

Original Research Article

Protective effects of *Chenopodium botrys* hydroalcoholic extract on thioacetamide-induced chronic hepatic encephalopathy in rats: changes in learning and memory, oxidative stress, and acetylcholinesterase activity

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Abstract

Objective: Hepatic encephalopathy (HE) is reported to impair cognition. This study aimed to evaluate the effects of hydroalcoholic extract of aerial parts of *Chenopodium botrys*, on acetylcholinesterase (AChE) activity, oxidative stress, and memory in thioacetamide (TAA)-induced HE in rats.

Materials and Methods: This study involved the random allocation of 40 rats into 5 groups: 1) Control; 2) TAA (300 mg/kg, twice a week, intraperitoneal); 3 and 4) TAA + Extract 12.5 mg/kg and TAA + Extract 25 mg/kg; and 5) TAA + Vitamin (Vit) C 100 mg/kg. The drugs were administered by oral gavage during 28 days. Passive avoidance (PA) and Morris water maze (MWM) assessments were conducted. Ultimately, AChE activity and oxidative stress status were assessed in the liver, cortex, and hippocampus.

Results: In the PA test, latency to enter the dark part and light spent time increased, whereas dark spent time and number of dark entries decreased in rats receiving the extract and Vit C compared to the TAA group. During the MWM test, the rats treated with extract and Vit C showed a reduced time and distance to reach the platform and spent more time and distance in the target quadrant. Furthermore, the extract and Vit C decreased levels of malondialdehyde and AChE while raising levels of superoxide dismutase and total thiol.

Conclusion: Our findings demonstrate that the *C. botrys* extract and Vit C can significantly enhance memory in a rat model of HE by improving cholinergic dysfunction and reducing oxidative stress.

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Introduction

Hepatic encephalopathy (HE) is a neuropsychological complication of liver damage such as cirrhosis and can happen in both cases of chronic and acute liver failure (Häussinger et al. 2022). Elevated blood ammonia is thought to cause neurological symptoms in HE patients, but the exact pathophysiology is unknown. Patients with HE and, similarly, animal models of HE demonstrate neuroinflammation and dysregulations of neurotransmitters in the brain, leading to impaired memory and cognition (Felipo et al. 2012; Izquierdo-Altarejos et al. 2020). The memory disruption in HE is accompanied by structural changes in the hippocampus (Garcia-Garcia et al. 2018). Neurotoxins (increased ammonia) and disruptions in neurotransmitter systems, especially the cholinergic system, are important variables in this disease. Other contributing factors include changes in brain metabolism, a disturbance in the blood-brain barrier, inflammatory responses, and oxidative stress (Ferenci 2017). As a result of HE, the endogenous antioxidants are decreased, and free radical substances are overproduced, which then lead to hepatic and cerebellar oxidative damage (Mousa et al. 2016). Possibly due to increased oxidative damage, liver damage can result in cholinergic dysfunction (García-Ayllón et al. 2008). Researchers also demonstrated that systemic oxidative stress and hyperammonemia interact synergistically to contribute to the development of HE (Bai et al. 2023; Görg et al. 2010). Oxidative stress leads to adverse effects, particularly on mitochondrial morphology and changes in neuronal function, which ultimately lead to deficits in memory and learning (Bai et al. 2023; Simicic et al. 2022). Previous studies have shown that mitigating oxidative damage can decrease the negative effects of HE (Mustafa et al. 2013).

The plant *Chenopodium botrys* (*C. botrys*) belongs to the Chenopodiaceae family. It grows in most parts of North America and is native to Europe and Asia

(Morteza-Semnani 2015). This plant contains components such as flavonoids, and several terpenoids (Bojilov et al. 2023). The compounds found in this plant possess anti-inflammatory, antifungal, antimicrobial, and antioxidant properties (Yilmaz and Kolak 2024). Additionally, a study indicates that the aqueous extract of *C. botrys* can inhibit acetylcholinesterase (AChE) activity (Ozer et al. 2016). Ascorbic acid, more commonly known as vitamin (Vit) C, functions as a potent antioxidant, which can work on both the liver and hippocampus and has desirable effects on memory; therefore, it has been used as a positive control in the context of HE in previous works (Behairy et al. 2021; Carr and Maggini 2017; Heo et al. 2013).

Due to the considerable antioxidant properties of *C. botrys* and Vit C, we evaluated their effects on learning and memory impairments in a thioacetamide (TAA)-induced model of HE. Oxidative stress markers were measured in the cortex and hippocampus as well as the liver. Cholinergic activity was also assessed using AChE in both the cortex and hippocampus.

Materials and Methods

Plant extract and chemicals

C. botrys specimens were identified by Dr. M.S. Amiri (No. 681) and sourced from the Zarrin-kuh protected area in Razavi Khorasan Province, located in northeastern Iran. Initially, the aerial parts of the plant were air-dried at 35 degrees Celsius inside an oven, and then it was grinded into a powder. The powder then was immersed in a hydroalcoholic solution (70%), which was maintained at room temperature for three consecutive days, and during this phase, the solution was kept inside a sealed container, and the solution was stirred twice a day. The solution was then filtered through a fine mesh and then was desiccated under vacuum using a rotary evaporator. The desired concentration was prepared by dissolving the desiccated

extract in distilled water and the extract was administered orally. All the materials used in the present study were obtained from Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany).

Animals and grouping

Forty male Wistar rats weighing 220–250 g were obtained from Mashhad University of Medical Sciences, with disease induction and treatment carried out over a 28-day period. Animals were randomly allocated into 5 groups (N=8 per group): (1) Control group: Animals received intraperitoneal administration of TAA vehicle (saline) twice a week and were gavaged (saline) daily. (2) TAA group: received intraperitoneal 300 mg/kg of TAA twice a week and was gavaged daily (saline). (3 and 4) *C. botrys*-extract groups: received intraperitoneal administration of 300 mg/kg TAA twice a week and daily oral gavage of the extract at doses of 12.5 and 25 mg/kg. (5) Positive control group: received intraperitoneal 300 mg/kg of TAA twice a week and daily oral gavage of 100 mg/kg Vit C. All animals were housed under standard light, humidity, and temperature conditions for the duration of the study, with unrestricted access to food and water. Experimental procedures followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and received approval from the Animal Ethics Committee of Mashhad University of Medical Sciences (Ethical Approved Number: IR.MUMS.REC.1402.336).

Behavioral evaluations

Passive avoidance (PA)

Memory retention was assessed using the PA test. A box with two compartments -one dark and one bright- was used. Between these two sections was a guillotine door. Stainless steel bars were placed on the bottom of the compartments. Rats received a foot shock from the metal bars under the compartment, and then the PA test was conducted 3, 24, 48, and 72 hr after the

shock. The test and the data recorded were done in adherence to a previous report (Rajabian et al. 2025).

Morris water maze (MWM)

The MWM was used to evaluate the spatial memory of the rats and consisted of two phases. The first phase was done on 5 days and assessed the learning ability of the animals. The second phase, a probe test, was conducted to evaluate the memory retention. The probe test was done after the first phase was over. The protocol of a previously published work was followed (Rajabian et al. 2025).

