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(RESEARCH ARTICLE)

Preclinical evaluation of thymol in alcohol withdrawal-induced behavioral and motor deficits in mice

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Abstract

Background: Alcohol withdrawal (AWD) induces central nervous system hyperexcitability, leading to anxiety, depression, and motor dysfunction, primarily via dysregulation of GABAergic, glutamatergic, and monoaminergic systems. Thymol, a monoterpenoid phenol from *Thymus vulgaris*, possesses documented $GABA_A$ receptor modulatory, anxiolytic, antioxidant, and anti-inflammatory properties. This study evaluated thymol's protective effects against AWD-induced neurobehavioral abnormalities in mice.

Material and Methods: Adult male Swiss albino mice were divided into six groups (n = 6/group): Control (CON) - vehicle-treated, Ethanol Withdrawal (EW) - 10% ethanol at 2 g/kg p.o. for 10 days, followed by abrupt withdrawal + Thymol 10 (EW-T10)- 10 mg/kg p.o. Thymol during withdrawal, EW+ Thymol 30 (EW-T30)-30 mg/kg p.o. thymol during withdrawal, Thymol 10 (T10) and Thymol 30 (T30) - Thymol only, no ethanol exposure. behavioural testing was performed 24 hours post-withdrawal using: Elevated Plus Maze (EPM), Open Field Test (OFT), and Light-Dark Test (LDT) for anxiety-like behaviour, Hole Board Test (HBT) for exploratory activity, Marble Burying Test (MBT) for compulsive/anxiety-linked behavior, Tail Suspension Test (TST) for depressive-like behavior, Stumbling and rotarod test for motor incoordination. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test ($p<0.05$).

Results: AWD significantly increased anxiety-like behavior (EPM, OFT, LDT), compulsive behavior (MBT), depressive-like behavior (TST), and motor incoordination, while reducing exploration (HBT). Thymol treatment (10 and 30 mg/kg) significantly reversed these alterations in a dose-dependent manner. The higher dose (EW-T30) showed near-complete behavioral restoration. Thymol alone caused no adverse effects.

Conclusion: Conclusion: Thymol exhibits significant neuroprotective effects against alcohol withdrawal-induced anxiety, depression, and motor deficits in mice. Its likely mechanism involves positive modulation of GABA-A receptors, attenuation of oxidative stress, and restoration of neurotransmitter homeostasis. These findings support thymol's potential as a phytopharmacological agent for managing alcohol withdrawal syndrome and its neuropsychiatric sequelae.

Keywords: Thymol; Alcohol withdrawal; Anxiety; depression; GABAergic modulation; Oxidative stress; Motor Coordination; Phytotherapy

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1. Introduction

1.1. Alcohol Use Disorder (AUD)& Alcohol withdrawal (AW)

Alcohol Use Disorder is a chronic medical condition characterized by a problematic pattern of alcohol consumption leading to significant distress or impairment. It involves compulsive alcohol use, loss of control, and negative emotional states when not drinking (Burnette et al., 2022). Craving or strong urge to drink, Loss of control over alcohol use

Continued use despite health or social problems, Tolerance (needing more to get the same effect)and Withdrawal symptoms when not drinking (Livne et al., 2022).

AUD is a chronic condition marked by difficulty controlling alcohol use, persistent cravings, and continued consumption despite negative outcomes (Patel & Balasanova, 2021). It often involves tolerance and withdrawal symptoms when alcohol use is reduced or stopped (McHugh & Weiss, 2019). Globally, around 76.3 million people suffer from AUD, contributing to 1.8 million deaths annually. Up to 42% of hospitalized patients and one-third of ICU cases involve AUD. Severe Alcohol Withdrawal Syndrome (AWS) may lead to seizures and delirium tremens (DT), with a mortality rate comparable to severe malignancies if untreated (Oluwoye et al., 2020). However, early intervention reduces mortality to below 1%. AUD is frequently linked to neurological issues like coma, seizures, dementia, and gait disturbances. (Sher et al., 2005);(Saitz, 1998).

Ethanol is a psychoactive substance with dependence liability. It ranks among the most widely abused drugs around the globe and its consumption contributes to 3 million deaths each year globally (Chavan et al., 2023).

Millions of people suffer from impairments and poor health as a result of alcohol misuse. 5.1% of all disease burden worldwide is attributable to alcoholism (Mohebbi et al., 2020). Alcohol consumption and abuse have been relevant to structural, functional, and neurochemical neuroadaptive alterations along with its two chief components i.e., dependence and withdrawal (Ruby et al., 2012).

Prolonged alcohol consumption disrupts a variety of excitatory and inhibitory neurotransmitters in the brain (Moroi et al., 2018), The neurobiological mechanisms behind alcohol withdrawal-induced anxiety and depression involve the complex interaction between neurotransmitters, such as gamma-aminobutyric acid (GABA) and glutamate. Alcohol typically has a depressant effect on the central nervous system, enhancing GABA activity and inhibiting glutamate. When alcohol use is abruptly stopped or reduced, this balance is disrupted, leading to hyperactivity of the central nervous system, which contributes to withdrawal symptoms like anxiety and depression (Becker & Mulholland, 2014); (Jyoti Borah et al., 2017) .

2. Materials and methods

2.1. Drugs and Chemicals

Thymol was purchased from Yucca Enterprises, India, & Ethanol (99%) of analytical grade was procured from Bio Liqua Research Pvt. Ltd., India. All chemicals used were of high quality.

2.2. Experimental Animals

Healthy adult male Swiss albino mice (30-40g) were procured from LACSMI Biofarms Pvt Ltd (Laboratory Animal Centre for Safe Medical Innovations), Alephata, Pune- 412411 (1277/PO/RcBt-S/RcNRc-L/2009/CCSEA). All mice were placed separately in cages made of polypropylene with paddy husk used as bedding material. The animals were maintained under controlled environmental conditions as specified in CCSEA guidelines, with temperature kept between 22-25-2 °C, relative humidity between 45-65 %, and a timer-controlled 12:12 hr light-dark cycle. Animals had free access to water and standard laboratory feed (Nutrivet Lab, Pune, India). All the animals were acclimatized for a week before being used in the study. The experimental protocol for this investigation was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Shriman Sureshdada Jain College of Pharmacy, Neminagar, Chandwad, bearing reference SSDJ/IAEC/24-25/01.

2.3. Selection of dose

Dose were selected on the basis of literature, i.e., Thymol 10, 30 mg/kg, i.p., ethanol administered 2 g/kg of 10% ethanol (v/v) intragastrically.

2.4. Preparation & Administration of EtOH & Thymol:

Ethanol (10% *v/v)* was prepared using 99.9% Ethanol, by dissolving it into 90 mL of solvent (water) & administered via oral route (p.o.) using an oral feeding needle at a constant dose, i.e., 2g/kg from day 1 to 6 & withdrawn on day 7 i.e., after 24 hrs. to the last dose of ethanol.

2.5. Experimental Design

The animals were divided into 5 groups containing 6 animals (n=6) in each group. Grouping of animals was done as follows;

- Group I – Control Group
- Group II – Ethanol-Withdrawal (EW) Group -10% ethanol at 2g/kg p.o. for 10 days.
- Group III – Ethanol -Withdrawal +Thymol 10 (EW-T10)- 10mg/kg p.o. thymol during withdrawal.
- Group IV – Ethanol-Withdrawal + Thymol 30 (EW- T30)- 30mg/kg p.o. thymol during withdrawal
- Group V – Thymol 30 (T30)-thymol only, no ethanol exposure.

Dose of Thymol was selected as 10mg/kg & 30mg/kg for T10 & T30 respectively given using oral feeding needle from day 1 to 6. Ethanol (10%v/v) was given at a dose of 2g/kg to animals from day 1 to 6 & withdrawal was done on day 7 i.e., after 24 hrs. to the last dose. All treatments were scheduled between 10.00 a.m. and 4.00 p.m.& doses were given accordingly from day 1 to 6

2.6. Study design

Acute study: Mice received 10 % ethanol 2 g/kg, intragastrically, after 30 min, its effect on different models of anxiety and depression was assessed.

Chronic study: Mice received 10% ethanol, 2 g/kg, intragastrically, twice a day on the 1st day and once a daily for a total six days. On the 7th day, mice were tested for withdrawal reactions. On the 7th day, the treatments were changed, the group chronically treated with ethanol received saline only. The animals that received Thymol (10 & 30 mg/kg, p.o) for longer period were challenged with normal saline only. The animals that received Thymol (10 & 30 mg/kg, p.o) alone with ethanol on day 1 through 6, were received only Thymol (10 & 30 mg/kg, p.o) on 7th day. Another group that received ethanol chronically was treated with Thymol (10 & 30 mg/kg, p.o).

2.7. Evaluation of Parameters

2.7.1. Assessment of Anxiolytic Activity using Elevated Plus Maze:

Principle

The EPM is a widely used behavioural test for anxiety research, originally developed for mice and rats. The EPM has been extensively utilized as a tool for investigating the psychological and neurochemical foundations of anxiety, as well as for screening drugs that modulate anxiety and studying different mouse genotypes. (Figueiredo Cerqueira et al., 2023).The EPM apparatus consists of two open arms (20 cm x 5 cm) positioned opposite each other and perpendicular to two closed arms (20 cm x 5 cm x 25 cm), with a central platform measuring. The open arms feature low walls designed to reduce the likelihood of falls, while the closed arms are enclosed by high walls for safety. The entire apparatus is elevated 20 cm above the floor.

