Pharmacological Effect of Thymoquinone and Divalproex in Alleviating Symptoms of Alcohol Withdrawal in Animal Model

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

Pharmacology

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LOVELY PROFESSIONAL UNIVERSITY
PUNJAB
2025

DECLARATION

I, hereby declared that the presented work in the thesis entitled "Pharmacological effect of Thymoquinone and Divalproex in alleviating symptoms of alcohol withdrawal in animal model" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Navneet Khurana, working as Professor and COD, in the Pharmacology, School of Pharmaceutical Sciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

(Signature of Scholar)

Name of the scholar: Mohd. Arsalan Sarmad Mohammad Ikram

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Pharmacological effect of Thymoquinone and Divalproex in alleviating symptoms of alcohol withdrawal in animal model" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Pharmacology, School of Pharmaceutical Sciences of Lovely Professional University, Punjab, India, is a research work carried out by Mohd. Arsalan Sarmad Mohammad Ikram (Registration No.) 41900312, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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Abstract

Introduction Alcohol withdrawal syndrome (AWS) involves severe neurological and physiological disturbances resulting from abrupt cessation of chronic alcohol use. Thymoquinone (THMQ), a bioactive constituent of *Nigella sativa*, possesses antioxidant and neuroprotective properties, whereas divalproex (DVPX) enhances GABAergic neurotransmission and reduces neuronal excitability. The present study evaluates the individual and combined effects of THMQ and DVPX in mitigating alcohol withdrawal-induced anxiety and craving in mice. **Methodology** Fourteen groups of mice (n=6/group) were used. Alcohol dependence was induced using an intermittent escalation model: 5% ethanol (days 1–5), 10% (days 8–12), 20% (days 15–19), and 35% (days 22–26), with intervening ethanol-free days to provoke craving. Treatment groups received THMQ (20 mg/kg), DVPX (30 mg/kg), or diazepam (1 mg/kg) on craving days. Behavioral assessments including the Elevated Plus Maze, Elevated Zero Maze, and Open Field Test were conducted on days 7, 14, 21, and 28 to evaluate anxiety-like activity and alcohol-seeking behaviour. **Results** Intermittent alcohol exposure produced significant anxiety-like behaviours and increased alcohol-seeking compared to controls ($p<0.01$). THMQ and DVPX treatment significantly reduced anxiety indices in EPM and EZM, decreased locomotor hyperactivity in OFT, and lowered withdrawal-related craving. Diazepam produced acute but short-lasting anxiolytic effects, whereas THMQ and DVPX demonstrated sustained therapeutic benefits during repeated withdrawal cycles. **Conclusion** THMQ and DVPX effectively attenuated behavioural manifestations of AWS, including anxiety and alcohol craving. Their prolonged protective effects suggest potential advantages over benzodiazepines for managing repeated withdrawal episodes.

Keywords: Alcohol withdrawal, Alcohol craving, Thymoquinone, Divalproex, Diazepam, Behavioural assessment, SK2, GluA1.

Acknowledgements

In The Name Of Allah the Most Merciful, the Very Merciful

If all the trees on earth were pens and the ocean was an ink pot, replenished with ink by seven more oceans, the writing of Allah's words would never exhaust. Indeed, Allah is all-mighty and all-wise." (Al-Quran, Chapter 31, Verses 27-28)

Looking back on this project, I am filled with immense gratitude for all the kindness, support, and encouragement I have received along the way. This thesis is not just my own work it is the result of countless contributions from so many people who have been there for me.

First and foremost, I want to extend my heartfelt thanks to my project supervisor, ***Dr. Navneet Khurana*** (Professor, School of Pharmaceutical Sciences, Lovely Professional University, Punjab). His deep knowledge and expert guidance have been absolutely crucial in shaping the direction of this project. His suggestions and advice have constantly pushed me to refine my work, ensuring that it has both quality and depth.

I am also incredibly thankful to my co-supervisor, ***Dr. M. Venkata Ramana*** (Principal, Azad College of Pharmacy, Hyderabad, India), for his steady support and availability. He helped me navigate the many complexities of this project with great expertise.

A big thank you goes to ***Mr. Ashok Mittal*** (Hon'ble Chancellor, Lovely Professional University, Punjab), ***Ms. Rashmi Mittal*** (Worthy Pro Chancellor, Lovely Professional University, Punjab), and ***Dr. Monica Gulati*** (Head of School, School of Applied Medical Sciences, Lovely Professional University, Punjab). Their generosity in providing crucial resources, including a fantastic laboratory environment, played a key role in helping me complete this thesis successfully.

I also owe so much to ***Salwa Fatema,*** my father ***Mohammad Ikram,*** my mother, and my siblings for their endless love and motivation throughout this entire journey. Their emotional and moral support has meant the world to me. I am equally grateful to my close friends Syed Shoeb Ahmed, Akib Shaikh, Syed Sharfuddin Ibrahim, and Riyaj Ahmad whose unwavering support and encouragement kept me going even when things were tough.

Any mistakes or shortcomings in this work are entirely mine. **Mohammad Arsalan Sarmad**

This work is Dedicated to Myself, My Passion And My Consistency.

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ABBRIVATIONS

ALC	Alcohol
THMQ	Thymoquinone
DVPX	Divalproex
DZPM	Diazepam
LD	Low Dose
HD	High Dose
i.p	Intera Peritoneal
p.o	per oral
GABA	Gamma Amino Butyric acid
5-HT	5-Hyderoxy tryptamine
R.U	Relative Expression value
WB	Western Blot
EPM	Elevated Plus Maze
EZM	Elevated Zero Maze
OFT	Open Field Test
SD	Standard Deviation
AWS	Alcohol withdrawal syndrome

Introduction

Alcohol dependence remains one of the most widely encountered and persistent forms of substance abuse across the world. It is recognized as a chronic, relapsing disorder that generates both physical and psychological dependence, leading to significant health and social consequences. When consumed in excess, Alcohol is not efficiently metabolized, resulting in a range of unpleasant physical and psychological symptoms that accompany alcohol craving. These symptoms can vary from mild tremors to severe hallucinations, creating substantial distress for affected individuals (Ruby B et al., 2012). Prolonged alcohol consumption disrupts the delicate neurochemical equilibrium of the brain (Bansal & Banerjee, 2016; Saitz, 1998), and as the brain adapts to the continuous presence of Alcohol, neurons develop hypersynchronous activity (Tunstall et al., 2019). This produces pronounced alterations in normal neurotransmission, ultimately pushing the brain towards dependence on Alcohol to maintain functional stability.

A sudden decline in alcohol levels can therefore precipitate a cascade of neurochemical disturbances, particularly affecting serotonin. Withdrawal from Alcohol leaves the brain in a hyperexcited state, activating auto-receptors and contributing to depressive symptoms (Haque et al., 2021). The underlying depression is largely associated with hypoactivity of neurotransmitters such as serotonin (5-HT), a critical modulator of mood (Farkhondeh et al., 2018). Stress further exacerbates these neurotransmitter abnormalities, accelerating the development of depressive features commonly observed in individuals with alcohol dependence (Tunstall et al., 2019).

Monitoring blood alcohol concentration (BAC) plays an essential role in assessing the severity of dependence and guiding detoxification strategies (Hedrich & Bullock, 2004). Chronic alcohol use also predisposes individuals to seizures and neurotoxicity, contributing to long-term cognitive and neurological deficits (Becker & Mulholland, 2014). These consequences highlight the complexity of alcohol dependence and the need for therapeutic interventions that can address its multifaceted nature.

Alcohol Dependence

Alcohol dependence arises from a combination of physical and psychological addiction, resulting in significant impairment and increased mortality risk (Becker & Mulholland, 2014). A major contributor to alcohol dependence is the imbalance between excitatory neurotransmitters such as glutamate and inhibitory ones such as gamma-aminobutyric acid (GABA) (Kayir & Uzbay, 2008). Under normal conditions, these systems maintain neural homeostasis (Panula, 2020), but chronic alcohol exposure disrupts this equilibrium, altering neurotransmitter release, receptor activity, and neuronal signalling (Becker & Mulholland, 2014).

In addition to GABA and glutamate, several other neurotransmitters—including dopamine, serotonin, and acetylcholine—play key roles in alcohol dependence (Hinton et al., 2012). Understanding these interactions is crucial for establishing therapeutic strategies for alcohol use disorders (Hirani et al., 2005).

Levels of Alcohol Withdrawal

Alcohol withdrawal manifests in progressive stages:

Level 1 – Minor withdrawal:

Mild anxiety, insomnia, headache, and nausea typically arise within 6–12 hours of cessation (Anton, 1999).

Level 2 – Moderate withdrawal:

Hallucinations, perceptual disturbances, and elevated pulse may appear 12–48 hours after cessation (Anton, 1999).

Level 3 – Severe withdrawal:

Severe tremors, autonomic instability, seizures, and delirium tremens may occur, sometimes becoming life-threatening in chronic drinkers (Saitz, 1998).

Neurochemical Changes During Alcohol Withdrawal

Alcohol withdrawal involves widespread disturbances in neurotransmission, affecting serotonin, dopamine, adrenaline, acetylcholine, GABA, and glutamate (Jung

& Metzger, 2010). Imbalances in these systems underlie both the psychological and physical symptoms of dependence and withdrawal (Yan et al., 2013).

Glutamate

Glutamate levels rise significantly during withdrawal, especially during intense withdrawal cycles (Overstreet et al., 2003). This contributes to CNS hyperactivity, anxiety, agitation, and increased seizure susceptibility (Kayir & Uzbay, 2008).

GABA

GABA is the primary inhibitory neurotransmitter (Addolorato et al., 2012). Chronic alcohol intake downregulates the GABAergic system, causing the brain to rely on Alcohol for inhibitory tone (Pineau et al., 2016). Withdrawal removes this compensatory mechanism, producing marked excitability and anxiety (Pineau et al., 2016).

Serotonin

Serotonin regulates mood and emotional responses, influencing sleep, cognition, temperature, and autonomic regulation (Alosetron et al., n.d.; Fahmy et al., 2020). Alcohol-induced disturbances in serotonin are linked to depression, anxiety, and behavioural dysregulation observed during withdrawal (Belmer et al., 2018).

Serotonin Receptors and 5-HT Agonists

Serotonin receptors are divided into seven major families, each influencing diverse behavioural and physiological responses (Belmer et al., 2018). Alterations in these receptors can negatively impact emotional stability, behaviour, and responses to substances of abuse (Alosetron et al., n.d.).

5-HT agonists modulate neuronal excitability and affect mood, learning, and stress responses (Kayir & Uzbay, 2008; Aquib et al., 2014). Serotonergic imbalance during alcohol dependence impairs cognition, increases irritability, and intensifies withdrawal symptoms (Belmer et al., 2018).

Pathophysiology of Alcohol Dependence

Chronic Alcohol exposure disrupts the GABAergic system through changes in gene expression and downregulation of GABAA_AA receptors (Heinz et al., 2003; Haque et al., 2021). Abrupt cessation leads to dysregulated excitatory–inhibitory signalling, contributing to withdrawal hyperexcitability. Additionally, chronic alcohol intake alters NMDA receptor activity, exacerbating CNS overstimulation during withdrawal (Shieh & Yang, 2020). Serotonergic receptor dysfunction further contributes to anxiety, mood disturbances, and addictive behaviours (Lucia et al., 2018; Owens & Nemeroff, 2003).

Difference Between Alcohol Withdrawal and Alcohol Craving

Withdrawal is characterised by CNS hyperexcitability due to physiological adaptation to chronic alcohol exposure (Tunstall et al., 2019). Symptoms may begin within six hours of cessation (Mendelson et al., 1978) and include nausea, tremors, insomnia, anxiety, cardiac palpitations, emotional instability, and cognitive disturbances.

Craving, in contrast, represents a psychological and physiological urge to consume Alcohol and is associated with dopamine, glutamate, GABA, serotonin, and endogenous opioid pathways (Heinz et al., 2003; Srinivasababu et al., 2014).

Acute Toxicity of Thymoquinone

Thymoquinone toxicity has been evaluated using dose-escalation studies in rodents, with olive oil used as the solvent (Hamdan et al., 2019). LD50_{50}50 determination was performed using the staircase method, followed by probit analysis (Farkhondeh et al., 2018; Firdaus et al., 2018). Histopathological examination of vital organs provided further insight into potential toxicity (Hamdan et al., 2019)

Review of Literature

1. B. Ruby et al. (2012)

In their study on Ashwagandha in the treatment of alcohol withdrawal, Ruby and Benson observed significant anxiolytic and adaptogenic effects. The herb modulated cortisol levels and improved behavioural responses in subjects undergoing Withdrawal. Their findings support the inclusion of herbal adaptogens in withdrawal management protocols, offering a non-sedative alternative to benzodiazepines.

2. Jürgen Rehm et al. (2015)

Their epidemiological analysis demonstrated that alcohol use disorder (AUD) contributes disproportionately to the global disease burden. They emphasized the lack of accessible, effective treatment strategies. Particularly in low- and middle-income countries, this highlighted the need to integrate pharmacological and psychosocial interventions to support long-term recovery.

3. Lorenzo Leggio et al. (2008)

These researchers emphasized the neurochemical dysregulation in AUD, particularly involving dopamine, glutamate, and opioid systems. They argued that targeted pharmacotherapy, rather than broad-acting CNS depressants, offers a better approach for preventing relapse. Their work laid the groundwork for glutamate modulators, such as acamprosate and lamotrigine.

4. Deborah A. Finn et al. (1997)

Their comprehensive review of alcohol withdrawal mechanisms discussed the kindling effect, where repeated withdrawals heighten the severity of symptoms. They developed rodent models demonstrating increased seizure susceptibility, laying the foundation for current pharmacological studies on withdrawal prevention.

5. Richard Saitz et al. (1998)

Saitz distinguished between acute withdrawal symptoms and post-acute withdrawal syndrome (PAWS), noting the persistence of anxiety, insomnia, and

depression for months after cessation. He advocated for long-term pharmacological and behavioral therapies that address both the acute and chronic phases of the condition.

6. Howard C. Becker et al. (1998)

Becker's work on kindling in alcohol withdrawal confirmed that neurotoxicity and excitotoxicity worsen with repeated episodes. His models revealed long-lasting neuroadaptive changes in glutamate and GABA signaling, reinforcing the importance of neuroprotective agents during detox.

7. David M. Lovinger et al. (1997)

Lovinger provided critical insights into serotonin's role in Alcohol's neurobehavioral effects. He highlighted serotonin's involvement in mood, impulsivity, and reward mechanisms, making it a target for therapeutic interventions aimed at reducing craving and relapse.

8. Valentina Vengeliene et al. (2007)

Their experiments with lamotrigine, a glutamate release inhibitor, showed reduced Alcohol seeking in rodent models. Their findings added strong support to the hypothesis that glutamatergic hyperactivity contributes to relapse, validating the use of glutamate modulators in withdrawal treatment.

9. Massimo Ubaldi et al. (2015)

This study investigated oxidative stress markers in alcohol-exposed brains. Their study concluded that oxidative damage to neurons plays a significant role in withdrawal severity. They proposed that antioxidant therapy could mitigate neurotoxicity and enhance recovery.

10. Vasilis Bozikas et al. (2002)

These researchers explored how chronic alcohol use alters prefrontal cortex function, which governs impulse control and decision-making. Their study suggested long-term alterations in executive functions that contribute to craving, relapse, and risky behaviors.

11. Katherine L. March et al. (2019)

This study examined the role of biomarkers, including brain-derived neurotrophic factor (BDNF) and pro-inflammatory cytokines, in the context of alcohol use disorder. They proposed that such markers could predict withdrawal severity and treatment response, offering a route to personalized medicine.

12. Edward G. Singleton et al. (1998)

Singleton and Gorelick investigated the multi-neurotransmitter model of craving, combining data on dopamine, serotonin, glutamate, and GABA pathways. They argued that alcohol craving is a distinct, measurable phenomenon and should be treated as a separate target.

13. Soheila Javidi et al. (2016)

Their work focused on the neurotherapeutic potential of Nigella sativa and its active compound thymoquinone. In rodent models of stress and neurodegeneration, Thymoquinone was shown to reduce oxidative damage and anxiety, making it a promising candidate for alcohol withdrawal management.

14. Farimah Beheshti et al. (2016)

This study elaborated on the capacity of Thymoquinone to suppress pro-inflammatory markers, such as TNF-α and IL-6. The neuroprotective effect extended to hippocampal neurons, supporting its use in conditions involving CNS excitability, such as alcohol withdrawal seizures.

15. Gilhotra et al. (2011)

These researchers provided direct evidence of GABAergic modulation by Thymoquinone in mice. Their anxiolytic test results (Elevated Plus Maze, Light-Dark Box) revealed that thymoquinone acts on GABA and nitric oxide pathways, resembling the action of conventional anxiolytics.

16. Vyawahare et al. (2007)

Their pharmacognostic survey on herbal anticonvulsants reviewed several natural agents, including Nigella sativa, Boerhaavia diffusa, and Bacopa monnieri. They recommended further research into polyherbal formulations for epilepsy and alcohol-related seizures.

17. Amir K. et al. (2012)

Their study on rats exposed to hyperlipidemia and oxidative stress showed that black caraway oil (Nigella sativa) significantly reduced lipid peroxidation and free radical activity. These antioxidant actions may counteract the oxidative cascade triggered by alcohol withdrawal.

18. Ahmed MM et al. (2010)

This research team evaluated pesticide-induced brain damage and found that Nigella sativa oil reversed neuronal oxidative stress, restoring normal levels of antioxidant enzymes. Their findings suggest that similar protective mechanisms may operate during alcohol-induced neurotoxicity.

19. Salman SA et al. (2014)

Studied Nigella sativa extract in the context of industrial toxicant exposure. Results showed restoration of antioxidant defenses and attenuation of neurobehavioral deficits. This supports its application in stress-related CNS conditions, including withdrawal states.

20. Sharrif MM (2011)

In a review of traditional uses of black seed (Nigella sativa), Sharrif documented centuries of its use for neurological disorders, including insomnia, convulsions, and anxiety. Modern science, he argued, is now validating these ancient observations with pharmacological evidence.

21 Chaudhary et al. (2014)

The study conducted by Scientists focused on Thymoquinone's potential to regulate the GABAergic System during alcohol withdrawal. Their study found

that Thymoquinone enhanced GABA receptor function, contributing to its ability to reduce withdrawal symptoms such as tremors, seizures, and anxiety. This suggests that Thymoquinone could be an effective treatment for managing the neurological symptoms of alcohol withdrawal syndrome by modulating GABAergic signalling.

22. Kumar et al. (2015)

Kumar and colleagues reviewed the potential of divalproex as an adjunctive treatment for alcohol withdrawal syndrome. Their animal studies demonstrated that divalproex, when used in combination with other treatments, significantly reduced withdrawal symptoms such as seizures and anxiety. The study suggests that divalproex may serve as an important adjunctive therapy in managing alcohol withdrawal and improving recovery outcomes.

23. Zhang et al. (2016)

Zhang and colleagues explored the cardioprotective effects of Thymoquinone in rats undergoing alcohol withdrawal. The study revealed that Thymoquinone significantly alleviated alcohol-induced cardiac damage, improving heart function and reducing markers of cardiovascular injury. These findings suggest that Thymoquinone may help prevent alcohol-induced cardiac complications during Withdrawal, highlighting its potential as a cardiovascular protectant.

24. Javed et al. (2017)

Javed et al. studied the neurochemical and behavioural effects of Thymoquinone in alcohol-induced anxiety models. Their research demonstrated that Thymoquinone reduced anxiety-like behaviour and restored neurotransmitter balance, leading to improved overall well-being in alcohol-withdrawing animals. These findings indicate that Thymoquinone may help alleviate both the neurochemical imbalances and behavioural disturbances that are commonly observed during alcohol withdrawal.

25. Ziaei et al. (2018)

Ziaei and colleagues examined the role of divalproex in preventing alcohol withdrawal seizures in rats. Their study found that divalproex was effective in reducing the incidence and severity of seizures during alcohol withdrawal, highlighting its potential therapeutic value. By modulating neuronal

excitability and stabilising neurotransmitter systems, divalproex shows promise as a treatment for seizure-related complications in alcohol withdrawal.

26. Hussain et al. (2015)

Hussain and colleagues explored the role of Thymoquinone in regulating the dopaminergic System in alcohol-withdrawing rats. Their study found that Thymoquinone significantly modulated dopamine levels, which helped alleviate the neuropsychiatric symptoms of alcohol withdrawal. Symptoms such as mood shifts and difficulties with cognitive function were observed. The results indicate that Thymoquinone could potentially function as a neuromodulator, offering therapeutic advantages in addressing the mental and emotional challenges associated with alcohol withdrawal syndrome.

Research Gap

Scholarly investigations have previously elucidated that the fundamental mechanism of alcohol withdrawal delirium tremens entails a substantial disturbance in the equilibrium of neurochemicals, including GABA, glutamate, serotonin, and dopamine. This imbalance is pivotal to the emergence of withdrawal symptoms. A potential method to reduce these effects is the utilisation of competitive agonists that target alcohol-binding areas in the brain, which may aid in restoring equilibrium and alleviating withdrawal symptoms. Furthermore, these approaches may effectively diminish alcohol cravings, a severe and potentially fatal illness. At present, Disulfiram is the sole widely acknowledged medicinal intervention for the management of alcohol cravings. This creates a void in pursuing alternative and possibly more efficacious therapies.

