J Herbmed Pharmacol. 2024; 13(4): 572-586.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2024.51455

Journal of Herbmed Pharmacology

Anxiolytic, memory improvement, hepatoprotective, and antioxidant effects of *Thymus vulgaris* on alcohol withdrawal syndrome in mice

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 9 February 2024
Accepted: 14 August 2024
epublished: 1 October 2024

Keywords:
Alcohol
Anxiety
Antioxidant
Memory
Liver
Thymus vulgaris

ABSTRACT

Introduction: *Thymus vulgaris* is a plant used in Cameroon to alleviate and treat hangover symptoms. This study was conducted to assess the anxiolytic, memory-improving, hepatoprotective, and antioxidant effects of *T. vulgaris* on alcohol withdrawal syndrome.
Methods: In this preclinical study 42 mice were grouped into seven equal sets of normal control, negative control, positive control (piracetam; 200 mg/kg), and test (*T. vulgaris*; 25, 50, 100, or 200 mg/kg) groups. All mice (except those of the normal control) were administered ethanol by gavage once daily for 28 days. After alcohol withdrawal, behavioral assessments (elevated plus maze, Y-maze, and open field) were performed from day 29 to day 31, one hour following appropriate treatments. Mice were euthanized on day 32 and the brains and the livers were used for histopathological assessment and oxidative stress parameters evaluation.
Results: In the elevated plus maze test, *T. vulgaris* at 100 and 200 mg/kg significantly ($P < 0.001$) increased the number of entries and the time spent in the open arms. The plant extract (200 mg/kg) significantly ($P < 0.001$) increased the spontaneous alternation percentage in the Y maze. *T. vulgaris* administration decreased malondialdehyde (MDA) and nitric oxide (NO) in the brain and liver and increased glutathione (GSH) and catalase (CAT). The plant extract reduced the neuronal degeneration and hepatocyte damage induced by chronic alcohol administration.
Conclusion: *T. vulgaris* had anxiolytic, memory-enhancing, and hepatoprotective properties against alcohol toxicity. These results may justify the use of thyme in traditional pharmacopeia in the management of alcohol use disorders (AUDs).

Implication for health policy/practice/research/medical education:
The aqueous extract of *Thymus vulgaris* protects the brain and liver against free radicals and oxidative damage due to chronic ethanol intake. Therefore, *T. vulgaris* might be a good alternative treatment for the management of some aspects of human alcoholism.
Please cite this paper as: Kouémou NE, Savo FM, Pale S, Noubissi AP, Dongmo SMN, Taiwe GS, Ngo EM. Anxiolytic, memory improvement, hepatoprotective, and antioxidant effects of *Thymus vulgaris* on alcohol withdrawal syndrome in mice. J Herbmed Pharmacol. 2024;13(4):572-586. doi: 10.34172/jhp.2024.51455.

Introduction

Alcohol use disorders (AUDs) are among the leading causes of mortality worldwide (1,2). More than 76.3 million people in the world suffer from AUDs, which, accounts for more than 1.8 million deaths each year (3). Alcohol withdrawal syndrome is a life-threatening condition that usually begins within 6 to 24 hours of initial reduction or total cessation of heavy drinking (4-6). The symptoms of alcohol withdrawal, which range from mild to severe, mainly include nausea/vomiting, hypertension, hyperthermia, tachycardia, anxiety, seizures, delirium tremens, and learning and memory deficits (3,6-8). Alcohol withdrawal plays a pivotal role in the establishment of alcohol addiction, thus making treatments of AUDs a greater challenge (9). The reason is that many patients self-administer ethanol to relieve

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the symptoms and discomfort associated with alcohol withdrawal (10).

Both glutamate and gamma amino butyric acid (GABA) neurotransmissions are involved in alcohol withdrawal's pathophysiology (6,7). Acute alcohol intake leads to central nervous system depression by increasing GABAergic neurotransmission and decreasing glutamatergic activity (11). After heavy and prolonged alcohol intake, there is a neuroadaptation in the brain, leading to a downregulation of GABA A receptors and an upregulation of glutamate (N-methyl-D aspartate) receptors (6). Thus, the sudden cessation of ethanol intake causes an imbalance originating from the acute reduction in GABA neurotransmission and an increase in glutamatergic activity. This imbalance leads to hyperactivity of the autonomic system and the development of ethanol withdrawal syndrome (12). The hyperactivation of the N-methyl-D aspartate receptors of glutamate resulting from alcohol withdrawal subsequently favors an increase in brain oxidative stress and neuroinflammation (13,14). This neurotoxicity, observed in different brain regions, leads to anxiety and memory impairment frequently observed during ethanol withdrawal (10,15,16).

The liver is the primary organ of alcohol metabolism and has a key role in detoxifying the body (17). The main mechanism by which alcohol leads to liver disease is through the increase of oxidative stress (18,19). Chronic or acute alcohol intake can lead to liver damage and alcohol liver disease (20). The damages caused by chronic alcohol intake to the liver may persist even after the termination of ethanol intake (21).

Currently, the most important medications used for the management of alcohol withdrawal are benzodiazepines. Benzodiazepines act on GABA A receptors and replace the inhibiting effect that has been lost during alcohol abstinence (6,22-24). In addition to their limited efficacy and high costs, the available drug therapies for the treatment of alcohol withdrawal have many side effects, including the risk of dependence and over-sedation (3). Thus, seeking new therapeutic sources involving different therapeutic pathways, such as those involved in oxidative stress, is crucial (14).

Numerous medicinal herbs, plant extracts, and plant molecules have shown their beneficial effects in the management of AUDs due to their potential to mitigate alcohol withdrawal-associated oxidative stress (10,11,14,16,25). *Thymus vulgaris* Linn, commonly called Thyme, is an odoriferous medicinal plant belonging to the Lamiaceae family. Thyme has a worldwide distribution (26,27) and has been intensively used in folk medicine to treat wounds, respiratory diseases, skin diseases, rheumatism, and body pains (27-29). Pharmacological studies of thyme have proven that it has sedative, antimicrobial, antiviral, antibacterial, antiseptic, anthelmintic expectorant, antifungal, anti-inflammatory, antioxidant, and anxiolytic effects (29-34). *T. vulgaris* leaves have phenolic compounds, including thymol, carvacrol, linalool, and camphor (34-37). The antioxidative activity of the plant is mainly attributed to carvacrol and thymol (27,37,38). Thyme is used in folk medicine to stimulate memory and treat gums, dental pain, and mouth inflammation (34,38). Thyme also works as a stomach alert, prevents fermentation, helps digestion and nutrient absorption, expels fungi from the stomach and intestines, and increases appetite (28). In Cameroon, *T. vulgaris* is generally cultivated in home gardens. Thyme is used by the local population to relieve symptoms associated with hangovers (39). Therefore, this study assessed the anxiolytic, memory improvement, hepatoprotective, and antioxidant effects of *T. vulgaris* on alcohol withdrawal syndrome in mice.

Materials and Methods

Plant material and extract preparation

Thymus vulgaris vulgaris dried leaves were bought from a neighboring market in Buea (South-West region, Cameroon) in April 2020. Professor George Chuyong identified species in the Department of Plant Science of the University of Buea (Cameroon). A plant specimen was registered under 25746/SRF/Cam at Cameroon's plant repository. *T. vulgaris* dried leaves were crushed into fine powder using an electric blender. With an electrical scale, 100 g of the dry powder was weighed, added into 1000 mL of boiled water, and left for 24 hours. After 24 hours, a Whatman No. 1 filter paper was plugged into a funnel and used to filter the solution. The filtrate obtained was freeze-dried by lyophilization. The weight of the brown lyophilized *T. vulgaris* was 8.4 g (yield = 8.4% w/w). The extract was freshly prepared each day before its administration to mice. The plant extract was administered in mice by gavage (10 mL/kg). The following doses of the extract were used: 25, 50, 100 and 200 mg/kg (27,30,40).

