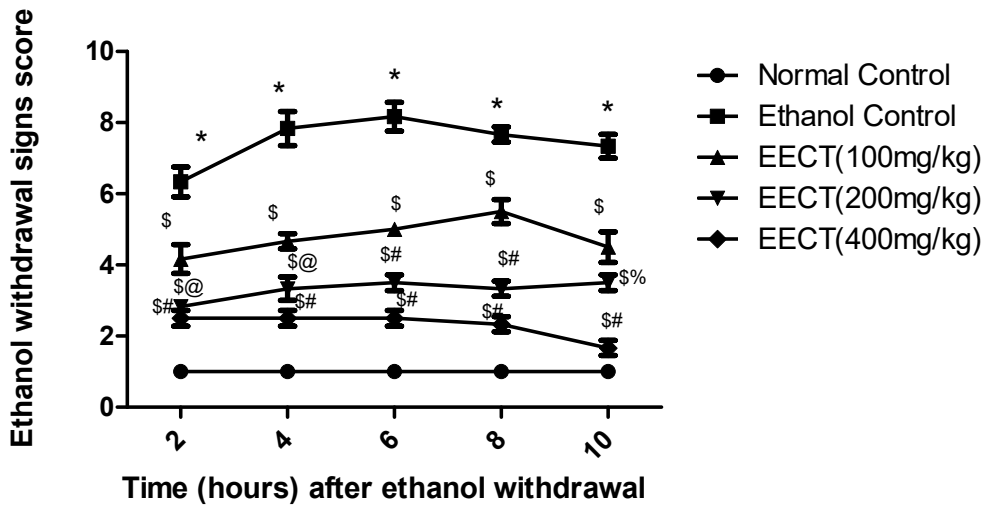


Figure 2: Effect of EECT on ethanol withdrawal scores

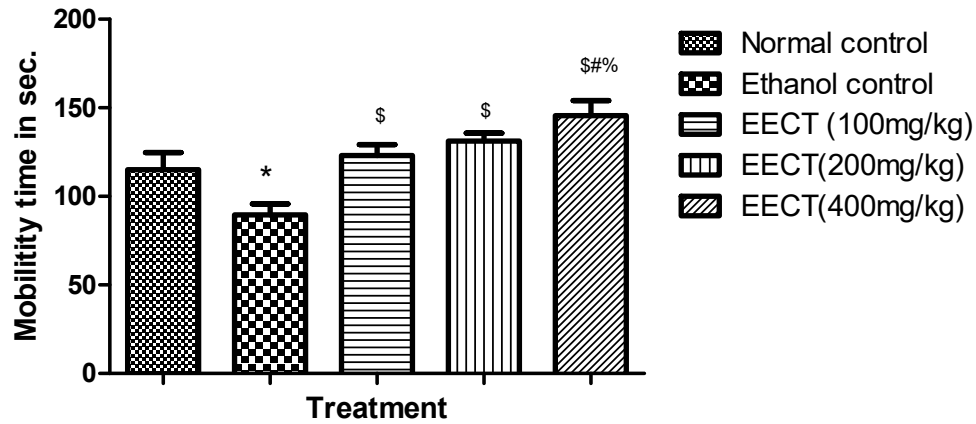


\*P<0.001 Vs. Normal control,

‡P<0.01 Vs. Ethanol Control, #P<0.001, @P<0.01, %P<0.05Vs. EECT 100mg/kg.

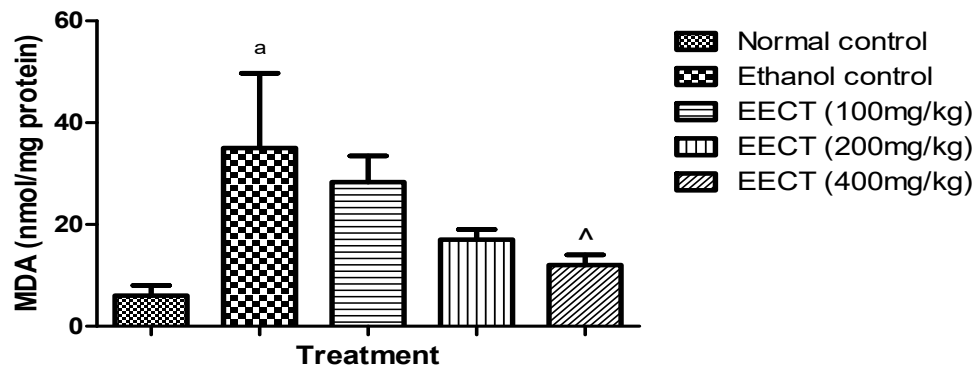


Figure 3: Effect of EECT on Mobility Time in Sec.