Evaluation Parameters:

- Number of entries in the open arm and closed arm.
- Total time spent in each arm (Pellow *et al.,* 1985).

2.7.2. Assessment of Anxiolytic Activity using Light and Dark Apparatus:

Principle

The light/dark test, introduced by Crawley and Goodwin in 1980, is used in experimental practice to assess anxiety in rodents. The test apparatus consists of two compartments: a completely dark compartment & a compartment with natural light. Originally developed with male mice, the light/dark test can be useful in predicting anxiolytic or anxiogenic activity in these animals. A typical apparatus, suitable for use in mice. The apparatus consisted of two boxes

(25x25x25cm) joined together; one box was made dark by covering its top & the other box was kept open with the natural light source.

Evaluation Parameters

- Time spent in the Light & Dark Compartment
- No. of Transitions (Campos-Cardoso *et al., 2023*).

2.7.3. Assessment of Locomotor Activity using the Open Field Test:

Principle

The open field test, originally developed by Hall for the study of rats, involves placing an animal within an unfamiliar environment enclosed by walls. This assessment aims to observe a variety of behavioural patterns, notably the tendency to remain near the edges of the area without entering into the center, behaviour referred to as thigmotaxis, which is frequently interpreted as indicative of anxiety. The open field apparatus is constructed from wood and measures 56 x 56 x 40 cm. The floor is partitioned into 16 equal squares of uniform dimensions. Each animal is carefully positioned in one of the corners of the apparatus at the commencement of the test.

Evaluation Parameters

- Time Spent (Centre & Periphery)
- No. of Squares Crossings (Centre & Periphery)
- No. of Rearing's (Voikar and Stanford, 2023) .

2.7.4. Assessment of Exploratory Behaviour in Hole Board Apparatus:

Principle

The concept of the hole board apparatus was first introduced by Bossier and Simon in 1962. The hole board test is a widely used method for screening the potential anxiolytic effects of drugs. This test is based on the assumption that the amount of head-dipping activity displayed by animals is inversely proportional to their level of anxiety. The instrument comprises a floor (40 X 40 cm), has 16 holes spaced at an equivalent 1.5 cm apart. The device was raised to 20 cm. The number of head dips was counted over the period five minutes.

Evaluation Parameters:

No. of Head Dips (Pisula et al.,2021).

2.7.5. Assessment of Compulsive-like Behaviour using the Marble Burying Test:

Principle

The burying behaviour of rodents was first described by Pinel and Tremblay in 1978. The inhibition of spontaneous burying of glass marbles by mice has been utilized as an index for assessing the effects of anxiolytic drugs in the marble burying test. This test serves as a model to evaluate the anti-anxiety effects associated with anxiety disorders and obsessive-compulsive behaviours, allowing researchers to identify phenotypes related to these conditions. A standard mouse cage or transparent plastic box, approximately 40cm x 30cm x 15cm, with a bedding of 4-5cm which is uniformly spread across the cage floor & should have high enough walls to prevent escape. Typically, 21 marbles are placed. Subsequently, the number of marbles buried to at least two-thirds of their surface area was counted.

Evaluation Parameters

No. of Marbles Buried/Unburied (Witkin and Smith, 2023)

2.7.6. Assessment of depressive-like behaviours in ethanol withdrawal mice;

Principle

The tail suspension test has become one of the most widely used methods for evaluating antidepressant-like activity in mice. During the tail suspension test, mice initially struggle when suspended by their tail, but this is followed by increasing periods of immobility throughout the six-minute duration of the test. The total amount of time spent

immobile is measured using a standardized method for assessing potential antidepressants (Guzmán *et al*.2014).The tail suspension test was conducted following the method developed by Steru. In this procedure, animals were suspended 50 cm above the floor using adhesive tape, which was placed approximately 1 cm from the tip of the tail. The duration of immobility was recorded over a test period of 5 minutes. Mice were considered immobile only when they remained passively suspended and completely still.

2.7.7. Assessment of Motor Coordination using Stumbling Test

Principle

The stumbling test is based on the concept that rodents display anxiety-like behaviours when placed in a novel or stressful environment. The foot fault test operates on the premise that mice with normal brain and motor function can place their paws correctly while walking. If a mice experiences neurological damage, stress, or anxiety, it may lose coordination, leading to its paw slipping through the grid this occurrence is known as a foot fault. By counting how many times this happens compared to normal steps taken, we can assess the extent of motor system impairment. Additionally, stress and anxiety may increase the frequency of foot faults, making this test useful for evaluating how emotional states impact movement, even though its primary focus is on motor performance.

A behavioural test is commonly used to assess exploration and anxiety-related behaviours in mice. Motor coordination is evaluated using an apparatus that measures 28 cm x 28 cm x 20 cm. This apparatus has a floor with 36 holes arranged in a 6x6 grid, with each hole spaced 2 cm apart and 1 cm deep.

2.7.8. Evaluation Parameters

No. of Foot Faults and Stumbles (Lepicard et al., 2023)

2.7.9. Assessment of Antidepressant Activity using the Tail Suspension Test:

Principle

The tail suspension test has become one of the most widely used methods for evaluating antidepressant-like activity in mice. This test is based on the observation that animals subjected to the short-term, inescapable stress of being suspended by their tail will adopt an immobile posture. The tail suspension test is a valuable tool in drug discovery, particularly for high-throughput screening of potential antidepressant compounds. During the tail suspension test, mice initially struggle when suspended by their tail, but this is followed by increasing periods of immobility throughout the six-minute duration of the test. The total amount of time spent being immobile is measured using a standardized method for assessing potential antidepressants.

The tail suspension test was conducted following the method developed by Steru. In this procedure, animals were suspended 50 cm above the floor using adhesive tape, which was placed approximately 1 cm from the tip of the tail. The duration of immobility was recorded over a test period of 5 minutes. Mice were considered immobile only when they remained passively suspended and completely still.

Evaluation Parameters:

Duration of Immobility (Pinto Brod *et al.,* 2021)

2.7.10. Assessment of motor coordination using Rota Rod Test

Principle

Since first described by Dunham and Miya in 1957, the rotarod test has become a conventional assessment tool for assessing motor coordination in rodents. It is widely believed that an accelerating rotarod test, in which the rod is linearly accelerated from 4-5 revolutions per minute (rpm) to 40 rpm or even a greater speed over a defined period, with the fall of time measured, can reliably reflect the motor coordination function in rodents. The five-compartment rotarod for mice is a wide-compartment rotarod apparatus with a rod. The rotation speed of the rod was 25-30 rpm. The timer for each animal compartment is automatically stopped once the animal falls on the base of the compartment, considered as the fall time.

Evaluation Parameter

Fall of Time (sec) (Keane *et al.,* 2024)

3. Results

3.1. Acute Study

3.1.1. Evaluation of Thymol on anxiety-like behaviour in ethanol withdrawal mice using EPM:

Acute administration of 10% ethanol produced a significant ($p<0.05$) increase in the duration of occupancy in open arms (115.5 ± 7.73) as compared with the control group. Percent preference for closed arm entry was reduced (40 and 20%) in acute treatment with T (T10 and T30). The mice treated with ethanol + T (T10 and T30) spent (121.3 ± 3.79 and 128.3 ± 8.89) significantly more time in the open arm as compared to control group. Mice treated with Drug (T30 mg Alone) (182.5 ± 12.51) also showed significant more time in open arm as compared with the ethanol withdrawal group and control group. (Figure 1).