Animals and ethical considerations

Two-month-old male Swiss mice (n=42) weighing 20-24 g were used in this study. They were from the animal house of the Faculty of Science of the University of Buea. The mice were humanely treated throughout the experiments. Mice were habituated to laboratory conditions one week before experimentations. The study protocol received consent from the University of Buea's ethical committee (UB-IACUC N°: 09/2020). The guidelines of the American Veterinary Association guide for the euthanasia of animals were followed in this experiment.

Drugs and chemicals

Ellman reagent, thiobarbituric acid, Trichloroacetic acid, phosphoric acid, naphthyl ethylenediamine, and sulphanilamide were obtained from Sigma Aldrich

(Germany). Piracetam was purchased from UCB Brussels (Belgium). Ethanol 95% and hydrogen peroxide were bought from a local pharmacy in Buea (Cameroon).

Animal grouping and experimental design

Forty-two young adult male mice were divided into 7 groups of 6 animals each, as presented in Table 1.

A mouse model of alcohol withdrawal was produced with little modification (41). Thus, ethanol (95%) was diluted in distilled water and administered orally to all the animals except those of the normal control, which received only distilled water. From the first week, mice in the alcohol groups received 10 mL/kg of 5% (v/v) ethanol by gavage once every 24 hours. During the second week, they received 10% ethanol by gavage, 20% ethanol the third week, and 35% ethanol the fourth week. Additionally, 5% alcohol was added to their drinking water, and this was made available to them *ad libitum* throughout alcohol administration. During the withdrawal period (days 29-31), mice in the alcohol groups were treated one hour before the behavioural tests with different doses of *T. vulgaris* (25, 50, 100, or 200 mg/kg), while the positive control received piracetam (200 mg/kg) (Figure 1) and the negative control received distilled water (10 mL/kg). The mice were sacrificed on day 32, and part of the liver and the whole brain were used for biochemical assessments.

Behavioural evaluation

All behavioural tests were performed after ethanol withdrawal between day 29 to day 31. Anxiety was evaluated using the elevated plus maze and open field paradigm, while the Y maze was used to assess spatial short-term memory.

Elevated plus maze (EPM) test

A locally made EPM with two open opposing branches (16 cm × 5 cm) and two closed opposing branches (16 cm × 5 cm × 10 cm) raised 50 cm above the ground was used to evaluate anxiety in mice 24 hours after alcohol withdrawal. Each mouse was placed at the center of the EPM opposite facing the direction of the open. The total exploration time for each animal was set at 5 minutes (42). During this period, the number of entries and the time spent in the branches of the EPM were registered by a well-trained researcher blind to the treatments (43,44). After each recording, the apparatus was thoroughly cleaned with 1% acetic acid.

Y-maze test

On day 30 of experimentation, a locally fabricated Y-maze apparatus was used to assess *T. vulgaris*'s effect on spatial

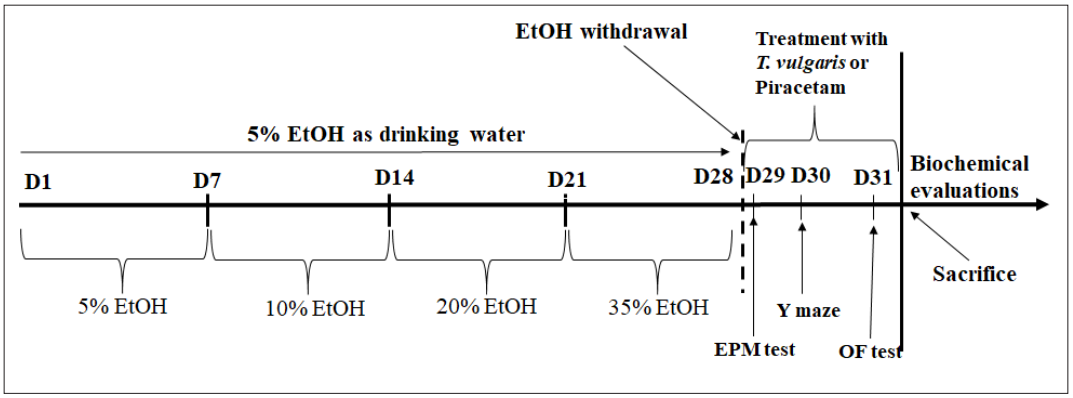

Figure 1. Overview of the experimental procedure. Ethanol was administered for 28 days followed by a three-day withdrawal period. *Thymus vulgaris* (25, 50, 100, or 200 mg/kg) or piracetam (200 mg/kg) was administered once daily before behavioural assessment. EPM: Elevated Plus Maze; OF: Open field.

Table 1. Animal grouping

Groups	Treatments/Doses
Normal control (DW + DW)	10 mL/kg of distilled water + distilled water as drinking water + 10 mL/kg of distilled water (gavage)
Negative control (ALC + DW)	10 mL/kg Alcohol (5%; 10%; 20% and 35%) + 5% alcohol in their drinking water + 10 mL/kg of water (gavage)
Test group I (ALC + Tv25)	10 mL/kg Alcohol (5%; 10%; 20% and 35%) + 5% alcohol in their drinking water + 25 mg/kg of *T. vulgaris* (gavage)
Test group II (ALC + Tv50)	10 mL/kg Alcohol (5%; 10%; 20% and 35%) + 5% alcohol in their drinking water + 50 mg/kg of *T. vulgaris* (gavage)
Test group III (ALC + Tv100)	10 mL/kg Alcohol (5%; 10%; 20% and 35%) + 5% alcohol in their drinking water + 100 mg/kg of *T. vulgaris* (gavage)
Test group IV (ALC + Tv200)	10 mL/kg Alcohol (5%; 10%; 20% and 35%) + 5% alcohol in their drinking water + 200 mg/kg of *T. vulgaris* (gavage)
Positive control (ALC + PIR 200)	10 mL/kg Alcohol (5%; 10%; 20% and 35%) + 5% alcohol in their drinking water + piracetam (200 mg/kg) (gavage)

ALC: Alcohol; DW: Distilled water (10 mL/kg); PIR200: Piracetam (200 mg/kg); Tv25, Tv50, Tv100, and Tv200: *Thymus vulgaris* aqueous extract at the doses 25, 50, 100 and 200 mg/kg, respectively.

short-term memory of ethanol-withdrawal animals (Figure 1) (45). The maze used was made of plywood and consisted of a Y-shaped paradigm, having three (3) identical arms. Each arm has the following dimensions: length of 35 cm, height of 8 cm, and width of 15 cm. The distance between the two arms of the maze was 120°. Letters A, B, and C were used to differentiate the three arms of the maze (45-47). One hour after treatment and 48 hours after alcohol withdrawal, each mouse naive to the paradigm was positioned in one of the arms for free exploration. During each 8-minute session, the arms entries and spontaneous alternations behaviors were registered for each animal. Spontaneous alternation corresponds to sequential entrance into three arms in one order: BCA, ABC, or CAB (45-47). After each recording, the apparatus was entirely cleaned with 1% acetic acid. Spontaneous alternation percentage was calculated using the following formula:

$$\frac{Number\ of\ alternation}{Total\ arms\ entries - 2} \times 100$$

Open field test

The open field (OF) apparatus used in this work had the following dimensions: 40 cm in length, 40 cm in width, and 45 cm in height. The floor of the open field was further divided into 16 smaller squares (10 cm length × 10 cm width). Seventy-two hours (72 hours) after alcohol withdrawal and 1 hour after different treatments, the animals were observed for 5 minutes to evaluate the effects of *T. vulgaris* on both exploration and anxiety (48,49). Hand-operated counters and stopwatches were used to score the number of crossings (number of square floor units entered), rearing (number of times that the animal stood on its hind legs), grooming, and defecation (49,50). Before the passage of the next animal, the maze was fully wiped with 1% acetic acid.