By rectifying the neurochemical imbalance associated with alcohol cravings, we may discover novel methods for addressing this problem. This may be accomplished by creating innovative medicines that focus on the identical neural pathways associated with alcohol craving. Hypothetically, as well as Diazepam, we might explore developing pharmacological medicines, such as Nigella sativa, lemon essential oil, and Divalproex, as prospective remedies. Moreover, specific potent 5-HT agonists, including triptans and lasmiditan, may also demonstrate efficacy. Numerous phytochemical substances have demonstrated potential in regulating neurochemical function. The list comprises Ashwagandha, Kava, Magnolia bark, Valerian, black seed oil, Lavender, Jasmine, and green tea. These natural compounds have shown promise in rectifying neurochemical imbalances, positioning them as viable options for mitigating alcohol cravings and facilitating withdrawal treatment. Therefore, examining these organic and artificial options might support the creation of more powerful therapies for addressing alcohol addiction and impulses.

Aim and Objectives

Aim: To evaluate the efficacy of Thymoquinone and Divalproex, alone and in combination, against Diazepam, in reducing alcohol craving and alleviating ethanol withdrawal symptoms in an animal model of alcohol dependence.

- To evaluate the effects of Thymoquinone and Divalproex on alcohol craving.
- To evaluate the effects of combining Thymoquinone and Divalproex with Diazepam in combating alcohol craving.

Animal models for evaluating alcohol Dependency

There are several approaches to creating physical alcohol dependence in animals, which help researchers evaluate the symptoms linked to alcohol addiction. One standard method involves providing a nutritionally complete liquid diet infused with Alcohol. Another approach uses alcohol vapours for inhalation, while a third method administers Alcohol through repeated injections or by inserting a tube into the animal's oesophagus for direct consumption. Each technique has its own set of benefits and limitations. However, they all aim to establish a steady level of Alcohol in the bloodstream, which is crucial for accurately assessing alcohol exposure. (Sharma et al., 2020)

The signs of alcohol dependence in animals typically manifest as motor dysfunction, tremors, and excessive neuronal activity, often referred to as hyper-synchronous firing. Among the various symptoms, seizures are the most frequently studied. The occurrence of seizures can vary in severity depending on the intensity of alcohol withdrawal and may either happen spontaneously or be triggered by physical handling of the animal. (Smith, 1977)

Plan of research work

Literature Survey

↓

Selection of Research Topic

↓

IAEC/CPSCEA approval

↓

Aim and Objectives

↓

Dosage Determination

Thymoquinone safe dose range

Divalproex therapeutic levels

↓

Behavioural Assessments

Elevated Plus Maze

Open Field Test

Elevated Zero Maze

↓

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Evaluation of Ethanol Withdrawal

Physical symptom scoring

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Pharmacological Evaluation

Thymoquinone properties.

Divalproex effects

↓

Collection Data

↓

Data Analysis

Statistical analysis using the SysStat software

↓

Result Writing

↓

Thesis Writing

↓

Submitting the Thesis to the University and the Supervisor

Methodology

Animal Grouping

A total of ten groups of mice were used in this study, with six animals in each group. The grouping consisted of a Negative Control, Alcohol Control, and treatment groups receiving Thymoquinone (THMQ), Divalproex (DVPX), Diazepam, and their respective combinations.

Alcohol Dependence Model

Alcohol dependence was induced using an intermittent escalating ethanol model adapted from (Xiao et al. 2018).

Mice received

- 5% ethanol in drinking water on days 1–5
- 10% ethanol on days 8–12
- 20% ethanol on days 15–19
- 35% ethanol on days 22–26

During each alcohol exposure phase, animals were also administered **0.2 mL** of the same ethanol concentration by gavage.

Intermittent **ethanol-free days** (days 6–7, 13–14, 20–21, 27–28) were introduced to induce craving and mimic repeated withdrawal cycles.

Drug Treatment

During ethanol-free withdrawal periods, the following treatments were administered once daily:

- Thymoquinone (THMQ): 20 mg/kg
- Divalproex sodium (DVPX): 30 mg/kg
- Diazepam: 1 mg/kg

Doses were selected based on established pharmacological studies indicating anxiolytic and neuroprotective effects.

Behavioral Assessments

Behavioral tests were conducted on days 7, 14, 21, and 28 to evaluate anxiety-like behavior, locomotion, and alcohol-seeking patterns.

Elevated Plus Maze (EPM)

The EPM apparatus consisted of two open and two closed arms elevated above the floor. Decreased time spent in open arms is indicative of anxiety (Carola et al., 2002).

Open Field Test (OFT)

The OFT assessed locomotor activity and exploratory behavior. Measurements included total distance travelled and time spent in center versus periphery, reflecting anxiety and hyperactivity (Shieh & Yang, 2020).

Elevated Zero Maze (EZM)

The EZM contained two open and two closed sections arranged in a circular elevated platform. Time spent in closed versus open areas was recorded during a 5-minute session to assess anxiety (Deal et al., 2021).

Physical Signs of Withdrawal

Withdrawal symptoms were assessed 12 hours after the final ethanol exposure by an observer blinded to treatment groups.

Symptoms such as:

Tremors

Tail stiffness

Touch sensitivity (vocalization)

Ventro-medial limb retraction

were scored from 0–2 per category, producing a total withdrawal severity score of 0–8 (Bleich & Degner, 2000; Jakaria et al., 2018).

Western Blot Analysis

Hippocampal tissue (dorsal and ventral regions) was isolated for molecular evaluation. Tissue homogenates were prepared in buffer and processed using SDS-PAGE (4–20% gradient gel). Proteins were transferred to nitrocellulose membranes and probed with: Rabbit anti-SK2, antibody, Mouse anti-actin antibody, Fluorescent secondary antibodies.

Visualization was performed using the **LICOR Odyssey CLx** imaging system. Band intensity was quantified via **ImageJ** software (Xue et al., 2021; Arafa et al., 2011; Deal et al., 2021).

Statistical Analysis

All values were expressed as **mean ± SD** (n = 6). Statistical analysis was performed using **one-way ANOVA,** followed by **Tukey's post-hoc test.** Levels of significance were indicated using the symbol legend described in the Results section.

Group No.	Group Name	Alcohol/vehicle administration	Drug/vehicle administration	No. of Swiss albino mice (20-30 g)
1	Vehicle control	The mice received their treatment from day 1 through day 5, which included their regular drinking water and 0.2 mL of water administered intragastrically (i.g.) each day. After day 5, they received only their standard drinking water in the water bottles until day 28.	0.5% CMC solution 5 ml/kg p.o. on days 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

2	Standard *per se*	The mice received their treatment from day 1 through day 5, which included their regular drinking water and 0.2 ml of water administered i.g., each day. After day 5, they received only their standard drinking water in the water bottles until day 28.	Diazepam 1 mg/kg i.p. [45] on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6
3	Test drug 1 *per se*	The mice received their treatment from day 1 through day 5, which	Thymoquinone 40 mg/kg p.o. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		included their regular drinking water and 0.2 mL of water administered intragastrically (i.g.) each day. After day 5, they received only their standard drinking water in the water bottles until day 28.		
4	Test drug 2 *per se*	The mice received their treatment from day 1 through day 5, which included their regular drinking water and	Divalproex 60 mg/kg p.o. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28[44]	6

		0.2 mL of water administered intragastrically (i.g.) each day. After day 5, they received only their standard drinking water in the water bottles until day 28.		
5	Negative Control	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the	0.5% CMC solution 5 ml/kg p.o. on days 6 and 7; 13 and 14; 20 and 21; 27 and 28[44]	6

		ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentrati on in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.[44]		
6	Positive Control	During the initial five	Diazepa m 1 mg/kg	6

		days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentrati on in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On	i.p. [45] on day 6 and 7; 13 and 14; 20 and 21; 27 and 28[44]	

		the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.		
7	Alcohol withdrawal + Test drug, one low dose	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was	Thymoquinone 20 mg/kg p.o. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		elevated to 10%. From days 15 to 19, the ethanol concentration in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.[44]		
8	Alcohol withdrawal + Test drug, one high dose	During the initial five days, the animals received a 5% ethanol	Thymoquinone 40 mg/kg p.o. on day 6 and 7; 13 and 14; 20	6

		solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentrati on in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely	and 21; 27 and 28	

		days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.		
9	Alcohol withdrawal Test drug 1+ Std drug	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the	Thymoquinone at 20 mg/kg was administered orally, and Diazepam at 1 mg/kg was administered intraperitoneally on days 6 and 7, 13 and 14, 20 and 21, and 27 and 28.	6

		ethanol concentration in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.		
10	Alcohol withdrawal + Test drug two low doses	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily	Divalproex 30 mg/kg p.o. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentrati on in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the		

		animals received standard water devoid of ethanol.		
11	Alcohol withdrawal + Test drug two high doses	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentration in the water bottle	Divalproex 60 mg/kg p.o. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.		
12	Alcohol withdrawal Test drug 2 + Std drug	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol	Divalproex 30 mg/kg p.o. + Diazepam 1 mg/kg i.p. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentrati on in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water		

		devoid of ethanol.		
13	Alcohol withdrawal Test drug 1 Test drug 2	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution. Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentration in the water bottle was	Thymoquinone 20 mg/kg p.o. + Divalproex 30 mg/kg p.o. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of ethanol.		
14	Alcohol withdrawal Test drug 1 +Test drug 2 + Std drug	During the initial five days, the animals received a 5% ethanol solution in their water bottles and a daily gavage of 0.2 mL of the same 5% ethanol solution.	Thymoquinone 20 mg/kg p.o. + Divalproex 30 mg/kg p.o. + Diazepam 1 mg/kg i.p. on day 6 and 7; 13 and 14; 20 and 21; 27 and 28	6

		Between days 8 and 12, the ethanol percentage in the water bottle was elevated to 10%. From days 15 to 19, the ethanol concentrati on in the water bottle was elevated to 20%, and from days 22 to 26, it was further increased to 35%. On the intervening days, namely days 6, 7, 13, 14, 20, 21, 27, and 28, the animals received standard water devoid of		

		ethanol.		
				84

Mice will be divided into the following groups

Animals

The study will involve adult male Swiss Albino mice, each weighing between 25 and 40 grams. These mice will be kept in a regulated environment where the temperature remains constant, and the light/dark cycle follows a 12-hour pattern (with light from 7:00 AM to 7:00 PM). Both ethanol exposure and all behavioral tests related to alcohol withdrawal will take place in the same controlled setting to ensure consistency in the experimental conditions.(Aron-Wisnewsky & Clément, 2016)

Pharmacology

Mechanism of Action of Thymoquinone

Thymoquinone influences NMDA receptors. Thymoquinone acts as a positive modulator of GABA within the GABAergic System and elevates serotonin levels. The aqueous and methanolic extracts of defatted *Nigella sativa* seeds exhibited notable depressant effects on the central nervous System, with the methanolic extract showing particularly strong activity. This extract functions as an anxiolytic medication by elevating 5-HT levels and reducing 5-HIAA levels in the brain. Prolonged treatment of Nigella sativa elevates 5-HT levels in the brain. Therefore, by stimulating the neurochemicals, we can achieve a reduction in alcohol cravings. (Mansour et al., 2002)

Mechanism of Action of Divalproex

Divalproex enhances the release of the inhibitory neurotransmitter GABA by preventing its breakdown via GABA-transaminase and promoting its synthesis from glutamic acid. Therefore, an increase in GABA levels in the brain facilitates the suppression of alcohol cravings. (Amato et al., 2011)

Side Effects of Diazepam

Drowsiness, dry mouth, tiredness, headache, constipation, and Muscle weakness.

Benefits of Thymoquinone over Diazepam

There are fewer side effects compared to Diazepam. It is a more potent, naturally obtained substance with multiple health benefits. Benefits of Divalproex over Diazepam

Inhibiting GABA degradation. Increase GABA synthesis. More

potent in action, Economical compared to Diazepam.

Selection of dose

The dosage of test medicines was determined based on prior literature and acute toxicity evaluation data concerning their pharmacological activity.

Animals models

- Chronic intermittent alcohol procedure

Animals can develop physical dependence on Alcohol through several methods, one of which includes incorporating it into their regular diet. To begin, we will use gavage (a method of oral administration) and ensure the bottle is securely locked to prevent free access. This approach allows us to directly observe the physical signs of alcohol dependence as they emerge. (Bansal & Banerjee, 2016)(Ruby et al., 2012)(Xiao et al., 2018)

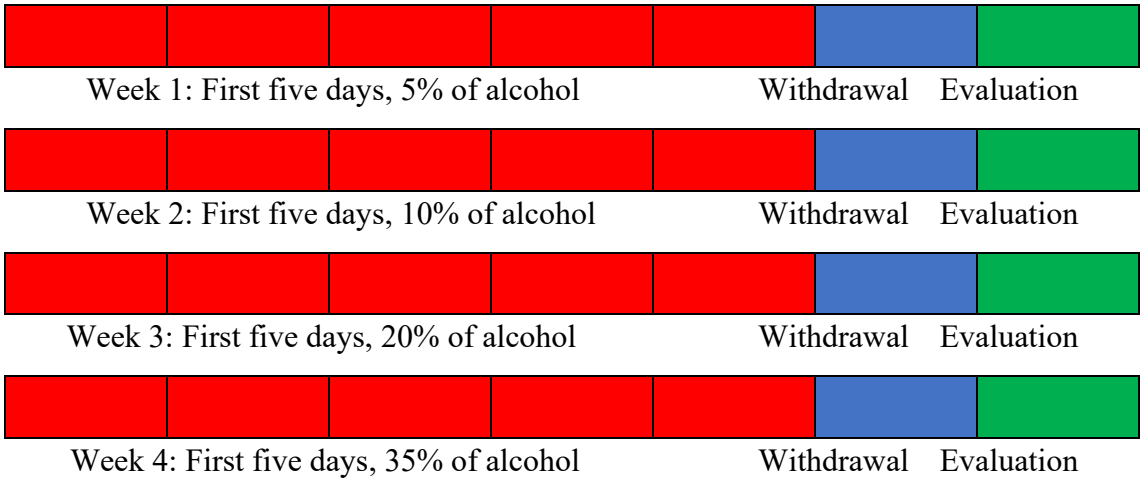

Figure 01: Alcohol Intake Procedure

Animal

Evaluating parameters

For Rodents

- Open field test (OFT)
- Elevated zero maze (EZM)
- Elevated plus maze (EPM)
- Western blot analysis

Result

Alcohol Drinking Data

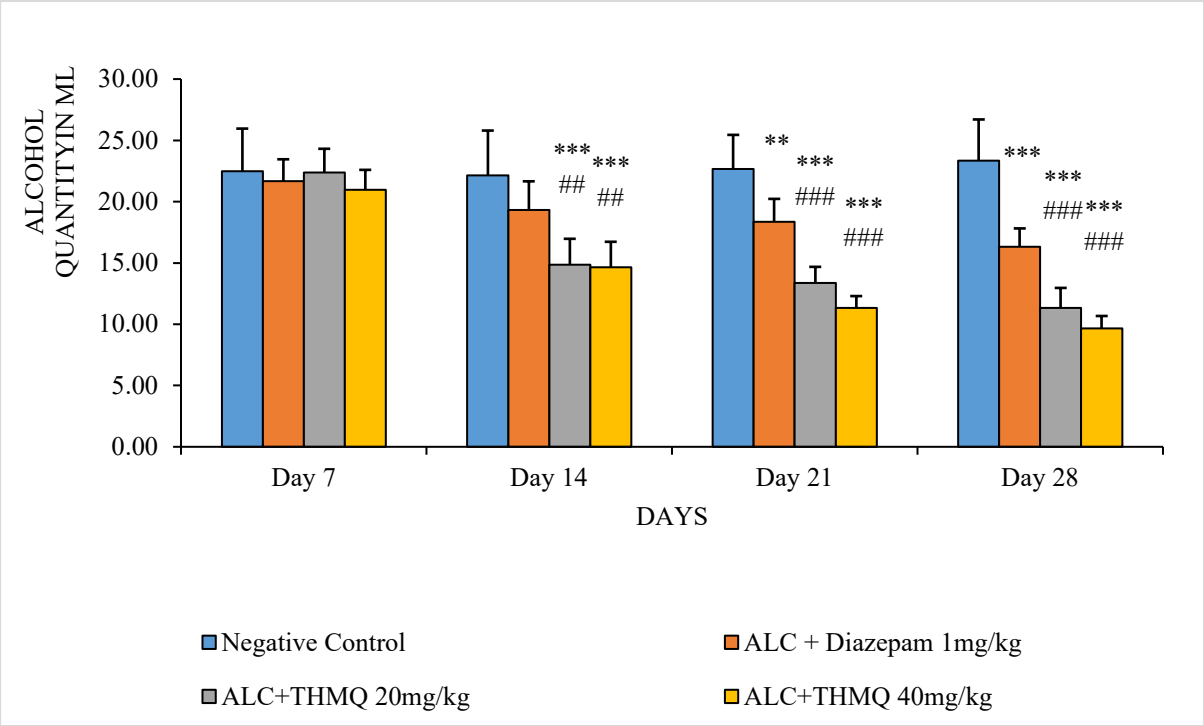

Figure 02 Alcohol Drinking Data Animals Treated by THMQ

Data are expressed as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance compared with the Negative Control is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001. Differences compared with the ALC + Diazepam (1 mg/kg) group are denoted by #p < 0.05, ##p < 0.01, and ###p < 0.001. Comparisons between THMQ (20 mg/kg) and DVPX (30 mg/kg) are represented by $p < 0.05, $$p < 0.01, and $$$p < 0.001. The symbol ^ indicates significant differences for the combination ALC + DVPX (30 mg/kg) + Diazepam (1 mg/kg) when compared with the corresponding single-drug treatments. The symbol ! denotes the difference between ALC + THMQ (20 mg/kg) and the combined treatment ALC + THMQ (20 mg/kg) + DVPX (30 mg/kg). The symbol % indicates significant variation between the combination ALC + THMQ (20 mg/kg) + DVPX (30 mg/kg) and the triple-therapy group receiving ALC + THMQ (20 mg/kg) + DVPX (30 mg/kg) + Diazepam (1 mg/kg).

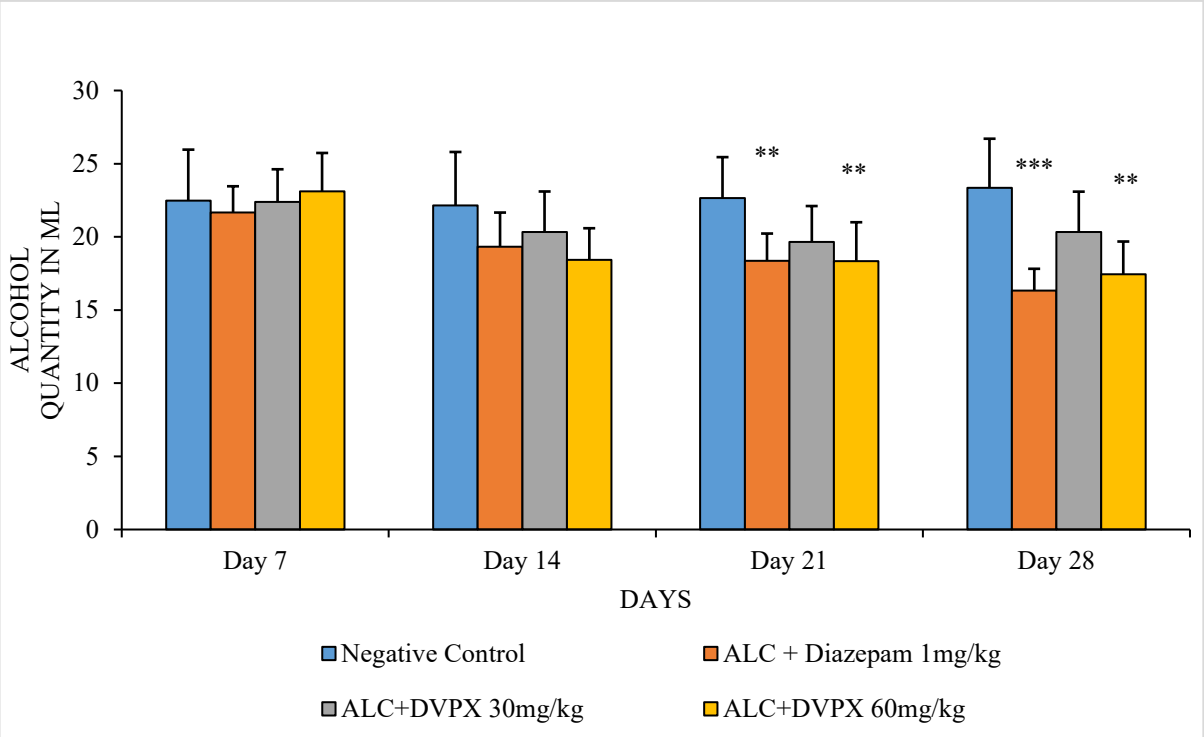

Figure 03 Alcohol Drinking Data Animals Treated by DVPX

Data are expressed as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance compared with the Negative Control is indicated by **p < 0.01 and ***p < 0.001, as shown on Days 21 and 28. No other comparison markers were observed in this figure.

