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UNCORRECTED PROOF

# Dodder (*Cuscuta* sp.) extract prevents cognitive deficits in a rat model of hepatic encephalopathy

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## ABSTRACT

**OBJECTIVE:** In our study, the protective effect of dodder plant extract against encephalopathy induced by cholestatic liver disease model was investigated.

**METHODS:** Spraque Dawley rats were used in the study. For the cholestatic liver disease model, the bile duct ligation (BDL) was applied. The groups were determined as control, *Cuscuta* sp. (CUS), BDL and BDL + CUS. Double ligation was performed in the bile duct in the BDL groups. For the applications, saline (SF) was administered to the control and BDL groups for 28 days while 250 mg/kg of *Cuscuta* sp. extract was given by oral gavage to the CUS and BDL + CUS groups. At the end of the experiment, cognitive evaluations were made by applying new object recognition and Morris water maze tests. After these tests, blood-brain barrier (BBB) measurements were made in half of the groups. In the other half of the groups, brain tissue samples were taken by decapitation and transforming growth factor-beta (TGF-β), 8-hydroxydeoxyguanosine (8-OHdG) and sodium-potassium adenosine triphosphatase ($Na^+/K^+$-ATPase) measurements were made in the tissues. Histological examinations of the tissues were also performed.

**RESULTS:** Cognitive performance was low, and BBB permeability was found to be increased in the group with bile duct ligation. In addition, TGF-β and 8-OHdG levels were increased in tissues, while $Na^+/K^+$-ATPase enzyme activity was suppressed. Treatment with *Cuscuta* sp. increased cognitive performance and decreased BBB permeability. Other biochemical parameters examined were significantly ($p<0.05–0.001$) reversed and supported by histological findings.

**CONCLUSION:** Our findings in the study suggest that dodder plant may be beneficial for the protection of cognitive performance and brain tissue in encephalopathy caused by cholestasis.

*Keywords: Cuscuta sp.; cholestasis; encephalopathy; fibrosis.*

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It is well known that cholestasis-induced liver dysfunction causes toxic substances accumulation such as bilirubin, urea, and ammonia in the blood, and hepatic fibrosis [1, 2]. Cholestasis also causes neurological and cognitive disorders known as hepatic encephalopathy (HE) [2]. Although the mechanisms responsible for hepatic encephalopathy have not been fully defined, the factors that trigger the development of neurological damage in patients with cirrhosis are increased toxic metabolites in the blood, especially ammonia [3–5]. Hyperammonemia induced by liver failure causes astrocyte swelling, brain edema, neurological dysfunction, changes in chemical homeostasis, and disorders in glutamatergic neurotransmission [5]. Suggestions propose that brain edema resulting from astrocyte swelling may be attributed to the disruption of aquaporin 4 (AQP4) channels, alterations in brain sodium and potassium homeostasis, changes in glutamine synthesis in the astrocyte, and an increase in extracellular glutamate levels [6]. Since ammonia ($NH_3$) is a weak base, it easily crosses the blood-brain barrier (BBB), via diffusion [7]. The cerebral detoxification pathway of ammonia is converted to glutamine by combining with glutamate in astrocytes [8]. According to biochemical evaluations of cerebrospinal fluid (CSF), it has been reported that there is a positive relationship between the severity of HE level and glutamine level [9]. Accumulated glutamine leads to astrocytic swelling through its osmotic effect and, also transported to presynaptic neurons, is converted into GABA or glutamate which plays a crucial role in the neurological pathways underlying HE [9].

Besides ammonia, other systemic factors such as increased bile acids and lactate, inflammation, and oxidative stress are also important factors in the development of HE [5]. Since oxygen is the main metabolic substrate in the central nervous system (CNS), disruptions in oxygen supply impair brain energy metabolism and ATP production, leading to oxidative stress [10]. In addition, activation of immune cells by proinflammatory cytokines also leads to overrun of reactive oxygen species (ROS) [11]. Although antibiotics and nutritional supplements are included in the treatment of HE today, the side effects of antibiotics limit their use [12]. Accordingly, searches for new alternatives that improve hepatic/neuronal functions with minimal side effects continue, and therapies that inhibit oxidative stress caused by hyperammonemia are targeted.