Biochemical evaluations

On day 32, all the animals were euthanized by cervical dislocation; brains and livers were harvested for biochemical estimations and histological studies.

Preparation of organ homogenates

The entire brain and a portion of the liver were weighted and crushed in a mortar containing Tris/HCl buffer. The homogenates (20%) were centrifuged at 10 000 revolutions per minute for 10 minutes. Supernatants were conserved in dried Eppendorf.

Determination of liver and brain nitrite oxide levels

Liver and brain nitrite contents were measured using the Greiss reagent (50,51). An equal volume (0.5 mL) of Greiss reagent and supernatant was pipetted into a test tube. Five minutes later, the absorbance was measured at 540 nm with a spectrophotometer (BK-UV1000 UV/VIS). Nitrite content (mol/g) was estimated using a standard curve for sodium nitrate.

Evaluation of liver and brain malondialdehyde (MDA) levels

Liver and brain MDA levels were estimated using thiobarbituric assay (52). Thiobarbituric acid (0.67%, 0.5 mL) and trichloroacetic acid (20%, 0.25 mL) were added to 0.5 mL of tissue sample in each testing tube. The tubes were heated in a water bath at 90 °C for 1 hour. The tubes were cooled with ice and centrifuged for 10 minutes at 3000 rpm. The absorbance of the stable pink compound obtained was read at 546 nm with a spectrophotometer (BK-UV1000 UV/VIS). Using Beer-Lambert's formula, the MDA levels for each sample were calculated and expressed in µmol/g (53).

Evaluation of liver and brain's reduced glutathione (GSH) levels

The liver and brain levels of GSH were determined using Ellman's reagent (54). Ellman's reagent (1.5 mL) and tissue sample (10 µL) were mixed in a test tube. The tubes were incubated for 1 hour. The absorbance was measured at 405 nm with a spectrophotometer (BK-UV1000 UV/VIS) (43).

Assessment of catalase (CAT) activity in the brain and the liver

An equal volume (125 µL) of tissue homogenate, 0.1 M phosphate buffer (pH 7.4), and 0.5 mL of 30 mM oxygenated water (H_2O_2) were mixed in a test tube. The absorbance was measured with a spectrophotometer (BK-UV1000 UV/VIS) at the wavelength 240 nm after 30, 60, and 90 seconds. CAT activity was expressed in µmol of H_2O_2 per mg of tissue (53).

Histopathological analysis of the brain and the liver

A portion of the liver and the entire brain were isolated and preserved in 10% formalin for two weeks. Coronal sections were made from the brain in the hippocampus region (dentate gyrus, CA1, CA2, and CA3), and a small portion of the liver was equally cut carefully. The procedures for histopathological evaluation were performed as reported by Kouémou et al (46). Tissue samples (brain and liver) were dehydrated in ascending ethanol concentrations (50%, 70%, 95%, and 100%) for 1 hour. After dehydration, the liver and brain tissues were 'cleared' in xylene for 150 minutes. The liver and brain tissues were immersed thrice in paraffin wax (58-60°C) for 4 hours and 30 minutes. The paraffin blocks were then cut into thin sections (~6 µ) using a microtome. The tissues were then spread on hot plates and mounted on glass slides. The tissues were carefully rehydrated in decreasing alcohol solutions (100%, 95%, 70%, and 50%) and stained with hematoxylin and eosin

stain (HE) to differentiate their components. Finally, the slides were observed using a microscope. Pictures of the tissues were taken with the aid of a digital camera.

Statistical analysis

Data are expressed as the means ± standard error of the means (SEM). Statistical disparities between controls and other groups were tested using ANOVA and Tukey's test. At $P \leq 0.05$, differences between treatments were considered significant. All data were analyzed with GraphPad Prism 8.4.3.

Results

Anxiolytic effects of Thymus vulgaris leaf extract on alcohol-withdrawn mice placed in the elevated plus maze

There was no significant difference among treatments concerning the total number of arm entries (Figure 2a).

One day after alcohol withdrawal, there was a significant difference ($P<0.05$) among treatments concerning the percentage of entries and time spent in the open arms of the EPM. The negative control group presented a lower percentage of entry into the open arms than the normal control. The percentage of entries into the open arms dropped from 53.9 ± 3.49% in the normal control to 22.2 ± 3.31% in the negative control. *T. vulgaris* at 50 and 100 mg/kg significantly ($P<0.01$) increased the percentage of entries into the open arms of the maze to 56.7 ± 3.49 and 59.9 ± 2.51%, respectively. Piracetam (200 mg/kg) also increased this percentage to 56.0 ± 4.29% (Figure 2b). The percentage of time spent in the open arms increased significantly from 25.0 ± 2.5% in the negative control to 39.0 ±1.87, 44.50 ± 1.56, and 51.7 ± 1.02 in the *T. vulgaris* groups at doses of 50, 100 and 200 mg/kg, respectively (Figure 2b).

Memory-enhancing effects of *Thymus vulgaris* on alcohol-withdrawn mice placed in the Y-maze

Alcohol withdrawal induced a significant reduction in spontaneous alternation in the Y-maze. Treatment with *T. vulgaris* at 50, 100, and 200 mg/kg significantly ($P<0.01$) reversed the effect of alcohol withdrawal and increased the percentage of spontaneous alternation from 12.90 ±