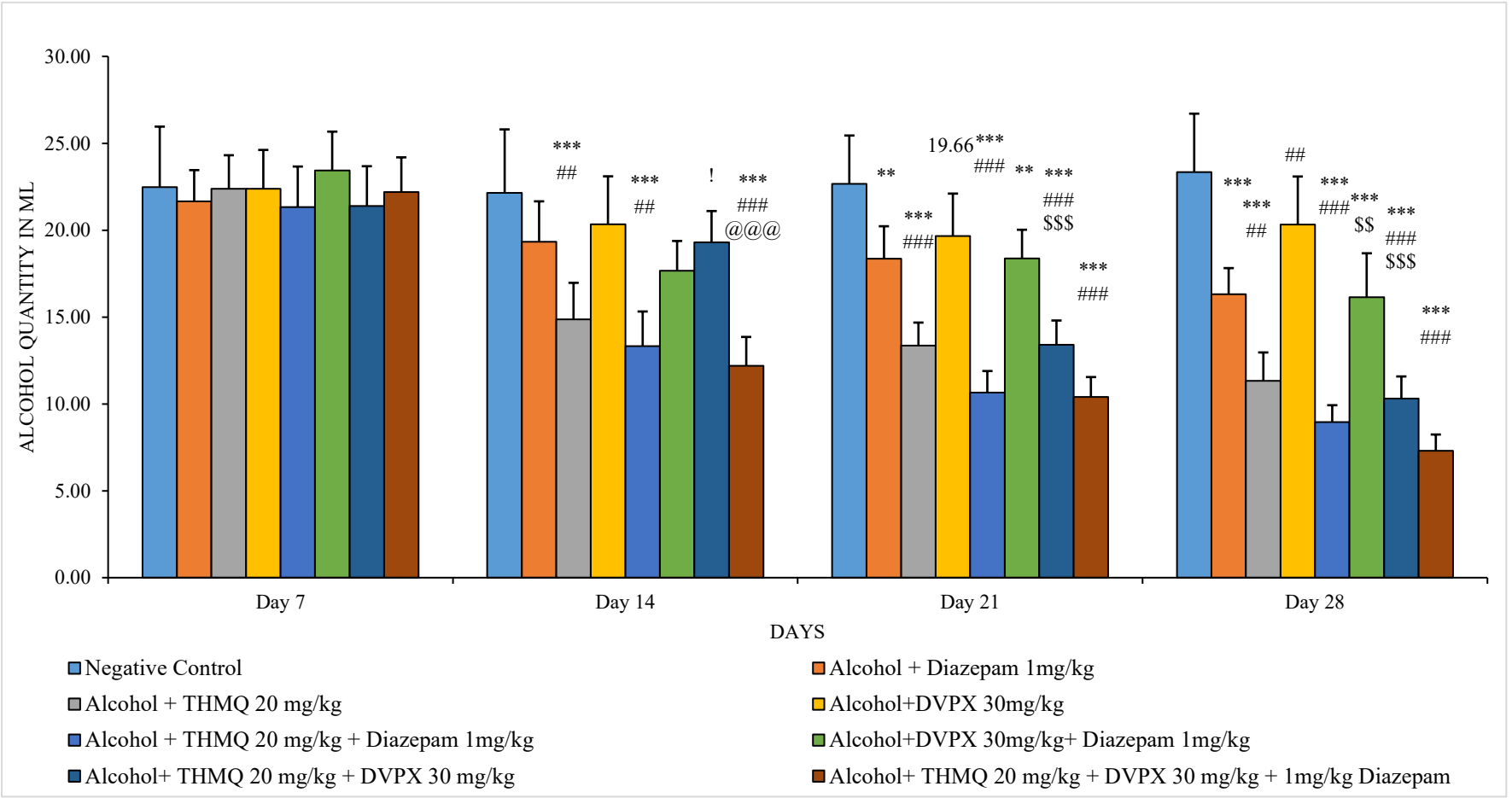

Figure 04 Alcohol Drinking Data Animals Treated by THMQ, DVPX vs Diazepam

Data are expressed as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance compared with the Negative Control is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001. Differences compared with the ALC + Diazepam (1 mg/kg) group are denoted by #p < 0.05, ##p < 0.01, and ###p < 0.001. Comparisons between THMQ (20 mg/kg) and DVPX (30 mg/kg) are represented by $p < 0.05, $$p < 0.01, and $$$p < 0.001. The symbol ! indicates a significant difference between THMQ (20 mg/kg) alone and the combined treatment THMQ (20 mg/kg) + DVPX (30 mg/kg). The symbol @@@ denotes significant differences between the combined THMQ (20 mg/kg) + DVPX (30 mg/kg) therapy and the triple-therapy group receiving THMQ (20 mg/kg) + DVPX (30 mg/kg) + Diazepam (1 mg/kg). All symbols correspond to the inter-group comparisons indicated in the statistical evaluation.

The daily alcohol-intake data (Figures 02–04) show distinct treatment-related differences across the 28-day experimental period. In Figure 02, THMQ-treated groups displayed progressive reductions in alcohol consumption compared with the Negative Control, with significant effects appearing on Days 14, 21, and 28. On Day 14, both THMQ 20 mg/kg and THMQ 40 mg/kg produced significant decreases relative to the Negative Control (**p < 0.01, ***p < 0.001). By Day 21, reductions in alcohol intake were more pronounced for both THMQ doses (***p < 0.001), and significant differences were also evident when compared with ALC + Diazepam, as indicated by # and ##. On Day 28, both THMQ doses and ALC + Diazepam showed strong reductions in intake (***p < 0.001), with additional differences between THMQ and Diazepam groups reflected by *, **, and ***.

Figure 03 illustrates the effects of DVPX treatment. No meaningful differences were seen on Days 7 or 14; however, by Day 21, significant reductions emerged in the ALC + Diazepam and ALC + DVPX 60 mg/kg groups compared with the Negative Control (**p < 0.01). On Day 28, both DVPX doses and Diazepam produced marked reductions in alcohol intake, with significance denoted by ** and ***. Comparisons between the two DVPX doses also showed differences, represented by ## and ###, while no major changes were noted between the DVPX-treated groups and THMQ 20 mg/kg groups on this figure.

Figure 04 shows the complete comparison including THMQ, DVPX, their combinations, and the triple-therapy group. On Day 14, the combinations involving THMQ and DVPX produced highly significant reductions compared with the Negative Control (***p < 0.001). Additional reductions relative to ALC + Diazepam were indicated by ## and ###. Differences between THMQ alone and THMQ + DVPX were represented by !, while the difference between THMQ + DVPX and the triple-therapy group (THMQ + DVPX + Diazepam) was strongly significant (***p < 0.001, @@@). On Day 21, nearly all treatment groups demonstrated highly significant reductions (***p < 0.001) compared with the Negative Control, with further reductions relative to Diazepam shown by ## and ###. Significant distinctions between THMQ and DVPX were denoted by , and differences involving the triple-therapy group remained consistent. By Day 28, most treatment groups again showed strong reductions versus the Negative Control (***p < 0.001), with further differences versus Diazepam indicated by ## and ###. Comparisons between THMQ and DVPX groups were represented by $$ and , while differences between combination therapies remained significant.

Overall, the alcohol-intake data across all figures indicate that THMQ, DVPX, and their combination therapies substantially reduce alcohol consumption during repeated withdrawal cycles, with combination treatments producing the strongest effects.

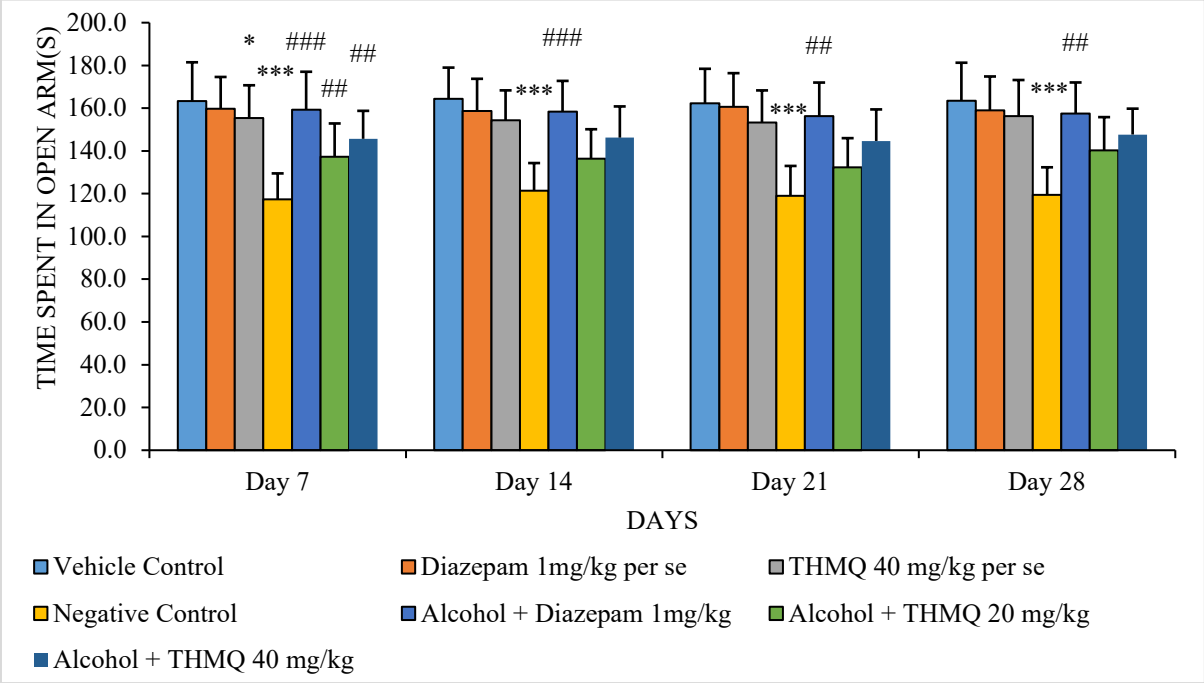

Figure 05 Time Spent in open arms treated by THMQ

Data are presented as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance compared with the Negative Control is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001. Differences compared with the ALC + Diazepam (1 mg/kg) group are denoted by #p < 0.05, ##p < 0.01, and ###p < 0.001. These symbols represent the comparisons made between the Negative Control and the treatment groups (ALC + Diazepam, ALC + THMQ 20 mg/kg, and ALC + THMQ 40 mg/kg), as well as comparisons between ALC + Diazepam and the two THMQ-treated groups.

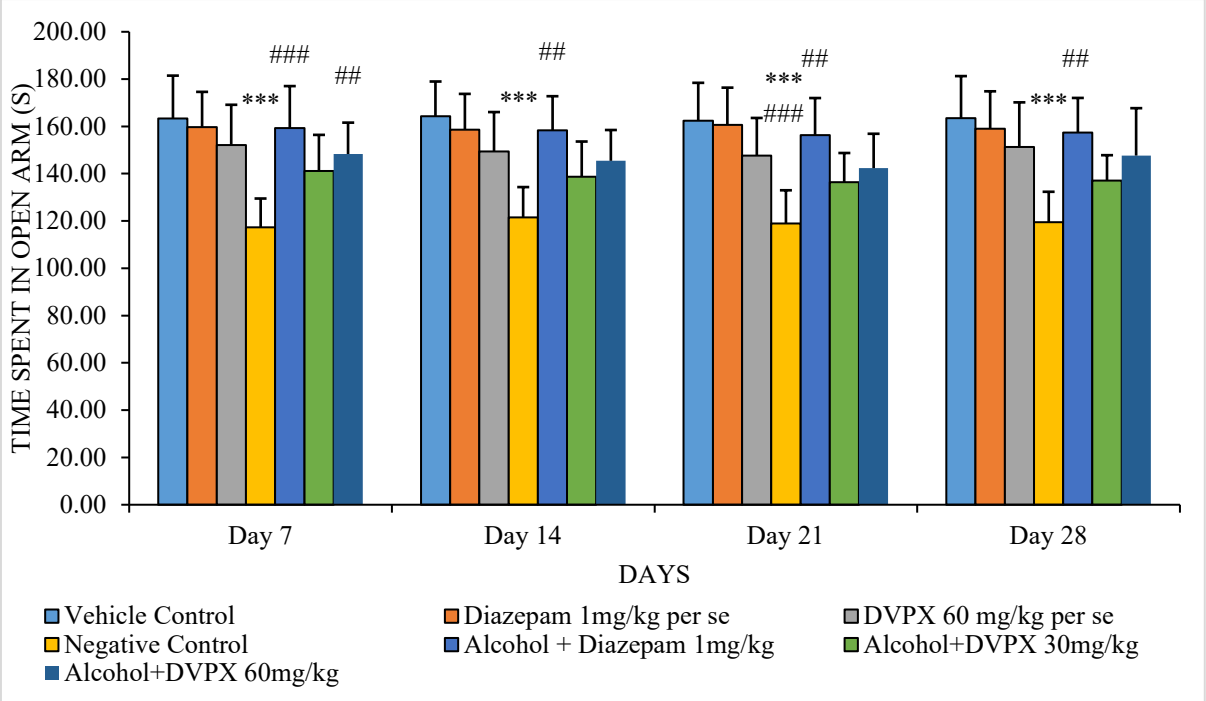

Figure 06 Time Spent in open arms treated by DVPX

Data are presented as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance compared with the Negative Control is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001. Differences compared with the ALC + Diazepam (1 mg/kg) group are represented by #p < 0.05, ##p < 0.01, and ###p < 0.001. These symbols correspond to the comparisons between the Negative Control and the treatment groups (ALC + Diazepam, ALC + DVPX 30 mg/kg, and ALC + DVPX 60 mg/kg), as well as comparisons between ALC + Diazepam and the DVPX-treated groups.

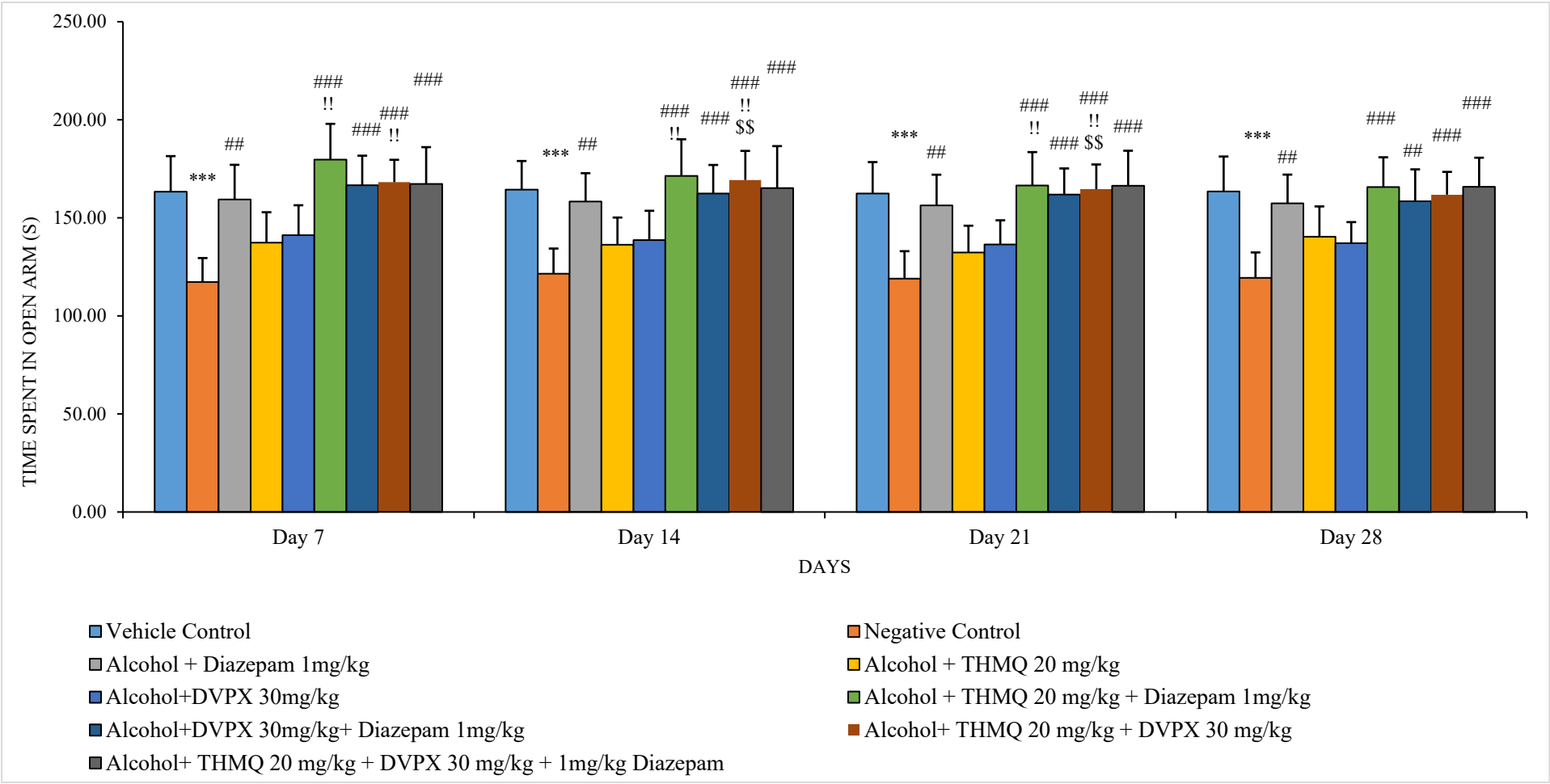

Figure 07 Time Spent in open arms treated by THMQ, DVPX vs Diazepam

Data are expressed as mean ± SD (n = 6) and were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance relative to the Negative Control is indicated by #, ##, and ### corresponding to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Differences compared with the Alcohol + Diazepam 1 mg/kg group are represented by ^, ^^, and ^^^ at the same significance levels. Comparisons involving Alcohol + THMQ 20 mg/kg versus its combination groups (Alcohol + THMQ 20 mg/kg + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are denoted by !, !!, and !!!. Differences between Alcohol + DVPX 30 mg/kg and its combination groups (Alcohol + DVPX 30 mg/kg + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are indicated by $, $$, and $$$. The comparison between Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg + Diazepam 1 mg/kg is shown by @, @@, and @@@. Higher repetitions of each symbol represent stronger levels of statistical significance ($p < 0.05$, $p < 0.01$, $p < 0.001$).

Figure 05 illustrates the effects of Alcohol + THMQ (20 mg/kg and 40 mg/kg) and Alcohol + Diazepam 1 mg/kg on time spent in the open arms across the withdrawal period. Significant differences were observed between the Negative Control and all treatment groups, indicated by *, , and *** ($p < 0.05$, 0.01, 0.001). Comparisons between Alcohol + Diazepam 1 mg/kg and the THMQ treatment groups are denoted by # and ##, reflecting significant improvements in open-arm activity. THMQ at both doses produced consistent anxiolytic effects from Day 7 to Day 28, demonstrating greater time spent in the open arms compared with the Negative Control. The pattern shows that THMQ 40 mg/kg exerted a slightly stronger behavioural effect than THMQ 20 mg/kg. Overall, the figure indicates that THMQ treatment significantly reduces anxiety-like behaviour during alcohol withdrawal, comparable to—but in some observations stronger than Diazepam.

Figure 06 presents the behavioural effects of Alcohol + DVPX (30 mg/kg and 60 mg/kg) and Alcohol + Diazepam 1 mg/kg on time spent in the open arms. Significant differences from the Negative Control are represented by *, , and *** across the four observation days ($p < 0.05$, 0.01, 0.001). DVPX at both doses demonstrated marked anxiolytic activity, with higher significance levels (## and ###) observed particularly for the 60 mg/kg group. Comparisons between Alcohol + Diazepam and DVPX groups are indicated by # and ##, showing that DVPX produced behavioural improvements comparable to or slightly stronger than Diazepam. These findings suggest that DVPX dose-dependently increases open-arm exploration, indicating reduced anxiety during

withdrawal.

Figure 07 summarises the behavioural outcomes of multiple combination therapies involving THMQ, DVPX, and Diazepam. Significant differences between the Negative Control and all Alcohol-treated groups are denoted by #, ##, and ### ($p < 0.05$, 0.01, 0.001). Comparisons between Alcohol + Diazepam 1 mg/kg and all other treatment groups are indicated by ^, demonstrating the relative behavioural strength of each regimen. THMQ 20 mg/kg showed significant improvements when combined with Diazepam or DVPX, marked by ! ($p < 0.05$ or $p < 0.01$), while DVPX 30 mg/kg exhibited notable changes in comparison with its Diazepam and THMQ combinations, represented by $. The strongest distinctions were observed between the THMQ + DVPX combination and the triple therapy (THMQ + DVPX + Diazepam), denoted by @. Overall, combination therapies, particularly THMQ + DVPX and the triple regimen, produced robust anxiolytic effects with higher open-arm activity than single-agent treatments.

Elevated Zero Maze

Time Spent in Open Section

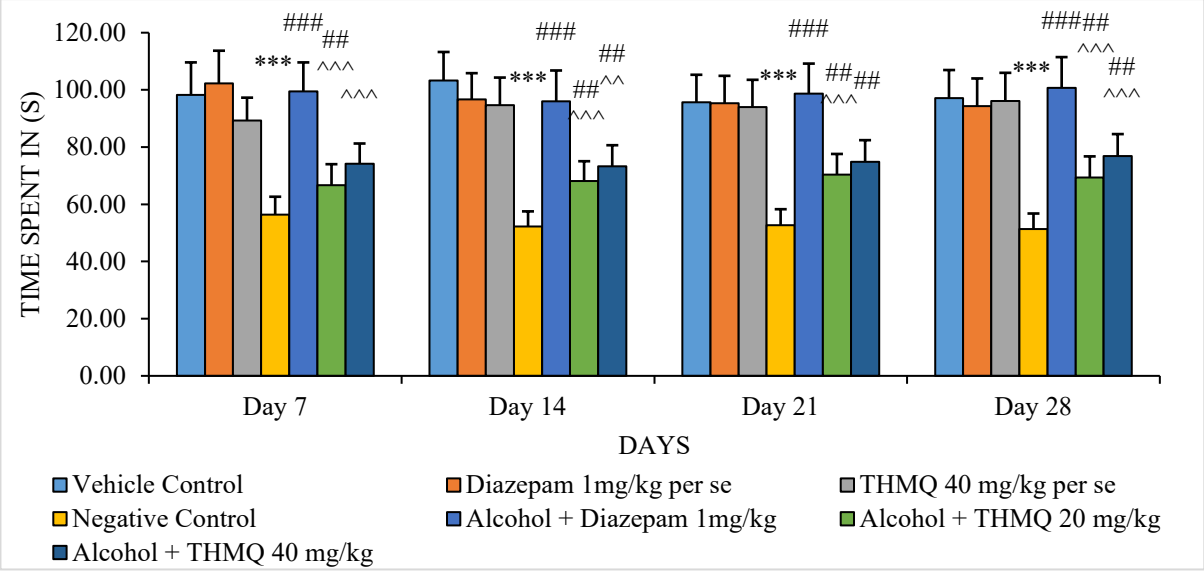

Figure 08 Time Spent in Open area Treated by THMQ

Data are expressed as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance relative to the Negative Control group is indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Differences compared with the Alcohol + Diazepam 1 mg/kg group are represented by #, ##, and ### for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Comparisons between Alcohol + THMQ 20 mg/kg and Alcohol + THMQ 40 mg/kg are denoted by ^, ^^, and ^^^. Overall, THMQ at both doses produced significant improvements in time spent in the open arms across Days 7, 14, 21, and 28, demonstrating a dose-dependent reduction in anxiety-like behaviour during alcohol withdrawal.

