Full Length Research Paper

Protective effects of *Ziziphus jujuba* fruit extract against ethanol-induced hippocampal oxidative stress and spatial memory impairment in rats

Majid Taati[1]*, Masoud Alirezaei[2], Mohamad Hadi Moshkatalsadat[3], Bahram Rasoulian[4], Mehrnoush Moghadasi[4], Farzam Sheikhzadeh[5] and Ali sokhtezari[6]

[1]Department of Physiology, School of Veterinary Medicine, Lorestan University, Khoramabad, Iran.
[2]Division of Biochemistry, School of Veterinary Medicine, Lorestan University, Khoramabad, Iran.
[3]Department of Chemistry, School of Basic Sciences, Lorestan University, Khoramabad, Iran.
[4]Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khoramabad, Iran.
[5]Department of Biotechnology, Agriculture Faculty, Tabriz University, Iran.
[6]Department of Clinical Sciences, School of Veterinary Medicine, Lorestan University, Khoramabad, Iran.

The current study assessed the effects of *Ziziphus jujuba* fruit extract on hippocampal oxidative stress and spatial memory of rats exposed to ethanol. Male Wistar rats were randomly divided into control, *Z. jujuba* treated (200 mg/kg, p.o.), ethanol (4 g/kg, p.o.) and *Z. jujuba* plus ethanol groups. The animals were treated daily by oral gavage for 8 weeks. The learning and memory performance was assessed using Morris water maze (from day 55 to 60). At the end of experiment, rats were sacrificed by decapitation and brains were dissected out for thiobarbituric acid reactive substances, and the activities of antioxidative enzymes (glutathione peroxidase and superoxide dismutase) determinations in the hippocampus. It was found that combined *Z. jujuba* extract-ethanol treatment significantly decreased TBARS level and increased glutathione peroxidase activity compared to the ethanol group (p<0.05), whereas no significant difference was observed in superoxide dismutase activity. In Morris water maze, rats treated with *Z. jujuba* prior to ethanol significantly shortened total path length during the acquisition period and enhanced the spent time in the correct quadrant on probe trial performance compared to ethanol treated rats (p<0.05). The results show that *Z. jujuba* fruit extract improved spatial memory impairment induced by ethanol, due in part, by its antioxidant activities.

Key words: *Zizyphus jujuba*, ethanol, oxidative stress, memory.

INTRODUCTION

Ethanol-induced neuropathology leads to a variety of behavioral problems, including hyperactivity, attention deficits, motor dysfunction, impairments in language and social skills, and learning deficits (Mattson et al., 2001; Riley and McGee, 2005). One of the principal cognitive effects of ethanol is disruption of learning and memory. Ethanol preferentially impairs hippocampal-dependent learning and memory tasks (Acheson et al., 2001). The pathogenesis of alcohol-induced injury of the central nervous system is a manifold process in which oxidative stress plays a pivotal role (Watts et al., 2005; Lee et al., 2007). Reactive oxygen species (ROS) are produced within the body during oxygen metabolism and living

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*Corresponding author. E-mail: Taatimajid@yahoo.com. Tel/Fax: +98-661-6200109.

organisms have developed several defense mechanisms to protect themselves from oxidative stress (Wickens, 2001). Under normal conditions, ROS and antioxidant systems are in balance. If any imbalance occurs between pro oxidant and antioxidant factors, it is called oxidative stress (Floyd and Hensley, 2002). Ethanol consumption enhances the generation of harmful reactive oxygen species (Lieber, 2000) and leads to a depletion of the antioxidant defenses in the brain (Nordmann, 1994). On the other hand, the brain is believed to be particularly vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of antioxidative defenses (Brewer, 1998).