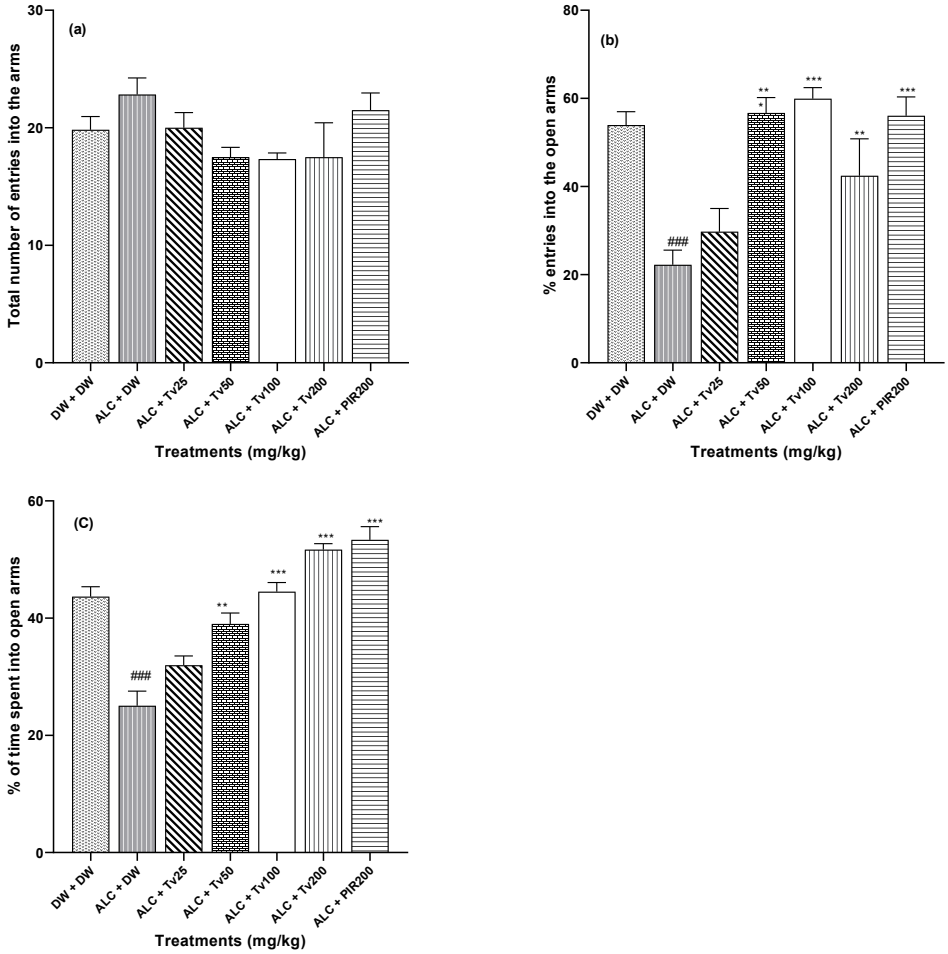

Figure 2. Effects of *Thymus vulgaris* on the number of arms entries, percentage of open arms entries, and percentage of time spent in the open arms on day 29. Results are expressed as the percentage mean ± SEM; (n= six mice). ### $P < 0.001$ compared to DW+DW; ** $P < 0.01$; *** $P < 0.001$ compared to ALC+ DW. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* 25 mg/kg, Tv50: *T. vulgaris* 50 mg/kg, Tv100: *T. vulgaris* 100 mg/kg, Tv200: *T. vulgaris* 200 mg/kg.

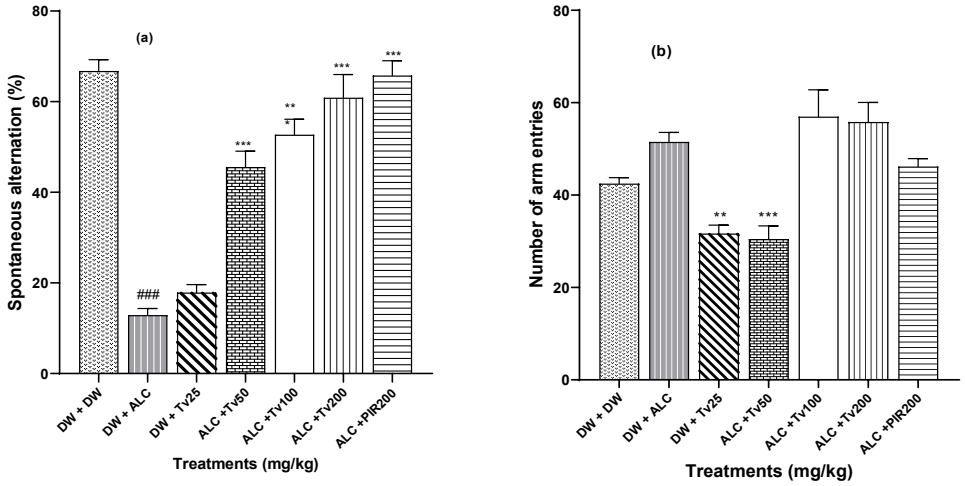

Figure 3. Effect of *Thymus vulgaris* of spontaneous alternation on the percentage (a) and number of arm entries (b) in the Y-maze test. The results are expressed as the percentage mean ± SEM (n= six mice). ### $P < 0.001$ compared to DW+DW; ** $P < 0.01$; *** $P < 0.001$ compared to ALC+ DW. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* 25 mg/kg, Tv50: *T. vulgaris* 50 mg/kg, Tv100: *T. vulgaris* 100 mg/kg, Tv200: *T. vulgaris* 200 mg/kg.

1.47% in the negative control group to 45.60 ± 3.49, 52.00 ± 3.41, and 60.90 ± 5.13.00%, respectively. Piracetam also increased the percentage of spontaneous alternations to 65.80 ± 3.25% ($P<0.001$) (Figure 3a). *T. vulgaris* extract at 25 and 50 mg/kg significantly decreased the locomotion of the alcohol-withdrawn mice. The number of arm entries was reduced from 51.50 ± 2.05 in the negative control to 31.70 ± 1.84 and 30.50 ± 2.81 at 25 and 50 mg/kg doses, respectively (Figure 3b).

Effects of *Thymus vulgaris* on anxiety and locomotion in the open field

Table 2 shows that *T. vulgaris* extract reduced rearing from 25.00 ± 0.58 in the negative control to 15.00 ± 0.86 ($P<0.01$), 12.83 ± 0.70 ($P<0.001$) and 15.17 ± 0.83 ($P<0.01$) in the groups treated with *T. vulgaris* at 25, 50 and 100 mg/kg, respectively. Similarly, piracetam dropped the rearing number to 10.67 ± 1.54 ($P<0.001$).

Crossing numbers were increased in both the plant and piracetam-treated mice. *T. vulgaris* at 25, 50, 100, and 200 mg/kg reversed the effect of alcohol withdrawal and increased the number of crossings from 87.50 ± 1.89 in the negative control to 171.33 ± 2.33 ($P<0.001$), 124.33 ± 1.94 ($P<0.01$) and 128.17 ± 1.45 ($P<0.01$) (Table 2).

Grooming events increased from 2.17 ± 0.70 in the negative control to 3.37 ± 0.70 and 3.53 ± 0.21 in mice treated with *T. vulgaris* at 50 and 200 mg/kg, respectively. The center time was raised from 1.00 ± 0.26 in the negative control to 1.67 ± 0.21 and 2.00 ± 0.52 in the plant group at 100 and 200 mg/kg, respectively.

Effects of *Thymus vulgaris* on nitric oxide (NO) levels in the liver and the brain

As presented in figure 4, ethanol withdrawal increased NO levels in both the liver and brain of animals. Treatment with *T. vulgaris* extract significantly suppressed this effect. NO level in the brain was reduced from 30.91 ± 2.90 mol/g tissue in the negative control to 22.48 ± 1.97 ($P<0.01$), 14.72 ± 1.26 ($P<0.1$), and 18.41 ± 2.27 ($P<0.01$) in the groups which received *T. vulgaris* 50, 100 and 200 mg/kg, respectively. Piracetam significantly reduced the brain's NO level to 8.65 ± 0.92 mol/g (Figure. 4).

Table 2. Effects of *Thymus vulgaris* on open field parameters: rearing, crossing, grooming, and center time

Treatments	Rearing (count)	Line crossing (count)	Grooming (count)	Centre time (s)
DW + DW	6.67 ± 0.67	115.67±2.06	4.00±0.63	2.67±0.67
ALC + DW	25.00 ± 0.58$^{###}$	87.50±1.89$^{##}$	2.17±0.70$^{#}$	1.00±0.26$^{#}$
ALC + Tv25	15.00±0.86**	173.33±1.84***	3.17±0.42*	1.17±0.48
ALC + Tv50	12.83±0.70***	171.33±2.33***	3.37±0.70*	1.17±0.17
ALC + Tv100	15.17±0.83**	124.33±1.94**	3.33±0.42*	1.67±0.21*
ALC + Tv200	20.50 ± 1.09*	128.17 ± 1.45**	3.53±0.21*	2.00±0.52*
ALC + PIR200	10.67±1.54***	124.33±1.76**	3.50 ± 0.33**	2.00±0.26*

Results are expressed as means ± SEM (n= six animals). $^{#}P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$ vs DW + DW. ** $P < 0.01$ and *** $P < 0.001$ vs DW + ALC. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* 25 mg/kg, Tv50: *T. vulgaris* 50 mg/kg, Tv100: *T. vulgaris* 100 mg/kg, Tv200: *T. vulgaris* 200 mg/kg.