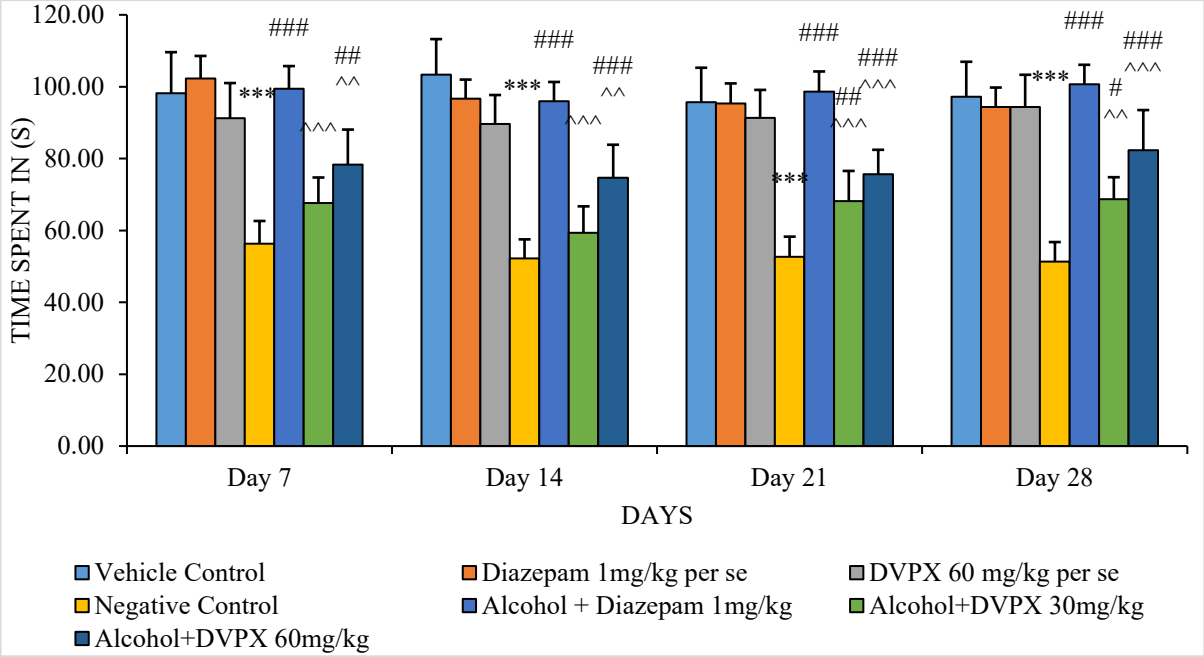

Figure 09 Time Spent in Open area Treated by DVPX

Data are presented as mean ± SD (n = 6) and were analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance relative to the Negative Control group is indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Comparisons with the Alcohol + Diazepam 1 mg/kg group are represented by #, ##, and ### for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Differences between Alcohol + DVPX 30 mg/kg and Alcohol + DVPX 60 mg/kg are denoted by ^, ^^, and ^^^ at corresponding significance levels. Overall, both DVPX doses produced marked improvements in open-arm exploration across all withdrawal days, indicating a dose-dependent anxiolytic effect superior to the Negative Control and comparable to Alcohol + Diazepam treatment.

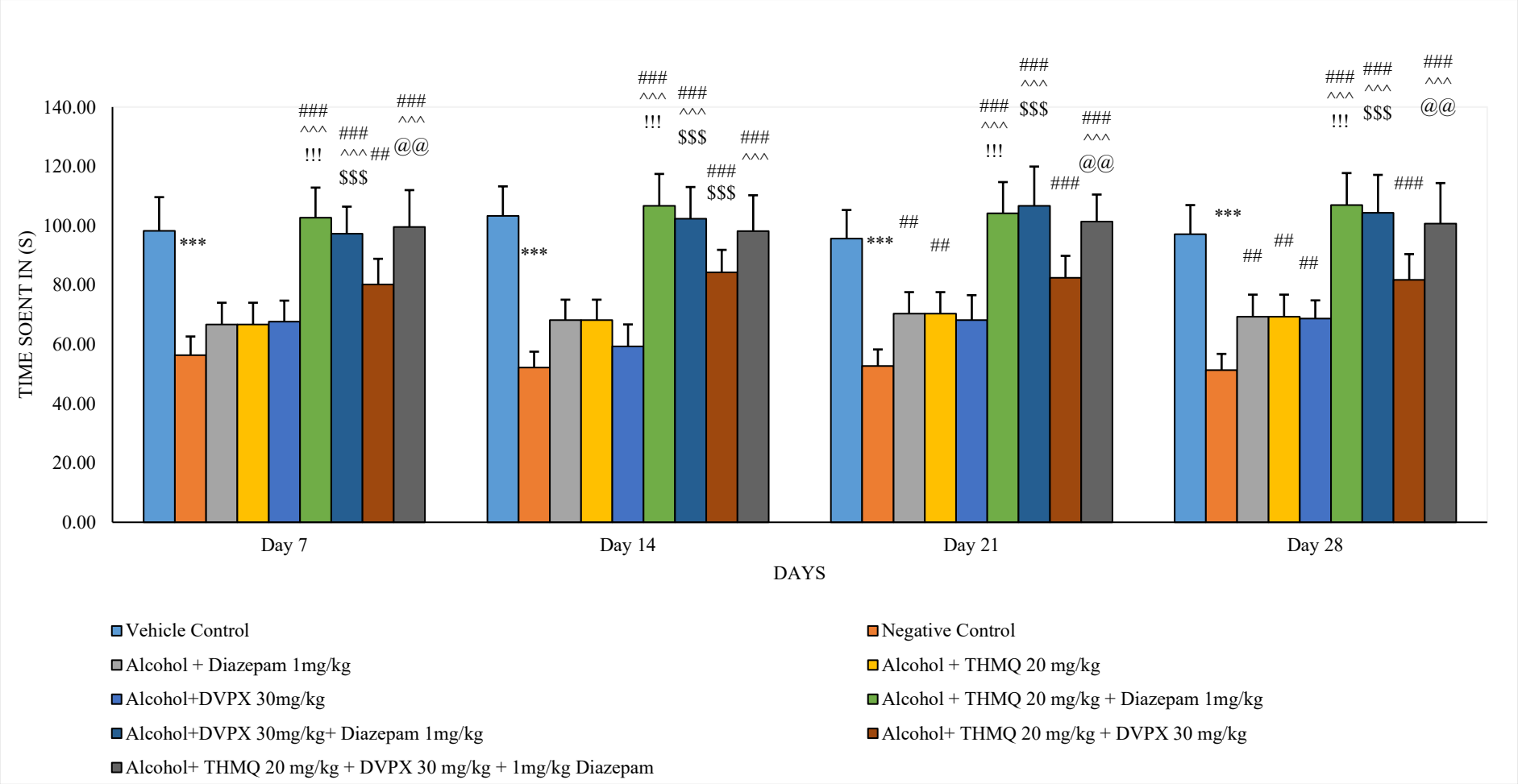

Figure 10 Time Spent in Open area Treated by THMQ, DVPX vs Diazepam

Data are expressed as mean ± SD (n = 6) and were analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance relative to the Negative Control is indicated by #, ##, and ### for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Differences compared with the Alcohol + Diazepam 1 mg/kg group are represented by ^, ^^, and ^^^ at the corresponding significance levels. Comparisons between Alcohol + THMQ 20 mg/kg and its combination groups (Alcohol + THMQ 20 mg/kg + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are denoted by !, !!, and !!!. Differences between Alcohol + DVPX 30 mg/kg and its combination treatments (Alcohol + DVPX 30 mg/kg + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are indicated by $, $$, and $$$. The comparison between the THMQ + DVPX combination group and the triple therapy (THMQ + DVPX + Diazepam) is shown by @ and @@. The overall results highlight strong, dose-dependent anxiolytic effects, with combination therapies producing greater open-arm activity than individual treatments.

Figure 08 shows the effect of Alcohol + THMQ 20 mg/kg and Alcohol + THMQ 40 mg/kg on anxiety-like behaviour during alcohol withdrawal. Across all observation days, the Negative Control group consistently displayed reduced time spent in the open arms, confirming withdrawal-related anxiety. Significant differences relative to the Negative Control are indicated by *, **, and *** ($p < 0.05$, 0.01, and 0.001). Both THMQ doses produced noticeable improvements in open-arm activity, with THMQ 40 mg/kg showing a stronger behavioural response than THMQ 20 mg/kg. Comparisons with the Alcohol + Diazepam group are denoted by #, ##, and ###, indicating that THMQ demonstrated anxiolytic activity comparable to Diazepam. Differences between the two THMQ doses are represented by ^, ^^, and ^^^, reflecting dose-dependent behavioural enhancement. Overall, THMQ treatment significantly alleviated anxiety-like responses associated with alcohol withdrawal in a dose-dependent manner.

Figure 09 illustrates the behavioural effects of Alcohol + DVPX 30 mg/kg and Alcohol + DVPX 60 mg/kg on open-arm exploration. The Negative Control group showed markedly reduced open-arm time, significantly different from Vehicle Control, confirming the presence of withdrawal-induced anxiety. Significant improvements relative to the Negative Control are indicated by *, **, and ***. Comparisons with Alcohol + Diazepam 1 mg/kg are represented by #, ##, and ###, demonstrating that DVPX produced anxiolytic effects comparable to Diazepam. Differences between DVPX 30 mg/kg and DVPX 60 mg/kg are shown by ^, ^^, and ^^^, which reflect a clear dose-dependent increase in open-arm activity. Across all days, DVPX 60 mg/kg

consistently produced the strongest anxiolytic response, suggesting significant behavioural benefits of higher-dose DVPX in mitigating alcohol-withdrawal-related anxiety.

Figure 10 presents the combined behavioural effects of THMQ, DVPX, and Diazepam administered individually or in combination. Significant differences relative to the Negative Control are represented by #, ##, and ###, indicating that all treatment groups showed improved open-arm exploration compared with the untreated withdrawal group. Comparisons with Alcohol + Diazepam 1 mg/kg are marked by ^, ^^, and ^^^, demonstrating varying degrees of behavioural improvement across treatment regimens.

Comparisons between Alcohol + THMQ 20 mg/kg and its combination treatments (with Diazepam or with DVPX) are indicated by !, !!, and !!!, highlighting significant additional benefit when THMQ is combined with either agent. Differences between Alcohol + DVPX 30 mg/kg and its combination groups (with Diazepam or THMQ) are denoted by $, $$, and $$$, showing enhanced anxiolytic effects when DVPX is used in combination. The contrast between the dual combination group (THMQ + DVPX) and the triple therapy (THMQ + DVPX + Diazepam) is expressed by @ and @@, indicating that triple therapy produced the most pronounced behavioural benefit. Overall, combination treatments produced superior anxiolytic effects compared with single-agent therapy, with the triple combination providing the strongest improvement in open-arm activity.

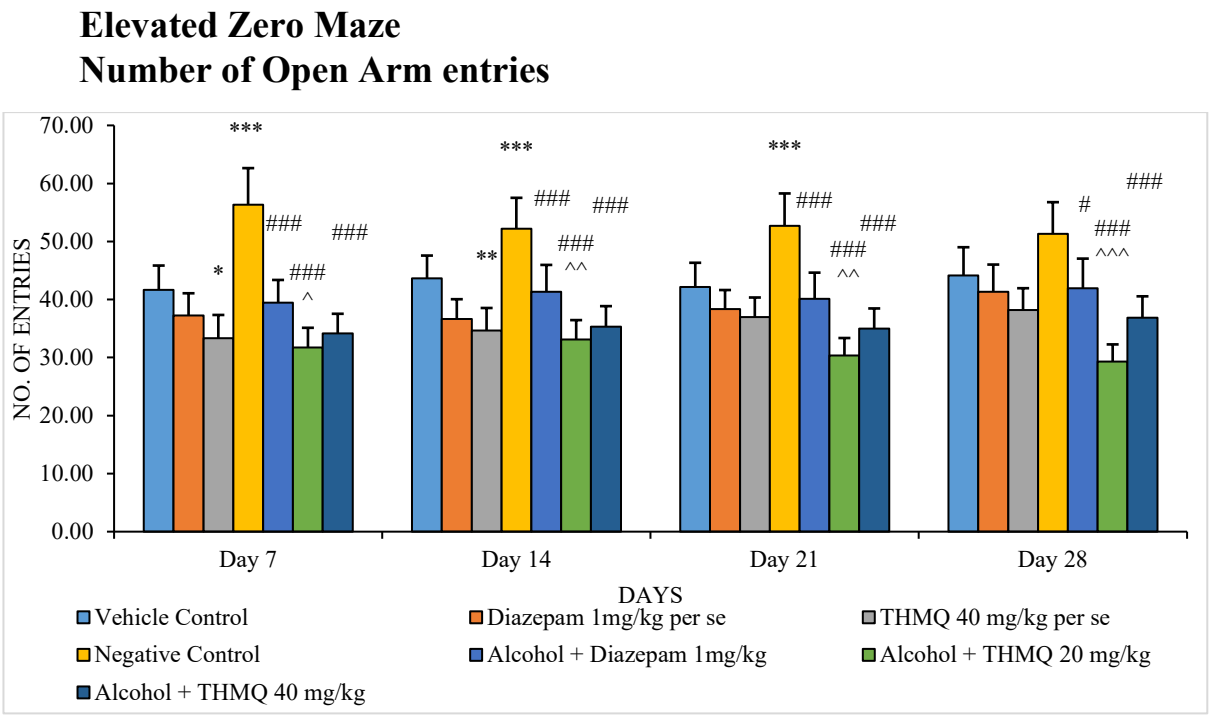

Figure 11 Number of Open Entries Treated by THMQ

Data are expressed as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance relative to the Negative Control group is indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Differences compared with the Alcohol + Diazepam 1 mg/kg group are represented by #, ##, and ###. Comparisons between Alcohol + THMQ 20 mg/kg and Alcohol + THMQ 40 mg/kg are denoted by ^, ^^, and ^^^ at corresponding significance levels. Overall, THMQ treatment at both doses significantly increased the number of entries into the open arms across all days, with THMQ 40 mg/kg producing a stronger behavioural improvement than the 20 mg/kg dose, indicating a dose-dependent reduction in anxiety-like behaviour during alcohol withdrawal.

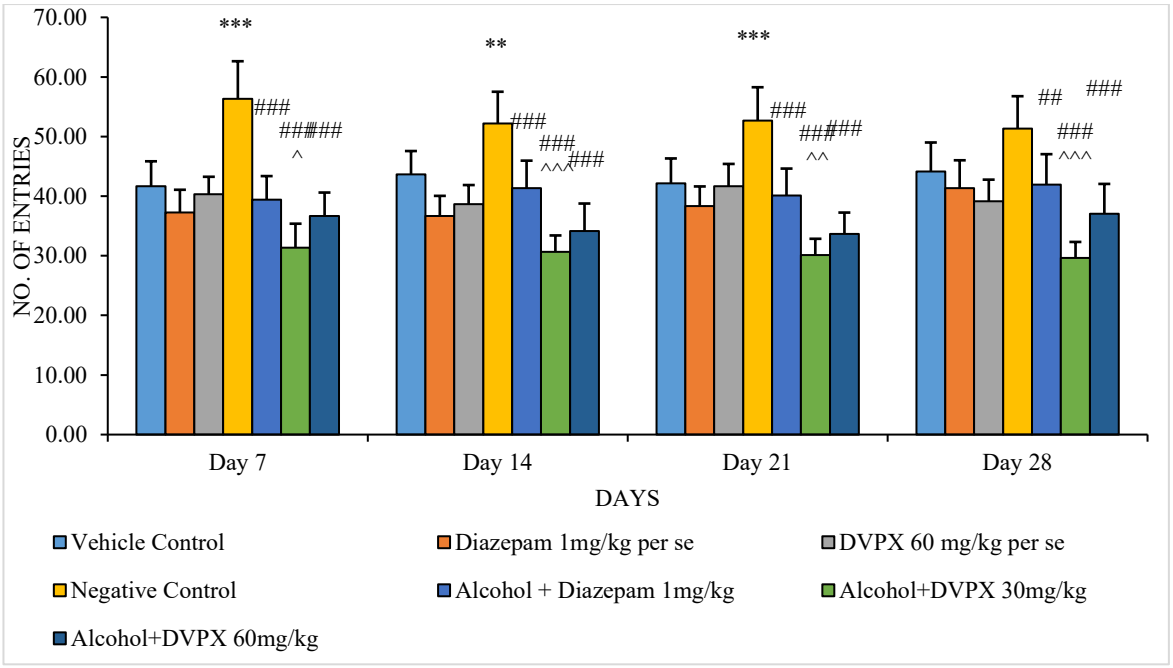

Figure 12 Number of Open Entries Treated by DVPX

Figure 12 presents the number of entries in the Open Field Test recorded on Days 7, 14, 21, and 28 following treatment with alcohol, Divalproex (DVPX), Thymoquinone (THMQ), Diazepam, and their combinations. All values are expressed as mean ± SD (n = 6), and statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Statistical significance in comparison with the Vehicle Control is denoted by *p < 0.05, **p < 0.01, and ***p < 0.001. Differences relative to the Negative Control are indicated by #, ##, and ###, corresponding to p < 0.05, p < 0.01, and p < 0.001, respectively. Comparisons with the Alcohol + Diazepam (1 mg/kg) group are represented by ^, ^^, and ^^^ (p < 0.05, p < 0.01, p < 0.001). Differences between Alcohol + DVPX (30 mg/kg) and its combination groups are marked by $, $$, and $$$, while comparisons involving Alcohol + THMQ (20 mg/kg) and its respective combination groups are denoted by !, !!, and !!!. Finally, the comparison between Alcohol + THMQ (20 mg/kg) + DVPX (30 mg/kg) and Alcohol + THMQ (20 mg/kg) + DVPX (30 mg/kg) + Diazepam (1 mg/kg) is represented by @, @@, and @@@. These symbols correspond to increasing levels of statistical significance and represent the predefined comparisons among treatment groups.

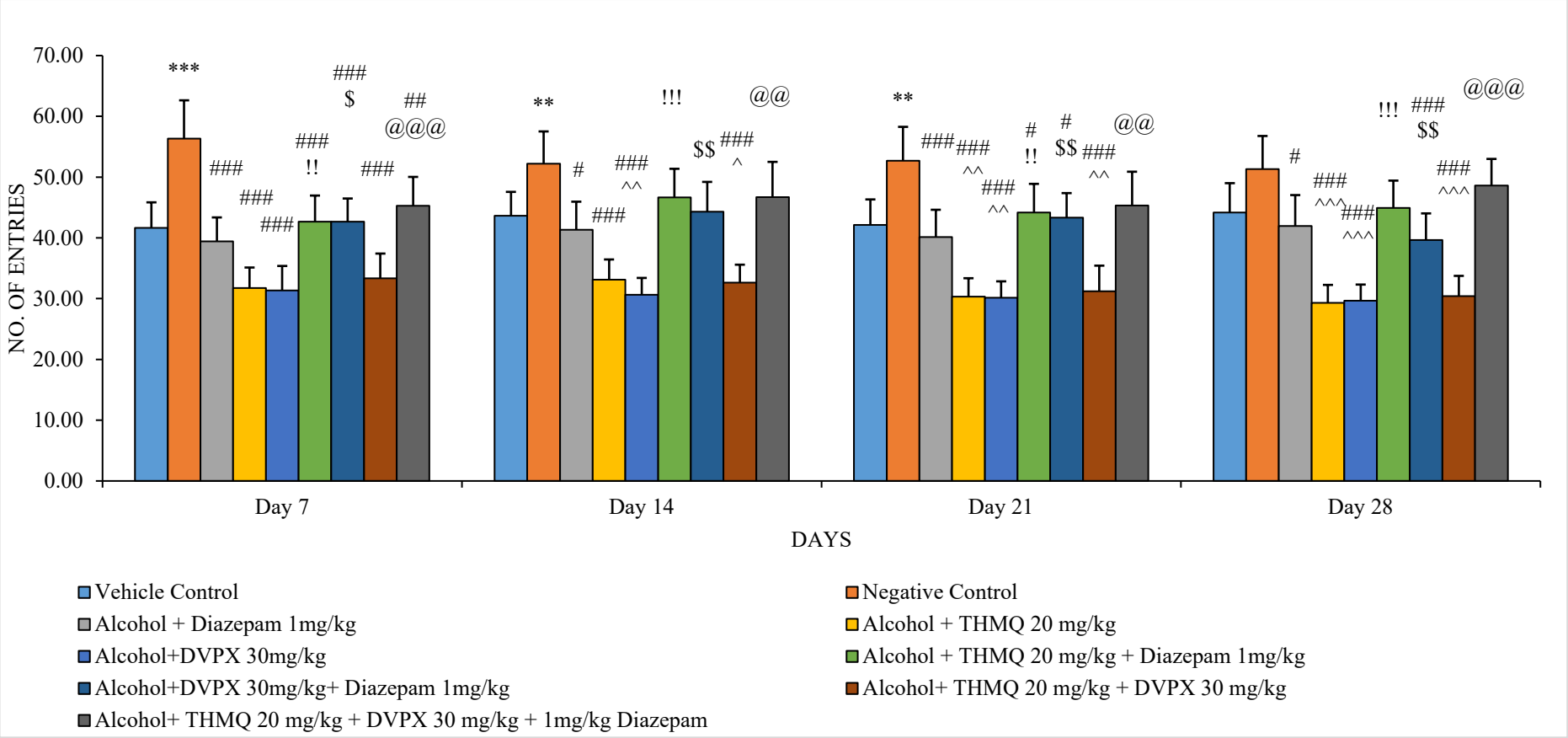

Figure 13 Number of Open Entries Treated by THMQ, DVPX vs Diazepam

Data are expressed as mean ± SD (n = 6) and analysed using one-way ANOVA followed by Tukey's post-hoc test. Statistical symbols represent comparisons between specific treatment groups as assigned in the experimental design.