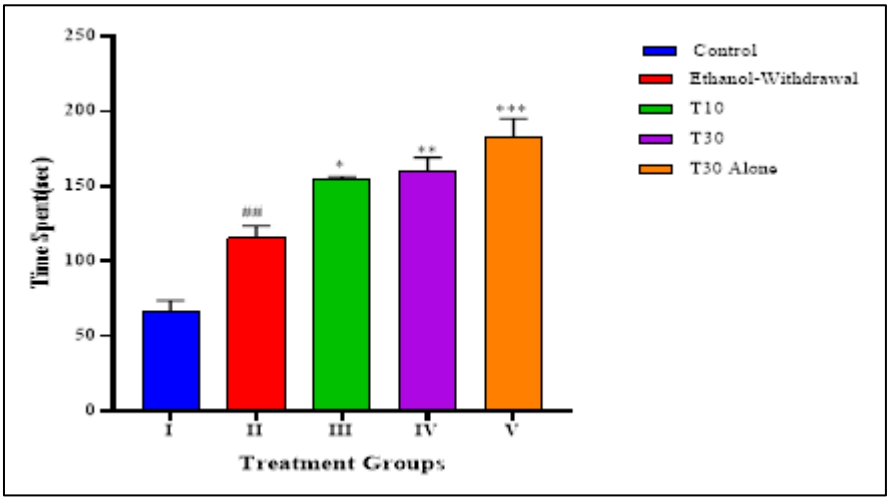


Figure 1 a) Time spent in open arm

Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

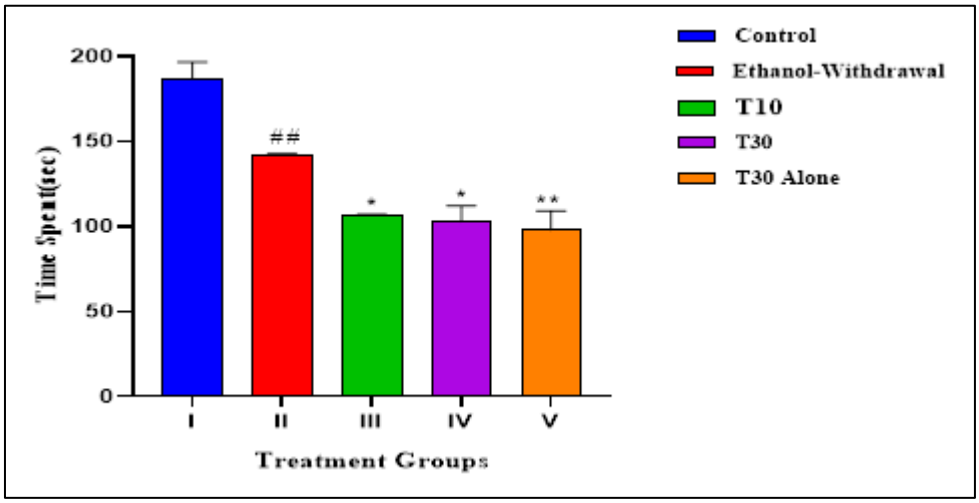
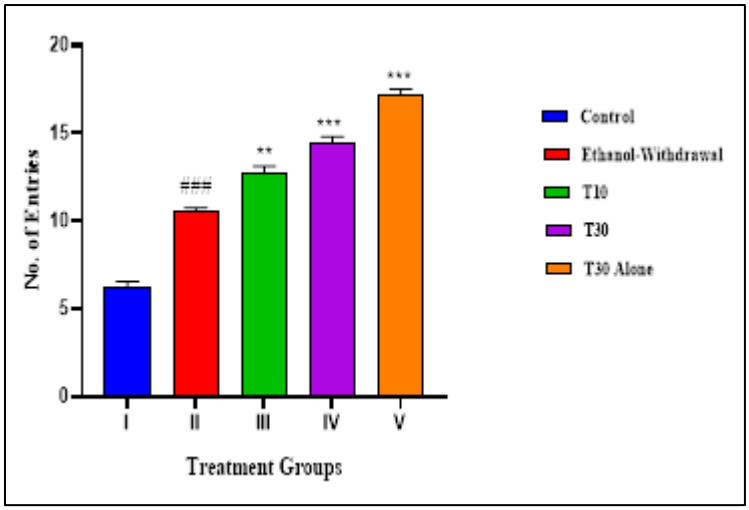


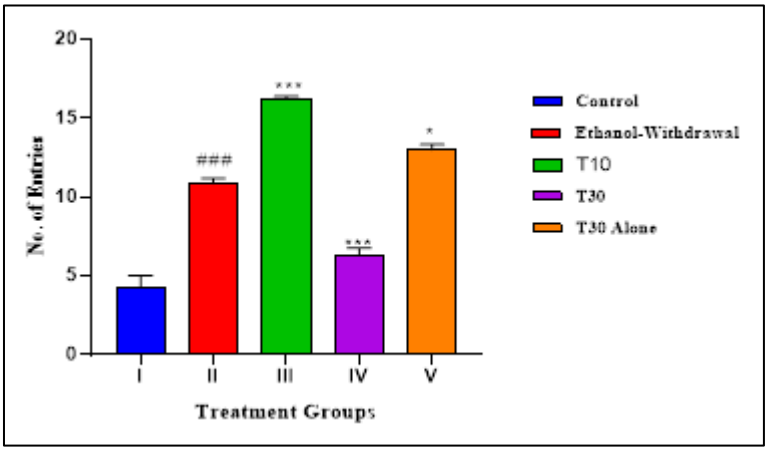
Figure 1(b) Time spent in closed arm

Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.



Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 1(c) No. of Entries in open arm

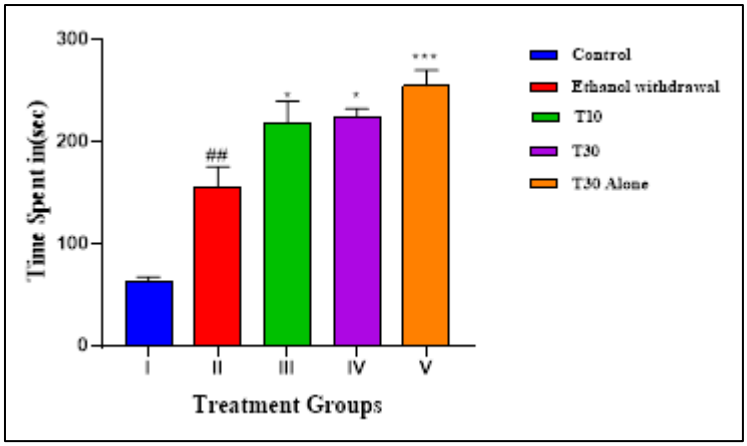


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 1(d) No. of Entries in closed arm

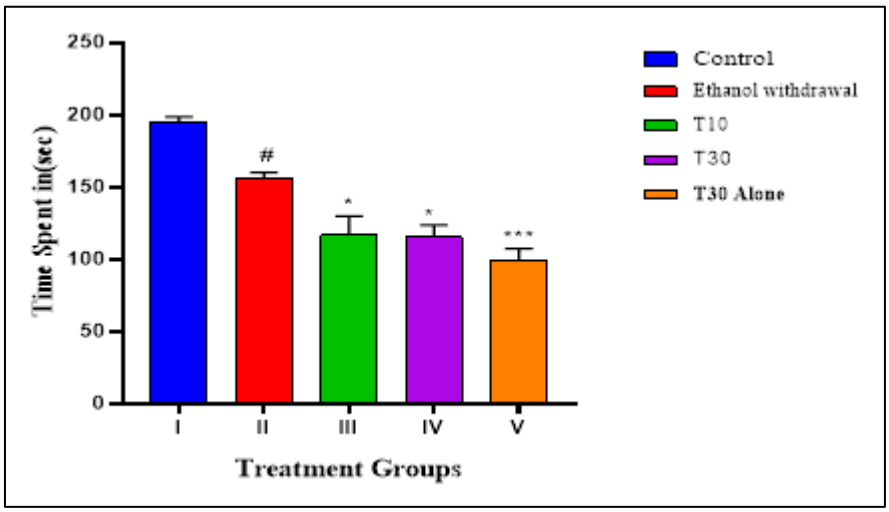
3.1.2. Evaluation of Thymol on anxiety-like behaviour in ethanol withdrawal mice using LDT

Acute administration of 10% ethanol produced a significant ($p<0.05$) increase in time spent in the light compartment (145.30 ± 20.37) compared with the control group (64.17 ± 3.609). The mice treated with 10% ethanol along with Thymol T10 (200.3 ± 20.37) shows significant ($p<0.05$) result as compared to EtOH-withdrawal group, & group treated with 10% ethanol along with Thymol (T30) Alone (225.0 ± 7.40) significantly ($p<0.05$) increased the time spent in the light compartment as compared to the EtOH-withdrawal group & mice treated with Thymol (T30) Alone only, showed a significant ($p<0.05$) increase in time spent in open compartment (235.1 ± 15.12) as compared to Ethanol-withdrawal group (Fig. 5.2 (a) & (b). Whereas all results were non-significant for No. of transitions.



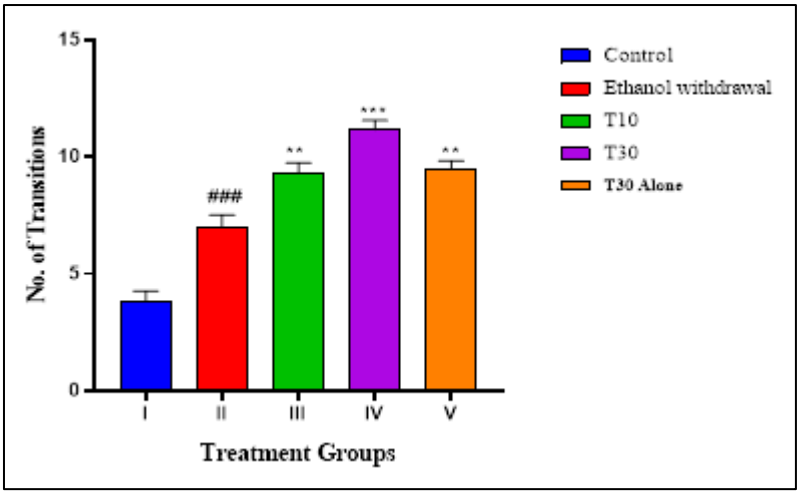
Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 2(a) Time spent in open compartment