Certain herbs have been employed in traditional medicine to address issues related to the liver and bile ducts. A particular plant species called *Cuscuta* is utilized for treating jaundice in traditional medicine and is commonly referred to as "Bostanbozan" in the local language of our country. This plant is annual or perennial, chlorophyll-free and parasitic plant and has also traditional uses such as a diuretic, carminative, and laxative. *Cuscuta* species contain flavonoids, alkaloids, steroids, sterols, triterpenes, carotenoids, and fatty acids and exhibit anti-inflammatory and antioxidant characteristics [13].

**Highlight key points**

- *Cuscuta* sp. extract improves cognitive performance in BDL-induced rats.
- *Cuscuta* sp. treatment decreases blood-brain barrier permeability in BDL-induced rats.
- *Cuscuta* sp. extract reverses brain tissue biochemical changes in BDL-induced rats.
- Histological tissue examinations confirm protective effects of *Cuscuta* sp.
- *Cuscuta* sp. shows promise in cholestatic encephalopathy management.

In the light of above knowledge, this study aimed to investigate the potential benefits of *Cuscuta* sp. extract against BDL-induced hepatic encephalopathy in rats, assessing both cognitive functions and biochemical parameters.

## MATERIALS AND METHODS

### Preparation of CUS Plant Extract

CUS plant samples were collected from Mardin-Midyat district and after identification, stored in Marmara University Herbarium under MARE no 22668. For extract preparation, the aerial parts of the plant were dried, powdered, and then macerated using methanol. The solvent of the methanol extract was evaporated. Extract was kept at + 4°C until the usage. After performing in vitro antioxidant and anti-inflammatory tests, the extract was included in the in vivo study [14].

### Animals, Ethics and Experimental Protocol

Male Sprague Dawley rats weighing between 200–300 g were housed under standard laboratory conditions with a constant temperature of 22±2°C, relative humidity of 50%±5, and a light/dark cycle of 12/12 h. All procedures for experimental protocols of the present study involving animals were performed in accordance with the ethical standards of the institution of practice at which the studies were conducted. This study was performed

in line with the principles of the Declaration of Helsinki. The research protocol was approved on 03.03.2021 by the Marmara University Animal Experiments Local Ethics Committee) (approval no: 40.2021mar).

Rats were divided into 4 groups, 12 rats in each. Sham-operated control (C), C + *Cuscuta* sp. methanol extract (CUS) (250 mg/kg, oral gavage in 1 mL), bile duct ligated (BDL), and BDL + CUS groups. The dose of *Cuscuta* sp. extract was based on an earlier study [13].

The C and BDL groups received 1 mL of saline, while the CUS and BDL + CUS groups were administered the methanolic extract of CUS at a dose of 250 mg/kg via oral gavage for 28 days. All treatments were started on the first day of BDL. At the end of the experimental period, in half of the animals of the groups BBB was measured, while the other half were subjected to cognitive tests and then decapitated to obtain brain tissue samples.

## Bile Duct Ligation for Induction of Cholestasis-Induced Encephalopathy

Under anesthesia induced by intraperitoneal injection of 100 mg/kg ketamine and 0.75 mg/kg chlorpromazine, a midline laparotomy was carried out to expose common bile duct. Using double ligation method,one ligature was placed below the junction of the hepatic ducts and the other was above the entrance of the pancreatic ducts. The bile duct was subsequently cut between the ligatures [15].

## Cognitive Tests

### Morris Water Maze Test

The water pool consists of a stainless-steel circular tank (160 cm in diameter, 35 cm in height). The tank is divided from 4 fixed points into form 4 quadrants. This pool included an escape platform measuring 10x10x10 cm in the same color as the pool (to eliminate any false positives related to the image). The platform was kept in a fixed quadrant of the pool and 1.5 cm below the water surface throughout the study. The rats were slowly released into the pool at the edge of a quadrant with no escape area. If the rat was unsuccessful to find the escape platform within 90 seconds, it was mildly directed to the platform and allowed to remain on it for 30 seconds. Animals were given 4 trials, spacing 10 minutes each for 5 consecutive days, during which time was taken to reach the platform.

On day 5, 24 hours after the previous trial, the escape platform was removed, and the probe studies were initiated by allowing the animals to swim freely for 75 seconds before terminating the study. In this probe study, the time the animal reached target quadrant and the time spent in target quadrant were calculated. This test has been accepted as a measure of spatial learning and memory [16].