Differences compared with the Negative Control are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Comparisons between Alcohol + Diazepam 1 mg/kg and all other treatment groups are indicated by ^, ^^, and ^^^, depending on the level of significance. The Alcohol + THMQ 20 mg/kg group compared with its combination treatments (Alcohol + THMQ 20 mg/kg + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) is represented by !, !!, and !!!. Comparisons between Alcohol + DVPX 30 mg/kg and its combined regimens (Alcohol + DVPX 30 mg/kg + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are represented by $, $$, and $$$. Finally, the difference between the Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg group and the triple-therapy group (Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg + Diazepam 1 mg/kg) is shown using @, @@, and @@@. These symbols indicate statistically significant variations across the treatments, demonstrating the modulatory effects of THMQ, DVPX, Diazepam, and their combinations on alcohol-induced behavioural alteration.

Figure 10 On Day 7, the Vehicle Control group showed strong differences from the Negative Control ($p < 0.001$) and a smaller difference from THMQ 40 mg/kg ($p < 0.05$). The Negative Control also differed significantly from Alcohol + Diazepam 1 mg/kg, Alcohol + THMQ 20 mg/kg, and Alcohol + THMQ 40 mg/kg (###, $p < 0.001$). A mild difference was seen between Alcohol + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg (^, $p < 0.05$). On Days 14 and 21, similar patterns continued, with Vehicle Control differing from Negative Control ($p < 0.001$) and the Negative Control showing strong differences from all THMQ and Diazepam groups (###, $p < 0.001$). Alcohol + Diazepam 1 mg/kg also differed from Alcohol + THMQ 20 mg/kg on both days (^^, $p < 0.01$).

By Day 28, differences between Vehicle Control and Negative Control became minimal, but the Negative Control still differed strongly from Alcohol + THMQ 20 mg/kg and THMQ 40 mg/kg (###, $p < 0.001$) and moderately from Alcohol + Diazepam 1 mg/kg (#, $p < 0.05$). Alcohol + Diazepam 1 mg/kg differed strongly from Alcohol + THMQ 20 mg/kg (^^^, $p < 0.001$).

Figure 11 On Day 7, Vehicle Control differed significantly from Negative Control ($p < 0.001$), while the Negative Control also differed strongly from Alcohol +

Diazepam 1 mg/kg, THMQ 20 mg/kg, and THMQ 40 mg/kg (###, $p < 0.001$). Alcohol + Diazepam 1 mg/kg showed a mild difference when compared with Alcohol + THMQ 20 mg/kg (^, $p < 0.05$).

On Days 14 and 21, Vehicle Control again differed from Negative Control, and the Negative Control continued to show strong differences from the THMQ and Diazepam groups (###, $p < 0.001$). Alcohol + Diazepam 1 mg/kg also differed from Alcohol + THMQ 20 mg/kg (^^^, $p < 0.001$ on Day 14; ^^, $p < 0.01$ on Day 21). On Day 28, differences between Vehicle Control and Negative Control were minimal, but Negative Control still differed significantly from Alcohol + THMQ 20 mg/kg and THMQ 40 mg/kg (###, $p < 0.001$) and moderately from Alcohol + Diazepam (##, $p < 0.01$). Alcohol + Diazepam 1 mg/kg again differed strongly from Alcohol + THMQ 20 mg/kg (^^^, $p < 0.001$).

Figure 12 On Day 7, Vehicle Control differed significantly from Negative Control ($p < 0.001$) and moderately from THMQ 40 mg/kg ($p < 0.05$). The Negative Control also differed strongly from Alcohol + Diazepam 1 mg/kg, THMQ 20 mg/kg, and THMQ 40 mg/kg (###, $p < 0.001$). A mild difference appeared between Alcohol + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg (^, $p < 0.05$). Strong differences were also found in multi-drug combinations, including Alcohol + DVPX 30 mg/kg + Diazepam vs. Alcohol + DVPX 30 mg/kg (###) and THMQ + DVPX combinations (@@). On Days 14 and 21, similar patterns continued, with Vehicle Control differing from Negative Control and the Negative Control showing strong differences from Alcohol + THMQ 20 mg/kg, Alcohol + DVPX 30 mg/kg, and combination treatments (###, $p < 0.001$). Several pairwise differences appeared between Diazepam groups, THMQ combinations, and DVPX combinations (^^, !!, , @@). On Day 28, the Negative Control differed strongly from Alcohol + THMQ 20 mg/kg, Alcohol + DVPX 30 mg/kg, Alcohol + DVPX 30 mg/kg + Diazepam, and THMQ–DVPX combinations (###, $p < 0.001$), and moderately from Alcohol + Diazepam (#, $p < 0.05$). Strong differences were also seen between Alcohol + Diazepam and THMQ/DVPX groups (^^^, $p < 0.001$), between THMQ and THMQ + Diazepam (!!!), between DVPX and DVPX + Diazepam (), and between THMQ + DVPX vs. the triple combination (@@@).

Open Field Test

Central Ambulation

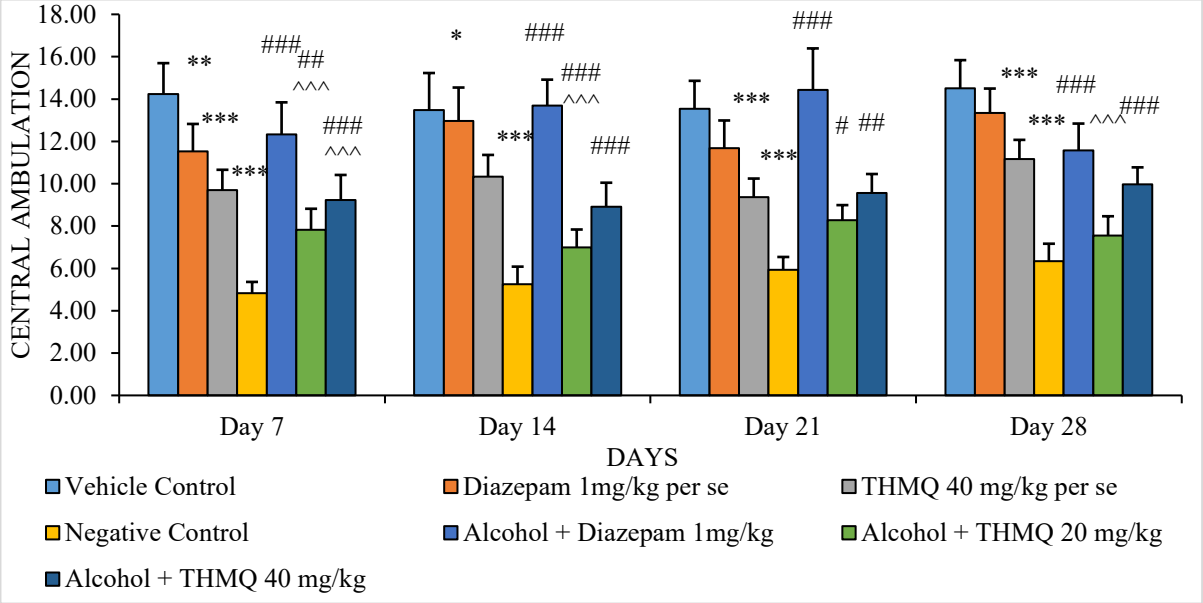

Figure 14 Central Ambulation treated by THMQ

Data are presented as mean ± SD (n = 6) and were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Significant differences compared with the Negative Control group are indicated by *, **, and *** ($p < 0.05$, 0.01, and 0.001). Comparisons between Alcohol + Diazepam 1 mg/kg and the THMQ-treated groups are denoted by #, ##, and ### ($p < 0.05$, 0.01, and 0.001). Differences between Alcohol + THMQ 20 mg/kg and Alcohol + THMQ 40 mg/kg are represented by ^, ^^, and ^^^. Across Days 7, 14, 21, and 28, the Negative Control group consistently showed significantly reduced central ambulation relative to the Vehicle Control group. All alcohol-treated groups receiving Diazepam or THMQ demonstrated increased central ambulation compared with the Negative Control. THMQ 40 mg/kg produced stronger anxiolytic-like effects, particularly on Days 14 and 21, as reflected by the prominent ### markers. Diazepam-treated mice showed significant improvement over Negative Control (***), while THMQ groups showed further separation from Diazepam (^^), indicating superior behavioural recovery. Overall, THMQ alone and in combination produced a progressive, dose-dependent elevation in central ambulation throughout the study.

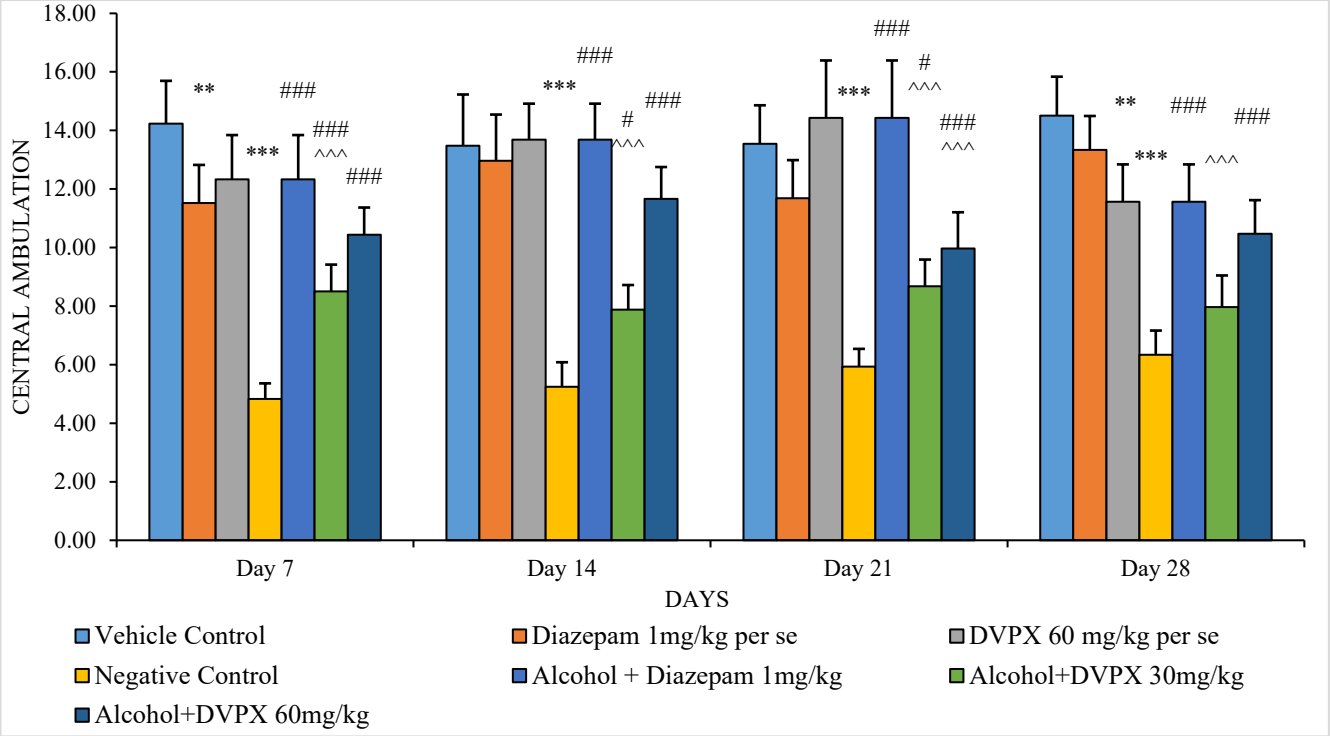

Figure 15 Central Ambulation treated by DVPX

In Figure 12, significant differences were observed across treatment groups. Comparisons made against the Negative Control are indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Differences relative to the Alcohol + Diazepam 1 mg/kg group are denoted by # ($p < 0.05$), ## ($p < 0.01$), and ### ($p < 0.001$). Comparisons of Alcohol + DVPX 30 mg/kg with its combination groups are marked by ^ ($p < 0.05$), ^^ ($p < 0.01$), and ^^^ ($p < 0.001$). Differences between Alcohol + DVPX 60 mg/kg and its related treatment combinations are shown by $ ($p < 0.05$), $$ ($p < 0.01$), and $$$ ($p < 0.001$). Finally, comparisons between Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg and the triple-therapy group (Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg + Diazepam 1 mg/kg) are represented by @ ($p < 0.05$), @@ ($p < 0.01$), and @@@ ($p < 0.001$). Data are presented as mean ± SD (n = 6) and analyzed using one-way ANOVA followed by Tukey's post-hoc test.

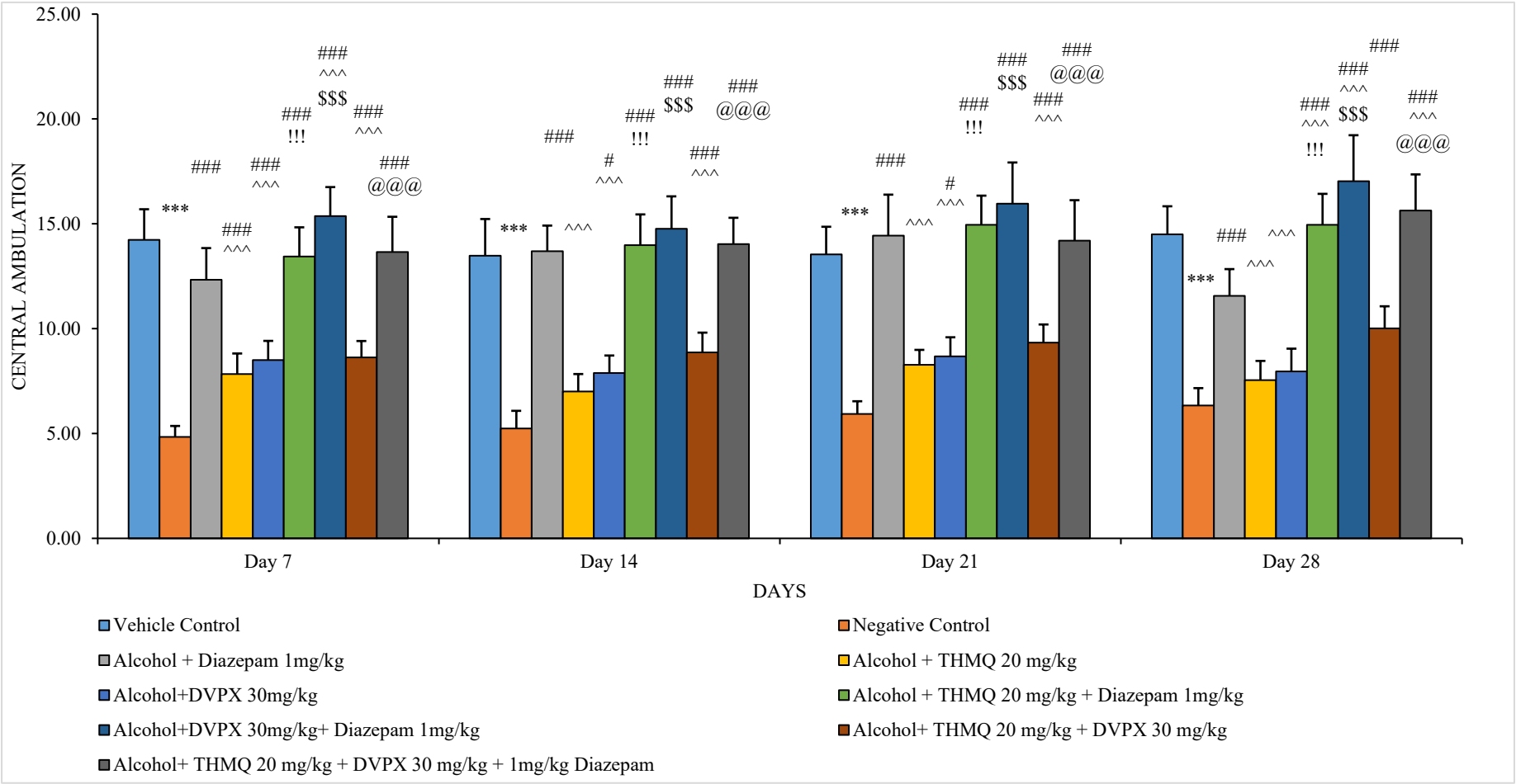

Figure 16 Central Ambulation treated by THMQ, DVPX vs Diazepam

Data are expressed as mean ± SD (n = 6) and analyzed using one-way ANOVA followed by Tukey's post-hoc test. Significant differences relative to the Negative Control group are indicated by #, ##, and ### ($p < 0.05$, 0.01, and 0.001, respectively). Comparisons of Alcohol + Diazepam (1 mg/kg) against all remaining treatment groups are marked with ^, ^^, and ^^^. Differences between Alcohol + THMQ 20 mg/kg and its combination groups (Alcohol + THMQ 20 mg/kg + Diazepam 1 mg/kg, and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are denoted by !, !!, and !!!. Variations between Alcohol + DVPX 30 mg/kg and its combination groups (Alcohol + DVPX 30 mg/kg + Diazepam 1 mg/kg, and Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg) are represented by $, $$, and $$$. Finally, statistical differences between the combined THMQ + DVPX group and the triple combination (THMQ + DVPX + Diazepam) are shown using @, @@, and @@@. These results demonstrate clear dose-dependent and interaction-dependent effects of THMQ, DVPX, and Diazepam on central ambulation behavior across all time points.

Figure 13 shows that on Day 7, Diazepam 1 mg/kg differed significantly from Vehicle Control ($p < 0.01$), while the Negative Control showed strong differences compared with Alcohol + Diazepam and both THMQ doses ($p < 0.001$; ###). Alcohol + Diazepam also differed markedly from both THMQ doses ($p < 0.001$; ^^^). On Day 14, the Negative Control again differed significantly from Alcohol + Diazepam and THMQ 20/40 mg/kg ($p < 0.001$; ###), and Alcohol + Diazepam differed from THMQ 20 mg/kg ($p < 0.001$; ^^^). On Day 21, strong differences persisted between the Negative Control and Alcohol + Diazepam ($p < 0.001$; ###) and between the Negative Control and THMQ 20/40 mg/kg. By Day 28, the Negative Control showed clear differences from Alcohol + Diazepam and THMQ 40 mg/kg ($p < 0.001$; ###), and Alcohol + Diazepam again differed from THMQ 20 mg/kg ($p < 0.001$; ^^^).

Figure 14 shows a similar pattern. On Day 7, Vehicle Control differed from Negative Control ($p < 0.001$) and from Diazepam ($p < 0.01$). The Negative Control differed strongly from Alcohol + Diazepam, THMQ 20 mg/kg, and THMQ 40 mg/kg ($p < 0.001$; ###), and Alcohol + Diazepam differed significantly from THMQ 20 mg/kg ($p < 0.001$; ^^^). On Days 14 and 21, the Negative Control continued to differ from Alcohol + Diazepam and both THMQ doses ($p < 0.001$; ###), with a moderate difference for THMQ 20 mg/kg ($p < 0.05$; #). Alcohol + Diazepam also differed strongly from THMQ 20 mg/kg ($p < 0.001$; ^^^). By Day 28, substantial differences remained between Negative Control

and Alcohol + Diazepam and THMQ 40 mg/kg ($p < 0.001$; ###), and Alcohol + Diazepam continued to differ from THMQ 20 mg/kg ($p < 0.001$; ^^^).

Figure 15 shows that on Day 7, the Negative Control differed markedly from Alcohol + Diazepam, THMQ 20 mg/kg, DVPX 30 mg/kg, and their combinations ($p < 0.001$; ###). Alcohol + Diazepam differed strongly from THMQ 20 mg/kg and combination treatments ($p < 0.001$; ^^^), and THMQ 20 mg/kg and DVPX 30 mg/kg each differed from their Diazepam combinations ($p < 0.001$; !!!, $$). THMQ + DVPX also differed strongly from its triple combination ($p < 0.001$; @@@).
On Day 14, the Negative Control again differed significantly from multiple treatments ($p < 0.001$; ###). Alcohol + Diazepam and THMQ/DVPX combinations continued to show strong differences between their paired forms. On Day 21, the Negative Control remained significantly different from Alcohol + Diazepam and THMQ 40 mg/kg ($p < 0.001$; ###). By Day 28, substantial differences persisted between Negative Control and Alcohol + Diazepam and THMQ 40 mg/kg ($p < 0.001$; ###), and Alcohol + Diazepam continued to differ strongly from THMQ 20 mg/kg ($p < 0.001$; ^^^).