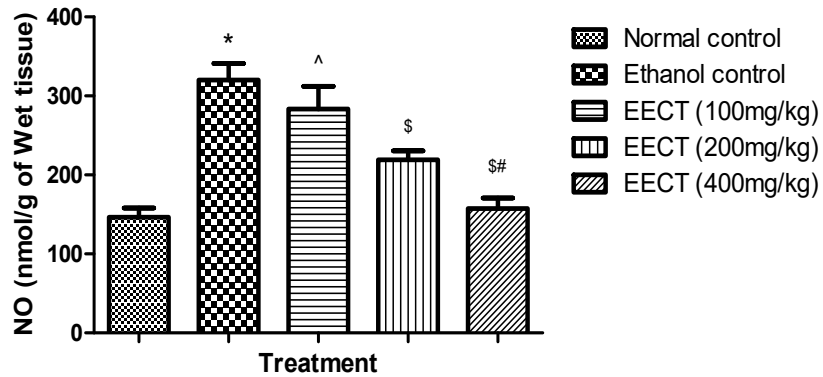
\* $P < 0.001$  Vs. Normal control,  $^{\$}$  $P < 0.001$  Vs. Ethanol control,  $^{\#}$  $P < 0.001$ ,  $^{\%}$  $P < 0.05$  Vs. EECT 100mg/kg.

Figure 4: Effect of EECT on ethanol withdrawal induced changes in Lipid peroxidation



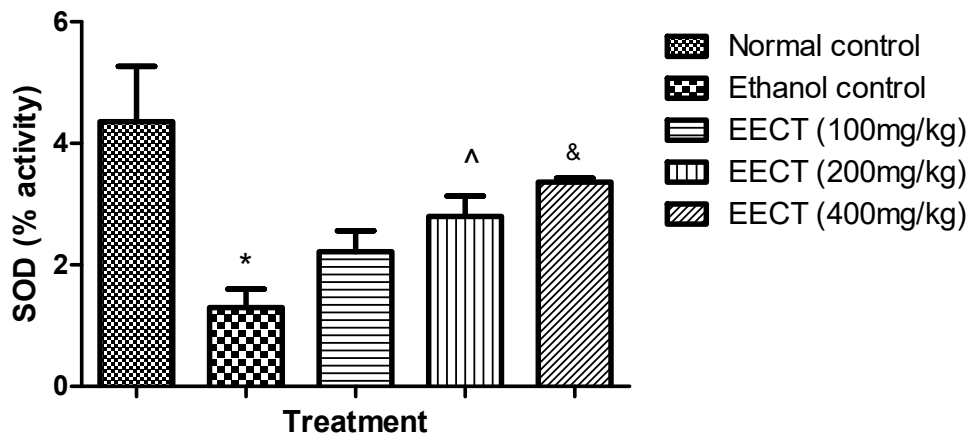
$^a$  $P < 0.01$  Vs Normal control,  $^{\wedge}$  $P < 0.05$  Vs. Ethanol control

Figure 5: Effect of EECT on ethanol withdrawal induced changes in nitric oxide (NO)



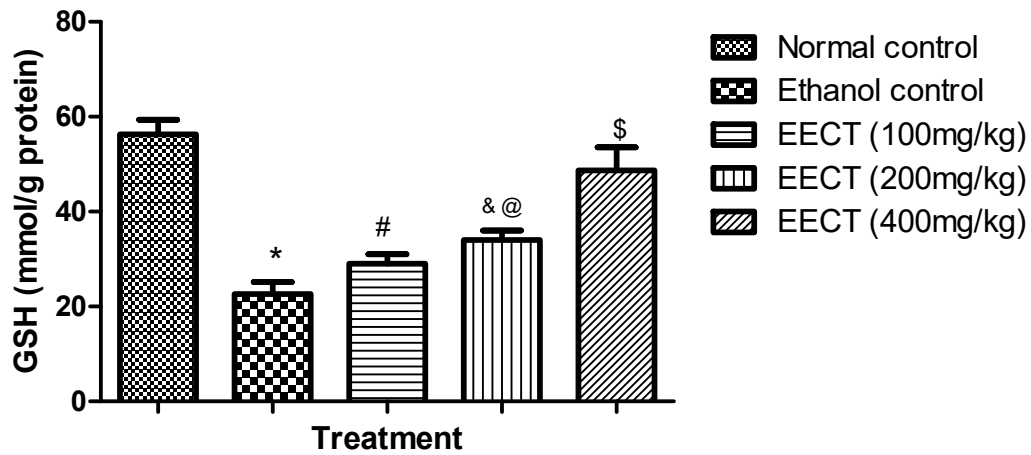
\* $P < 0.001$  Vs. Normal control, ^ $P < 0.05$  \$ $P < 0.001$  Vs. Ethanol control. # $P < 0.001$  Vs. 100mg/kg.