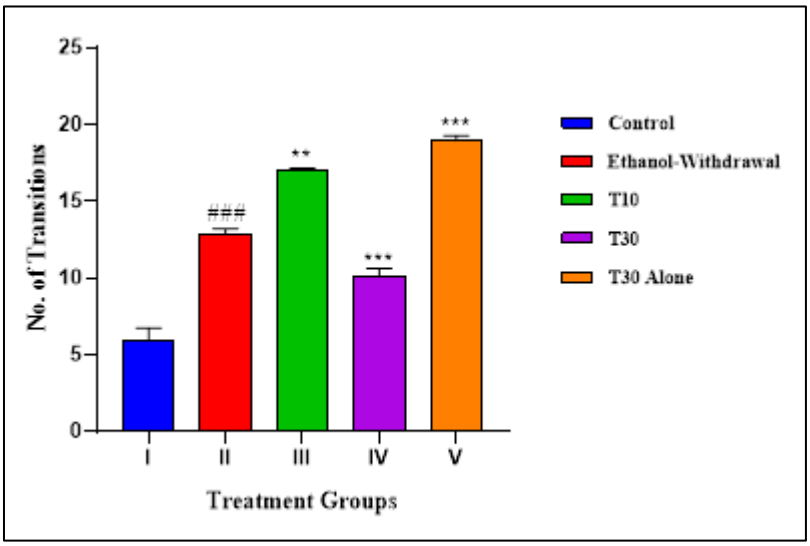
Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 2 (b) Time spent in closed compartment



Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 2(c) No. of Transitions in open Compartment

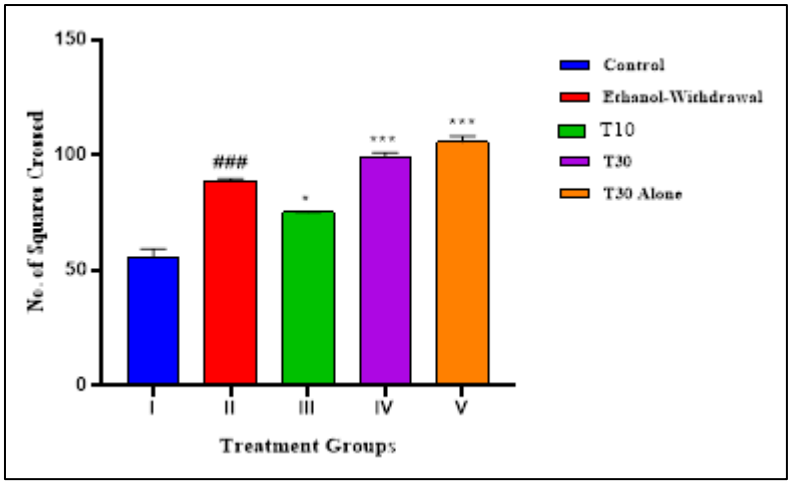


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 2(d) No. of Transitions in closed Compartment

3.1.3. Evaluation of Thymol on anxiety-like behaviour in ethanol withdrawal mice using OFT:

Acute administration of 10% ethanol showed a significant increase in no. of squares crossed ((62.50 ± 3.31)) compared to the control group (44.4 ± 6.61). The mice treated with 10% ethanol along with Thymol (T1) (55.5 ± 3.91) showed significant ($p<0.05$) effects & mice treated with 10% ethanol along with Thymol (T2) (67.8 ± 1.62*) significant ($p<0.05$) increased the no. of squares crossings as compared to the EtOH-withdrawal group & mice treated with Thymol (T2) only, showed a significant ($p<0.05$) increase in the no. of squares crossed (79.16 ± 6.30) as compared to the Ethanol-withdrawal group.Mice treated with T (T2) alone with ethanol significantly (15.00 ± 3.84) decreased number of transitions as compared to vehicle treated group (Figure 3).

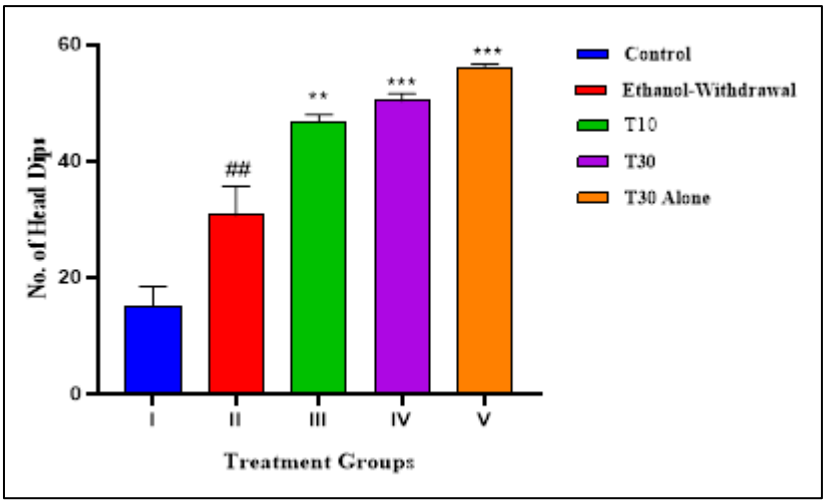


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 3 No. of Squares crossings

3.1.4. Evaluation of Thymol on exploratory activity in ethanol withdrawal mice using HBT:

Acute administration of 10% ethanol significantly (p<0.05) increased the no. of head dips ((30.50 ± 1.99)) compared with the control group (22.50 ± 1.08). The mice treated with 10% ethanol along with Thymol (T10 & T30) (26.45 ± 1.55 &33.71 ± 1.01) significantly (p<0.05) increased the number of head dips, as compared to the EtOH-withdrawal group & mice treated with Thymol (T30) Alone only, showed a significant (p<0.05) increase in the no. of head dips (42.51 ± 3.08) as compared to Ethanol-withdrawal group (Figure 4)

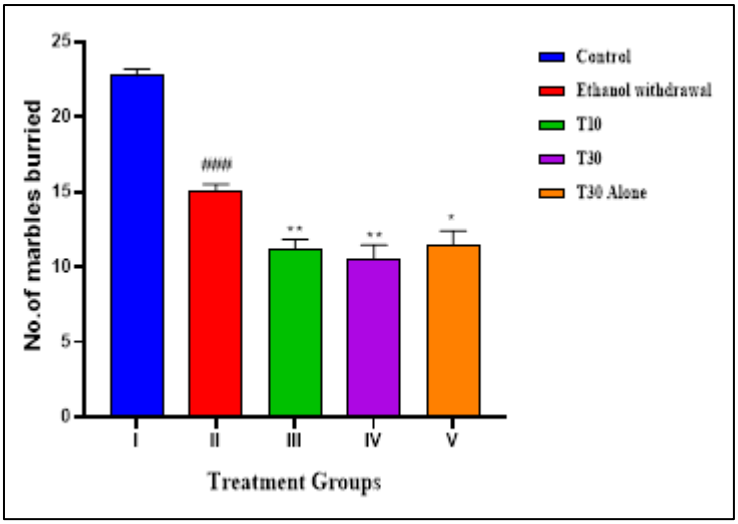


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 4 No. of Head Dips

3.1.5. Evaluation of Thymol on compulsive/anxiety-linked behaviour in ethanol withdrawal mice using MBT:

Acute administration of 10% ethanol showed a significant decrease in no. of marbles buried (8.33 ± 0.33) compared to the control group (10.72 ± 0.47). The mice treated with 10% ethanol along with Thymol (T10 & T30) (9.01 ± 0.85 &9.6 ± 0.57) showed significant (p<0.05) results as compared to the EtOH-withdrawal group & mice treated with Thymol (T30) Alone only showed a significant (p<0.05) decrease in no. of marbles buried (7.16 ± 1.022*) as compared to the Ethanol-Withdrawal group (Figure 5).

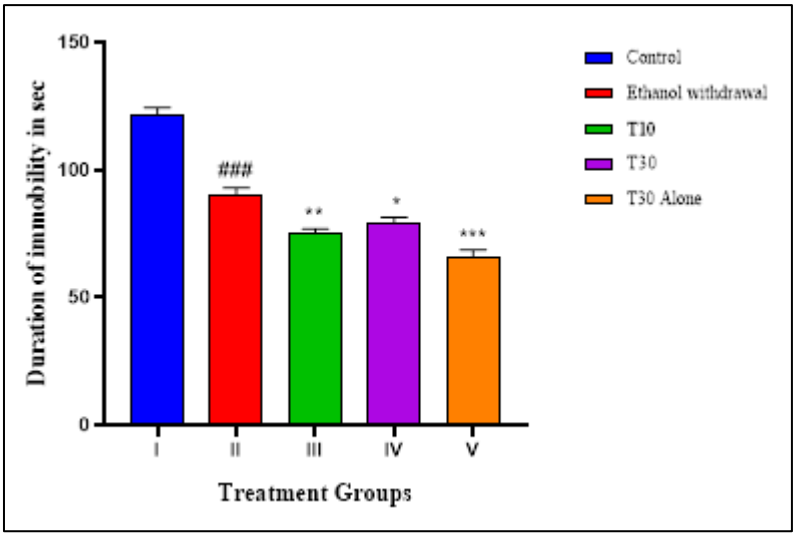


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 5 No. of marble buried in MBT

3.1.6. Evaluation of Thymol on depressive-like behaviour in ethanol withdrawal mice using TST:

Acute administration of 10% ethanol showed a significant (p<0.05) decrease in duration of immobility (118.6 ± 2.52) compared to the control group (141.8 ± 10.18). The mice treated with 10% ethanol along with Thymol (T10 & T30) (114.1 ± 4.21& 103.3 ± 3.32) significantly (p<0.05) decreased duration of immobility as compared to the EtOH-withdrawal group & mice treated with Thymol (T30) Alone only, showed a significant (p<0.05) decrease in duration of immobility (90.6 ± 17.11) as compared to Ethanol-Withdrawal group (Fig. 6).