Sample collection

Animals were deeply anesthetized using ketamine and xylazine (100 and 10 mg/kg, respectively), and they were then sacrificed following the behavioral tests, and samples of the blood, liver, cortex, and hippocampus were obtained for further assessments. Serum was separated from the whole blood sample via centrifuge. Tissue samples were also homogenized and centrifuged, and the resulting supernatant was collected. All samples were stored at -80 °C until further analysis.

Biochemical assessments

The serum samples were evaluated in a medical laboratory and were used to measure alkaline phosphatase (ALKP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The tests were performed according to the kit manufacturer's protocol (Beheshti et al. 2021).

5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) was used to evaluate the thiol content, which reacts with DTNB and forms a yellow solution. The absorbance was read following the protocol of a previous report (Sabeti et al. 2023).

2-thiobarbituric acid (TBA) was mixed with tissue samples to react and generate a pink liquid substance. This reaction happened between malondialdehyde (MDA) and TBA. The level of MDA was

then measured with a colorimetric technique that was previously described (Saber *et al.* 2023).

The activity of superoxide dismutase (SOD) was tested by mixing the tissue samples with pyrogallol. The SOD content in the sample can inhibit the autoxidation of pyrogallol. The absorbance was read following a previously reported methodology (Saber *et al.* 2023).

The samples from brain tissue (cortex and hippocampus) were mixed with DTNB and acetylthiocholine iodide to measure the amount of AChE in the tissue. We followed a previously established methodology for this purpose (Ellman *et al.* 1961; Saber *et al.* 2023).

Statistical analysis

Statistical analysis was performed with IBM SPSS software (version 27) following verification of normal distribution. Escape latency and swim distance across the five acquisition days in the MWM were assessed by repeated-measures ANOVA, and probe trial performance was assessed by one-way ANOVA; both were followed by Tukey's post hoc test for multiple comparisons. Biochemical parameters were compared using one-way ANOVA with Tukey's post hoc test. Given the non-parametric distribution of PA data, group differences were assessed using the Kruskal-Wallis test with Dunn's post hoc test. Data are presented as mean $\pm$ SEM, with the exception of PA data expressed as median and interquartile range. A *p* value below 0.05 was considered statistically significant.

Results

PA test

TAA group had lower latency to enter the dark compartment in comparison to the control and this was the case for all the sessions after the shock ($p < 0.01$). In contrast, latency was increased in all

treatment groups (TAA+ Extract 12.5, TAA+ Extract 25, and TAA+ Vit C) relative to the TAA group at 3 and 24 hr post-shock ($p < 0.05$ – $p < 0.001$). Furthermore, time latency in the TAA+ Extract 25 and TAA+ Vit C groups was higher than in the TAA group at 48 hr post-shock ($p < 0.01$ and $p < 0.05$, respectively) (Figures 1A-1D).

Significant differences in time spent in the light chamber were observed between the TAA and Control groups at 3, 24, 48, and 72 hr post-shock ($p < 0.01$). At 24 hr post-shock, treatment groups (TAA+ Extract 12.5, TAA+ Extract 25, and TAA+ Vit C) spent more time in the light chamber than the TAA group ($p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively). Additionally, the TAA+ Extract 25 and TAA+ Vit C groups spent significantly more time in the light chamber than the TAA group at 3, 48, and 72 hr post-shock ($p < 0.05$ – $p < 0.001$) (Figures 1E-1H).

As shown in Figure 2, rats in the TAA group spent more time in the dark chamber than those in the Control group at 3, 24, 48, and 72 hr post-shock ($p < 0.05$ and $p < 0.01$). At 3, 24, 48, and 72 hr post-shock, animals in the TAA+ Extract 25 and TAA+ Vit C groups spent less time in the dark chamber than those in the TAA group ($p < 0.05$ – $p < 0.001$). Additionally, 24 hr after the shock, the TAA+ Extract 12.5 group spent considerably less time in the dark chamber than the TAA group ($p < 0.05$) (Figures 2A-2D).

The findings further indicate that the TAA group entered the dark chamber more frequently than the Control group at 3, 24, and 48 hr post-shock ($p < 0.01$). In contrast, the TAA+ Extract 12.5 group showed fewer entries into the dark chamber at 3 and 24 hr post-shock compared to the TAA group ($p < 0.05$), while the TAA+ Extract 25 and TAA+ Vit C groups showed significantly fewer entries at 3, 24, and 48 hr post-shock relative to the TAA group ($p < 0.05$ – $p < 0.001$) (Figures 2E-2H).