Studies have shown potential neuroprotective effects of plant origin antioxidants such as flavonoid compounds against alcohol-induced injury (Antonio and Druse, 2008) and their beneficial effects on cognition have been demonstrated in animal studies (Kumar el., 2009; Khalili et al., 2009; Farshchi et al., 2010; Divyaet al., 2010). *Zizyphus jujuba* (*Rhamnaceae*) is widely distributed in Iran and fruit of this plant has gained wide attention in native herbal medicine for treatment of a broad range of disorders. Chemical analysis of its fruit has shown the presence of flavonoids (quercetin and kaempferol) and phloretin derivatives (Pawlowska et al., 2009). However, its antioxidative activity in central nervous system and its effects on spatial memory deficits induced by ethanol have not been scientifically documented so far. Thus the aims of this study were (1) to investigate the effect of ethanol on superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and on thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation, in the hippocampal area of the rat brains and (2) to determine the effects of aqueous extract of *Z. jujuba* fruit on ethanol-induced impairment of spatial learning and memory as well as on antioxidant enzymes activities and TBARS levels.

MATERIALS AND METHODS

Preparation of extract

Fresh ripened fruits of *Z. jujuba* were purchased from local herbal shops of Khoramabad, Iran during the months of October to November, 2008. Fruits were authenticated at the botany department of Lorestan University. Seeds were separated from fruits and about 700 g of pulp material was extracted three times with distilled water (1500 ml totally) by grinding with a mechanical set. It was centrifuged at 4°C for 20 min at 4000 g, and the supernatant was collected, lyophilized and stored at -20°C until use. A solution was prepared with distilled water at a concentration of 100 mg/ml on the day of experiment.

Animals and treatment

We used twenty eight male Wistar rats, 220 to 250 g in our study. These animals were kept under standard laboratory conditions with a 12 h light/dark cycle and *ad libitum* food and water throughout the experiments which were approved by Khorram Abad University

ethical committee. All animals were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University of Medical Sciences (Khorram Abad, Iran). All of experimental procedures were carried out between 04.00 to 08.00 pm. The animals were randomly divided into four groups, with seven rats in each. One group received ethanol (Ethanol group), the second group was treated with extract of *Z. jujuba* fruit (Extract group), the third group received extract 30 min before the ethanol administration (Extract plus ethanol group), and control rats received equivalent volumes of saline at the respective time-points (Control group).

Ethanol was diluted to 40% in saline and ingested at a dose of 4 g/kg. Fruit extract of *Z. jujuba* administrated in a dose of 200 mg/kg. The animals were treated daily by oral gavage for eight weeks. The doses of ethanol and aqueous extract were chosen based on our previous research on liver and kidney (unpublished data). In that study, after administration of *Z. jujuba* fruit extract to different groups at the increasing doses of 50, 100 and 200 mg/kg, rats were allowed food and water *ad libitum* and all animals were observed for possible mortality cases and behavioral changes for 72 h. we did not observe any mortality case up to the dose of 200 mg/kg of *Z. jujuba* fruit extract.

Morris water maze testing

To assess hipocampal dependent spatial learning and memory, all rats were trained in a standard Morris water maze task (Morris et al., 1982; Stackman et al., 2002). The rats performed four trials per day for four consecutive days. In the swimming trials, each individual rat was released gently into the water at a randomly chosen quadrant. The rat swam and learned how to find the hidden platform within 60 s. After reaching, the rat was allowed to stay on the platform for 10 s and was then taken back into the cage. The rats were placed on the platform by hand for 10 s if they could not escape to the platform within 60 s by themselves, and their escape latency was accepted as 60 s. During the inter-trial intervals, animals were kept in a dry home cage for 60 s. The time to reach the platform (latency), the length of swim path, and the swim speed were recorded semi-automatically with a video tracking system. Twenty-four hours after the last day of training, subjects were tested on a probe trial, during which the escape platform was removed and the time spent in the correct quadrant was measured for a 60 s trial.

Biochemical estimations

The rats were sacrificed by cervical dislocation under ether anesthesia after the probe trial and hippocampus was dissected on an ice-cold surface. Tissue homogenates were prepared as described by Carrillo et al. (1991). Both homogenate and supernatant liquated and stored at -70°C until TBARS levels, an indicator of lipid peroxidation, and SOD and GPx enzyme activities were determined.