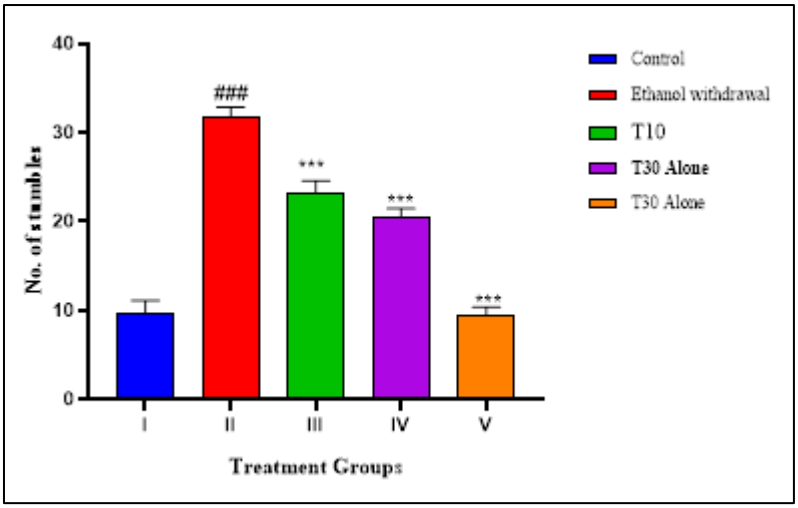


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 6 Duration of immobility in TST

3.1.7. Evaluation of Thymol on motor coordination in ethanol withdrawal mice using the stumbling test:

Acute administration of 10% ethanol showed a significant (p<0.05) increase in no. of stumbles ((31.81 ± 1.04) compared to the control group (9.60 ± 1.50). The mice treated with 10% ethanol along with Thymol (T10 & T30) (23.2± 1.37&27.1 ± 1.0) More significant (p< 0.05) effect was obtained with mice treated with T1 indicating anti-depressant potential of the Thymol in ethanol withdrawal mice. significantly (p<0.05) decreased no. of stumbles as compared to the EtOH-withdrawal group & mice treated with Thymol (T30) only, showed a significant (p<0.05) decrease in no. of stumbles (9.42 ± 0.94) as compared to the EtOH-withdrawal group (Figure 7).

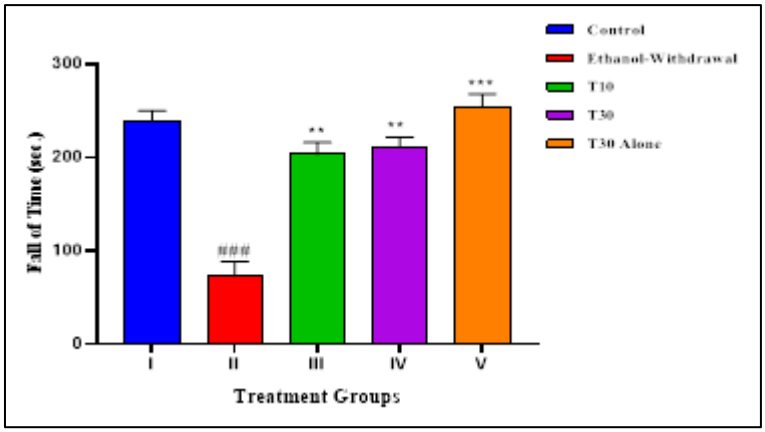


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 7 No. of Stumbles

3.1.8. Evaluation of Thymol on motor coordination in ethanol withdrawal mice using the Rota rod test:

Acute administration of 10% ethanol showed a significant (p<0.05) decrease in no. of stumbles (129.8 ± 15.8) compared to the control group (238.6 ± 11.6). The mice treated with 10% ethanol along with Thymol (T10 & T30) (203.4±12.7 & 226.8±10.9) significantly (p<0.05) increase in fall of time as compared to the EtOH-withdrawal group & mice treated with Thymol (T30) only, showed a significant (p<0.05) increase in fall of time (254.7±13.4) as compared to the EtOH-withdrawal group (Fig. 8).



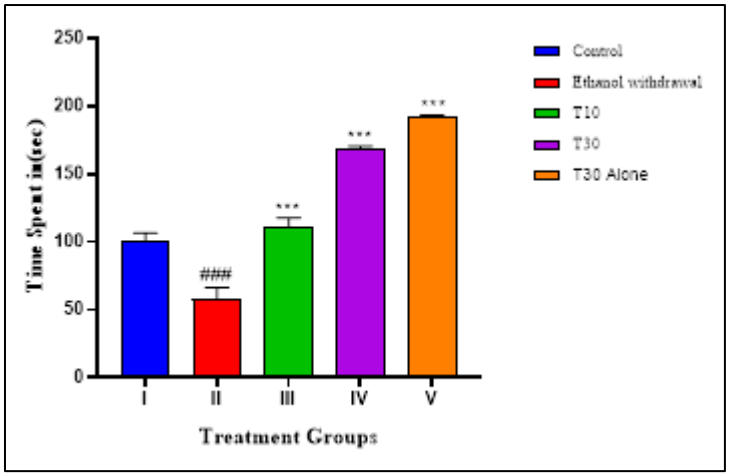
Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 8 Fall of Time (sec.)

3.2. Chronic Study

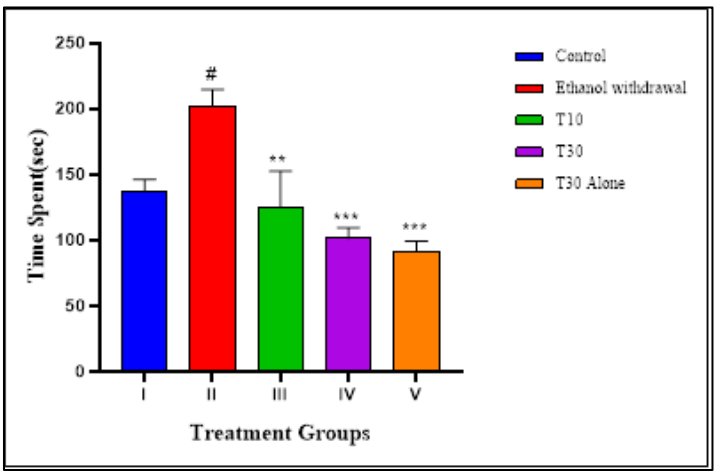
3.2.1. Evaluation of Thymol on anxiety-like behaviour in ethanol withdrawal mice using EPM:

Chronic ethanol consumption led to significant (p<0.05) (57.50 ± 8.82) decreased in time spent in open arm and increased time spent in closed arm (202.5±12.18) compared to control group in mice and mice treated with 10% ethanol & Thymol (T10) shows significant (p<0.05) (110.6±7.17) as compared to the EtOH-withdrawal group & mice treated with 10% ethanol & Thymol (T30) as found to relieve ethanol abstinence-induced anxiety in mice as indicated by significantly (p<0.05) (168.8±1.94) increased open arm time in comparison with Ethanol-withdrawal group. Mice treated with Thymol (T30) Alone only showed a significant (p<0.05) increase in time spent in the open arm (192.1±1.41) as compared to the Ethanol-Withdrawal group [Fig. 5.8]. Whereas, mice treated with 10% ethanol with Thymol (T10 & T30) show significantly higher entries than the Ethanol-withdrawal group. ethanol for day 1-6 and challenged with test drug (T) on 7th day showed anxiolytic effect as compared with ethanol treated group (Figure 9)



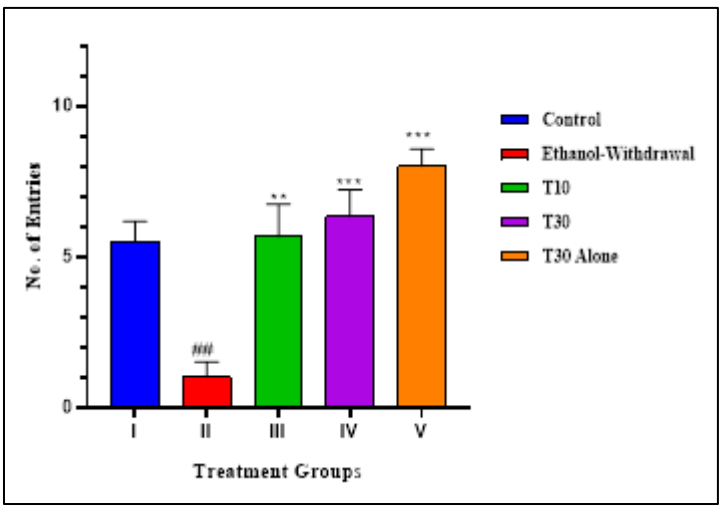
Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group