Open Field Test

Time Spent in the centre

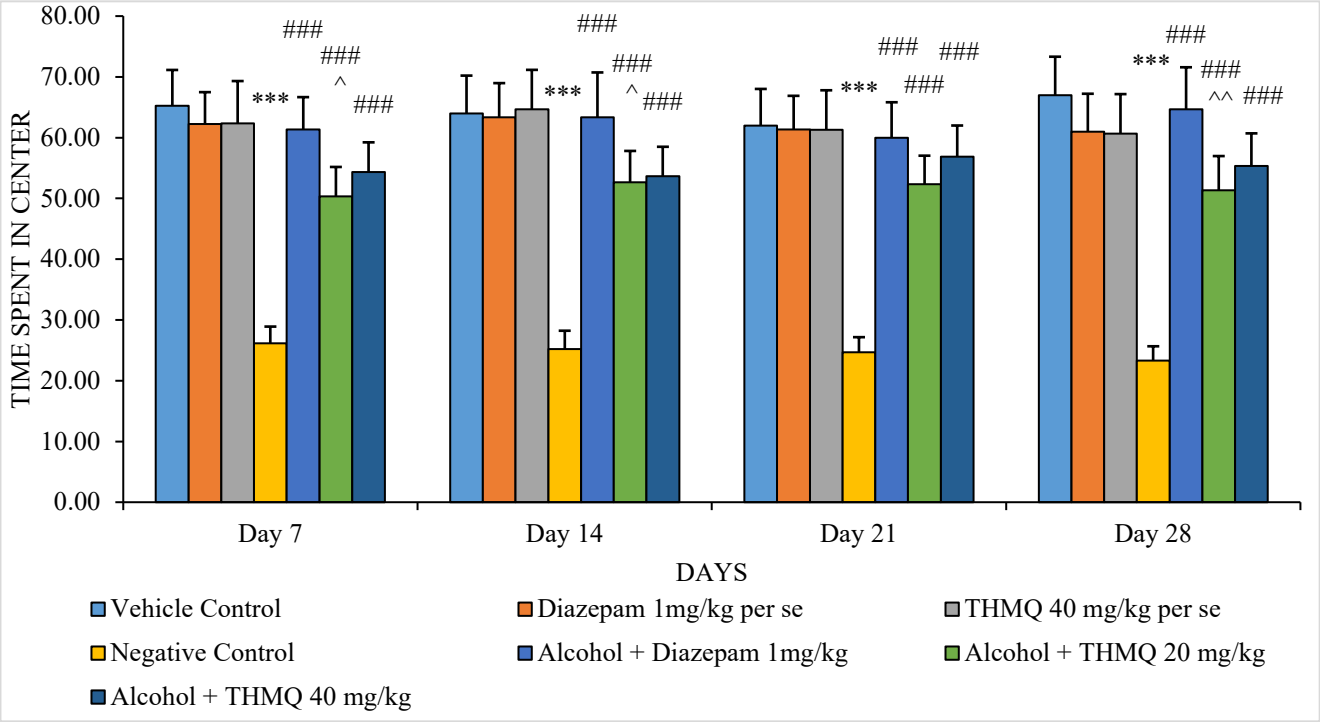

Figure 17 Time Spent in Centre Treated by THMQ

The data are presented as mean ± SEM (n = 6). Significant differences compared with the Negative Control group are marked by *** ($p < 0.001$). Comparisons with the Alcohol + Diazepam 1 mg/kg group are shown by ^ ($p < 0.05$) and ^^ ($p < 0.01$). Differences relative to the Alcohol + THMQ 40 mg/kg group are indicated by ### ($p < 0.001$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

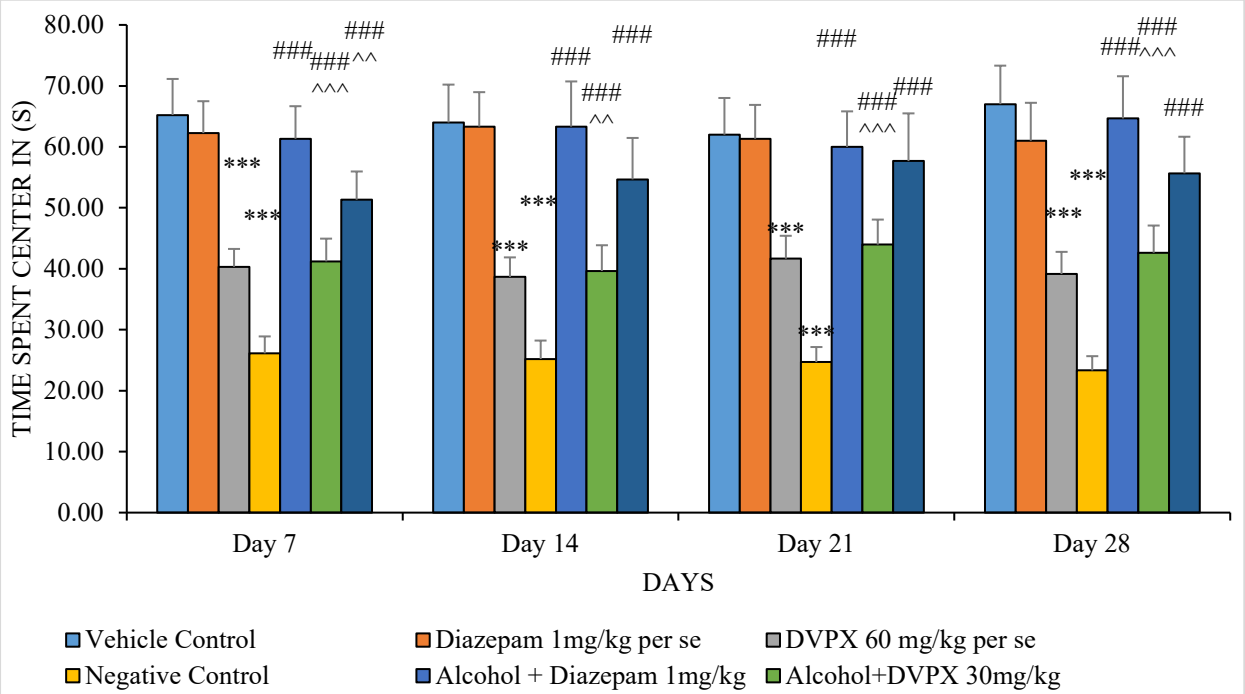

Figure 18 Time Spent in Centre Treated by DVPX

Data are expressed as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Significant differences relative to the Negative Control group are indicated by ***p < 0.001, **p < 0.01, and *p < 0.05. Comparisons relative to the Alcohol + Diazepam 1 mg/kg group are indicated by ###p < 0.001, ##p < 0.01, and #p < 0.05. Differences between Alcohol + DVPX 30 mg/kg and Alcohol + DVPX 60 mg/kg groups are denoted by ^^^p < 0.001 and ^^p < 0.01.

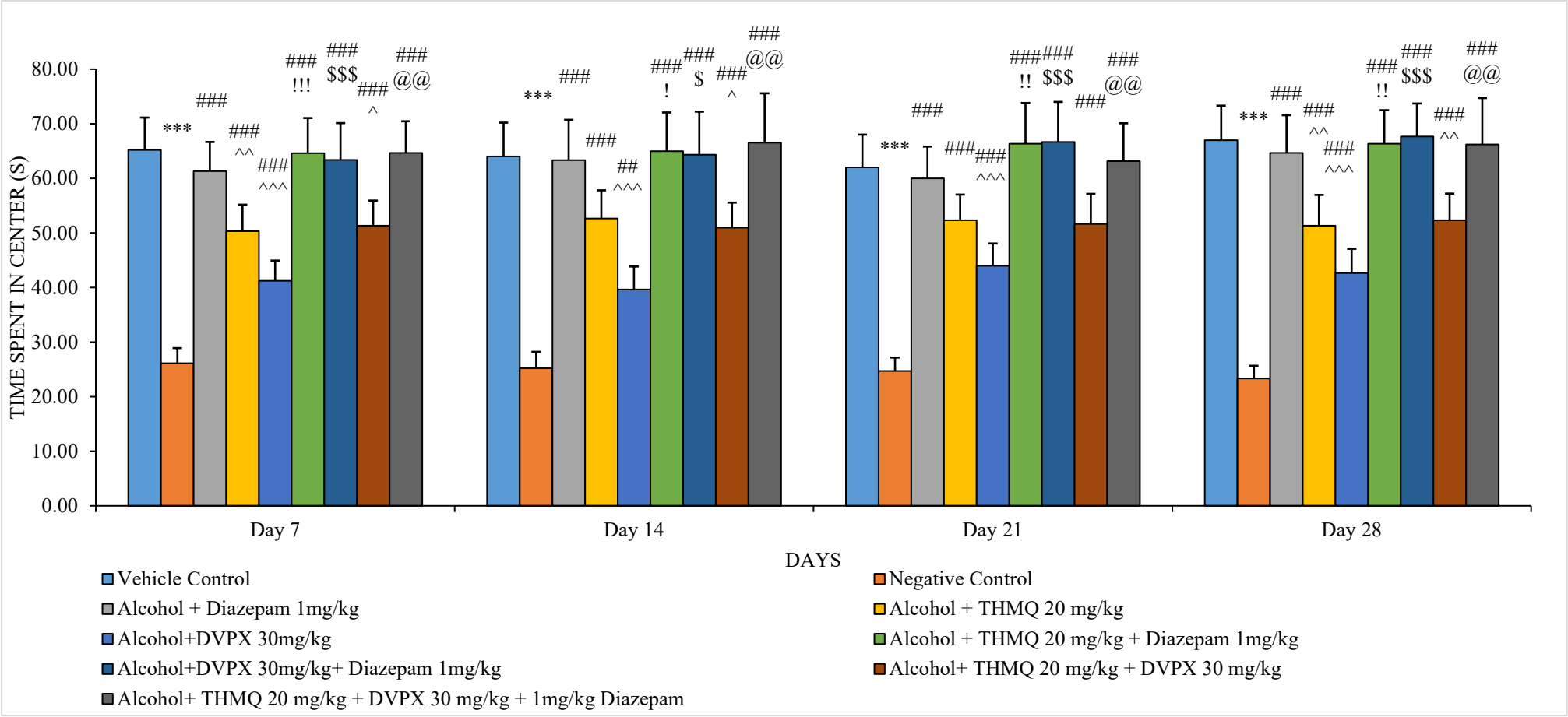

Figure 19 Time Spent in Centre Treated by THMQ, DVPX vs Diazepam

Data were analysed using one-way ANOVA followed by Tukey's post-hoc test, and values are expressed as mean ± SD (n = 6). Statistical significance is represented by the symbols shown above the bars in the figure.

Asterisks (***, **, *) indicate significant differences when compared with the Vehicle Control group, whereas hashes (###, ##, #) denote differences relative to the Negative Control group. Caret symbols (^^^, ^^, ^) mark comparisons with the Alcohol + Diazepam 1 mg/kg group, dollar signs ($$$, $$, $) denote comparisons with the Alcohol + THMQ 20 mg/kg group, and at-signs (@@@, @@, @) represent differences relative to the Alcohol + DVPX 30 mg/kg group. Statistical differences between combination-treated groups (Alcohol + THMQ 20 mg/kg + DVPX 30 mg/kg and its Diazepam-augmented form) are also shown by the corresponding symbols above the respective bars. A lower p-value corresponds to a greater level of statistical significance.

Figure 16 shows that on Day 7, Vehicle Control and THMQ 40 mg/kg showed no meaningful difference, while Vehicle Control differed significantly from Diazepam 1 mg/kg ($p < 0.01$). The Negative Control also showed highly significant reductions when compared with Alcohol + Diazepam 1 mg/kg, Alcohol + THMQ 20 mg/kg, and Alcohol + THMQ 40 mg/kg ($p < 0.001$, ###), with a moderate difference against Alcohol + Diazepam 1 mg/kg ($p < 0.01$, ##). Alcohol + Diazepam 1 mg/kg differed markedly from both THMQ-treated groups ($p < 0.001$, ^^^). Similar trends continued on Day 14, with strong differences between Vehicle Control and Negative Control ($p < 0.001$) and between Vehicle Control and Diazepam 1 mg/kg ($p < 0.05$). The Negative Control remained significantly different from Alcohol + Diazepam 1 mg/kg and both THMQ doses ($p < 0.001$, ###), and Alcohol + Diazepam 1 mg/kg again differed sharply from Alcohol + THMQ 20 mg/kg ($p < 0.001$, ^^^). On Day 21, Vehicle and Negative Controls remained significantly different ($p < 0.001$), and the Negative Control again differed from all Alcohol-treated groups ($p < 0.001$, ###), although Alcohol + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg showed negligible difference. By Day 28, significant variations persisted between Vehicle and Negative Control ($p < 0.001$, ***). The Negative Control again differed markedly from Alcohol + Diazepam 1 mg/kg and both THMQ doses ($p < 0.001$, ###), with a moderate difference against Alcohol + Diazepam 1 mg/kg ($p < 0.01$, ^^).

Figure 17 demonstrates similar outcomes. On Day 7, Vehicle Control differed significantly from both THMQ 40 mg/kg and the Negative Control ($p < 0.001$), while the Negative Control differed strongly from Alcohol + Diazepam 1 mg/kg and both

THMQ doses ($p < 0.001$, ###). Alcohol + Diazepam 1 mg/kg displayed significant differences relative to Alcohol + THMQ 20 mg/kg ($p < 0.001$, ^^^) and THMQ 40 mg/kg ($p < 0.01$, ^^). Day 14 again showed strong differences between Vehicle Control, THMQ 40 mg/kg, and Negative Control ($p < 0.001$), while the Negative Control remained significantly different from all Alcohol-treated groups ($p < 0.001$, ###). Alcohol + Diazepam 1 mg/kg differed moderately from THMQ 20 mg/kg ($p < 0.01$, ^^). On Day 21, Vehicle Control again differed from THMQ 40 mg/kg and Negative Control ($p < 0.001$). The Negative Control continued to differ from Alcohol + Diazepam 1 mg/kg and both THMQ doses ($p < 0.001$, ###), and Alcohol + Diazepam 1 mg/kg remained significantly different from Alcohol + THMQ 20 mg/kg ($p < 0.001$, ^^^). On Day 28, strong differences remained between Vehicle Control, THMQ 40 mg/kg, and the Negative Control ($p < 0.001$). The Negative Control again differed significantly from all THMQ- and Diazepam-treated groups ($p < 0.001$, ###), and Alcohol + Diazepam 1 mg/kg continued to show strong differences from THMQ 20 mg/kg ($p < 0.001$, ^^^).

Figure 18 further supports these findings. On Day 7, Vehicle Control showed significant differences from the Negative Control ($p < 0.001$). The Negative Control differed markedly from Alcohol + Diazepam 1 mg/kg, Alcohol + THMQ 20 mg/kg, Alcohol + DVPX 30 mg/kg, and combined treatments ($p < 0.001$, ###). Alcohol + Diazepam 1 mg/kg significantly differed from Alcohol + THMQ 20 mg/kg ($p < 0.01$, ^^) and Alcohol + DVPX 30 mg/kg ($p < 0.001$, ^^^). Marked differences also appeared between Alcohol + THMQ 20 mg/kg and its Diazepam combination ($p < 0.001$, !!!), and between DVPX 30 mg/kg and its Diazepam combination ($p < 0.001$,). On Day 14, Vehicle and Negative Controls remained significantly different ($p < 0.001$), and the Negative Control differed significantly from Alcohol + Diazepam 1 mg/kg, Alcohol + THMQ 20 mg/kg, and Alcohol + THMQ 40 mg/kg ($p < 0.001$, ###). Additional differences appeared between Alcohol + Diazepam 1 mg/kg and DVPX-treated combinations ($p < 0.001$, ^^^), and between THMQ- and DVPX-based combinations ($p < 0.05$ to < 0.001). Day 21 showed strong, continued differences between Vehicle and Negative Controls ($p < 0.001$), with the Negative Control differing from all Alcohol-treated groups ($p < 0.001$, ###). Diazepam- and THMQ-based combinations continued to show significant distinctions (^^^, !!!, , @@). By Day 28, profound differences were observed between Vehicle and Negative Control ($p < 0.001$). The

Negative Control differed significantly from all Diazepam-, THMQ-, and DVPX-based treatments ($p < 0.001$, ###). Alcohol + Diazepam 1 mg/kg also differed strongly from several THMQ and DVPX combinations ($p < 0.001$, ^^^), and combination therapies showed further significant contrasts (!!!, $$, @@).

Western Blot

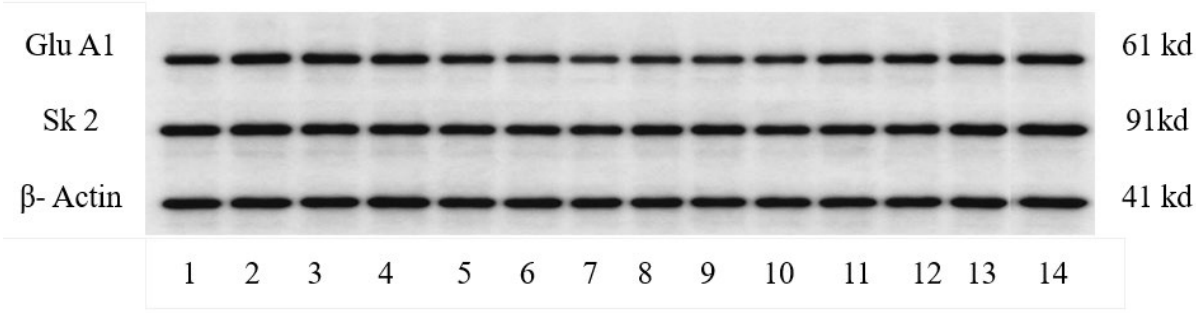

Figure No. 20 Western Bolts

Group No.	Group Name
1	Normal Control (Vehicle only)
2	Diazepam per se (1 mg/kg)
3	THMQ per se (40 mg/kg)
4	DVPX per se (60 mg/kg)
5	Alcohol only (Negative Control)
6	Alcohol + Diazepam i.p (1 mg/kg)
7	Alcohol + THMQ p.o (20 mg/kg)
8	Alcohol + THMQ p.o (40 mg/kg)
9	Alcohol + DVPX p.o (30 mg/kg)
10	Alcohol + DVPX p.o (60 mg/kg)
11	Alcohol + THMQ p.o (20 mg/kg) + Diazepam i.p (1 mg/kg)
12	Alcohol + DVPX p.o (30 mg/kg) + Diazepam i.p (1 mg/kg)
13	Alcohol + THMQ p.o (20 mg/kg) + DVPX p.o (30 mg/kg)
14	Alcohol + THMQ p.o (20 mg/kg) + DVPX p.o (30 mg/kg) + Diazepam i.p (1 mg/kg)

Protein Sk2 vs β-Actin

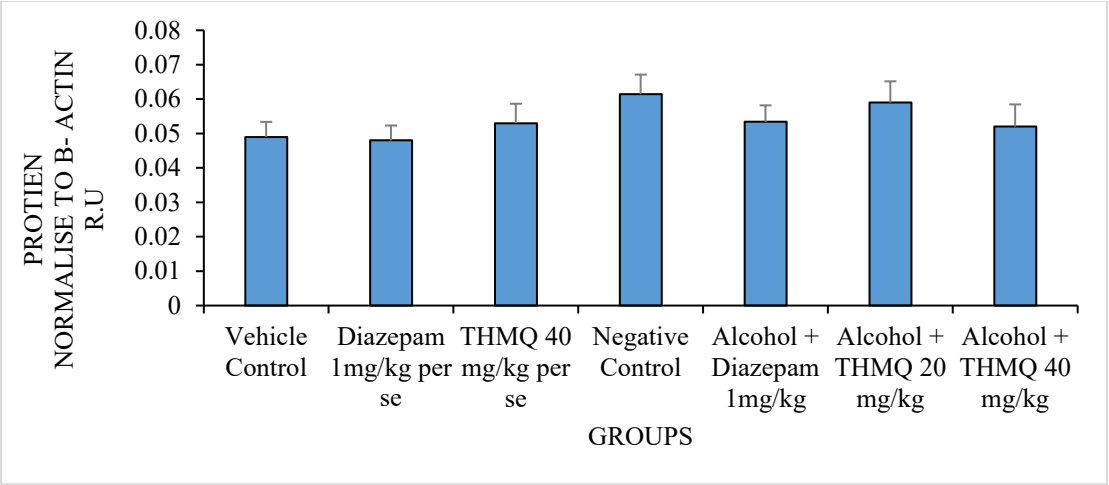

Figure 21 Protein Sk2 vs β-Actin Treated by THMQ

Numerical results were evaluated using variance analysis (ANOVA), subsequently followed by Tukey's method for post hoc multiple group comparison. Findings are displayed as average values ± standard deviation (n = 6). Significance thresholds were interpreted as follows: ***P < 0.001, *P < 0.05, and **P < 0.01, in comparison with the untreated Control group.