Measurement of lipid peroxidation

The level of lipid peroxidation was indicated by the content of TBARS in the hippocampus. Tissue TBARS was determined by following the production of thiobarbituric acid reactive substances as described by Subbarao et al. (1990). In short, 40 µl of homogenate was added to 40 µl of 0.9% NaCl and 40 µl of deionized H_2O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37°C for 20 min and stopped by the addition of 600 µl of cold 0.8 M hydrochloride acid, containing 12.5% trichloroacetic acid. Following the addition of 780 µl of 1%

Table 1. Mean ± SEM of hippocampal antioxidant enzyme activities (GPx and SOD) as well as TBARS levels after *Z. jujuba* fruit extract and/or ethanol treatment in rats.

	SOD (U/mg protein)	GPx (U/mg protein)	TBARS (nmol/mg protein)
Control	5.62 ± 0.39^a	0.15 ± 0.08^a	1.04 ± 0.06^a
Ethanol	2.79 ± 0.24^b	0.06 ± 0.004^b	1.9 ± 0.53^b
Z. jujuba plus ethanol	3.26 ± 0.59^b	0.11 ± 0.039^c	1.3 ± 0.24^c
Z. jujuba fruit extract	6.01 ± 0.14^a	0.21 ± 0.06^d	0.87 ± 0.04^a

Means with different superscripts (a, b, c) within each column are significantly different ($p < 0.05$).

Table 2. Mean ± SEM of latency times (sec) during the days of training trials after *Z .jujuba* fruit extract and/or ethanol treatment in rats.

	Day 1	Day 2	Day 3	Day 4
Control	46.29 ± 2.34^a	34.91 ± 1.42^b	28.5 ± 2.67^c	25.45 ± 1.61^c
Ethanol	50.64 ± 2.56^a	42.89 ± 3.71^b	40.60 ± 1.33^b	32.75 ± 2.07^c
Z. jujuba plus ethanol	51.00 ± 1.64^a	48.6 ± 2.19^a	35.10 ± 2.11^b	25.14 ± 1.86^c
Z. jujuba fruit extract	43.64 ± 2.63^a	30.53 ± 1.51^b	22.39 ± 0.38^c	19.53 ± 0.75^c

Means with different superscripts (a, b, c) within each row are significantly different ($p < 0.05$).

TBA, the reaction was boiled for 20 min and then cooled at 4°C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1500 g in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using and extinction coefficient of 1.56×10^5. The blanks for all of the TBARS assays contained an additional 40 µl of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nmol per milligram of tissue protein (nmol/mg protein).

Determination of GPx and SOD activities

SOD and GPx activities were measured in the supernatant by using RANSOD and RANSEL kits (Randox Labs, UK). The results were expressed as units/mg protein.

Protein measurement

Protein content of supernatants for enzyme analysis and TBARS level was determined using a colorimetric method of Lowry with bovine serum albumin as standard (Lowry et al., 1951).

Statistics

For each parameter of the water maze task, and to compare the data on SOD or GPx enzyme activities and TBARS levels, one-way ANOVA and post-hoc Tukey's test was used. The results of the experiments were expressed as means ± SEM. $p < 0.05$ value was considered to be statistically significant.

RESULTS

Table 1 presents the GPx and SOD activities and TBARS levels in the rat hippocampus. Chronic administration of ethanol significantly decreased GPx and SOD activities and increased TBARS levels in the hippocampus of ethanol group compared to the control rats ($p < 0.05$). Combined *Z. jujuba* extract plus ethanol treatment caused a significant decrease of TBARS levels in the hippocampus as compared to the ethanol group ($p < 0.05$). While *Z. jujuba* extract administration prior to ethanol significantly enhanced GPx activity compared to the ethanol group ($p < 0.05$), its effect on SOD activity compared to the ethanol group was not statistically significant. The *Z. jujuba* extract administered significantly increased GPx activity compared to the control group ($p < 0.05$), but there was not any significant difference in SOD activities and TBARS levels between control and rats received *Z. jujuba* extract. As shown in Tables 2 and 3, all groups showed significant decreases in distance traveled and latency to find the escape platform across four consecutive training days. While ethanol group took significantly longer total path length and total latency to find the escape platform compared to the controls ($p < 0.05$; Figures 1 and 2), administration of *Z. jujuba* extract significantly shortened these parameters compared to the controls ($p < 0.05$; Figures 1 and 2).