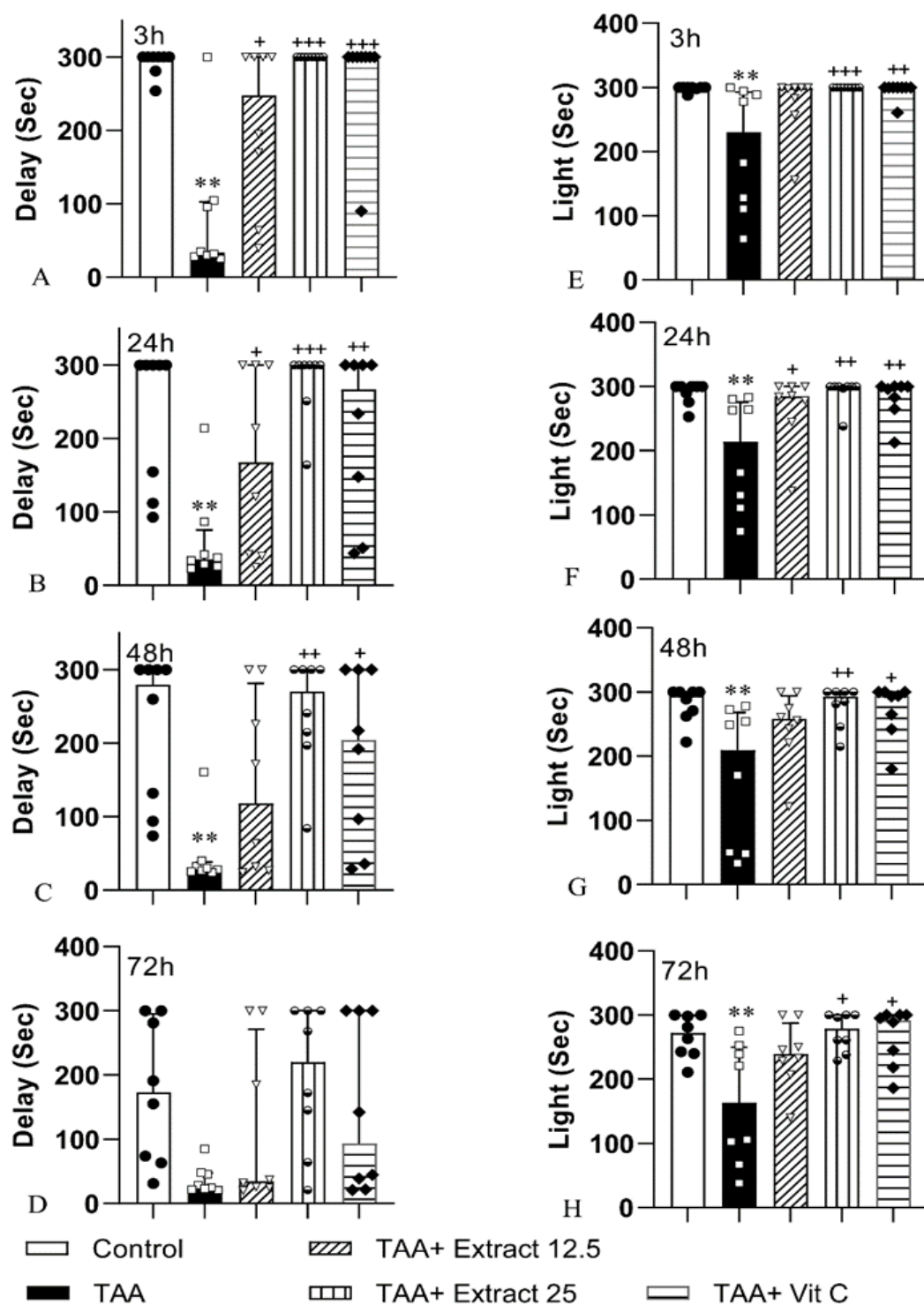


Figure 1. The effects of *C. botrys* and Vitamin C on the latency to enter the dark compartment (A, B, C, and D) and the time spent in the light compartment (E, F, G, and H) in the passive avoidance test. The data is presented as median and interquartile range (n = 8). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, and * is used as a comparison to the control group. TAA: thioacetamide, Vit: vitamin.

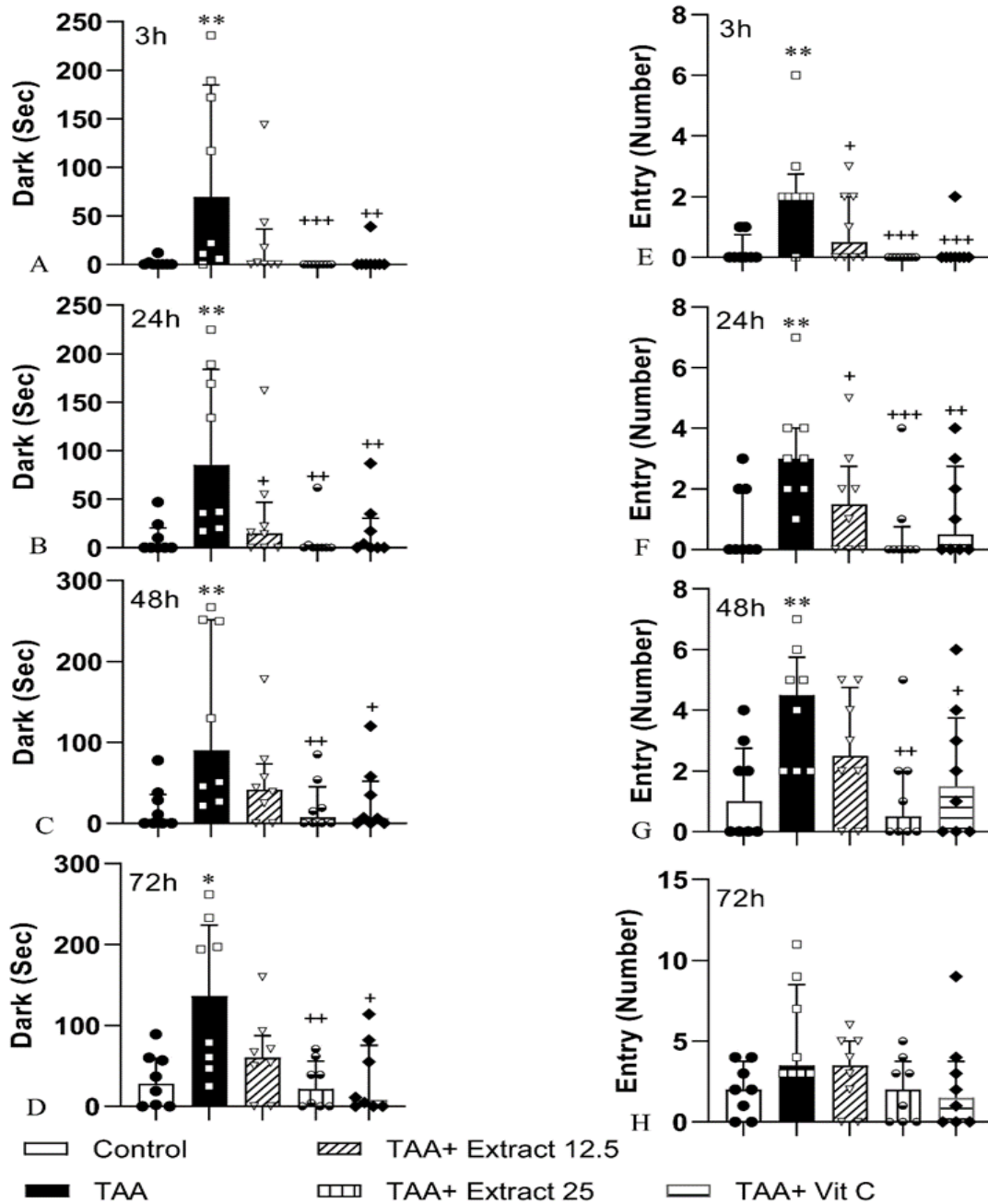


Figure 2. Effect of *C. botrys* extract and Vit C on total time spent in the dark chamber (A, B, C, and D) and number of entries to the dark chamber (E, F, G, and H) in the passive avoidance test. The data is presented as median and interquartile range ($n = 8$). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, and * is used as a comparison to the control group. TAA: Thioacetamide, Vit: vitamin.

MWM test

In the first five days of the MWM test, as illustrated in Figure 3, consistently more time was spent finding the platform by TAA ($p < 0.05$ for the 2nd and 3rd days, $p < 0.01$ on the 4th day, and $p < 0.001$ on the 5th day) compared to control except on the

first day. On day 1, the TAA group required 38.76 ± 3.71 sec to locate the platform, which decreased to 13.22 ± 2.06 sec by day 5. As for the control group, on the first day, animals spent 25.90 ± 3.61 sec, and this value was reduced to 4.03 ± 0.49 sec. The TAA group also exhibited a longer travel

distance at different time points on the 4th and 5th days when compared to the control group ($p < 0.01$ and $p < 0.001$ respectively). TAA group travelled 975.30 ± 130.89 cm on the first day, and this distance was reduced to 611.07 ± 152.94 cm on the 5th day. The distance traveled by the control group also reduced from 933.71 ± 123.87 cm on the first day to 124.44 ± 24.87 cm on the 5th day.