Figure 9 (a) Time spent in open arm



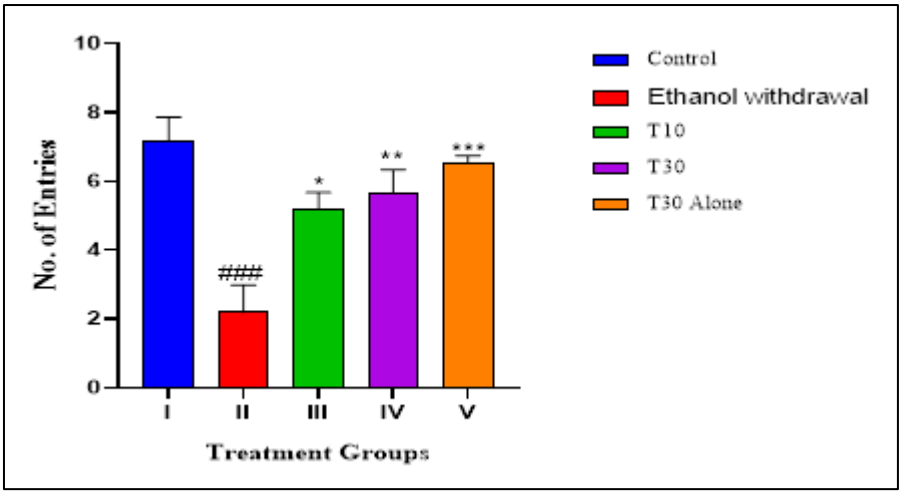
Values are expressed as mean ± SEM (n=6) analyzed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 9(b) Time spent in closed arm



Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 9 (b) No. of Entries in open arm

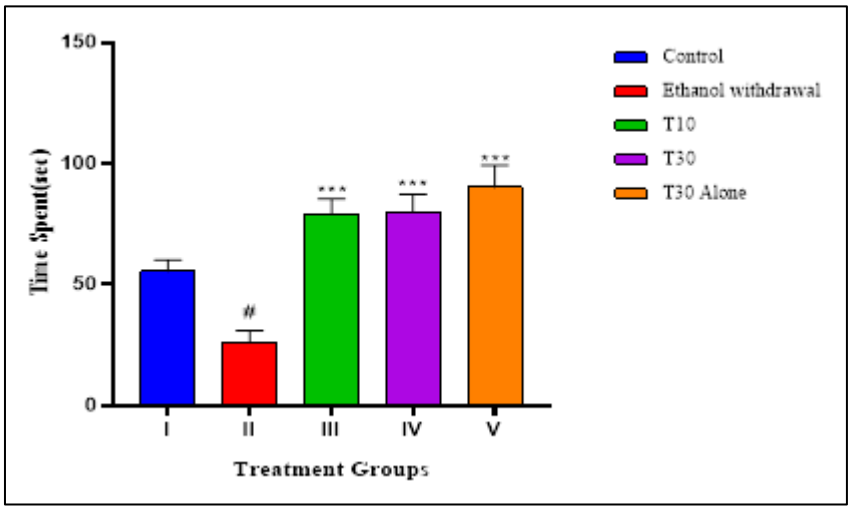


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 9 (c) No. of Entries in closed arm

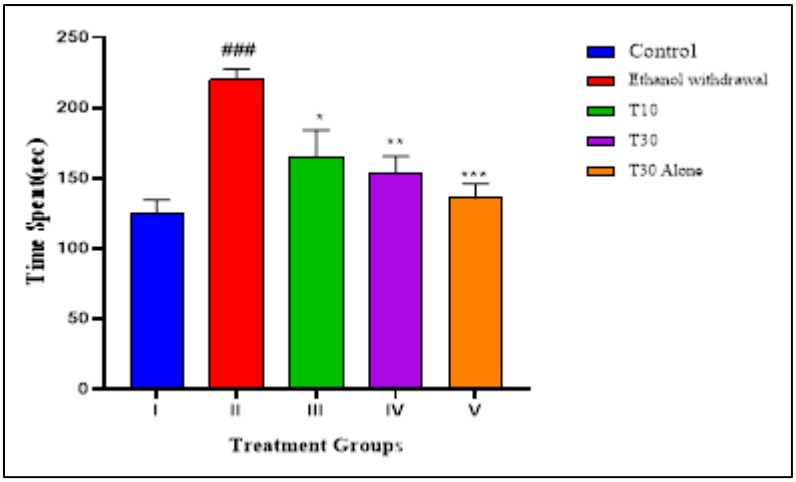
3.2.2. Evaluation of Thymol on anxiety-like behaviour in ethanol withdrawal mice using LDT

Mice withdrawn from chronic administration of 10% ethanol showed a significant (p<0.05) decrease in time spent in the open arm (26.98 ±5.00) compared to the control group (36.54 ±4.67). The mice treated with 10% ethanol along with Thymol (T10) (71.25 ±6.15) showed non-significant effects & mice treated with ethanol along with Thymol (T30) (80.19 ±7.12) significantly (p<0.05) increased the time spent in open arm as compared to the Ethanol-withdrawal group & mice treated with Thymol (T30) Alone only, showed a significant (p<0.05) increase in the time spent in open arm (90.28 ±9.12) as compared to Ethanol-withdrawal group (Fig. 10)



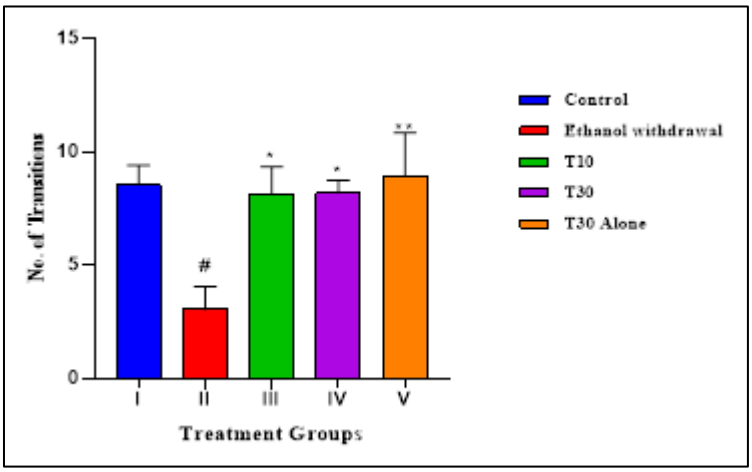
Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 10 (a) Time spent in Light compartment



Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 10 (b) Time spent in closed compartment

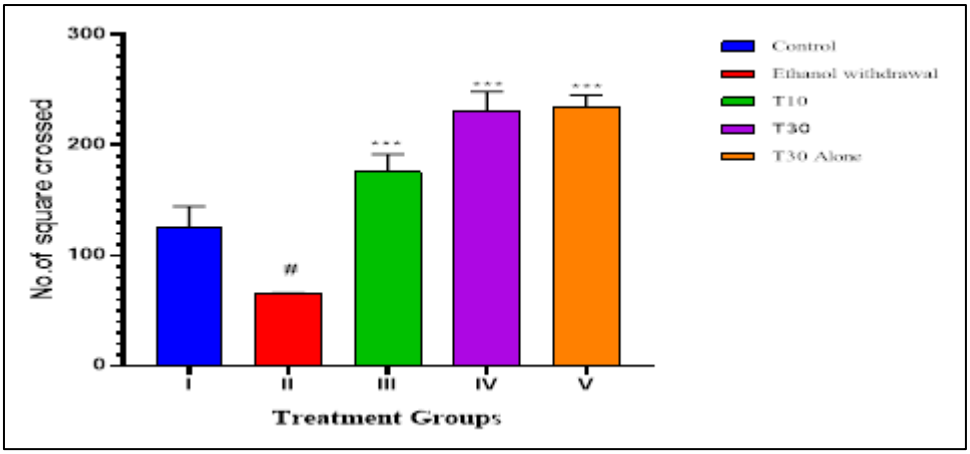


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 10(c) No. of Transitions in closed compartment

3.2.3. Evaluation of Thymol on anxiety-like behaviour in ethanol withdrawal mice using OFT:

Mice withdrawn from chronic administration of 10% ethanol on 7th day and challenged with dosed T (T1 and T2) resulted in significantly increased number of square crossing (195.50±16.12 and 200.30±17.09) indicating anxiolytic behaviour as compared to ethanol treated group. Mice treated with 10% ethanol for 1-6 days showed significant (87.91 ± 17.12) decreased number of square crossings as compared to group. Mice Control treated with dosed with 10% ethanol for 1-6 days challenged with T (T1 and T2) on 7th day shown significant (4.33 ± 2.01 and 7.05 ± 1.39) increase in rearing in comparison with ethanol treated group (Figure 11).