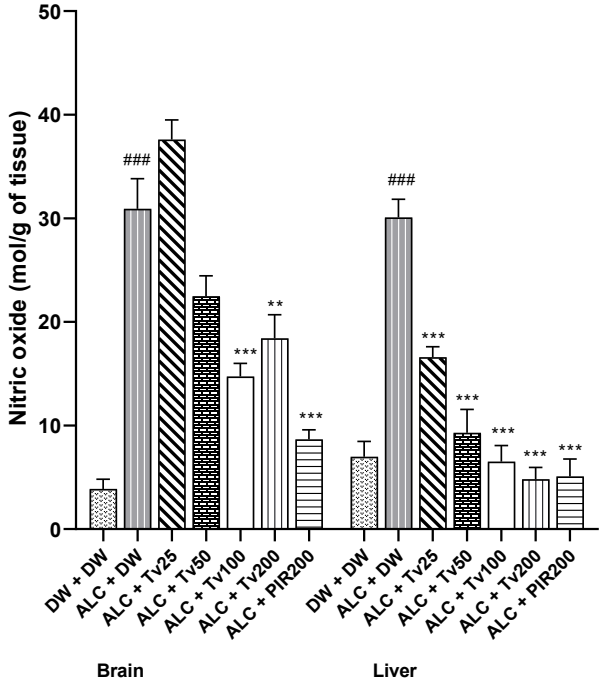

Figure 4. Effects of *Thymus vulgaris* extracts on nitric oxide concentrations in the brain and liver of ethanol-withdrawn mice. Results are expressed as mean ± SEM. (n= six animals). ### $P < 0.001$ vs DW + DW. ** $P < 0.01$ and *** $P < 0.001$ vs DW + ALC. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* (25 mg/kg), Tv50: *T. vulgaris* (50 mg/kg), Tv100: *T. vulgaris* (100 mg/kg), Tv200: *T. vulgaris* (200 mg/kg).

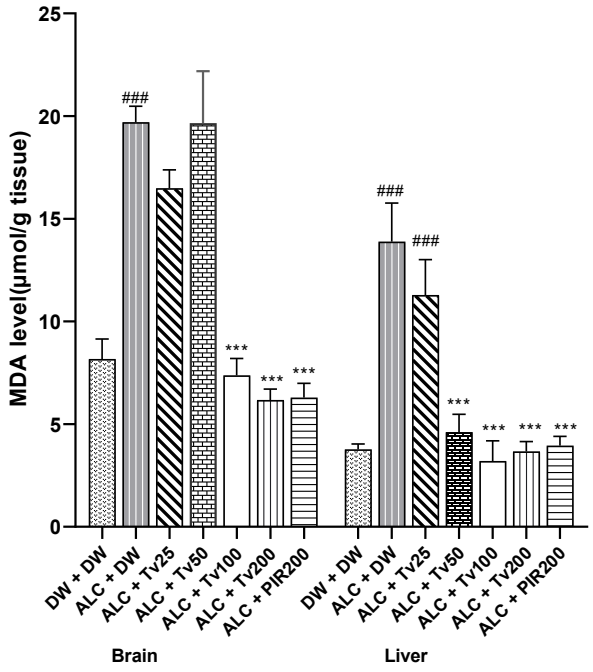

Figure 5. Effects of *Thymus vulgaris* extract on malondialdehyde concentration in the brain and liver of ethanol-withdrawn mice. Results are presented as mean ± SEM (n = six mice). ### $P < 0.001$ vs DW + DW. *** $P < 0.001$ vs DW + ALC. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* 25 mg/kg, Tv50: *T. vulgaris* 50 mg/kg, Tv100: *T. vulgaris* 100 mg/kg, Tv200: *T. vulgaris* 200 mg/kg.

In the liver, NO concentration dropped from 30.07 ± 1.76 mol/g in the negative control to 9.30 ± 2.55 ($P<0.001$), 6.51 ± 1.23 ($P<0.001$), and 4.80 ± 1.15 mol/g tissue ($P<0.001$) in groups treated with 50, 100 and 200 mg/kg of *T. vulgaris*, respectively. NO concentrations also dropped to 5.08 ± 1.60 ($P<0.001$) mol/g tissue in the piracetam group.

Effects of *Thymus vulgaris* extract on MDA levels in the brain and liver

In both the brain and the liver, alcohol withdrawal increased MDA levels. In the brain, MDA increased from 8.17 ± 0.35 µmol/g in the normal control to 19.70 ± 0.77 µmol/g in the negative control. *T. vulgaris* decreased MDA levels to 7.37 ± 0.82 and 6.16 ± 0.53 µmol/g of tissue ($P<0.001$) µmol/g at doses of 100 and 200 mg/kg, respectively. Piracetam significantly ($P<0.001$) reduced MDA levels to 6.29 ± 0.69 µmol/g (Figure 5).

In the liver, MDA levels increased from 3.76 ± 0.26 µmol/g in the normal control to 13.89 ± 1.87 µmol/g in the negative control. *T. vulgaris* extract lowered MDA levels. MDA levels dropped from 13.89 ± 1.87 µmol/g in the negative control to 3.19 ± 0.98 and 3.67 ± 0.47 ($P<0.01$) µmol/g of tissue in the group treated with 100 and 200 mg/kg of *T. vulgaris*, respectively. Piracetam significantly ($P<0.001$) reduced MDA levels to 3.94 ± 0.45 µmol/g (Figure 5).

Effects of *Thymus vulgaris* aqueous extract on the concentration of reduced GSH in the brain and liver of ethanol-withdrawn mice

Figure 6 shows that withdrawal from ethanol resulted in GSH reduction levels in the brain and liver. Brain GSH significantly decreased from 5.33 ± 0.48 in the normal control to 2.16± 0.50 ($P<0.01$) µmol/g in the negative control. The GSH concentration increased to 4.41 ± 0.18 ($P<0.01$) and 4.80 ± 0.48 ($P<0.001$) µmol/g in *T. vulgaris* groups 100 and 200 mg/kg, respectively. Piracetam increased the brain GSH levels to 4.60 ± 0.56 µmol/g.

In the liver, GSH levels decreased from 5.66 ± 0.61 in the normal control to 2.87 ± 0.60 µmol/g ($P<0.01$) in the negative control group. *T. vulgaris* increased the level of GSH in the liver, from 2.87 ± 0.60 µmol/g of tissue in the negative control to 4.61 ± 0.34 ($P<0.01$) and 5.62 ± 0.24 µmol/g of tissue at the doses of 100 and 200 mg/kg, respectively (Figure 6).

Effects of *Thymus vulgaris* extract on CAT activity in the liver and the brain of alcohol-withdrawn mice

The brain's CAT activity decreased from 5.98 ± 0.05 µmol/g in the normal control to 1.87 ± 0.38 µmol/g in the negative control. CAT activity significantly increased to 4.05 ± 0.78 ($P<0.01$) and 5.52 ± 0.52 ($P<0.01$) µmol/g in *T. vulgaris* groups at 100 and 200 mg/kg, respectively.

Piracetam increased CAT activity to 5.56 ± 0.73 ($P < 0.05$) µmol/g (Figure 7).

In the liver, CAT activity significantly decreased from 8.50 ± 0.59 µmol/g in the normal control to 2.88 ± 0.41 µmol/g in the negative control. Interestingly, *T. vulgaris* extract increased CAT activity to 4.38 ± 0.87 ($P < 0.05$) and 6.60 ± 0.59 ($P < 0.01$) µmol/g of tissue at 100 and 200 mg/kg, respectively (Figure 7). Piracetam increased CAT activity to 6.67 ± 0.54 µmol/g (Figure 7).