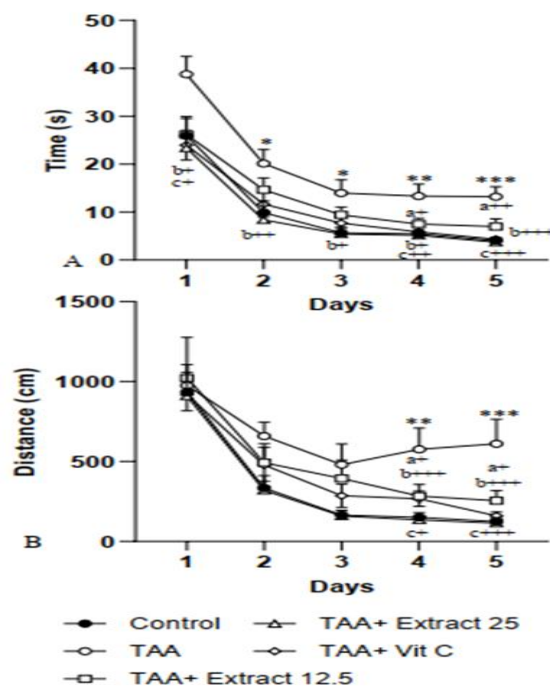


Figure 3. Effect of *C. botrys* extract and Vit C on the time (A) and distance (B) spent in the Morris water maze during a five-day training period. The data are presented as Mean $\pm$ SEM ($n=8$). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$ and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group and * is used as a comparison to the control group. TAA: thioacetamide, Vit: vitamin. The letter "a" represents the TAA+ Extract 12.5 group, the letter "b" represents the TAA+ Extract 25 group, and the letter "c" represents the TAA+ Vit C group.

TAA+ Extract 12.5 was able to reduce the time on the 4th and 5th days ($p < 0.05$ and $p < 0.001$, respectively) as well as reducing the distance ($p < 0.05$ for both). TAA+ Extract 25 reduced the time spent compared to TAA every day ($p < 0.05$ - $p < 0.001$) and the time spent on the first day was 23.35 ± 3.47 sec and was reduced to 3.69 ± 0.56 sec on the fifth day. TAA+ Extract 25 resulted in a shorter distance traveled on the last two days ($p < 0.001$ for both). TAA+ Extract 25 traveled 909.75 ± 119.31 cm on the first day and progressed to 115.08 ± 24.12 cm on the

last day. TAA + Vit C also reduced time spent on the 1st, 4th and 5th days ($p < 0.05$ - $p < 0.001$) and the distance traveled was shorter on the 4th and 5th days ($p < 0.05$ and $p < 0.001$ respectively).

Both time and distance traveled in the target quarter are presented in Figure 4 and both factors were significantly decreased in the TAA group in comparison to control ($p < 0.01$ for both). TAA+ Extract 12.5 and TAA+ Extract 25 both increased times spent in the target area significantly ($p < 0.01$ and $p < 0.001$ respectively) compared to TAA (28.49 ± 1.87 sec for TAA+ Extract 12.5, 33 ± 1.53 sec for TAA+ Extract 25, and 20.93 ± 1.12 sec for TAA), and TAA+ Vit C also exhibited marked improvement over TAA ($p < 0.001$) (31.94 ± 1.48 sec). TAA+ Extract 25 and TAA+ Vit C both increased the distance traveled in the target area ($p < 0.001$) (815.80 ± 44.26 cm for TAA+ Extract 25 and 722.82 ± 34.44 cm for TAA+ Vit C), and TAA+ Extract 25 also significantly improved this factor compared to TAA+ Extract 12.5 ($p < 0.001$). TAA+ Extract 12.5 failed to be different from TAA when the distances were compared.

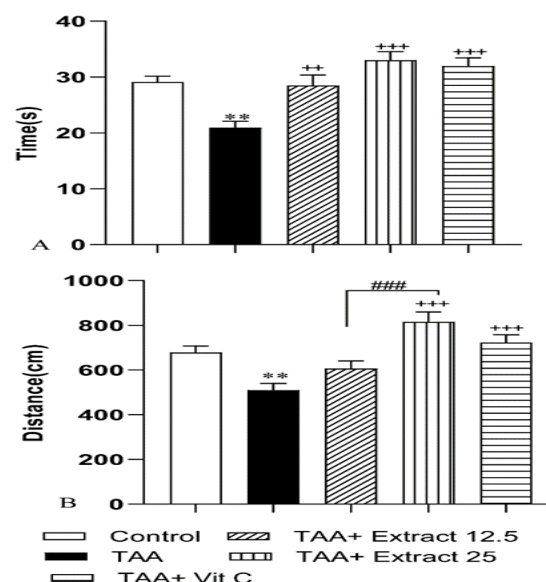


Figure 4. Effect of *C. botrys* extract and Vit C on duration (A) and distance (B) traversed during the probe trial of the Morris water maze. The data are presented as Mean $\pm$ SEM ($n=8$). 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, * is used as a comparison to the control group, and # is used as a comparison between treatment groups. TAA: thioacetamide, Vit: vitamin.

Liver enzymes

Liver function was evaluated by measuring serum AST, ALT, and ALK-P levels (Figure 5), with TAA administration resulting in elevated levels of all of these enzymes compared to the Control group ($p < 0.05$ and $p < 0.01$). AST levels in the control were 136.28 ± 9.15 U/L, ALT was 53.12 ± 3.99 U/L, and ALKP was 348 ± 21.38 U/L, and these factors in the TAA group were increased and were as follows:

AST, 242 ± 27.46 U/L, ALT 103.16 ± 12.49 U/L, and ALKP, 522.37 ± 41.94 U/L. By contrast, administration of Extract 25 mg/kg significantly reduced AST levels in the TAA+ Extract 25 group ($p < 0.05$) compared to the TAA group, and the values were 117 ± 17.57 U/L. It is also worth noting that AST levels in the TAA+ Extract 25 group were significantly lower than in the TAA+ Extract 12.5 group ($p < 0.05$).