### Novel Object Recognition Test

Novel object recognition test (NORT) was carried out in a semi-dark environment in a setup made of 50x50x30 cm black plexiglass. Before each animal was tested, the objects and apparatus were cleaned with ethanol. One day before starting NORT, pre-training was done and the next day the test was started. On the training day, the animals in the same cage were placed in the plexiglass assembly without the object and allowed to acclimate to the test apparatus for 60 minutes. The acclimation process was applied to all animals. On the test day, each animal was placed in the setup for 3 minutes, 60 minutes apart. The first insertion was called the recognition phase. In this process, the same two objects will be placed in the test setup and the animal was permitted to wander liberally in the setup for 3 minutes and recognize these objects. After 3 minutes, the animal was taken from the apparatus which was made of plexiglass and located in its cage (T1). After 60 minutes, the same animal was rearranged and one of the objects it had seen in T1 was changed, allowing it to wander liberally for 3 minutes (T2). Both periods were recorded with the help of a computer-aided camera system and the animals' interest in both objects was scored in T2. The ratio of the animal's interest in the novel object to its interest in the old object has been accepted as a measure of the learning and memory function of this animal [17].

## Blood-Brain Barrier Measurement Method

Six animals from each group were used for BBB permeability assessment. BBB integrity was assessed using Evans blue, which was used as an indicator of albumin extravasation. Following the induction of anesthesia, Evans blue (4 mL/kg in 2% saline) was infused into the jugular vein and was allowed to circulate for 30 minutes. Then thorax was exposed, and the rats were perfused transcardially with 250 mL of saline at 110 mm Hg for around 15 minutes. After guillotining, whole brain tissues were detached for quantitative measurement of Evans blue albumin extravasation. Brain samples were homogenized in 2.5 mL of phosphate-buffered saline and mixed by vortexing for 2 minutes. To precipitate the proteins 2.5 mL of 60% trichloroacetic acid was added and then cen-

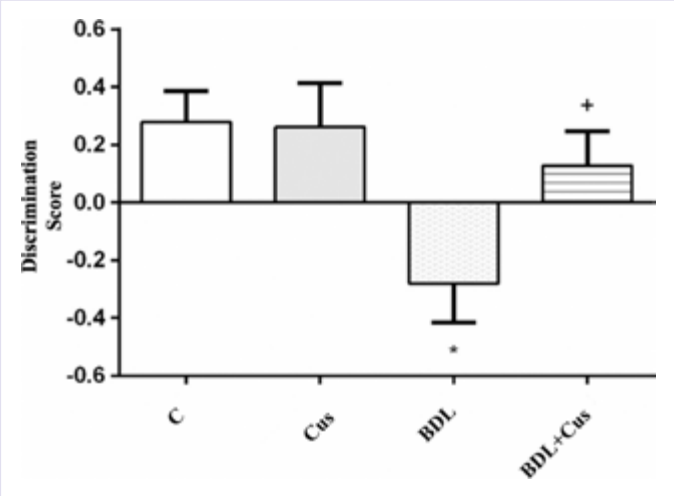


FIGURE 1. Discrimination score of groups.

C: Control; BDL: Bile duct ligation; CUS: *Cuscuta* sp. extract. * : P<0.05: Compared to control group; +: P<0.05: Compared to BDL group.

trifuged at 1000 g for 30 minutes. Absorption of Evans blue dye in the supernatant was measured at 620 nm using a spectrophotometer (Shimadzu UV1208, Japan). By using a standard curve which is stated as mg/g, absorption of Evans blue dye was analyzed for brain tissue [18].

### Biochemical Analysis

In brain tissues, using commercial kits (AFG BIOSCIENCE) transformed growth factor-β (TGF-β; EK720060), 8-hydroxyguanosine (8-OHdG; EK720424), and sodium-potassium ATPase ($Na^+/K^+$-ATPase; EK720668) were analyzed in accordance with the manufacturer's procedures.

### Light Microscopic Preparation

Brain tissue samples were fixed in 10% formaldehyde, followed by clearance with diluted alcohol using toluene. The samples were then fixed in paraffin and cut into 5 μm thickness. To evaluate histopathology, the brain tissue slices were marked with hematoxylin and examined under a light microscope (Olympus BH-2, Tokyo, Japan).

### Statistical Analysis

Statistical evaluations were executed by using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean±SEM. ANOVA followed by Tukey multiple comparison tests were used to compare the data of the groups except for cognitive tests where Mann-Whitney U non-parametric test was used. $p<0.05$ values were considered significant.