Effect of *Thymus vulgaris* extract on the histopathology of the liver of mice treated with alcohol

Figure 8 shows the histological profile of the liver of mice treated with alcohol. Histological analysis of the liver revealed a normal liver architecture with the presence of the Hepatic artery (Ha), the hepatic portal vein (Hpv), the sinusoidal capillary (Sc), and the Bile canaliculi (Bc) with no leucocyte's infiltrations in the normal control (distilled water alone-treated mice) group. After prolonged alcohol exposure, there was a marked distortion of the liver cytoarchitecture. Compared to the normal control, the negative control (alcohol-alone treated mice) exhibited histological changes mainly marked by a pronounced leukocyte infiltration (Li). *Thymus vulgaris* administration at 100 and 200 mg/kg resulted in a structural improvement of the liver architecture, close to normal, with no leucocyte infiltrations. Piracetam group also showed a structural improvement in the liver architecture (Figure 8).

Effect of *Thymus vulgaris* extract on the histopathology of the brain of mice treated with alcohol

Figure 9 shows the histopathological profile of the brains of mice treated with alcohol. Histopathological analysis of the normal control group shows a hippocampal architecture with apparently intact neurons in the different layers of the hippocampus (DG, CA1, CA2 and CA3). In negative control, several pathological alterations were noted in the different areas of the hippocampus. The hippocampal sections of the alcohol-treated group show a reduction in the density of neuronal cells in all the dentate gyrus layers associated with damaged cells. Interestingly, there is also a change in the organization of neuronal cells in the various sections of the hippocampus (DG and CA3) of the alcohol group in comparison to the distilled water group (Figure 9). Treatment with *T. vulgaris* aqueous extract reversed alcohol's effect on the hippocampus. Indeed, treatment with *T. vulgaris*, at doses of 100 and 200 mg/kg, restored the neuronal density of the brain. In addition, treatment with *T. vulgaris* extract shows a normal architecture and thickness of the cell layer of the dentate gyrus when compared to the negative control. Piracetam-treated mice show the dentate gyrus and the different sections of the *Cornu ammonis* (CA1, CA2, and CA3) without any sign of cell injury (Figure 9). The effect

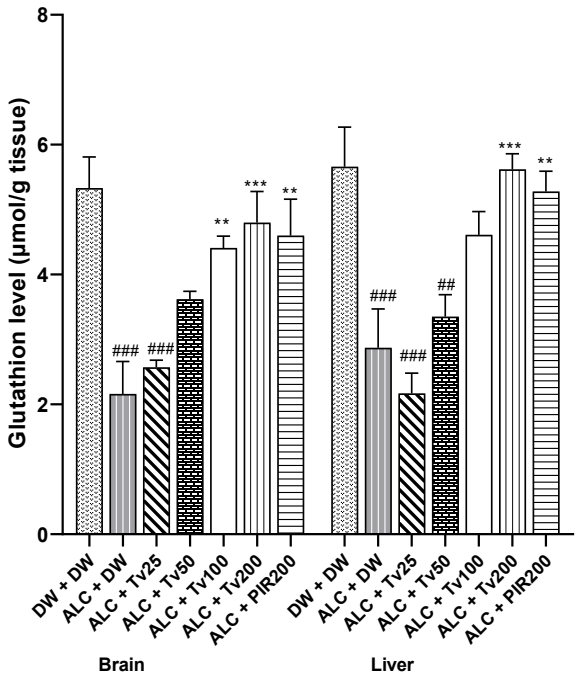

Figure 6. Effects of *Thymus vulgaris* extract on glutathione reduced concentration in the brain and the liver of ethanol-withdrawn mice. Results are presented as the means ± SEM (n= 6 mice) $^{\#\#}P < 0.01$; $^{\#\#\#}p < 0.001$ compared to DW+DW; ** $P < 0.01$; *** $P < 0.001$ compared to ALC+ DW. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* 25 mg/kg, Tv50: *T. vulgaris* 50 mg/kg, Tv100: *T. vulgaris* 100 mg/kg, Tv200: *T. vulgaris* 200 mg/kg.

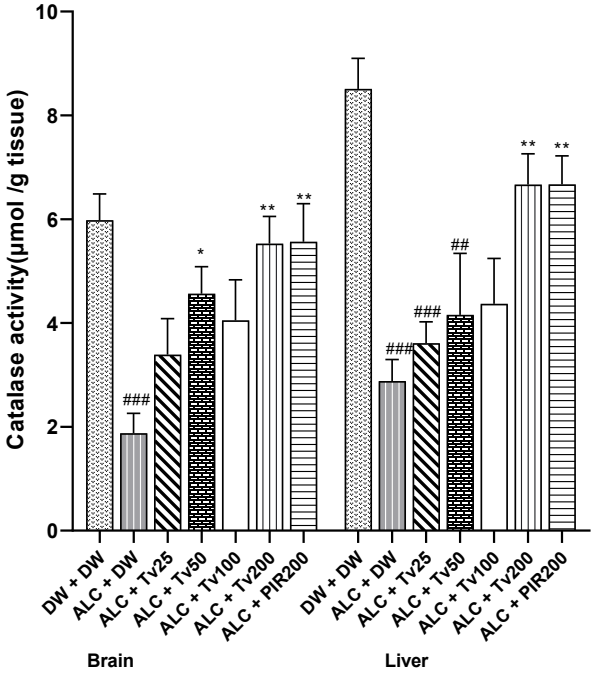

Figure 7. Effects of *Thymus vulgaris* extract on catalase activity in the brain and liver of ethanol -withdrawn mice. Results are expressed as means ± SEM (n= 6 mice). $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared to DW+DW; ** $P < 0.01$; *** $P < 0.001$ compared to ALC+ DW. ALC: alcohol; DW: distilled water; PIR200: piracetam 200 mg/kg; Tv25: *T. vulgaris* 25 mg/kg, Tv50: *T. vulgaris* 50 mg/kg, Tv100: *T. vulgaris* 100 mg/kg, Tv200: *T. vulgaris* 200 mg/kg.

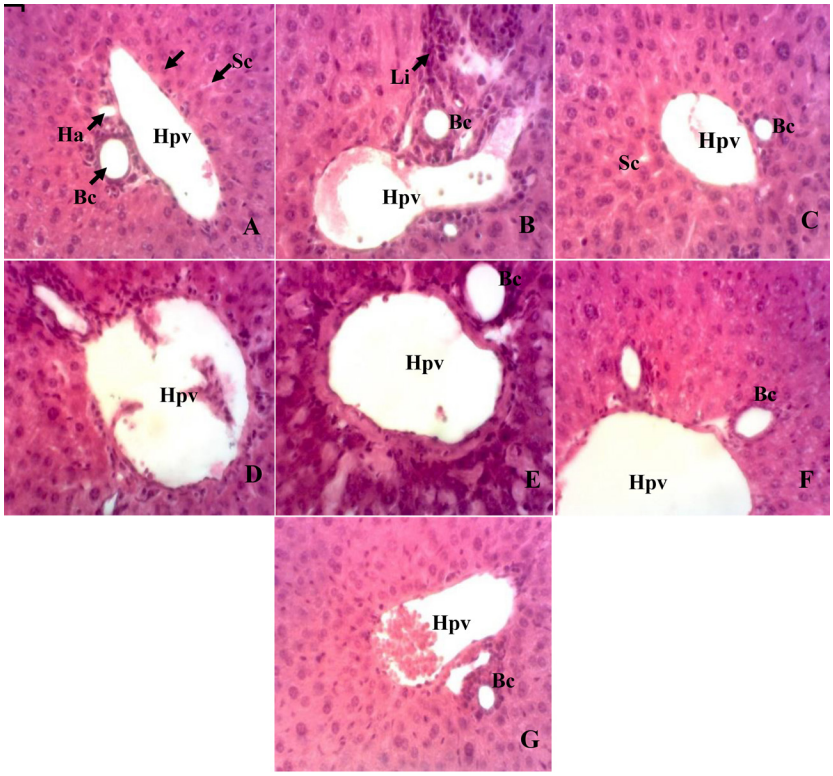

Figure 8. Photomicrographs of the liver of mice treated with alcohol (×200 magnification, hematoxylin-eosin stain). **A** = Normal control; **B** = Negative control; **C**= *Thymus vulgaris* 25 mg/kg, Tv200: **D**= *Thymus vulgaris* 50 mg/kg, **E**= *Thymus vulgaris* 100 mg/kg, **F** = *Thymus vulgaris* 200 mg/kg, **G**= Piracetam 200 mg/kg; **Bc** = Bile canaliculi; **Ha** = hepatic artery; **He** = Hepatocyte; **Hpv**= Hepatic portal vein; **Li** = Leukocyte infiltration; **Sc** = Sinusoidal capillary.