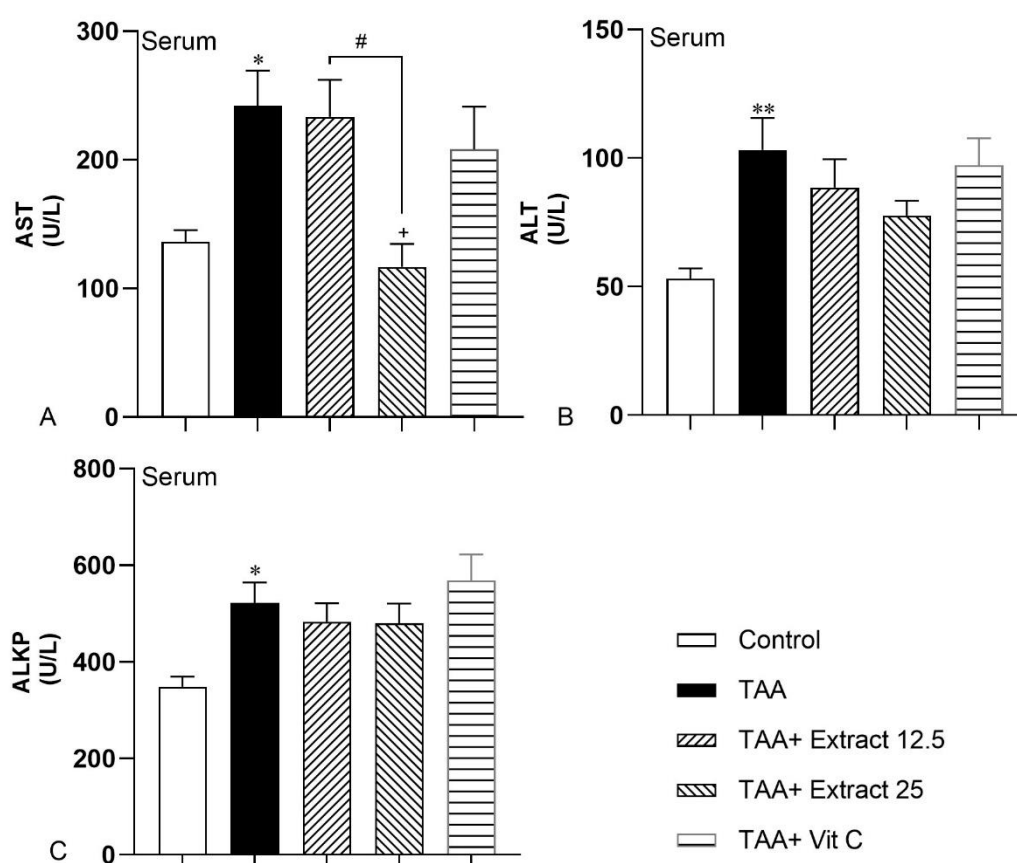


Figure 5. Effect of *C. botrys* extract and Vit C on liver enzymes. Blood serum levels of AST (A), ALT (B), and ALK-P (C). The data are presented as Mean $\pm$ SEM ($n=8$). 1 symbol represents $p < 0.05$, and 2 symbols represent $p < 0.01$. + is used as a comparison to the TAA group, * is used as a comparison to the control group, and # is used as a comparison between treatment groups. TAA: thioacetamide, Vit: vitamin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, and ALKP: alkaline phosphatase.

Oxidative stress markers in the liver

Animals in TAA group had higher levels of MDA in the liver tissue compared to control ($p < 0.001$) as shown in Figure 6A. MDA concentration was 10.33 ± 0.65 nmol/g tissue for control and 40.40 ± 3.96 nmol/g tissue for TAA. When 12.5 mg/kg of extract was administered, the liver's

MDA concentration was considerably higher than in the Control group ($p < 0.05$). In contrast to the TAA group, the MDA level in liver tissue was significantly reduced by the treatment with the extract of *C. botrys* in the TAA+ Extract 12.5 and TAA+ Extract 25 groups ($p < 0.05$ and $p < 0.001$, respectively) and was reduced to 26 ± 4.73 and 15.60 ± 1.19 nmol/g tissue,

respectively. Vit C in the TAA+ Vit C group also decreased this factor to 26.69 ± 3 nmol/g tissue ($p < 0.05$).

Thiol concentration of the liver tissue was also decreased compared to control (Figure 6B) ($p < 0.001$), TAA administration reduced this factor to 7.01 ± 0.41 $\mu\text{mol/g}$ tissue, whereas in the control group the concentration was 14.25 ± 1.17 $\mu\text{mol/g}$ tissue. Also, the total thiol concentration in the liver of rats in the TAA+ Extract 12.5 group decreased significantly compared to the control group and the amount was 8.54 ± 1.10 $\mu\text{mol/g}$ tissue ($p < 0.01$). Notably, the TAA+ Extract 25 (13.09 ± 1.18 $\mu\text{mol/g}$ tissue) and TAA+ Vit C (11.43 ± 1.19 $\mu\text{mol/g}$ tissue) groups had higher liver thiol concentrations than the TAA group ($p < 0.01$ and $p < 0.05$, respectively). A notable

disparity in thiol content in the liver was observed between the TAA+ Extract 12.5 and TAA+ Extract 25 groups ($p < 0.05$).

Hepatic SOD (Figure 6C) activity was lower in the TAA group than in the Control group ($p < 0.05$) and the concentrations of SOD in TAA were 12.8 ± 1.09 U/g tissue and they were 18.46 ± 0.30 U/g tissue in control. The values were similarly reduced in the TAA+ Extract 12.5 group relative to the Control group ($p < 0.01$). Nevertheless, the TAA+ Extract 25 group exhibited a significantly higher activity of the SOD enzyme in the liver than the TAA group ($p < 0.01$) the value of SOD was 18.01 ± 0.55 U/g tissue. Additionally, the TAA+ Extract 25 group revealed a considerably higher activity of the SOD enzyme in the liver than the TAA+ Extract 12.5 group ($p < 0.01$).

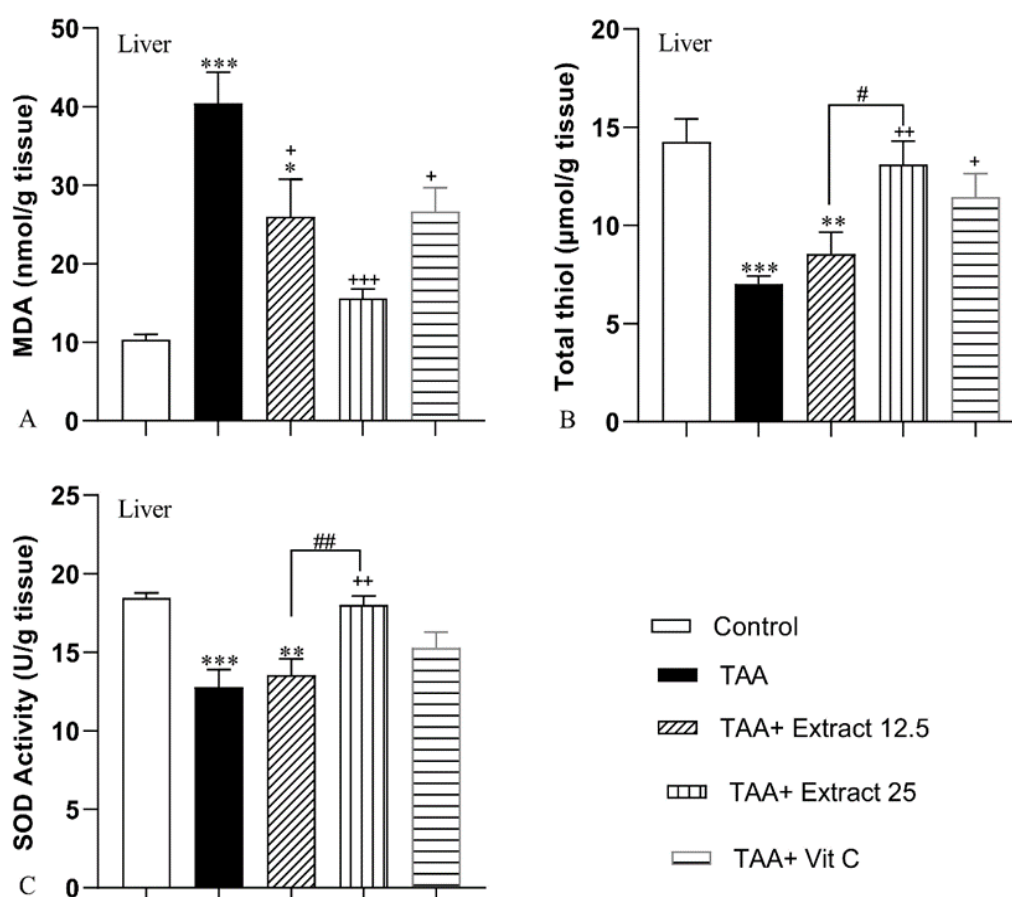


Figure 6. Effect of *C. botrys* extract and Vit C on liver oxidative stress markers. MDA concentration (A), total thiol concentration (B), and SOD activity (C). The data are presented as mean $\pm$ SEM ($n=8$). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, * is used as a comparison to the control group, and # is used as a comparison between treatment groups. TAA: thioacetamide; Vit: vitamin, MDA: malondialdehyde; SOD: superoxide dismutase.