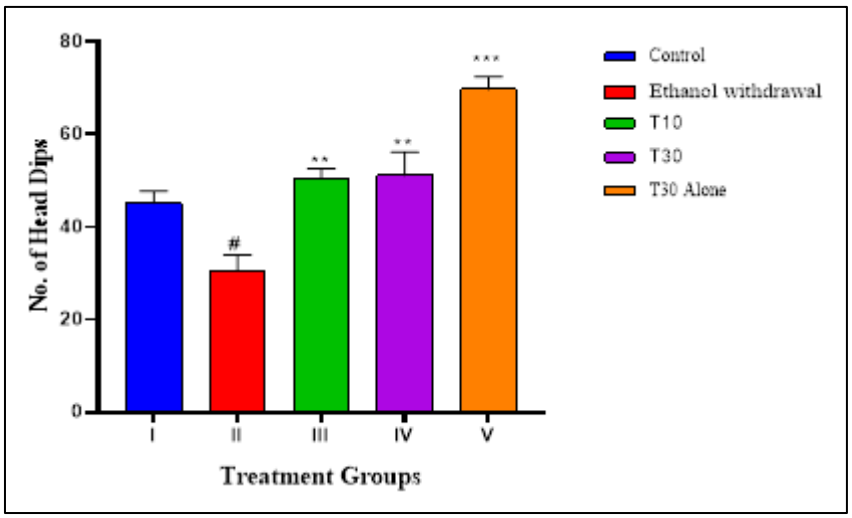


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 11 No. of Squares Crossed

3.2.4. Evaluation of Thymol on exploratory activity in ethanol withdrawal mice using HBT:

Chronic 10% ethanol administration resulted in significant (30.60 ± 3.36) decreased no. of head dips in HBT in mice in comparison to the Control group (45.17±2.58). Animals dosed with 10% ethanol for 1-6 days challenged with T (T30) on 7th day shown significant (51.16 ± 5.08) increase in head dips in comparison with ethanol treated and Control group. More significant effect was obtained with mice treated with T2 indicating anti-anxiety activity of the Thymol on ethanol withdrawal mice. Mice treated with Thymol (T30) Alone showed more significant (69.83 ± 2.67) effect as compared to ethanol treated and Control group. Mice withdrawn from 10% ethanol administration on 7th day and treated with T (T1 and T2) significantly (5.16 ± 0.79 and 4.33 ± 0.21) decreased grooming as compared to ethanol treated group (Figure 12).

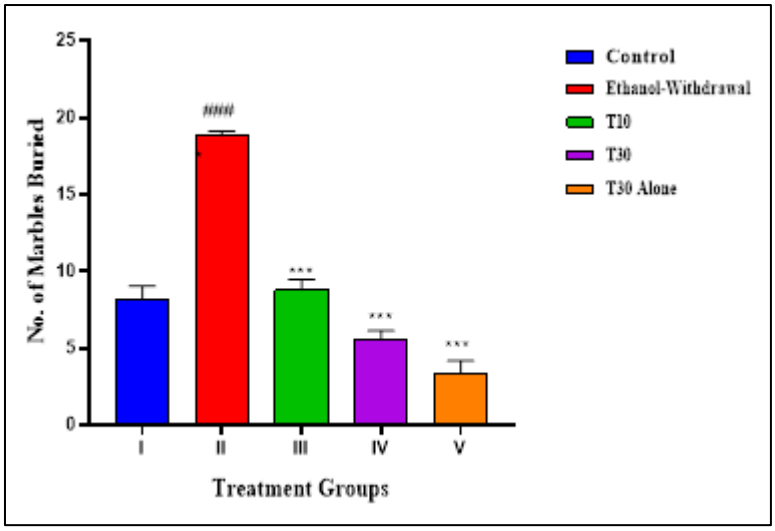


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 12 No. of Head Dips

3.2.5. Evaluation of Thymol on compulsive/anxiety-linked behaviour in ethanol withdrawal mice using MBT:

Mice withdrawn from chronic administration of 10% ethanol on 7th day and challenged with dosed T (T1 and T2) resulted in significantly decreased number of marbles buried (8.8 ± 0.66 and 5.6 ± 0.54) indicating anxiolytic behaviour as compared to ethanol treated group. Mice chronically treated with 10% ethanol on day 1-6 showed significant increase in marble buried shows anxiogenic behaviour due to abstinence. Mice treated with *Thymol* showed significant ($p < 0.05$) decreased marble burying in mice shows anxiolytic activity. More significant effect was shown in T2 received mice when compared to Control group and ethanol withdrawal groups (Figure 13).

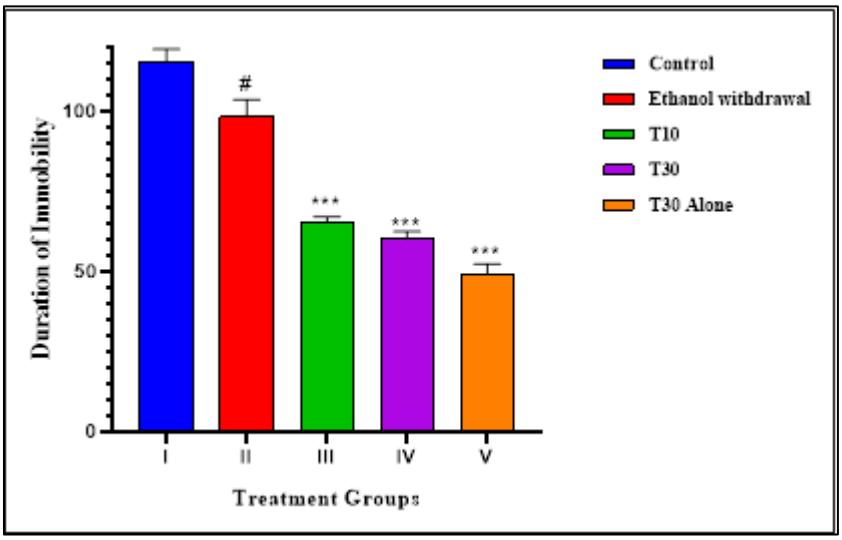


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 13 No. of Marbles Buried

3.2.6. Evaluation of Thymol on depressive-like behaviour in ethanol withdrawal mice using TST:

Chronic ethanol administration resulted in increased (98.50± 5.43) duration of immobility in TST using mice in comparison to the Control group. Animals dosed with T (T1 and T2) shown significant (65.70 ± 1.57) and 60.4± 2.30) decreased in duration of immobility as compared to the ethanol treated group. More significant (p< 0.05) effect was obtained with mice treated with T2 indicating anti-depressant potential of the *Thymol* in ethanol withdrawal mice. Mice treated with Thymol T30 Alone showed significantly (49.30 ± 3.18) decreased duration of immobility as compared to ethanol and Control group (Figure 14).

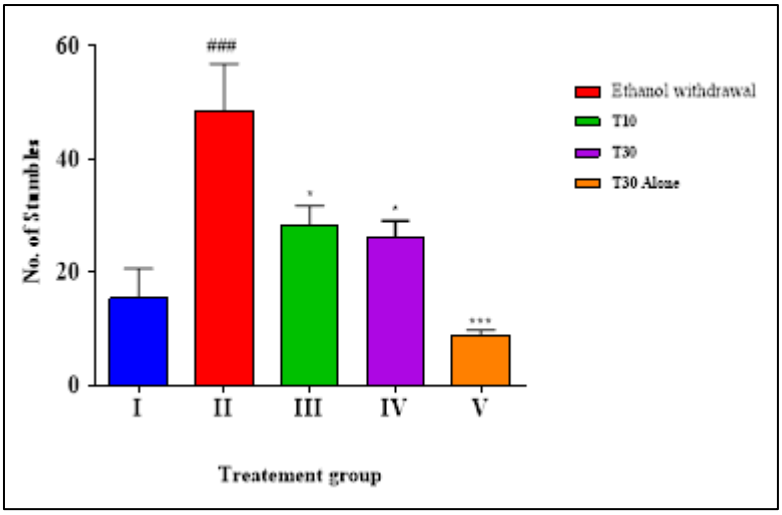


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 14 Duration of Immobility in TST

3.2.7. Evaluation of Thymol on motor coordination in ethanol withdrawal mice using the stumbling test:

Chronic ethanol administration resulted in increased (42.40 ± 1.04) No. of stumbles in the Stumbling Test, using mice in comparison to the Control group. Animals dosed with T (T1 and T2) shown significant (29.69 ± 1.37 and 24.88 ± 1.07) decreased in No. of stumbles as compared to the ethanol treated group. More significant (p< 0.05) effect was obtained with mice treated with T1 indicating anti-depressant potential of the *Thymol* in ethanol withdrawal mice. Mice treated with Thymol T30 Alone showed significantly (8.75 ± 0.94) decreased No. of stumbles as compared to ethanol and Control group (Figure 14).

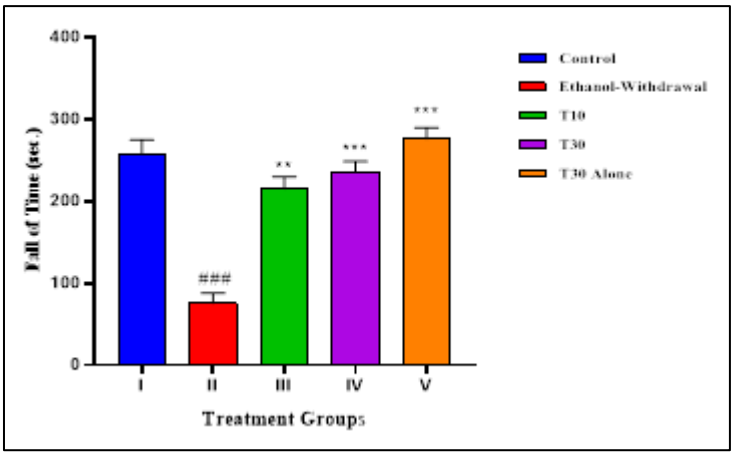


Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 15 No. of Stumbles in Stumbling Test

3.2.8. Evaluation of Thymol on motor coordination in ethanol withdrawal mice using the Rota rod test:

Mice withdrawn from chronic administration of 10% ethanol showed a significant (p<0.05) decrease in fall of time (103.7 ± 12.1) compared to the control group (257.4 ± 17.5). The mice treated with 10% ethanol along with Thymol (T10 & T30) (194.5 ± 14.4 & 208.9 ± 12.8) significantly (p<0.05) increase the fall of time, compared to the Ethanol-withdrawal group & mice treated with Thymol (T30) only, showed a significantly (p<0.05) increase in fall of time (276.4 ± 13.6) as compared to the Ethanol-withdrawal group (Fig. 16).