of *Thymus vulgaris* and alcohol withdrawal on the number of neurons in the hippocampus was significantly different in all the hippocampal regions. In the dentate gyrus, CA1, CA2, and CA3 regions of the hippocampus, alcohol withdrawal led to a reduction in the number of neurons. Neurons numbers in the CA1, CA2, CA3 and the dentate gyrus were significantly raised, respectively from 15.80 ± 2.16, 20.80 ± 4.96, 14.20 ± 1.04, and 21.80 ± 3.36 neuron per visualized field in the negative control to 25.00 ± 3.20 ($p < 0.05$), 30.80 ± 1.84 ($P<0.05$), 30.00 ± 2.40 ($P<0.05$), and 40.20 ± 1.52 ($P<0.05$) neurons per visualized field in *T. vulgaris* groups at the dose of 200 mg/kg (Figure 10).

Discussion

The present study was developed to evaluate the effect of *T. vulgaris* leaves on a mouse model of alcohol withdrawal. In humans, anxiety is a key component of alcohol withdrawal syndrome (9,10). In this study, the anxiety of ethanol-withdrawn mice was assessed using the EPM. Developed by Handley and Mithani in 1984, the EPM is the most commonly used behavioral paradigm to evaluate anxiety and for the screening of anxiolytic drugs in rodents (55,56). EPM test results showed that alcohol withdrawal led to a reduction in time and percentage of time spent in the EPM. Our results are in line with those of Jiao et al and Qiao et al (9,57). *T. vulgaris* administration during the withdrawal period increased the time spent and the number of entries in the open arms of the EPM. It is well known that an increase in open-arm activity and a reduction of exploration and stay in the closed arms in mice reflects a reduction in stress (58). The findings mentioned above, therefore, suggest that *T. vulgaris*'s leaves aqueous extract has anxiolytic potential against alcohol withdrawal anxiety. *T. vulgaris*'s effect is similar to that of piracetam, which is an approved non-sedative anxiolytic compound (59). The anxiolytic properties of *T. vulgaris* extract could be mediated by the Gabaergic neurotransmission since EPM is a test sensitive to benzodiazepines and non-sedative anxiolytic compounds (42). Furthermore, *Thymus vulgaris* contains thymol, which is a positive allosteric modulator of the GABA A receptor (60). *T. vulgaris* also contains bioactive monoterpenes, such as carvacrol and linalool, which have been shown to relieve anxiety (30). Therefore, these components may be responsible for the anxiolytic properties of *T. vulgaris* observed in the EPM test.

Anxiety and locomotion in ethanol-withdrawn mice were evaluated in the open field paradigm. Ethanol termination significantly reduced the time spent in the center of the open field and reduced grooming and line crossings. Our results corroborate those of Bonasolli et al and Shoja et al, who found a reduction in exploratory behavior in ethanol-withdrawn mice (15,61). Similar to piracetam, *T. vulgaris* administration raised the number

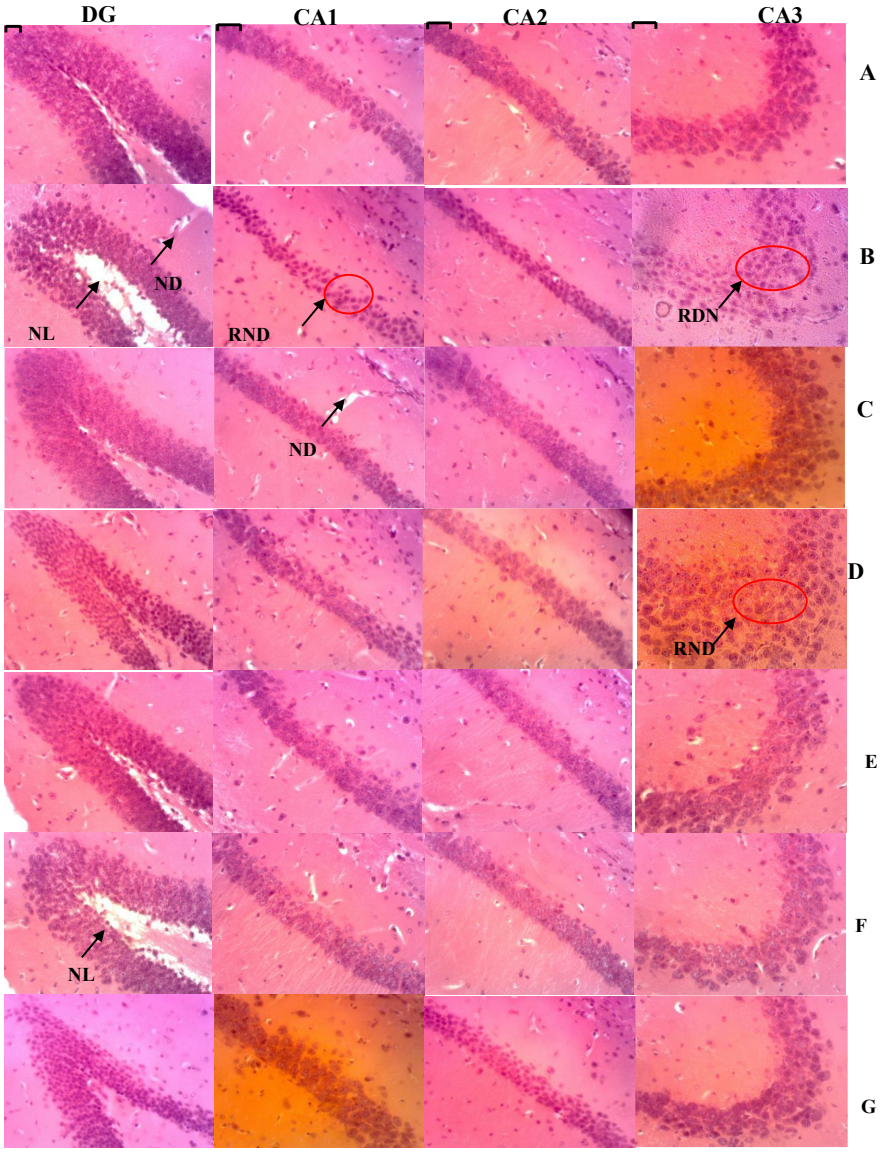

Figure 9: Photomicrographs of the dentate gyrus (x100) and the *Cornu ammonis* 1, 2, and 3 (×200 magnification) of the hippocampus; Haematoxylin-eosin staining. **A**= Normal control; **B** = Negative control; **C**= *Thymus vulgaris* 25 mg/kg, Tv200: **D**= *Thymus vulgaris* 50 mg/kg, **E**= *Thymus vulgaris* 100 mg/kg, **F** = *Thymus vulgaris* 200 mg/kg, **G**= Piracetam 200 mg/kg. **CA1**= *Cornu ammonis* 1, **CA2**= *Cornu ammonis* 2, and **CA3**= *Cornu ammonis* 3; **DG**: Dentate gyrus; **ND:** Neurodegeneration; **NI***:* Neuronal loss; **RND**: Reduction in neuron density.

of crossings, center time, and grooming behaviors, suggesting theanxiolytic effects of thyme. The results of the open field test additionally validated the anxiolytic properties of thyme against ethanol discontinuation.