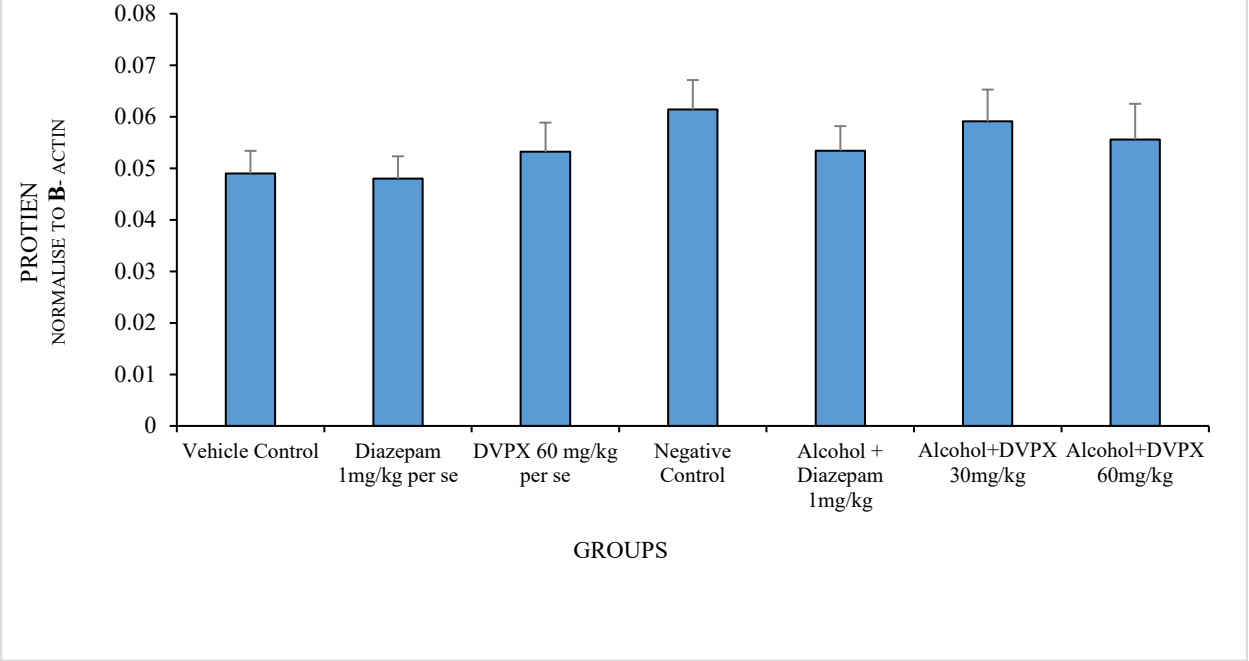

Figure 22 Protein Sk2 vs β-Actin Treated by DVPX

Numerical results were evaluated using variance analysis (ANOVA), subsequently followed by Tukey's method for post hoc multiple group comparison. Findings are displayed as average values ± standard deviation (n = 6). Significance thresholds were interpreted as follows: ***P < 0.001, *P < 0.05, and **P < 0.01, in comparison with the untreated Control group.

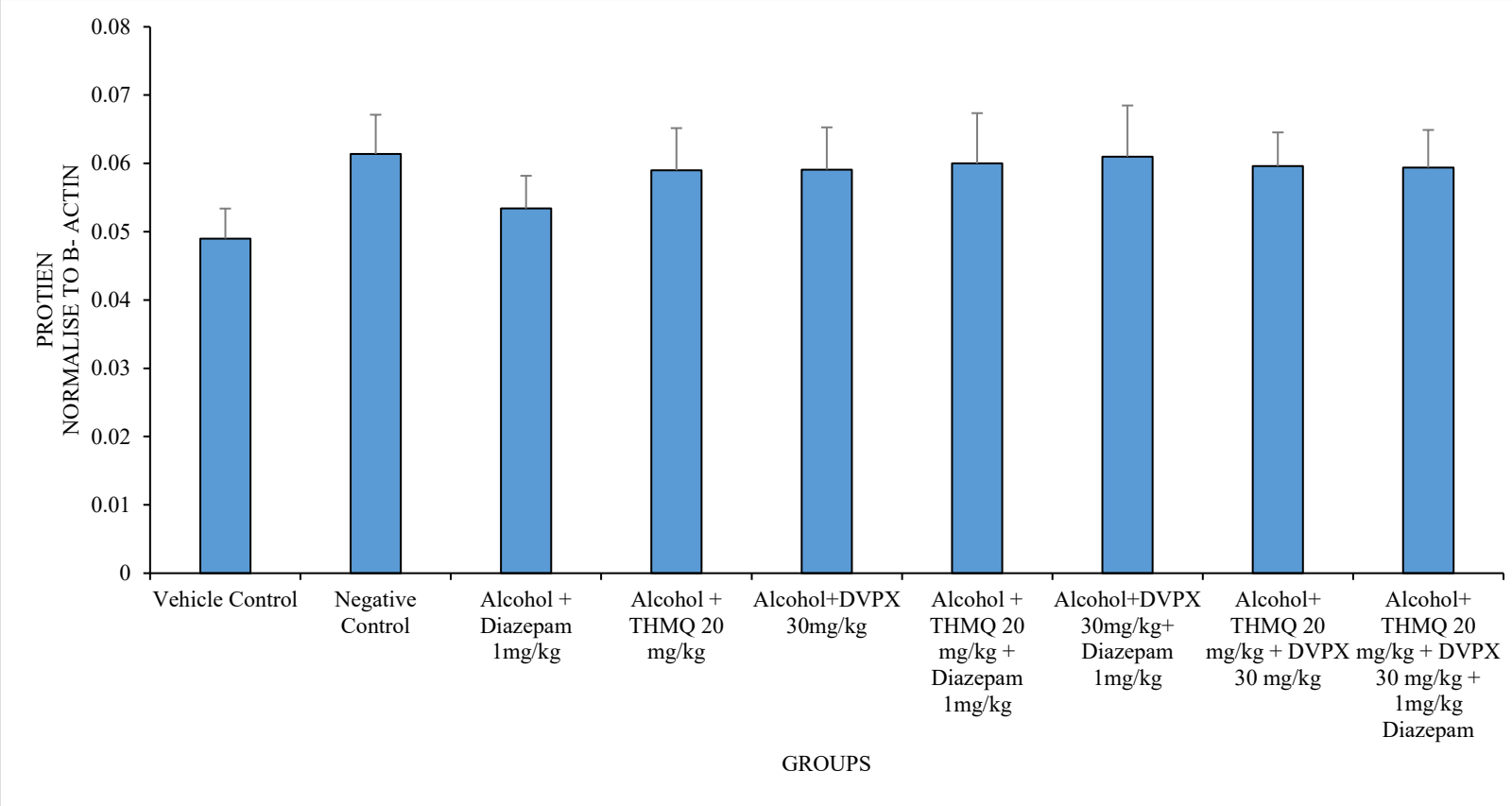

Figure 23 Protein Sk2 vs β-Actin Treated by THMQ, DVPX vs Diazepam

Numerical results were evaluated using variance analysis (ANOVA), subsequently followed by Tukey's method for post hoc multiple group comparison. Findings are displayed as average values ± standard deviation (n = 6). Significance thresholds were interpreted as follows: ***P < 0.001, *P < 0.05, and **P < 0.01, in comparison with the untreated Control group.

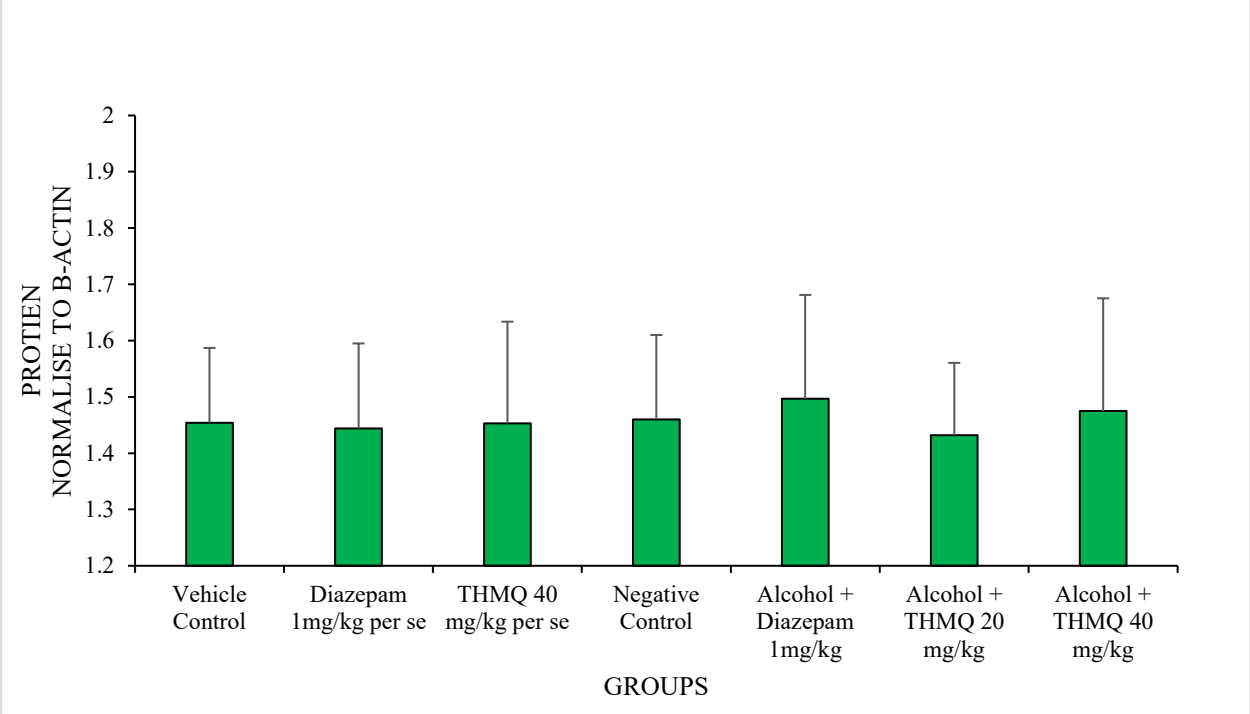

Figure 24 Protein Glu- A1 vs β-Actin Treated by THMQ

Numerical results were evaluated using variance analysis (ANOVA), subsequently followed by Tukey's method for post hoc multiple group comparison. Findings are displayed as average values ± standard deviation (n = 6). Significance thresholds were interpreted as follows: ***P < 0.001, *P < 0.05, and **P < 0.01, in comparison with the untreated Control group.

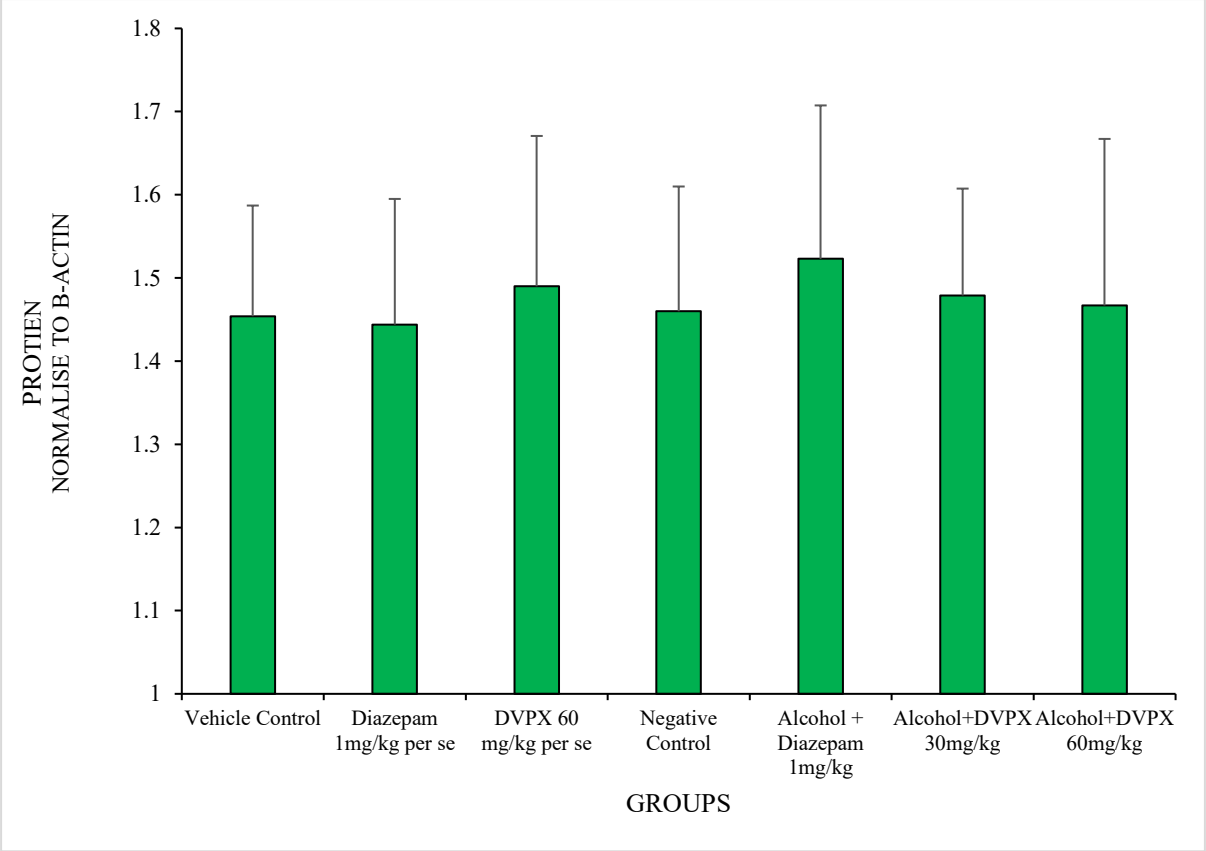

Figure 25 Protein Glu- A1 vs β-Actin Treated by DVPX

Numerical results were evaluated using variance analysis (ANOVA), subsequently followed by Tukey's method for post hoc multiple group comparison. Findings are displayed as average values ± standard deviation (n = 6). Significance thresholds were interpreted as follows: ***P < 0.001, *P < 0.05, and **P < 0.01, in comparison with the untreated Control group.

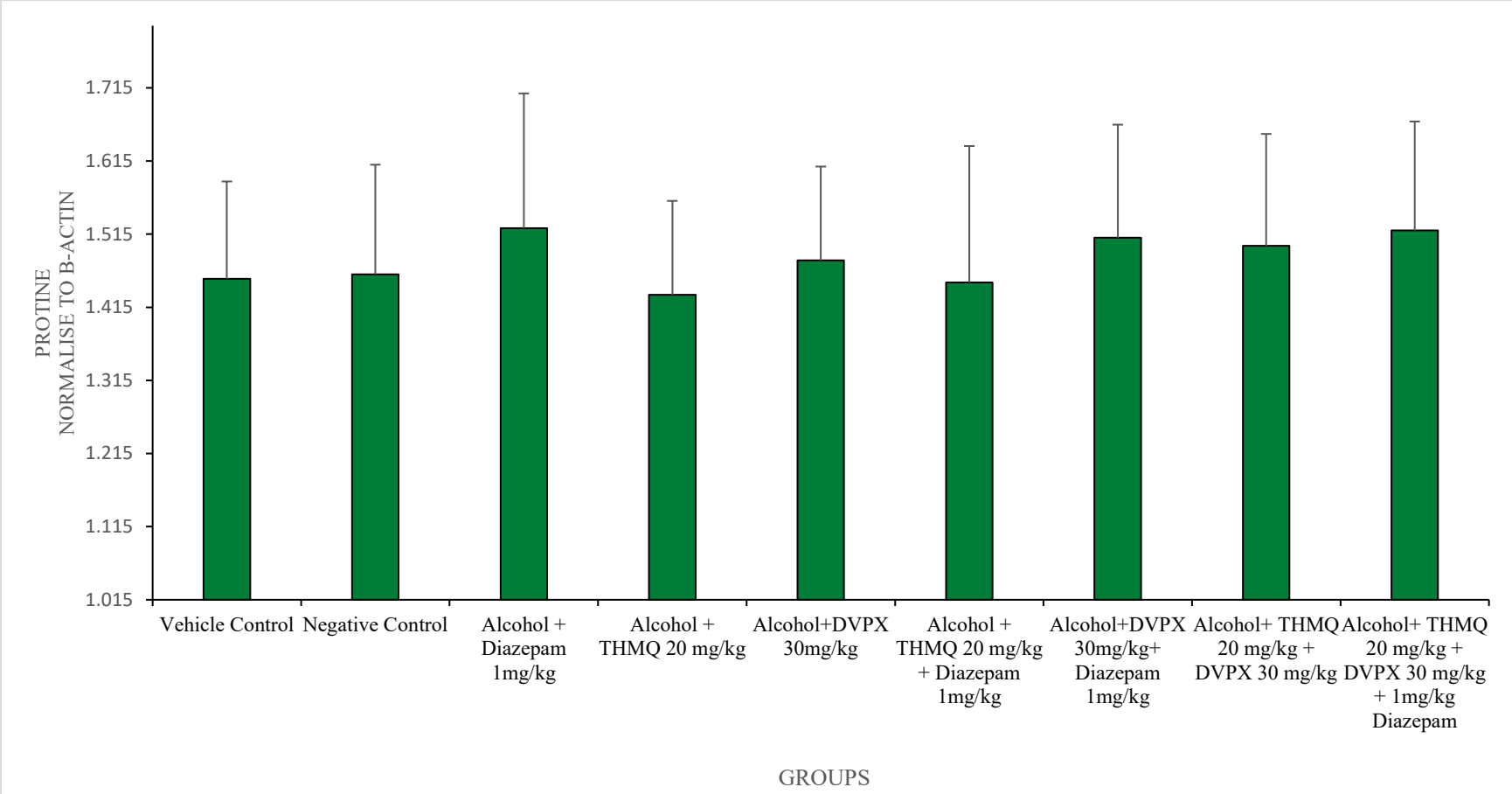

Figure 26 Protein Glu- A1 vs β-Actin Treated by THMQ, DVPX vs Diazepam

Numerical results were evaluated using variance analysis (ANOVA), subsequently followed by Tukey's method for post hoc multiple group comparison. Findings are displayed as average values ± standard deviation (n = 6). Significance thresholds were interpreted as follows: ***P < 0.001, *P < 0.05, and **P < 0.01, in comparison with the untreated Control group.

No significant difference was observed in protein Sk2 and Glu A1 vs β-Actin.

Discussion

The present study investigated the effects of combined pharmacological treatments Alcohol + Diazepam, Alcohol + THMQ, and Alcohol + DVPX—on behavioral and physiological outcomes in an experimental model. These treatments were selected to explore how different neuroactive drug combinations modulate behavioral patterns and neurobiological markers. As reported by Hedrich & Bullock (2004), alterations in neural activity are closely associated with changes in synaptic plasticity, making this an important area of investigation.

To evaluate molecular correlates of these behavioral effects, GluA1 (an AMPA receptor subunit) and SK channels (small-conductance calcium-activated potassium channels) were assessed using Western blot analysis. Although behavior was significantly altered across treatment groups, no measurable differences were observed in GluA1 or SK protein expression. While initially unexpected, this finding suggests that the behavioral effects induced by the treatments may involve alternative or parallel molecular pathways rather than direct modulation of these proteins.

The primary objective of the study was to assess behavioral responses following administration of Alcohol + Diazepam, Alcohol + THMQ, and Alcohol + DVPX across multiple time points (Day 7, Day 14, Day 21, and Day 28), allowing both short-term and progressive effects to be captured (Xiao et al., 2018). Clear and consistent differences were observed between the Vehicle Control and Negative Control groups throughout the study, demonstrating that the experimental conditions themselves produced significant behavioral alterations. These differences often at ***$p < 0.001$ confirm the robustness of the model and support the validity of the study design (Mansour et al., 2002).

Pharmacological treatments produced distinct behavioral modifications when compared to the Negative Control, particularly in groups receiving Alcohol + Diazepam 1 mg/kg, Alcohol + THMQ 20 mg/kg, Alcohol + THMQ 40 mg/kg, and Alcohol + DVPX 30 mg/kg. Comparisons between Alcohol + Diazepam 1 mg/kg and Alcohol + THMQ 20 mg/kg revealed highly significant differences (^^^ $p < 0.001$), indicating that the two compounds exerted different pharmacological influences. Diazepam, known for its sedative and anxiolytic actions mediated through GABAergic

modulation, predictably altered central inhibition (Woo et al., 2012). THMQ, with reported neuroprotective potential, may act through different neurochemical pathways, producing distinct behavioral outcomes.

Synergistic interactions were also observed, particularly in the Alcohol + Diazepam 1 mg/kg + DVPX 30 mg/kg combination, which produced pronounced effects ($$$ $p < 0.001$). These findings highlight the importance of assessing pharmacodynamic interactions, as combined treatments may exhibit synergistic or antagonistic properties that differ significantly from the effects of individual drugs.

Western blot analyses further examined GluA1 and SK channel expression, both of which are integral to neuronal excitability and synaptic plasticity (Jakaria et al., 2018). Despite clear behavioral alterations, no significant changes were detected in these protein levels. Several explanations are plausible. First, the evaluated drugs may not directly modulate pathways governing GluA1 or SK expression (Aquib et al., 2014). Diazepam acts primarily on GABA-A receptors, whereas THMQ and DVPX may engage alternative neurochemical systems. Second, time-dependent protein regulation may not have been captured within the sampling windows (Xiao et al., 2018). Longer exposure or later time points may be required to detect molecular changes. Finally, the behavioral effects may involve other systems dopaminergic, serotonergic, or adrenergic pathways which were not assessed but may account for the observed outcomes (Ruby B et al., 2012).

The absence of detectable GluA1 and SK modulation does not diminish the value of the findings. Instead, it underscores the complexity of neuropharmacological mechanisms and the need to examine broader molecular networks. As Haque et al. (2021) suggest, multidrug interactions may involve extensive signaling cascades not easily captured by isolated protein assays. Future studies employing transcriptomic or proteomic approaches may provide a more comprehensive understanding of these processes.

The progressive divergence between Vehicle Control and Negative Control groups by Day 28 further emphasizes the importance of longitudinal research (Kurhe et al., 2014). Time-dependent behavioral and physiological alterations suggest that chronic drug exposure may lead to delayed or cumulative neurobiological changes not

immediately reflected in the molecular markers assessed.