MDA levels in the brain

As shown in Figure 7, MDA levels in the hippocampus and cortex were higher in the TAA group than in the Control group ($p < 0.001$), MDA content in the cortex of TAA group was 50.09 ± 3.51 nmol/g tissue and was 50.04 ± 2.90 nmol/g tissue in the hippocampus; these values were respectively 17.29 ± 0.96 and 22 ± 1.08 nmol/g tissue in the cortex and hippocampus of the control group. Significant differences were also observed between the TAA+ Extract 12.5 and Control groups in both regions ($p < 0.05$ and $p < 0.01$, respectively). Additionally, the cortex of the TAA+ Extract 25 and Control groups exhibited a substantial difference in MDA content ($p < 0.01$) and the MDA content in TAA+ Extract 25 was 34.74 ± 2.50 nmol/g tissue and 31 ± 2.99 nmol/g tissue in the cortex and hippocampus, respectively. However, the concentration of MDA in both the cortex and hippocampus was significantly reduced in all treatment groups (TAA+ Extract 12.5, TAA+ Extract 25, and TAA+ Vit C) in comparison to the TAA group ($p < 0.05$ - $p < 0.001$).

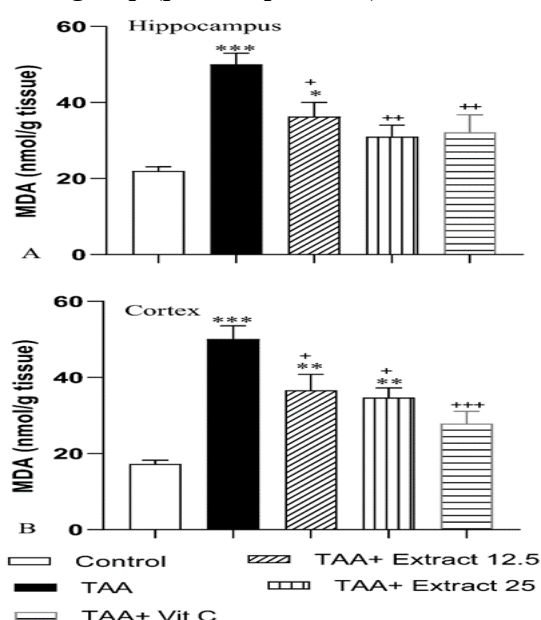


Figure 7. Effect of *C. botrys* extract and Vit C on MDA levels in the hippocampus (A) and cortex (B) tissues. The data are presented as mean $\pm$ SEM (n=8). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, and * is used as a comparison to the control group. TAA: thioacetamide, Vit: vitamin, MDA: malondialdehyde

SOD activity in the brain

Data in Figure 8 indicate that SOD activity in the cortex and hippocampus was significantly lower in the TAA group (10.52 ± 0.95 U/g tissue in the cortex and 10.65 ± 0.86 U/g tissue in the hippocampus) than in the control group (18.28 ± 0.12 U/g tissue in the cortex and 17.74 ± 0.49 U/g tissue in the hippocampus) ($p < 0.001$), with significant differences also observed between the TAA+ Extract 12.5 and Control groups in both regions ($p < 0.05$ and $p < 0.01$, respectively). Cortical SOD also differed significantly between the TAA+ Vit C and Control groups ($p < 0.05$) and the SOD concentration in TAA+ Vit C was 14.72 ± 1.02 U/g tissue. By contrast, all treatment groups (TAA+ Extract 12.5, TAA+ Extract 25, and TAA+ Vit C) showed significantly higher SOD activity than the TAA group in both the cortex and hippocampus ($p < 0.01$ and $p < 0.001$). The SOD value was measured at 16.30 ± 0.70 U/g tissue in the cortex and 16.63 ± 0.37 U/g tissue in the hippocampus of the TAA+ Extract 25 group.

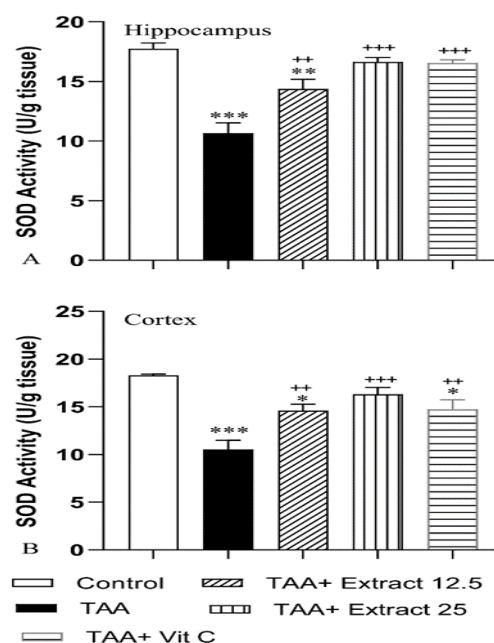


Figure 8. Effect of *C. botrys* extract and Vit C on SOD activity in the hippocampus (A) and cortex (B) tissues. The data are presented as mean $\pm$ SEM (n=8). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, and * is used as a comparison to the control group. TAA: thioacetamide, Vit: vitamin, SOD: superoxide dismutase.

Total thiol concentrations in the brain

As shown in Figure 9, total thiol concentrations in the hippocampus and cortex were significantly lower in the TAA group than in the Control group ($p < 0.001$), thiol concentration in the cortex of the TAA group was $4.16 \pm 0.76 \mu\text{mol/g}$ tissue and $4.63 \pm 0.36 \mu\text{mol/g}$ tissue in the hippocampus, whereas the concentrations in the control group were $12.42 \pm 1.49 \mu\text{mol/g}$ tissue and $11.23 \pm 1.44 \mu\text{mol/g}$ tissue in the cortex and hippocampus, respectively. Significant differences in total thiol were also observed between the TAA+ Extract 12.5 and Control groups in both regions ($p < 0.05$ and $p < 0.01$, respectively). Nevertheless, total thiol concentrations in the cortex and hippocampus were considerably greater in the TAA+ Extract 25 ($8.76 \pm 1.01 \mu\text{mol/g}$ tissue in the cortex and $9.17 \pm 1.07 \mu\text{mol/g}$ tissue in the hippocampus) and TAA+ Vit C ($8.43 \pm 0.34 \mu\text{mol/g}$ tissue in the cortex and $8.81 \pm 0.92 \mu\text{mol/g}$ tissue in the hippocampus) groups than in the TAA group ($p < 0.05$).