Values are expressed as mean ± SEM (n=6) analysed by one-way ANOVA followed by Tukey's post hoc test. # p < 0.05, ## p < 0.01, ### p < 0.01 compared to control group and *p < 0.05, **p < 0.01, ***p < 0.01 compared to Ethanol-withdrawal group.

Figure 16 Fall of Time (sec.)

4. Discussion

This study provides strong evidence that thymol, a key compound in thyme oil, effectively reduces the behavioral problems caused by alcohol withdrawal in mice. After long-term alcohol use followed by sudden stopping, mice showed increased anxiety, depression-like symptoms, and poor motor coordination. Importantly, thymol treatment reversed these problems in a dose-dependent way, showing its potential as an anxiolytic, antidepressant, and motor-protective agent. This supports earlier findings about thymol's effects on the brain's GABA system and its antioxidant properties (Albert & Lemonde, 2004).

The assessment of anxiety-like behavior is a cornerstone in preclinical models of AWS, as anxiety is a prominent and distressing symptom in individuals undergoing alcohol withdrawal (Koob & Le Moal, 2001). Our results from the

Elevated Plus Maze (EPM), a widely validated ethological assay employed to assess anxiety-like behavior in rodents(Gautam et al., 2024) (Walsh & Cummins, 1976); (Lister, 1987), provided the initial compelling evidence. The ethanol withdrawal group (ETWD) displayed a statistically significant reduction in both the time spent in and the number of entries into the open arms of the maze. This pattern of behavior is a classic indicator of increased anxiety and fear, reflecting a heightened state of apprehension and risk aversion in an open, unprotected environment. This observation is remarkably consistent with the well-documented anxiogenic profile routinely observed during periods of alcohol abstinence in various rodent models, often attributed to dysregulation of neurotransmitter systems and neuronal hyperexcitability (Poudel et al., 2020); (Crabbe, 2008); (Tsai et al., 1995).

In stark contrast, thymol treatment significantly increased both the time spent in and entries into the open arms, thereby providing robust evidence for its anxiolytic activity. This observation aligns seamlessly with a growing body of preclinical literature that has consistently reported the anxiolytic capabilities of thymol. For instance, studies have shown that thymol can modulate anxiety-related behavior by acting on the GABAergic system, particularly by positively modulating GABAA receptors, similar to benzodiazepine (Goldschen-Ohm, 2022). Given that alcohol withdrawal is characterized by a significant decrease in GABAergic inhibition and resultant neuronal hyperexcitability, thymol's ability to enhance GABAA receptor function could directly counteract the anxiogenic effects of withdrawal (Davies, 2003) Furthermore, other research suggests thymol's potential anti-inflammatory properties (Nagoor Meeran, Javed, Al Taee, et al., 2017),(Galovičová et al., 2021), which could contribute to anxiety reduction by mitigating neuroinflammation, a known contributor to anxiety during AWS (Crews et al., 2023). The restoration of exploratory behavior in the EPM by thymol underscores its potential as a therapeutic agent to alleviate the severe anxiety that frequently drives relapse in individuals undergoing alcohol withdrawal

Anxiety is a major symptom of alcohol withdrawal. In the Elevated Plus Maze (EPM) test, withdrawn mice spent less time and entered fewer times into open arms, indicating high anxiety and fear (Koob & Le Moal, 2001; Gautam et al., 2024). Thymol treatment increased open arm time and entries, confirming its anxiety-reducing effects likely through positive modulation of GABAA receptors (Goldschen-Ohm, 2022). Thymol's anti-inflammatory effects may also reduce neuroinflammation linked to withdrawal anxiety (Nagoor Meeran et al., 2017; Crews et al., 2023).

The Light/Dark Box (LDB) test also showed withdrawal mice avoided the light area and explored less, indicating anxiety (Takao & Miyakawa, 2006). Thymol reversed this behavior, increasing time spent in the light and the number of transitions, confirming anxiolytic activity across multiple tests.

The Open Field Test (OFT) further supported these findings. Withdrawal mice spent less time in the centre and showed reduced overall movement, signs of anxiety and low motivation (Prut & Belzung, 2003). Thymol increased central activity and exploration, likely by enhancing GABA signalling and reducing oxidative stress (Liu et al., 2022; Lee et al., 2016).

Exploratory behavior, measured by the Hole Board Test, was reduced in withdrawal mice, seen by fewer head dips (File & Wardill, 1975). Thymol increased head-dipping in a dose-dependent manner, indicating less anxiety and improved curiosity, consistent with its anxiolytic effects.

In the Marble Burying Test, withdrawal mice buried more marbles, indicating increased anxiety or compulsive-like behavior (Thomas et al., 2009). Thymol decreased this behavior, suggesting it helps reduce stress-induced repetitive actions, likely due to its anti-anxiety and neuroprotective effects (Islam et al., 2024).

Depression is another key symptom of alcohol withdrawal. The Tail Suspension Test (TST) showed withdrawal mice had increased immobility, reflecting depressive-like states (Koob & Volkow, 2016). Thymol significantly reduced immobility, indicating antidepressant-like effects. While its exact action on serotonin or dopamine is unclear, thymol's antioxidant and anti-inflammatory properties may reduce brain inflammation and oxidative damage linked to depression (Faleiro et al., 2005; Kim et al., 2018). Its combined anxiolytic and antidepressant effects make it promising for mood regulation in withdrawal.

Motor coordination problems, including tremors and ataxia, are common in alcohol withdrawal. The Stumbling Frequency Test revealed increased motor deficits in withdrawal mice (Swift & Rosse, 1998). Thymol improved coordination, reducing stumbling events, likely due to its neuroprotective antioxidant effects that protect motor control brain regions from withdrawal-induced damage (Javed et al., 2019; Kamal et al., 2020).

Similarly, the Rotarod test showed thymol improved motor skills by increasing the time mice stayed on the rotating rod, confirming motor benefits (Keane et al., 2024).

Though this study focused on behavior, the results support prior research showing thymol's broad neuropharmacological effects. Alcohol withdrawal disrupts multiple brain systems, and thymol's multiple actions modulating GABA, reducing inflammation, and oxidative stress make it an especially suitable treatment for this complex syndrome (Li et al., 2023). The current findings highlight the effectiveness of the rotarod test in evaluating Thymol as a viable alternative for addressing motor coordination issues associated with alcohol withdrawal (Keane et al., 2024a). The animals were placed at the rotating rod & then the rod was rotated at a constant speed & The timer for each animal compartment is automatically stopped by the falling animal on the bottom of each chamber under the corresponding compartment, with the latency to fall automatically registered in the generated data after falling. Notably, treatment with berberine resulted in a significant and dose-dependent improvement in motor coordination, as demonstrated by a marked decrease in fall time among the ET-WD mice. (González-cabrera et al., 2024)

While the current study primarily focused on behavioral phenotypes, the comprehensive and consistent behavioral data strongly align with and provide *in vivo* behavioral validation for previous mechanistic studies that support thymol's broad pharmacological actions. This polypharmacological profile makes thymol particularly appealing for a complex syndrome like AWS, which involves disturbances across multiple neurobiological systems (Li et al., 2023 for general concept).

5. Conclusion

In conclusion, the present study provides compelling and unequivocal evidence that thymol effectively ameliorates a broad and debilitating range of behavioural symptoms that manifest during alcohol withdrawal. Our comprehensive preclinical investigation, utilizing a well-validated murine model of alcohol withdrawal, revealed that thymol significantly mitigated the distinct and interconnected clusters of symptoms, including pronounced anxiety-like behaviours (as evidenced by its effects in the EPM, OFT, LDB, hole board, and marble burying tests), marked depressive-like states (demonstrated in the TST), and significant motor coordination deficits (quantified by the SFT) and Rotarod.

These robust findings unequivocally validate thymol's inherent anxiolytic, antidepressant, and neuromotor-protective properties specifically within the challenging and neurobiologically complex context of alcohol withdrawal syndrome (AWS). The consistent and dose-dependent reversal of these pathologies across diverse behavioural paradigms strongly suggests that thymol's therapeutic action is not narrowly targeted but rather stems from a likely polypharmacological profile. This multifaceted efficacy is critically attributed to its capacity to engage in the positive modulation of the GABAergic system (a primary mechanism for its anxiolytic and hyperexcitability-reducing effects), its potent attenuation of oxidative stress (a major contributor to neuronal damage and dysfunction during withdrawal), and its significant reduction of neuroinflammation (an increasingly recognized driver of withdrawal-induced psychopathology and neuronal vulnerability). By addressing these core neurobiological imbalances and detrimental processes, thymol offers a promising and holistic approach to managing the complex symptomatology of alcohol withdrawal.

Compliance with ethical standards

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Disclosure of conflict of interest

The author states that there is no conflict of interests.

Statement of ethical approval

The experimental procedures and protocols have been reviewed & approved by the Institutional Animal Ethics Committee (IAEC) at SSDJ College of Pharmacy in Neminagar, Chandwad (SSDJ/IAEC/24-25/01)

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Data Availability

The findings reported in this study were performed by the authors, and all referenced data has been correctly cited.

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