Figure 6: Effect of EECT on ethanol withdrawal induced changes in superoxide dismutase (SOD).



\* $P < 0.001$  Vs Normal control, ^ $P < 0.05$  & $P < 0.01$  Vs Ethanol control.

Figure 7: Effect of EECT on ethanol withdrawal induced changes in reduced glutathione (GSH)

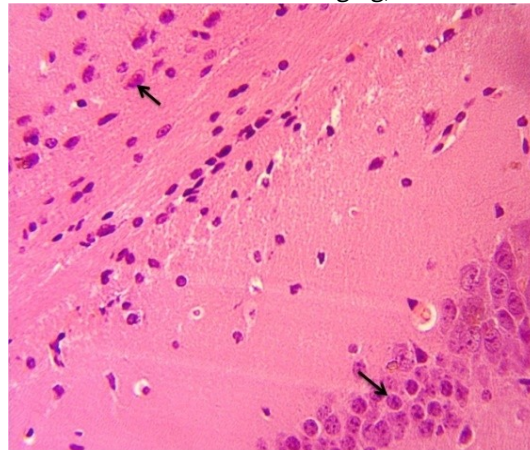


\* $P < 0.001$  Vs. Normal control,  $^{\$}P < 0.001$ ,  $^{\&}P < 0.01$ , Vs. Ethanol control,  $^{\#}P < 0.001$   $^{\@}P < 0.01$  Vs. EECT 100mg/kg.

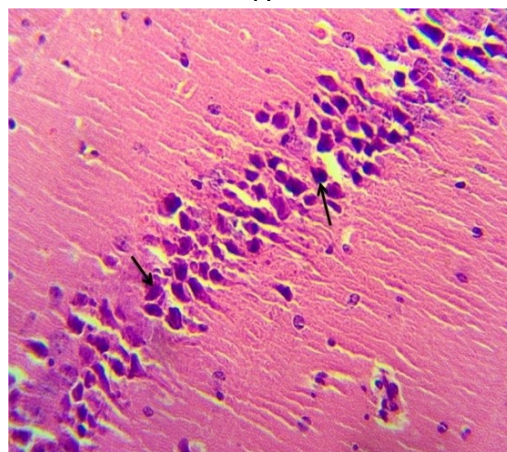
Figure 8: Histopathological sections of mice brain

Hematoxylin and eosin stained histopathological sections of mice brain

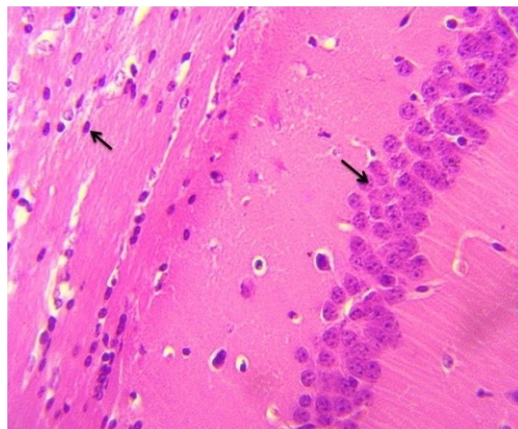
(A=Normal control, B- Ethanol control C- EECT 100mg/kg, D- EECT 200mg/kg, EECT-400mg/kg)



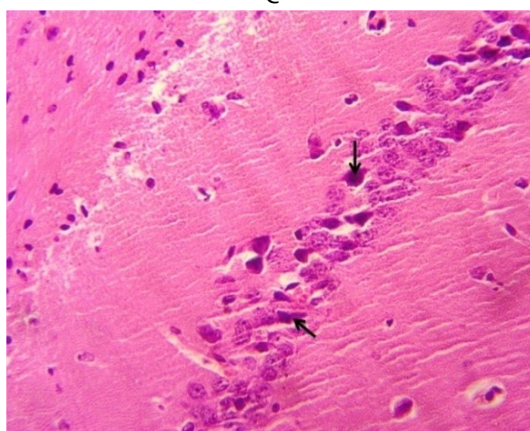
A



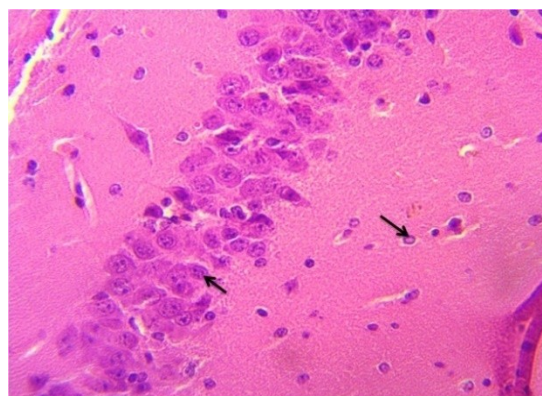
B



C



D



E