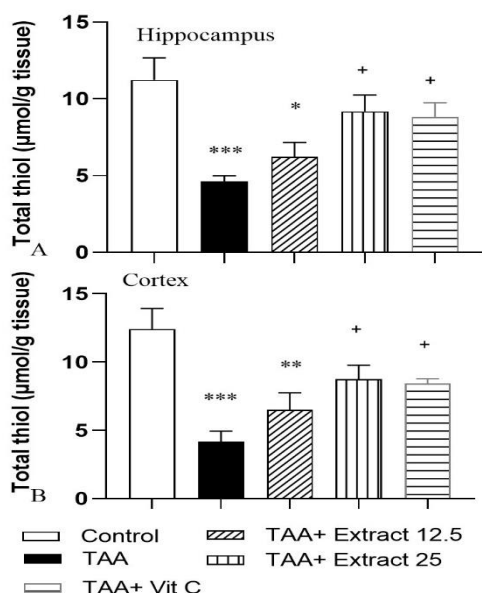


Figure 9. Effect of *C. botrys* extract and Vit C on total thiol levels in the hippocampus (A) and cortex (B) tissues. The data are presented as mean $\pm$ SEM (n=8). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, and * is used as a comparison to the control group. TAA: thioacetamide, Vit: vitamin.

AChE activity in the brain

Results indicated that AChE activity in the cortex and hippocampus was higher in the TAA group ($225.65 \pm 23.71 \text{ nmol/min/g}$ tissue in the cortex and $209.29 \pm 20.41 \text{ nmol/min/g}$ tissue in the hippocampus) than in the Control group ($99.15 \pm 5.42 \text{ nmol/min/g}$ tissue in the cortex and $86.96 \pm 11.93 \text{ nmol/min/g}$ tissue in the hippocampus) (Figure 10, $p < 0.001$), with a significant difference also observed between the TAA+ Extract 12.5 and Control groups in both regions ($p < 0.05$ and $p < 0.01$, respectively). The effect of TAA was reversed by administering 25 mg/kg of extract ($129.29 \pm 19.10 \text{ nmol/min/g}$ tissue in the cortex and $129.29 \pm 18.50 \text{ nmol/min/g}$ tissue in the hippocampus) and Vit C ($128.79 \pm 12.28 \text{ nmol/min/g}$ tissue in the cortex and $109.41 \pm 15.28 \text{ nmol/min/g}$ tissue in the hippocampus), as evidenced by a decrease in AChE activity in the cortex and hippocampus of the TAA+ Extract 25 and TAA+ Vit C groups ($p < 0.05$ and $p < 0.01$).

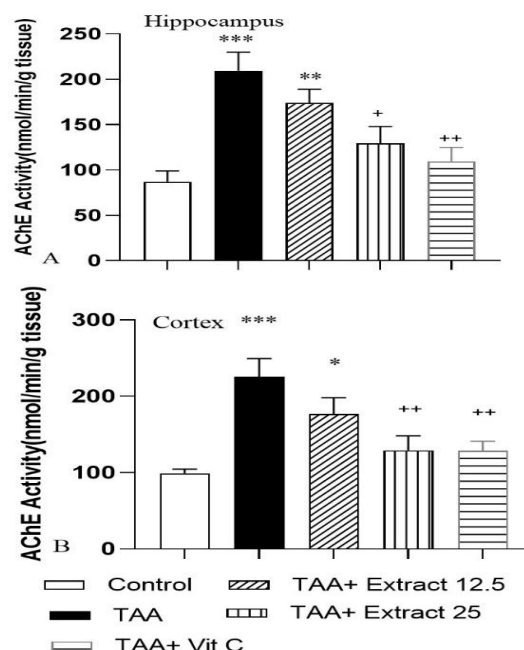


Figure 10. Effect of *C. botrys* extract and vitamin C on AChE activity in the hippocampus (A) and cortex (B) tissues. The data are presented as mean $\pm$ SEM (n=8). 1 symbol represents $p < 0.05$, 2 symbols represent $p < 0.01$, and 3 symbols represent $p < 0.001$. + is used as a comparison to the TAA group, and * is used as a comparison to the control. TAA: thioacetamide, Vit: vitamin, AChE: acetylcholinesterase

Discussion

This study found that *C. botrys* is able to mitigate the learning and memory impairment associated with TAA-induced HE and can improve the behavioral performance in rats with HE. These beneficial effects may occur due to the antioxidant activities of *C. botrys* and AChE inhibition by this extract.

The hepatotoxic potential of TAA has been thoroughly documented in the literature, and it is utilized in medical research to develop an animal model of HE (Farjam et al. 2012). Elevated levels of liver enzymes (ALT, AST, and ALKP) were observed in the blood serum of the TAA group. Similar findings were previously reported as well (Moustafa et al. 2014). TAA animals had lower latencies in the PA test and spent longer in the dark compartment and exhibited an increased number of entries to the dark. TAA animals also spent a longer duration and traveled longer in order to find the platform in the MWM. During the probe, they spent less time in the target quarter and traveled less as well. All of these together suggest impaired learning and a deficit of memory. These findings corroborate the detrimental effects of TAA-induced HE on learning and memory, aligning with prior research (Hajipour et al. 2023).

HE has a multifaceted and complex pathophysiology and may influence the learning and memory process with different mechanisms. These mechanisms include proinflammatory cytokine activation, blood-brain barrier disruption, altered energy metabolism, oxidative stress, direct toxic effects on astrocytes and neuronal membranes, and changes in glutamate concentration (Ciećko-Michalska et al. 2012; Ochoa-Sanchez and Rose 2018). Additionally, research showed that hyperammonemia-induced astrocyte swelling and oxidative/nitrosative stress led to RNA oxidation and protein modifications, changes in gene expression, and signaling pathways that altered cortical excitability. Following that, these situations

involve cognitive deficits (Lu 2023). Brain ammonia levels can increase as the result of blood ammonia increase since ammonia can readily cross the blood-brain barrier (Lu 2023). Studies show that hyperammonemia causes HE-related brain abnormalities. We did not evaluate ammonia levels in the current study; therefore, we cannot determine its role in the cognitive deficit, but TAA administration resulted in liver impairment, which can be corroborated with high levels of liver enzymes. Hepatic damage was further corroborated by evidence of oxidative stress in the liver, manifested as elevated lipid peroxidation alongside reduced thiol concentration and SOD activity in the TAA group.

In this study, *C. botrys* extract, particularly at the 25 mg/kg dose, along with Vit C, attenuated TAA-induced memory impairment in the PA test, as reflected by longer latency to enter the dark chamber, extended time spent in the light chamber, reduced time spent in the dark chamber, and fewer entries into the dark chamber relative to the TAA group. *C. botrys* and Vit C both alleviated the spatial memory impairment caused by TAA during the MWM test. This was evident by decreased escape latency and traveled distance. Both *C. botrys* extract and Vit C increased time and distance in the target quarter, suggesting an improvement of memory retrieval and recall. The effects of *C. botrys* were dose-dependent, and the higher dose produced better results than the lower dose but were comparable to Vit C.