Although, rats that received *Z. jujuba* extract prior to ethanol tended to have shorter total latency and total path length than the ethanol-exposed group, pre-treatment with extract reached statistically to significant level only with respect to total path length ($p < 0.05$; Figures 1 and 2). Neither ethanol exposure nor extract treatment had significant effects on total swimming speed (data not shown). Figure 3 shows the percent of total time that rats spent in correct quarter. While data analyses have shown that ethanol-treated rats spent significantly less time in

Table 3. Mean ± SEM of path length (cm) to locate on the hidden escape platform for each day after *Z. jujuba* fruit extract and/or ethanol treatment in rats.

	Day 1	Day 2	Day 3	Day 4
Control	1186.12 ± 46.04[a]	1036.87 ± 47.46[a]	737.58 ± 48.66[b]	505.95 ± 45.49[c]
Ethanol	1326.46 ± 39.84[a]	1120.5 ± 50.66[b]	899.75 ± 41.71[c]	813.64 ± 48.67[c]
Z. jujuba plus ethanol	1272.57 ± 48.11[a]	915.07 ± 43.26[b]	828.53 ± 48.16[b]	631.25 ± 42.16[c]
Z. jujuba fruit extract	1119.39 ± 52.17[a]	848.25 ± 44.43[b]	573.82 ± 46.56[c]	419.89 ± 39.3[c]

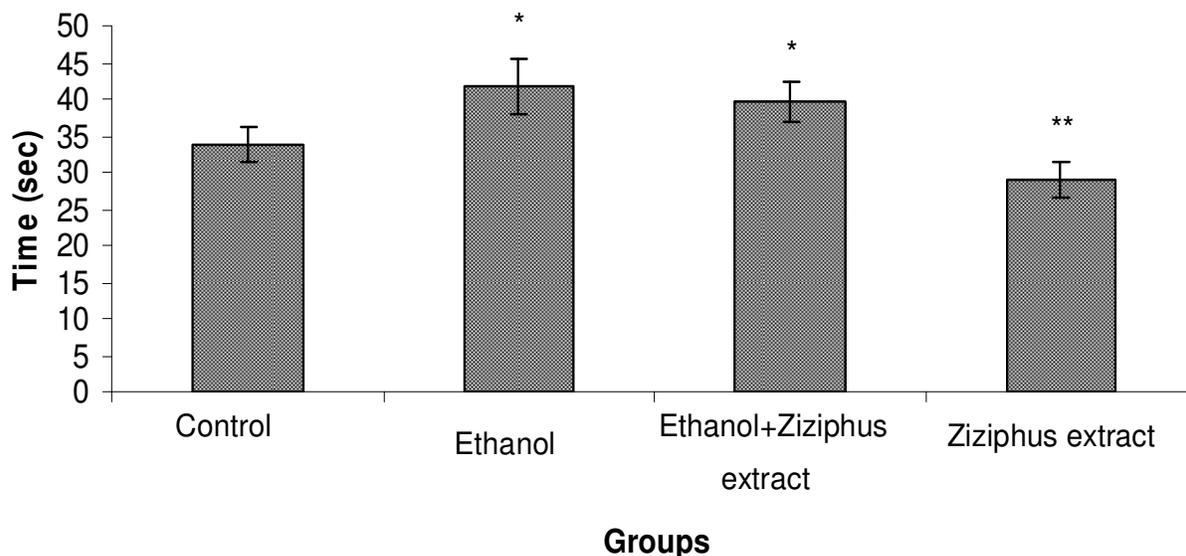Means with different superscripts (a, b, c) within each row are significantly different ($p < 0.05$).