## RESULTS

### Novel Object Recognition Test

The new object test results indicated that discrimination index levels were significantly decreased ($p<0.05$) in the BDL group than that of both control and control + CUS group. On the other hand, it was found to be increased in BDL + CUS group ($p<0.05$, Fig. 1).

### Morris Water Maze (MWM) Test

Morris water maze test learning habituation period findings showed that the time to find the platform decreased significantly in the in-group comparisons of each group.

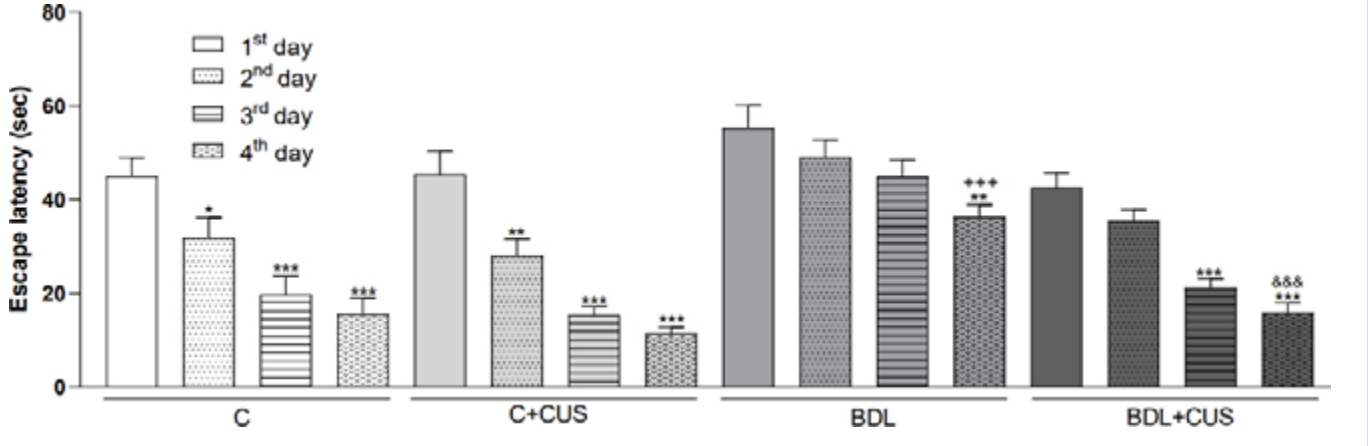


FIGURE 2. Time taken to find the platform (Escape latency) in the learning phase (1–4 days) in the Morris water maze test.

C: Control; BDL: Bile duct ligation; CUS: *Cuscuta* sp. extract. *: P<0.05; ***: P<0.001 according to the 1st day of each group; +++: P<0.001 is comparison for the 4th day of each group to the 4th day of the control group; &&&: P<0.001 is comparison for the 4th day of the BDL + CUS group to the 4th day of the BDL group.

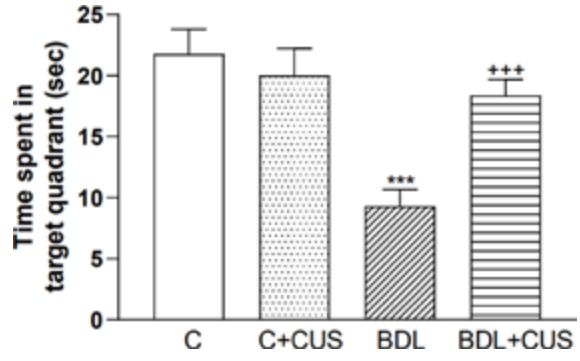


FIGURE 3. Time spent in the target quadrant in the Morris water maze test.

C: Control; BDL: Bile duct ligation; CUS: *Cuscuta* sp. extract. ***: P<0.001 Compared to the control group; +++: P<0.001 Compared to the BDL group.

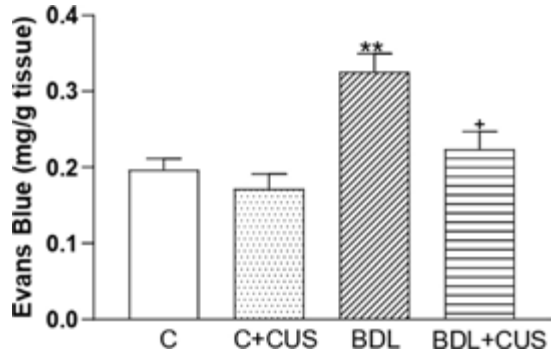


FIGURE 4. Blood-brain permeability results measured by Evans blue absorbance.