Overall, this study provides important insights into the behavioral consequences of Alcohol combined with Diazepam, THMQ, and DVPX. While GluA1 and SK protein expression remained unchanged, the significant behavioral alterations indicate that these treatments influence neural function through other molecular pathways. These findings highlight the complexity of drug interactions and underscore the need for broader, multidimensional analyses in future research. Continued exploration may help clarify how these pharmacological combinations affect brain function and contribute to their potential therapeutic applications.

Conclusion

This study investigated the behavioral and physiological effects of combined pharmacological treatments Alcohol + Diazepam, Alcohol + THMQ, and Alcohol + DVPX across four experimental intervals (Day 7, Day 14, Day 21, and Day 28). Behavioral assessments were accompanied by Western blot analysis of GluA1, a subunit of the AMPA receptor involved in excitatory neurotransmission, and SK channels, which regulate calcium-activated potassium currents. β-actin served as the internal loading control to ensure accurate protein quantification.

Across all behavioral parameters, consistent and statistically significant differences were observed between the Vehicle Control and Negative Control groups (***$p < 0.001$). The Vehicle Control group provided a stable baseline, while the Negative Control reflected the impact of experimental stress or alcohol exposure, thereby validating the model. These persistent differences throughout the study period confirm that the experimental conditions exerted strong and progressive physiological effects.

Marked differences were also observed between the Negative Control and the treatment groups including Alcohol + Diazepam 1 mg/kg, Alcohol + THMQ 20 mg/kg, Alcohol + THMQ 40 mg/kg, and Alcohol + DVPX 30 mg/kg (### $p < 0.001$) demonstrating that each pharmacological intervention significantly altered behavioral outcomes. Of particular importance were the highly significant distinctions between Alcohol + Diazepam and Alcohol + THMQ treatments (^^^ $p < 0.001$), reflecting the differing pharmacological actions of Diazepam, a GABAergic modulator, and THMQ, which likely exerts neuroprotective or neuromodulatory effects.

A strong interaction was also evident in the Alcohol + Diazepam + DVPX group ($$$ $p < 0.001$), suggesting a synergistic influence when DVPX was combined with Diazepam. This highlights the complexity of multi-drug interactions and supports the need for further investigation into their combined pharmacodynamics.

By Day 28, the separation between Vehicle Control and Negative Control remained significant (***$p < 0.001$), demonstrating that treatment effects were not only immediate but cumulative over time. These findings underscore the value of

longitudinal assessment for understanding the temporal progression of drug-induced behavioral changes.

Despite these robust behavioral outcomes, Western blot analysis did not reveal significant alterations in GluA1 or SK protein expression across treatment groups. This indicates that the behavioral differences observed may not be mediated through changes in these specific synaptic proteins. Instead, the treatments may act through other neurotransmitter systems such as GABAergic, dopaminergic, or serotonergic pathways or through intracellular signaling cascades that were not evaluated in the present study. It is also possible that molecular changes occur at time points not captured by the sampling schedule or involve protein families beyond GluA1 and SK.

Overall, the findings support the conclusion that Alcohol in combination with Diazepam, THMQ, or DVPX produces pronounced behavioral alterations through mechanisms that may extend beyond GluA1 or SK channel modulation. These results highlight the complexity of pharmacological interactions and emphasize the need for broader molecular analyses, including the evaluation of additional biomarkers and signaling pathways. Continued research in this direction may help clarify the mechanisms through which these compounds influence brain function and contribute to their potential therapeutic or clinical applications.

Reference

Addolorato, G., Leggio, L., Hopf, F. W., Diana, M., & Bonci, A. (2012). Novel therapeutic strategies for alcohol and drug addiction: Focus on GABA, ion channels and transcranial magnetic stimulation. In *Neuropsychopharmacology* (Vol. 37, Issue 1, pp. 163–177). https://doi.org/10.1038/npp.2011.216

Alosetron, •, Dolasetron, •, Granisetron, •, Ondansetron, •, Palonosetron, •, Tropisetron, •, Almotriptan, •, Eletriptan, •, Frovatriptan, •, Naratriptan, •, Rizatriptan, •, Sumatriptan, •, Zolmitriptan, •, Glennon A N D, R. A., & Dukat, M. (n.d.). *Antiemetic drugs (5-HT 3 receptor antagonists) Drugs for the treatment of migraine (5-HT 1D/1F receptor agonists) Serotonin Receptors and Drugs Affecting Serotonergic Neurotransmission*.

Amato, L., Minozzi, S., & Davoli, M. (2011). Efficacy and safety of pharmacological interventions for the treatment of the Alcohol Withdrawal Syndrome. *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.cd008537.pub2

Anton, R. F. (1999). *What Is Craving? Models and Implications for Treatment* (Vol. 23, Issue 3).

Aquib, M., Najmi, A. K., & Akhtar, M. (2014). Antidepressant Effect of Thymoquinone in Animal Models of Depression. *Drug Research*, *65*(9), 490–494. https://doi.org/10.1055/s-0034-1389920

Arafa, E. S. A., Zhu, Q., Shah, Z. I., Wani, G., Barakat, B. M., Racoma, I., El-Mahdy, M. A., & Wani, A. A. (2011). Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *706*(1–2), 28–35. https://doi.org/10.1016/j.mrfmmm.2010.10.007

Arentsen, T., Raith, H., Qian, Y., Forssberg, H., & Heijtz, R. D. (2015). Host microbiota modulates development of social preference in mice. *Microbial Ecology in Health & Disease*, *26*(0). https://doi.org/10.3402/mehd.v26.29719

Aron-Wisnewsky, J., & Clément, K. (2016). The gut microbiome, diet, and links to cardiometabolic and chronic disorders. *Nature Reviews*

Nephrology, *12*(3), 169–181. https://doi.org/10.1038/nrneph.2015.191

Bansal, P., & Banerjee, S. (2016). Effect of Withinia Somnifera and Shilajit on Alcohol Addiction in Mice. *Pharmacognosy Magazine*, *12*(Suppl 2), S121. https://doi.org/10.4103/0973-1296.182170

Becker, H. C., & Mulholland, P. J. (2014). Neurochemical mechanisms of alcohol withdrawal. In *Handbook of Clinical Neurology* (Vol. 125, pp. 133–156). Elsevier B.V. https://doi.org/10.1016/B978-0-444-62619-6.00009-4

Belmer, A., Patkar, O. L., Lanoue, V., & Bartlett, S. E. (2018). 5-HT1A receptor-dependent modulation of emotional and neurogenic deficits elicited by prolonged consumption of alcohol. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-20504-z

Bleich, S., & Degner, D. (2000). Reversal of ethanol-induced hepatic steatosis and lipid peroxidation by taurine: A study in rats. *Alcohol and Alcoholism*, *35*(2), 215. https://doi.org/10.1093/alcalc/35.2.215

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., & Renzi, P. (2002). Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behavioural Brain Research*, *134*(1–2), 49–57. https://doi.org/10.1016/S0166-4328(01)00452-1

Deal, A., Cooper, N., Kirse, H. A., Uneri, A., Raab-Graham, K., Weiner, J. L., & Solberg Woods, L. C. (2021). Early life stress induces hyperactivity but not increased anxiety-like behavior or ethanol drinking in outbred heterogeneous stock rats. *Alcohol*, *91*, 41–51. https://doi.org/10.1016/j.alcohol.2020.11.007

Fahmy, H. M., Khardrawy, Y. A., Abd-El Daim, T. M., Elfeky, A. S., Abd Rabo, A. A., Mustafa, A. B., & Mostafa, I. T. (2020). Thymoquinone-encapsulated chitosan nanoparticles coated with polysorbate 80 as a novel treatment agent in a reserpine-induced depression animal model. *Physiology and Behavior*, *222*(March), 112934. https://doi.org/10.1016/j.physbeh.2020.112934

Farkhondeh, T., Samarghandian, S., Shahri, A. M. P., & Samini, F. (2018). The neuroprotective effects of thymoquinone: A review. In *Dose-*

Response (Vol. 16, Issue 2). SAGE Publications Inc. https://doi.org/10.1177/1559325818761455

Firdaus, F., Zafeer, M. F., Ahmad, M., & Afzal, M. (2018). Anxiolytic and anti-inflammatory role of thymoquinone in arsenic-induced hippocampal toxicity in Wistar rats. *Heliyon*, *4*(6), e00650. https://doi.org/10.1016/j.heliyon.2018.e00650

Goyal, S. N., Prajapati, C. P., Gore, P. R., Patil, C. R., Mahajan, U. B., Sharma, C., Talla, S. P., & Ojha, S. K. (2017). Therapeutic potential and pharmaceutical development of thymoquinone: A multitargeted molecule of natural origin. In *Frontiers in Pharmacology* (Vol. 8, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fphar.2017.00656

Hamdan, A. M., Al-Gayyar, M. M., Shams, M. E. E., Alshaman, U. S., Prabahar, K., Bagalagel, A., Diri, R., Noor, A. O., & Almasri, D. (2019). Thymoquinone therapy remediates elevated brain tissue inflammatory mediators induced by chronic administration of food preservatives. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-43568-x

Haque, I. M., Mishra, A., Kalra, B. S., & Chawla, S. (2021). Role of standardized plant extracts in controlling alcohol withdrawal syndrome—an experimental study. *Brain Sciences*, *11*(7). https://doi.org/10.3390/brainsci11070919

Hedrich, H. J., & Bullock, G. R. (2004). *The laboratory mouse*. Elsevier Academic Press.

Heinz, A., Löber, S., Georgi, A., Wrase, J., Hermann, D., Rey, E. R., Wellek, S., & Mann, K. (2003). Reward craving and withdrawal relief craving: Assessment of different motivational pathways to alcohol intake. *Alcohol and Alcoholism*, *38*(1), 35–39. https://doi.org/10.1093/alcalc/agg005

Hinton, D. J., Lee, M. R., Jacobson, T. L., Mishra, P. K., Frye, M. A., Mrazek, D. A., Macura, S. I., & Choi, D. S. (2012). Ethanol withdrawal-induced brain metabolites and the pharmacological effects of acamprosate in mice lacking ENT1. *Neuropharmacology*, *62*(8), 2480–2488. https://doi.org/10.1016/j.neuropharm.2012.02.022

Hirani, K., Sharma, A. N., Jain, N. S., Ugale, R. R., & Chopde, C. T. (2005). Evaluation of GABAergic neuroactive steroid 3α-hydroxy-5α-pregnane-20-one as a neurobiological substrate for the anti-anxiety effect of ethanol in rats. *Psychopharmacology*, *180*(2), 267–278. https://doi.org/10.1007/s00213-005-2169-7

Jakaria, M., Cho, D. Y., Haque, M. E., Karthivashan, G., Kim, I. S., Ganesan, P., & Choi, D. K. (2018). Neuropharmacological potential and delivery prospects of thymoquinone for neurological disorders. *Oxidative Medicine and Cellular Longevity*, *2018*. https://doi.org/10.1155/2018/1209801

Jung, M. E., & Metzger, D. B. (2010). Alcohol withdrawal and brain injuries: Beyond classical mechanisms. In *Molecules* (Vol. 15, Issue 7, pp. 4984–5011). https://doi.org/10.3390/molecules15074984

Kayir, H., & Uzbay, T. (2008). Effects of clozapine on ethanol withdrawal syndrome in rats. *Alcohol and Alcoholism*, *43*(6), 619–625. https://doi.org/10.1093/alcalc/agn052

Kurhe, Y. V., Radhakrishnan, M., Thangaraj, D., & Gupta, D. (2014). Anti-anxiety effect of a novel 5-HT3 receptor antagonist N-(benzo[d]thiazol-2-yl)-3-ethoxyquinoxalin-2-carboxamide (6k) using battery tests for anxiety in mice. *Indian Journal of Pharmacology*, *46*(1), 100–104. https://doi.org/10.4103/0253-7613.125186

Lucia, T. C. A., Jesus, H. R. J., Carolina, Z. Z., Carlos, P. M., & Alberto, S. G. (2018). Antidepressant and antipsychotic-like activity of the ethanolic extract of the leaves of Maytenus macrocarpa. *Pharmacognosy Journal*, *10*(6), S33–S37. https://doi.org/10.5530/pj.2018.6s.6

Mansour, M. A., Nagi, M. N., El-Khatib, A. S., & Al-Bekairi, A. M. (2002). Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and dt-diaphorase in different tissues of mice: A possible mechanism of action. *Cell Biochemistry and Function*, *20*(2), 143–151. https://doi.org/10.1002/cbf.968

Mendelson, W. B., Majchrowicz, / Edward, Mirmirani, N., Dawson, S., Gillin, J. C., & Wyatt, R. J. (1978). Sleep During Chronic Ethanol Administration and Withdrawal in Rats. In *Journal o! Studies on Alcohol*

(Vol. 39, Issue 7).

Overstreet, D. H., Knapp, D. J., Moy, S. S., & Breese, G. R. (2003). A 5-HT1A agonist and a 5-HT2C antagonist reduce social interaction deficit induced by multiple ethanol withdrawals in rats. *Psychopharmacology*, *167*(4), 344–352. https://doi.org/10.1007/s00213-003-1425-y

Owens, M. J., & Nemeroff, C. B. (2003). Pharmacology of valproate. *Psychopharmacology Bulletin*, *37 Suppl 2*, 17–24.

Panula, P. (2020). Histamine, histamine H3 receptor, and alcohol use disorder. *British Journal of Pharmacology*, *177*(3), 634–641. https://doi.org/10.1111/bph.14634

Pineau, S., Legros, C., & Mattei, C. (2016). The Medical use of Lemon Balm (Melissa officinalis) and Valerian (Valeriana officinalis) as Natural Sedatives: Insight into their Interactions with GABA Transmission. *International Journal of Clinical Pharmacology & Pharmacotherapy*, *1*(2), 1–8. https://doi.org/10.15344/2456-3501/2016/112

Ruby, B., Benson, M. K., Kumar, E. P., Sudha, S., & Wilking, J. E. (2012). Evaluation of Ashwagandha in alcohol withdrawal syndrome. *Asian Pacific Journal of Tropical Disease*, *2*(SUPPL2), 3–7. https://doi.org/10.1016/S2222-1808(12)60279-5

Ruby B, Mk, B., Ep, K., Sudha S, & Je, W. (2012). Evaluation of Ashwagandha in alcohol withdrawal syndrome Asian Pacific Journal of Tropical Disease. In *Asian Pacific Journal of Tropical Disease*. www.elsevier.com/locate/apjtd

Saitz, R. (1998). *Introduction to Alcohol Withdrawal* (Vol. 22, Issue 1).

Sharma, R., Sharma, A., Sahota, P., & Thakkar, M. M. (2020). Orexin gene expression is downregulated in alcohol dependent rats during acute alcohol withdrawal. *Neuroscience Letters*, *739*. https://doi.org/10.1016/j.neulet.2020.135347

Shieh, K. R., & Yang, S. C. (2020). Formosan wood mice (Apodemus semotus) exhibit more exploratory behaviors and central dopaminergic activities than C57BL/6 mice in the open field test. *Chinese Journal of Physiology*, *63*(1), 27–34. https://doi.org/10.4103/CJP.CJP_47_19

Smith, C. M. (1977). The Pharmacology of Sedative/Hypnotics, Alcohol,

and Anesthetics: Sites and Mechanisms of Action. *Drug Addiction I*, 413–587. https://doi.org/10.1007/978-3-642-66612-4_8

Srinivasababu, N., Kumar, S., & Vijaya Kumar Reddy, K. (2014). An experimental observation of disparity in mechanical properties of turmeric fiber reinforced polyester composites. *Biomaterial Applications: Micro to Nanoscales*, 123–134. https://doi.org/10.1201/b17860

Tabassum, S., Rosli, N., Ichwan, S. J. A., & Mishra, P. (2021). Thymoquinone and its pharmacological perspective: A review. *Pharmacological Research - Modern Chinese Medicine*, *1*(November), 100020. https://doi.org/10.1016/j.prmcm.2021.100020

Tunstall, B. J., Lorrai, I., McConnell, S. A., Gazo, K. L., Zallar, L. J., De Guglielmo, G., Hoang, I., Haass-Koffler, C. L., Repunte-Canonigo, V., Koob, G. F., Vendruscolo, L. F., & Sanna, P. P. (2019). Probenecid Reduces Alcohol Drinking in Rodents. Is Pannexin1 a Novel Therapeutic Target for Alcohol Use Disorder? *Alcohol and Alcoholism*, *54*(5), 497–502. https://doi.org/10.1093/alcalc/agz054

Woo, C. C., Kumar, A. P., Sethi, G., & Tan, K. H. B. (2012). Thymoquinone: Potential cure for inflammatory disorders and cancer. In *Biochemical Pharmacology* (Vol. 83, Issue 4, pp. 443–451). Elsevier Inc. https://doi.org/10.1016/j.bcp.2011.09.029

Xiao, H. wen, Ge, C., Feng, G. xing, Li, Y., Luo, D., Dong, J. li, Li, H., Wang, H., Cui, M., & Fan, S. jun. (2018). Gut microbiota modulates alcohol withdrawal-induced anxiety in mice. *Toxicology Letters*, *287*, 23–30. https://doi.org/10.1016/j.toxlet.2018.01.021

Xue, M., Teng, X., Liang, H., Zhao, J., Jiang, Y., Qiu, X., Zhang, Z., Pei, Z., Zhang, N., & Qin, Y. (2021). Neuroprotective effect of fucoidan by regulating gut-microbiota-brain axis in alcohol withdrawal mice. *Journal of Functional Foods*, *86*. https://doi.org/10.1016/j.jff.2021.104726

Yan, Y., Jiang, W., Spinetti, T., Tardivel, A., Castillo, R., Bourquin, C., Guarda, G., Tian, Z., Tschopp, J., & Zhou, R. (2013). Omega-3 fatty acids prevent inflammation and metabolic disorder through inhibition of NLRP3 inflammasome activation. *Immunity*, *38*(6), 1154–1163. https://doi.org/10.1016/J.IMMUNI.2013.05.015

Ind. J. Pharm. Edu. Res., 2025; 59(1):101-111.
https://www.ijper.org

Original Article

Assessing the Pharmacological Potential of Thymoquinone for Managing Alcohol Craving and Withdrawal Syndrome in Mice

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ABSTRACT

Aim/Background: The aim is to evaluate THMQ's impact on alcohol withdrawal and alcohol craving using an animal model. Mice were exposed to increasing ethanol doses to simulate dependency. Researchers assessed anxiety levels during withdrawal using behavioural and biochemical tests. THMQ was administered orally at 20 mg/kg and 40 mg/kg, compared to diazepam (1 mg/kg). **Materials and Methods:** Model Creation Mice received escalating ethanol doses (5% to 35% v/v) over specific days. Regular water was given during withdrawal periods. Assessment Tools Elevated Plus Maze (EPM), Elevated Zero Maze (EZM), Open Field Test (OFT), and Western Blot analysis. Treatment Intervention: THMQ (20 mg/kg and 40 mg/kg) vs. diazepam (1 mg/kg). **Results and Conclusion:** THMQ-treated group showed reduced anxiety and alcohol desire. Alcohol consumption decreased in THMQ-treated mice. No changes in GluA1 and sk2 protein levels. THMQ holds promise as a remedy for alcohol cravings and withdrawal symptoms

Keywords: Alcohol Craving, Alcohol Withdrawal, Thymoquinone, Western blot, Open Field Test.

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Received: 27-06-2024;
Revised: 22-08-2024;
Accepted: 14-11-2024.

INTRODUCTION

Alcohol is considered the most widely abused substances globally due to its propensity for inducing tolerance and dependency with extended usage, observed in both animals and humans. For the context herein, "alcohol" pertains specifically to ethanol (C_2H_5OH), as it is the predominant type employed in the concoction of alcoholic beverages.[1,2] Alcohols emit an aroma often delineated as "pungent" and "persistent" in the nasal cavity.[3]

Alcohol dependence denotes a state of substance misuse wherein an individual develops a tolerance to alcohol and persists in consumption despite adverse outcomes. For a diagnosis of alcohol dependence, a minimum of three out of seven specified symptoms must manifest consistently over a duration of at least one year.[4] These symptoms encompass consuming more alcohol than intended, experiencing prolonged intoxication beyond anticipated durations, attempting unsuccessfully to curtail consumption, dedicating significant time to obtaining alcohol or recovering from its effects, and diminishing engagement in social, professional, and leisure pursuits as a consequence of excessive alcohol usage.[5]

The alcohol withdrawal syndrome encompasses a constellation of distressing physical and psychological manifestations that may arise when an individual abruptly discontinues alcohol consumption. Symptoms can vary from subtle tremors to severe manifestations like hallucinations and seizures. Repeated episodes of withdrawal can contribute to the development of alcoholism and lead to adverse health ramifications. A deeper understanding of the genetic and physiological underpinnings of alcoholism holds promise for the advancement of efficacious treatments targeting withdrawal and other facets of the disorder.[6,7] Withdrawal, a pivotal stage in the neural modulation cycle induced by alcohol, is a tangible phenomenon. Evidence suggests that acute alcohol intake can perturb the release of neurotransmitters and disrupt the functioning of neuronal membrane proteins, including receptor proteins binding neurotransmitters and ion channels facilitating ion influx (e.g., sodium or calcium.[8] Over time, the brain acclimates to alcohol, thereby diminishing its once-disruptive effects a phenomenon known as tolerance. Prolonged heavy drinking may potentially alter the structure and function of brain neurons to the extent that they require alcohol to maintain normal function, a condition termed physical dependence. Abrupt cessation of alcohol intake among heavy drinkers may precipitate rebound hyperexcitability, colloquially referred to as

DOI: 10.5530/ijper.20250194

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Date of Issue : 19-10-2024
Place : Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

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