Figure 1. Latency time (Mean ± SEM) to find and locate on the hidden platform for total acquisition trials.* = significantly different from control group,** = significantly different from all other groups.
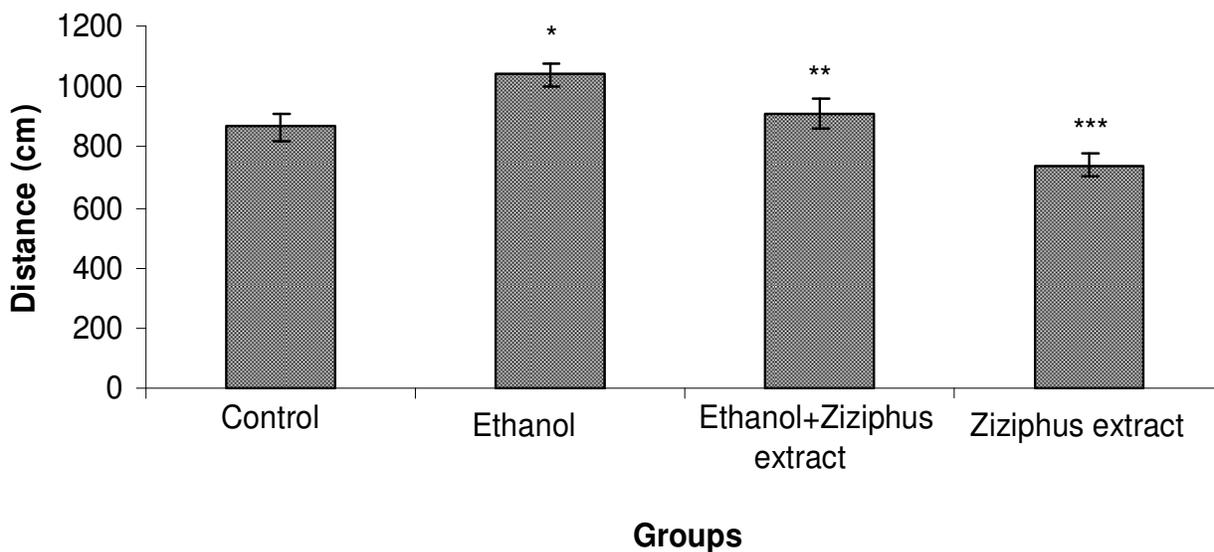

Figure 2. Path length (Mean ± SEM) to find and locate on the hidden platform for total acquisition trials. * = significantly different from control group, ** = significantly different from ethanol group,*** = significantly different from all other groups.
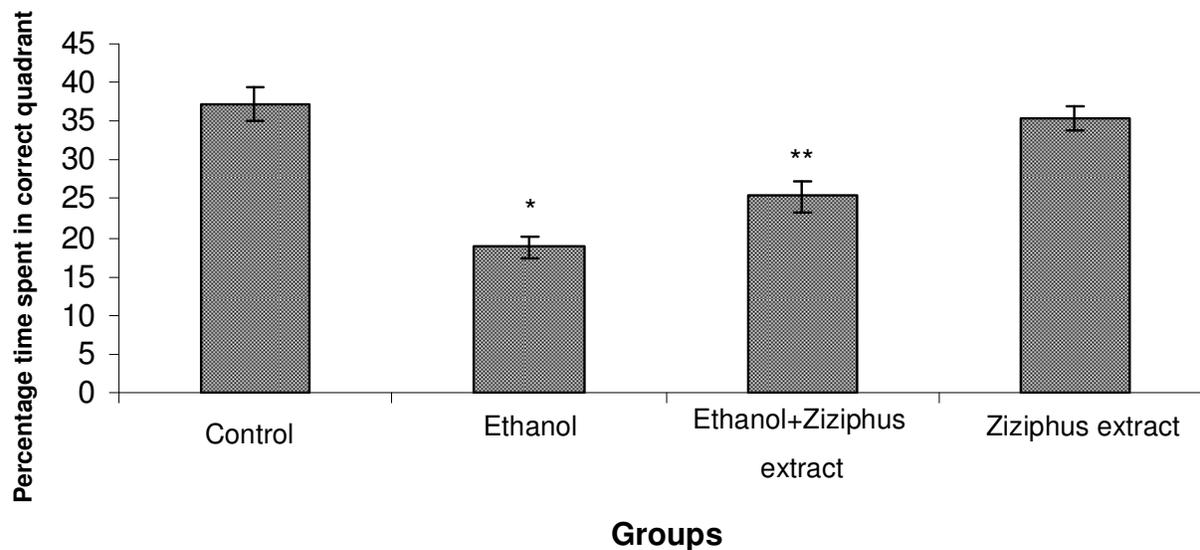
Figure 3. Percent of total time (Mean ± SEM) spent by rats in correct quarter for probe trial at the fifth day after *Z. jujuba* fruit extract and/or ethanol treatment in rats.* = significantly different from all other groups,** = significantly different from ethanol and control groups.