After withdrawal from heavy and constant alcohol exposure, neuronal damage and neurochemical changes occur in the brain, leading to working memory impairment (62,63). The Y maze paradigm was used in this study to evaluate spatial short-term memory. The natural tendency of animals to explore new environments and the degree of spontaneous alternation is the basis of the Y-maze task (64). The administration of *T. vulgaris* extract significantly counteracted the reduction in the percentage of spontaneous alternation induced by alcohol weaning. In rodents, spontaneous alternation is an indicator of spatial working memory performance (46,65). An increase in spontaneous alternation in the Y-maze suggests a significant improvement in spatial short-term memory produced by both piracetam and *T. vulgaris* extract (46). Our results were in line with the results of Rabiei et al in 2015, who found that *T. vulgaris* extract had reparative effects against memory impairment induced by scopolamine (40). Different brain regions involved in memory processes, such as the prefrontal cortex and the hippocampus, appear to be very sensitive to ethanol toxicity (66,67). It has also been postulated that through the exacerbation of the excitatory neurotransmission system and the reduction of GABA neurotransmission, alcohol withdrawal leads to memory disturbances via the inhibition of long-term potentiation in the hippocampus

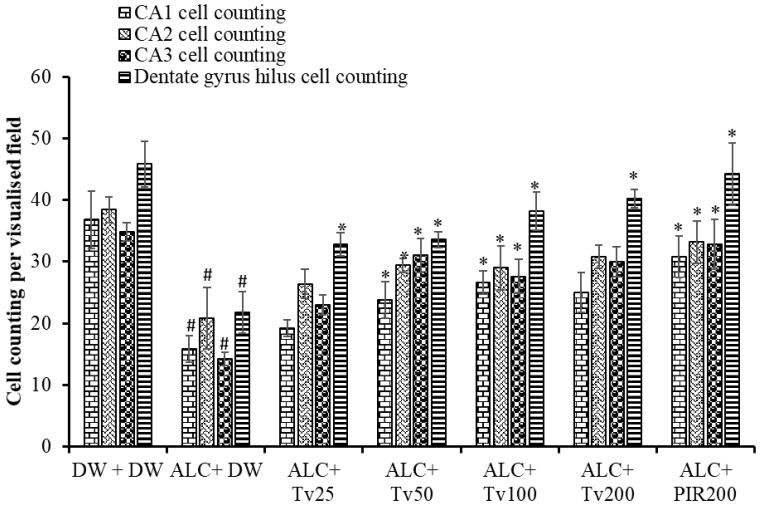

Figure 10. Effects of *Thymus vulgaris* extract on neuronal cell counting per visual field of alcohol-withdrawn mice (n = 6 mice per group). The results show the quantifications of haematoxylin and eosin staining of neurons in hippocampal Dentate gyrus CA1, CA2, and CA3. $^{\#\#}P < 0.01$ compared to DW+DW; $^{*}P < 0.05$ compared to ALC+ DW. ALC: alcohol; DW: distilled water; PTM: Piracetam 200 mg/kg; Tv25, Tv50, Tv100, and Tv200: *Thymus vulgaris* aqueous extract at the doses of 25, 50, 100, and 200 mg/kg, respectively.

(14,68). The inhibition of the effect of alcohol withdrawal-induced memory impairment by thyme further favors its possible action on GABA A receptors, as previously reported (60). Several lines of evidence support that ethanol termination leads to brain damage triggered by oxidative stress (13,14). Free radicals interact with neuronal pathways regulating the gene transcription of inflammatory mediators associated with the state of anxiety (14). In addition, during the metabolism of ethanol in the liver, free radicals are generated, which can also affect different organ systems (69,70). The overall manifestations of this oxidative stress are manifested by an increase in pro-oxidant factor generation and a reduction in the antioxidant enzymatic system (71,72). Therefore, in this study, to further elucidate the mechanism by which thyme antagonizes anxiety and short-term memory defects, we evaluated some brain and liver biomarkers of oxidative damage, namely, NO, MDA, and antioxidant enzymes (reduced GSH and CAT).

NO is an abundant reactive nitrogen radical that acts as an important oxidative signaling molecule in biological activities. Alcohol withdrawal or chronic ethanol administration activates NO production in the brain and in the liver (15,69). These previous findings corroborate the results obtained during this study. Alcohol withdrawal raised NO's level in the liver and brain tissues. Administration of *T. vulgaris* reversed the effect of ethanol, thus conferring more validity to the antioxidant activity of the plant extract against alcohol withdrawal toxicity. A high level of MDA, is an indicator of cellular damage due to oxidative stress generation (42,73). In this study, alcohol withdrawal resulted in an increase in MDA levels in both the liver and the brain. This increase in MDA concentration in ethanol-withdrawn mice was reversed by post-administration of *T. vulgaris*. This effect is in accordance with a previous report (28).

The ability of thyme to combat oxidative stress has been mentioned by several authors, and it is attributed to its rich content of molecules such as carvacrol, thymol, monoterpene phenolic compounds, and flavonoids (31,38,74-76). To further validate the involvement of oxidative stress in the results obtained in this study, CAT activity and reduced GSH were evaluated. Alcohol withdrawal led to a significant reduction in CAT activity and reduced GSH content. Low CAT activity in an organ is a sign of cellular damage and implies the destructive effect of alcohol withdrawal in both the liver and the brain. *T. vulgaris* significantly raised CAT activity in the liver and the brain. This result is similar to previous reports, according to which the administration of *T. vulgaris* significantly protected liver cells from alcohol damage (76). Hence, it can be deduced that treatment with *T. vulgaris* is effective in counteracting the effect of alcohol intoxication.

Alcohol withdrawal depleted GSH concentration. Decreased levels of GSH are associated with enhanced lipid peroxidation in alcohol-treated mice. Administration of *T. vulgaris* extract significantly increased GSH concentrations in the brain and liver of alcohol-treated mice. Increased GSH activity indicates significant antioxidant activity (46,77). *Thymus vulgaris* aqueous extract demonstrated neuroprotective and hepatoprotective properties due to its ability to increase GSH levels in the brain and liver of alcohol-treated mice. These results are in accordance with previous works on the pharmacological activities of thyme (28,78).

The results of histopathological studies of the liver and the brain showed that thyme administration protected the liver and the hippocampus against ethanol-induced cell death in these organs. Thymol, polyphenols, and flavonoids are known plant constituents with neuroprotective and hepatoprotective properties (30). Therefore, it can be suggested that the neuroprotective and hepatoprotective activities shown by the *T. vulgaris* extract can be due to these compounds.

Conclusion

This study was conducted to test the efficacy of an aqueous extract of *T. vulgaris* on alcohol withdrawal-induced anxiety and spatial short-term memory deficits. The results obtained highlight that thyme has anxiolytic and memory-enhancing effects and protects the brains and livers of ethanol–withdrawn mice against oxidative stress damage. The pharmacological effects of this plant could be attributed to its rich phytochemical composition. This study gives additional credit to the use of thyme in traditional medicine in the management of some aspects of ethanol withdrawal syndrome. Nevertheless, more studies are required to better elucidate the mechanisms of actions of the plant extract.

Acknowledgments

The authors are grateful to Dr. Teh Rene Ning of the University of Buea for the proofreading and editing of the manuscript.

Author contributions

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Conflict of interests

The authors declare that they have no conflict of interest.

Data availability statement

The datasets recorded and analyzed during this study are available from the corresponding author upon reasonable request.

Ethical considerations

This study was approved by the University of Buea-Institutional Animal Care and Use Committee (UB-IACUC) with permit number N°: UB-IACUC N°010/2020.

Funding/Support

This research received no external funding.

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