C: Control; BDL: Bile duct ligation; CUS: *Cuscuta* sp. extract. **: P<0.01; Compared to the control group; +: P<0.05; Compared to BDL group.

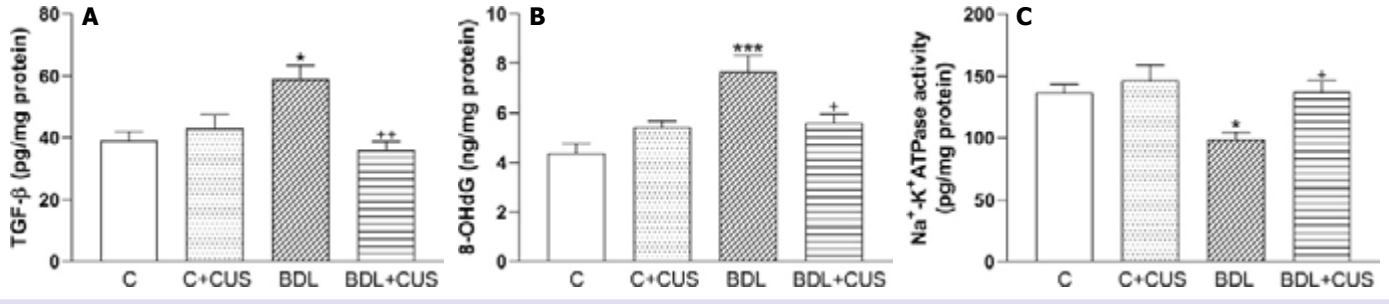


FIGURE 5. TGF-β, 8-OHdG and $Na^+/K^+$-ATPase results of brain tissue.

TGF-β: Transforming growth factor; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; $Na^+/K^+$-ATPase: Sodium-potassium adenosine triphosphatase; C: Control group; BDL: Bile duct ligation; CUS: *Cuscuta* sp. extract. One-way analysis of variance ANOVA was used for statistical analysis. *: P<0.05; ***: P<0.001; Compared to the control group; +: P<0.05; ++: P<0.01; Compared to BDL group.

This decrease has been started on the 2nd day in the control groups, on the 4th day in the BDL group and on the 3rd day in the BDL + CUS group. The time to find the platform at day 4 recorded in the BDL group was higher than that of the three others (p<0.01), while in the BDL + CUS group, this value was found to be significantly lower than the value in the BDL group (p<0.001, Fig. 2).

According to the test day findings, there was a significant decrease in the time that spent in target quadrant of BDL group compared to the C group (p<0.001). On the other hand, when compared to the BDL group, a significant (p<0.001) increase was observed in BDL + CUS group (Fig. 3).

## Findings of Blood-Brain Barrier Measurement

Evans blue absorbance levels, indicating BBB, in the BDL group were found to be significantly higher (p<0.01) than the C group. Furthermore, these levels were significantly lower (p<0.05) in the BDL + CUS group than in the BDL group (Fig. 4).

## Biochemical Findings of Brain Tissues

TGF-β, 8-OHdG levels were significantly increased in the brain tissues of the BDL group compared to the C group, while in BDL + CUS group, these levels were found to be significantly lower compared to the BDL group (p<0.05–0.001, Fig. 5A, B).

$Na^+/K^+$-ATPase levels in the brain tissues of the BDL group were significantly lower compared to the C group, and $Na^+/K^+$-ATPase levels were found to be higher in the CUS-treated-BDL group compared to the BDL group (p<0.05, Fig. 5C).