Cholinergic neurotransmission role in cognitive function has been extensively studied (Sarter and Parikh 2005). In certain regions of the brain in rats with HE, the activity of AChE is affected (Méndez et al. 2011). Following the induction of TAA, AChE activity in the brain increased significantly in the current study, which is in line with findings from earlier investigations (García-Ayllón et al. 2008; Mladenović et al. 2015). It was reported that *C. botrys* extracts had significant

cholinesterase inhibitory abilities and may be useful in the treatment of Alzheimer's disease (AD) (Iqbal et al. 2020). In another study, the water extract of *C. botrys* showed inhibiting action on AChE (Ozer et al. 2016). These results are consistent with the present study's findings. The AChE activity of HE rats was suppressed in both the cortex and hippocampus tissues by *C. botrys* extract and Vit C in the current study.

The effects on the AChE activity were dose-dependent in the case of *C. botrys*, where the higher dose was similar to Vit C in terms of effectiveness and the lower dose was not effective. Even though we evaluated the activity of AChE, we did not measure the levels of acetylcholine itself, which we suggest should be measured in the future studies. Considering the findings of the present work, the improvement of memory and learning performance in the behavioral tests might be due to modulation of cholinergic activity and, specifically, AChE. The decrease in AChE and increase of acetylcholine might be the reason for memory improvement, but acetylcholine needs to be measured to confirm the hypothesis. Since oxidative stress is another possible mechanism contributing to learning and memory impairment in HE (Lemberg and Fernández 2009), we also measured oxidative stress markers in the cortex and hippocampus. Several neurodegenerative diseases, such as HE and AD, are linked to oxidative stress. Oxidative stress also greatly impacts neurodegenerative disease progression. Oxidative damage can occur in the brain due to an imbalance between reactive oxygen species (ROS) generation and neutralization. Overproduction of ROS can cause cellular dysfunction, apoptosis, and neurodegeneration (Simicic et al. 2022). Some of the most prominent biomarkers for oxidative stress are MDA as a marker for lipid peroxidation and SOD and thiol content as natural antioxidant defenses (Rajabian et al. 2025). We found increased MDA levels and decreased thiol content and SOD in both the hippocampus and

cortex of animals in the TAA group. Previous studies also report similar changes in the oxidative stress markers (Abdel Salam et al. 2013; Mousa et al. 2019).

Rats with TAA-induced HE showed decreased MDA and increased SOD and thiol content when treated with *C. botrys* and Vit C. This was the case in both the hippocampus and the cortex.

C. botrys and Vit C fortified antioxidant defense and reduced oxidative damage done by TAA administration as suggested by our findings. Vit C and the high dose of *C. botrys* improved thiol concentration in the hippocampus and cortex, but the lower dose was not effective in that regard. In the case of SOD and MDA, all treatment groups, including both doses of *C. botrys* and Vit C, were effective, and there was no difference between treatments. The effects of the plant extract on thiol content in the hippocampus were dose-dependent. The *C. botrys* plant is a substantial source of antioxidant compounds (Arvin and Firouzeh 2022), similar to Vit C (Padayatty et al. 2003). In a study consistent with the results of the current research, conducted on acute rhabdomyolysis-associated kidney injury in rats, it was shown that *C. botrys* extract could increase the total antioxidant capacity in kidney tissue (Momeni-Moghaddam et al. 2025). Another study utilized *C. botrys* hydroalcoholic extract as an analgesic in mice due to its anti-inflammatory and antioxidant effects (Mohebbati et al. 2023). Vit C, as a well-known antioxidant agent, has been frequently shown to have some beneficial effects (Beheshti et al. 2017; Esmailizadeh et al. 2020; Ghasemi et al. 2017; Hosseini et al. 2018; Khordad et al. 2018; Mustafa et al. 2013). Vit C was reported to be protective in HE (Mustafa et al. 2013; Patel et al. 2022) and it was used as a positive control in the current research however, it was better to use the standard drugs such as lactulose and rifaximin as positive controls. We found that the antioxidant effects of *C. botrys* were comparable to Vit C when it comes to brain. The results found in the

present work indicates that the beneficial effects of *C. botrys* extract on HE might partially be due to the antioxidant effects.

Previous works report a link between oxidative stress and the cholinergic system. The study suggests that oxidative stress can upregulate the AChE mRNA expression, which subsequently results in increased AChE (Evans et al. 2024). Therefore, it seems that a part of the effects of the plant extract and Vit C on the cholinergic system is indirectly related to its antioxidative effects; however, it needs to be investigated more.

Analysis of oxidative stress markers in liver tissue revealed that Vit C and both doses of the plant extract attenuated MDA levels. Vit C and the higher extract dose also improved thiol content, with the high dose producing significantly greater effects than the low dose. Additionally, the high dose of the extract improved SOD activity, whereas Vit C and the low dose showed no significant effect. As indicated by our finding, both Vit C and *C. botrys* extract have hepatoprotective effects, and this might be a contributing factor to the memory and learning improvement witnessed in the present study. However, the results of liver function markers did not fully confirm this hypothesis, and the high dose of the extract was able to attenuate only AST, but the low dose of the extract and Vit C were not effective. In addition, neither the plant extract nor Vit C affected ALT and ALKP in the serum.

The responsible components of the extract for the results of the present study were not determined in the current research. The polyphenolic composition of *C. botrys* was reported to reduce oxidative stress and nitrosative stress (Bojilov et al. 2023) and therefore might be responsible for beneficial effects of the plant, which was seen in the present study. Quercetin is a main flavonoid compound present in *C. botrys* (Bojilov et al. 2023). Quercetin was previously reported to attenuate inflammation and oxidative stress in a rat model of HE (Baraka et al. 2020)

terpenoids that are also present within *C. botrys* have anti-inflammatory effects (Bergman et al. 2019), and all of these substances can contribute to the overall antioxidant defense.

Our study had some limitations that should be addressed. The effects of the treatment were not compared with standard treatments of HE such as lactulose, so we do not know how effective the *C. botrys* is in comparison with these drugs. The active compounds of the plant extract were not identified, and it is suggested to be determined in the future studies. Ammonia levels were not assessed as well, and we did not examine how treatments affected inflammation and inflammatory markers. This work also did not provide any histopathological data, and future studies should examine how this treatment affects the histology of the liver and brain, inflammation, and systemic ammonia levels. The effects of the active compounds should also be studied.

The extract of *C. botrys* and Vit C were found to reduce AChE activity and oxidative stress while enhancing memory in rats with induced HE. The findings of this study indicate that *C. botrys* extract and Vit C may serve as therapeutic agents for mitigating neurodegeneration and memory impairment associated with HE.

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Conflicts of interest

Authors declare no conflict of interests.

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Ethical Considerations

The procedures performed in this study have been approved by the ethical committee of Mashhad University of Medical Sciences.

Code of Ethics

Ethical approved number:
IR.MUMS.REC.1402.336

Authors' Contributions

RS and SM conducted the behavioral tests and biochemical assessments.
FS and KhKh helped with behavioral tests.
AR assisted with the biochemical assessments.
SH, FA, MNS, AKhR contributed to the manuscript.
MH conceptualized and finalized the manuscript.

Abbreviations

HE = Hepatic encephalopathy; TAA = Thioacetamide; AChE = Acetylcholinesterase; *C. botrys* = *Chenopodium botrys*; Vit C = Vitamin C; PA = Passive avoidance; MWM = Morris water maze; ALKP = Alkaline phosphatase; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase; MDA = Malondialdehyde; SOD = Superoxide dismutase; ROS = Reactive oxygen species;

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