## Histological Evaluations

Light microscopic evaluation of the control (Fig. 6A) and

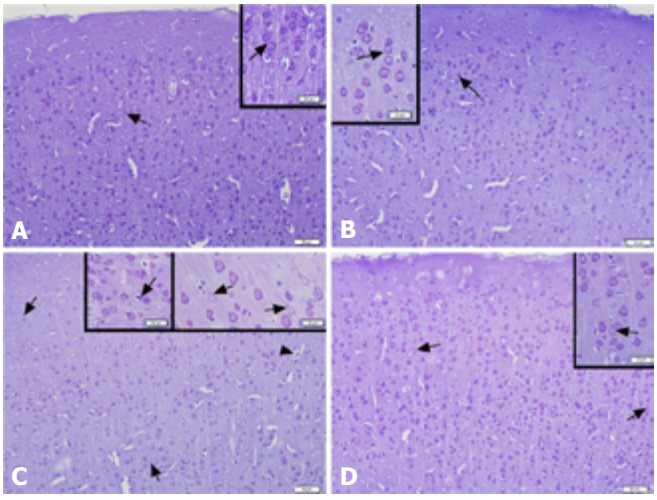


Figure 6. Representative light micrographs of the cerebral cortex samples in the experimental groups. Regular morphology of the cerebral cortex with neurons (arrow) and capillaries are seen in the control **(A)** and C + CUS **(B)** groups. Severe increase of degenerated neurons (arrow) and subcapillary edema (arrowhead) are seen in the BDL group **(C)**. Moderate decrease of degenerated neurons (arrow) is seen in the BDL + CUS group **(D)**. Cresyl violet staining, Scale bars: 50 µm, insets: 20 µm.

C + CUS (Fig. 6B) groups showed a regular morphology of the cerebral cortex with neurons and capillaries. Severe increase of degenerated neurons and subcapillary edema were observed in the BDL group (Fig. 6C).

On the other hand, in the BDL + CUS group, moderate decrease of degenerated neurons and subcapillary edema were observed (Fig. 6D).

## DISCUSSION

Hepatic encephalopathy (HE), a significant neurological condition, induces cognitive, psychiatric, and motor impairments, contributing to a substantial number of hospitalizations in the USA, exerting a heavy burden on the healthcare system [19–21]. Cognitive alterations in HE patients are closely linked to heightened brain ammonia levels, influencing multiple neurotransmitter systems, leading to astrocyte swelling and, ultimately, cerebral edema [22]. In this study, we examined the potential protective effects of *Cuscuta* sp. against BDL-induced brain tissue damage and the accompanying changes in cognitive functions.

Morris water maze test was designed as a method to assess spatial or location learning [23]. According to the MWM test findings of BDL studies, cognitive decline in animals has been reported [24, 25]. In agreement with the mentioned studies, in our study significant decreases were observed in the learning performance of animals in the BDL group compared to the control group. Based on the Morris Water Maze (MWM) end-of-task results, the decrease in the time spent in the target quadrant by the BDL group animals compared to the control rat suggested that the animals' 4-day learning performance was unsuccessful. The BDL group treated with CUS exhibited a significant improvement in both their performance and the time spent in the target quadrant compared to the BDL group.

It is known that cognitive functions were impaired in patients with hepatic encephalopathy that develops with cholestasis. In experimental studies, short-term memory functions are examined with the novel object recognition test (NORT) [26, 27]. Accordingly, we examined the changes in memory in rats with cholestasis and the effects of CUS treatment on these changes using NORT. When the results were examined, as expected, cholestasis significantly reduced cognitive performance. In our previous study, we showed that cholestasis impairs liver functions and treatment with CUS extract protects the liver. In this study, the improvement in cognitive functions caused by CUS treatment is thought to be probably due to the liver-protective effect of CUS and also its protective effect on the BBB.

The hepatoprotective effect of different types of CUS has been demonstrated in different studies. Koca-Caliskan et al. [13] reported that *Cuscuta* arvensis Beyr extract protected the liver tissue with its antioxidant effects in paracetamol toxicity, and the deteriorated liver enzyme values were significantly improved. Similarly, in the study by Yen et al. [28] the protective effect of the seeds of *Cuscuta* chinensis Lam, which is utilized in traditional Chinese medicine, against acetaminophen toxicity was demonstrated.

Increased ammonia levels in the circulation of rats with liver damage impair BBB integrity and may ultimately cause HE [29, 30]. Indeed, when the pathophysiology of HE due to chronic liver diseases is examined, it has been understood that hyperammonemia causes swelling in astrocytes and brain edema, and as a result BBB is impaired [5]. Ammonia is a neurotoxin that causes a wide variety of dysfunction in the central nervous system (CNS). It is well known that HE is associated with increased inflammation, ROS formation, impaired neurotransmission, astrocyte

swelling, and cerebral edema [31]. In our earlier study, BDL increased oxidative damage in brain tissues, and the BBB was disrupted due to increased ROS [32]. Similarly, in the present study, increasing absorbance values indicated heightened BBB permeability, signifying damage to the barrier. On the other hand, CUS prevented the deterioration of BBB due to its hepatoprotective, antioxidant, anti-inflammatory and neuroprotective effects.

The most harmful targets of ROS are nucleic acids (macromolecules such as DNA), membrane lipids and proteins. The sodium/potassium adenosine triphosphatase ($Na^+/K^+$-ATPase), is a structural enzyme found in all cell membranes. A decrease in enzyme activity serves as an indicator of membrane damage. The enzyme is crucial for maintaining ion balance in the brain. Reduced enzyme activity leads to astrocytic swelling, brain edema, inflammation, and neuronal damage, contributing significantly to the development of HE [33]. Toklu et al.'s study [32] demonstrated that nigella sativa oil protects brain tissue from oxidative damage by reducing oxidative stress and inflammation in the BDL-induced cholestasis model. They also showed that decrease in $Na^+/K^+$-ATPase enzyme activity in the brain was accompanied by oxidative injury; since there was a significant increase in malondialdehyde levels, a lipid peroxidation index, and a decrease in glutathione levels, an important antioxidant. Our study yielded similar results. While $Na^+/K^+$-ATPase activity in the brain tissues of the BDL group significantly decreased compared to the control group, the enzyme activity increased in the treatment group subjected to CUS.

As mentioned above, ROS damage cell structures and thus their functions. The most commonly used marker for assessing damage to DNA structure is 8-hydroxy-2'-deoxyguanosine (8-OHdG). More recently, Pierzchala et al. [34] emphasized the role of inflammation in the development of HE, by showing an increase in both CNS and systemic oxidative stress in BDL-treated rats. Indeed, utilizing immunohistochemical methods, they demonstrated an increase in both 8-OHdG, a marker of oxidative DNA/RNA modification, and the proinflammatory cytokine interleukin-6 in the brain tissues of BDL rats. Similarly, in our study, indicating oxidative damage, 8-OHdG levels exhibited a marked increase in the brain tissues of rats in the BDL group, whereas a significant reduction in 8-OHdG levels was observed in the BDL + CUS group.

Transforming growth factor (TGF-β) is a multifunctional cytokine that plays a fundamental function in numerous biological processes, involving embryonic development, cellular maturation, differentiation, and wound healing. Thus, it plays a regulatory role in the physiological and pathological changes associated with tissue damage, inflammation, and fibrosis. In the study of McMillin et al. [35] it was indicated that liver failure increased the expression of TGF-β in both the liver and brain tissue. The study suggested that the elevated cytokine in the brain leads to neuroinflammation, neurological deficits, and ultimately HE. Similarly, in our study, BDL caused an increase in TGF-β levels in the brain, which agrees with the literature. On the other hand, CUS treatment reversed these cytokine levels, likely through its anti-inflammatory and antioxidant properties. Our histological findings also support the biochemical findings; accordingly, there was significant damage to the brain tissue of the BDL group, whereas this damage was ameliorated in CUS-treated BDL rats.

## Conclusion

As a conclusion, CUS has a possible protective effect against cholestasis-induced oxidative brain damage. The observed beneficial effects of CUS on liver fibrosis and hepatic encephalopathy could be attributed to its anti-inflammatory, antioxidant, and neuroprotective properties. Therefore, based on these findings, CUS may suggest a future role in the treatment of cognitive dysfunction, and HE induced by cholestasis.

**Ethics Committee Approval:** The Marmara University Animal Experiments Ethics Committee granted approval for this study (date: 03.03.2021, number: 40.2021mar).

**Authorship Contributions:** Concept – GS, OA, DO, AS; Design – GS, OA, BNH, AS; Supervision – GS, FE, OC, AS; Fundings – GS, OC, DO, OA, SEP; Materials – GS, DO, FE, TK, AD, AS; Data collection and/or processing – GS, OC, OA, BNH, DO, AD, FE, TK, FK; Analysis and/or interpretation – GS, OC, OA, BNH, DO, FK; Literature review – GS, OA, AS, BNH, SE, DO; Writing – GS, OA, AS, BNH, SE, DO; Critical review – GS, OA, AS, BNH, SE, DO, FE.

**Conflict of Interest:** No conflict of interest was declared by the authors.

**Use of AI for Writing Assistance:** Artificial intelligence (AI) assisted technologies (such as Large Language Models [LLMs], chatbots or image generators, ChatGPT) were not used in our study.

**Financial Disclosure:** The authors declared that this study has received no financial support.

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