the correct quadrant compared to all other groups ($p<0.05$; Figure 3), Pretreatment with extract significantly attenuated ethanol's effects on probe trial performance ($p<0.05$; Figure 3). Performance of extract group was not significantly different from that of control (Figure 3).

DISCUSSION

In the present study, it was revealed that chronic administration of ethanol decreases GPx and SOD activities and induces the lipid peroxidation (as shown by TBARS levels) in the rat hippocampus. These findings are in agreement with other studies that have shown chronic and acute ethanol consumption leads to significant decreasing of antioxidant enzymes and increasing lipid peroxidation in the whole brain or in the hippocampus (De Freitas et al., 2004; Gonenc et al., 2005; Crews and Nixon, 2009).

It is well known that, ethanol intake increases the formation of harmful ROS and decreases the antioxidant defense system of the brain, thus causing oxidative stress and lipid peroxidation in brain (Reiter, 1995; Calabrese et al., 1998). Free radicals are highly reactive molecules that may be formed during various biochemical reactions in the cell. Many of these free radicals contain oxygen and are called ROS. Typically, the levels of ROS and other free radicals are controlled by various scavenger molecules, known as antioxidants, that are normally found within the cell and which eliminate free radicals. The antioxidant defense mechanisms include antioxidant enzymes like SOD, GPx and several non-enzymatic free radical scavengers (Wickens, 2001). If

ROS levels exceed the cell's ability to eliminate them or if the normal antioxidant levels within the cell are reduced due to a toxic agent such as alcohol, then oxidative stress can occur. This oxidative stress can cause damage to cellular components, such as membranes, DNA, and proteins (Qian et al., 2008).

The brain is more vulnerable to oxidative stress than other organs due to its low antioxidant protection system and increased exposure of target molecules to reactive oxygen species. The nervous tissue has a high content of polyunsaturated fatty acids (Sun et al., 2002), which are easy targets to oxidative damage by free radicals due to the unsaturated bonds they contain (Reiter, 1995). On the other hand it has been revealed that brain structures supporting memory are uniquely sensitive to oxidative stress due to their elevated demand for oxygen (Floyd, 1999). Renis et al. (1996) showed that hippocampus is a brain area particularly susceptible to ethanol-induced oxidative stress.

The present study demonstrated that *Z. jujuba* fruit extract-ethanol co administration increased GPx activity and decreased TBARS level in the hippocampus compared to ethanol-treated rats. This is the first report indicating that *Z. jujuba* fruit extract decreases ethanol-induced lipid peroxidation and increases GPx activities in the rat hippocampus. *Z. jujuba* extract administration alone increased GPx activity without any significant effects on SOD activity or TBARS levels in the hippocampus compared to control rats. Flavonoid compounds such as quercetin, kaempferol and phloretin derivatives found in *Z. jujuba* fruit might contribute to these effects (Pawlowska et al., 2009). Flavonoids are a ubiquitous group of polyphenolic substances which are

present in most plants, extensively existing in fruits (Jiancai et al., 2006). Several reports suggest that phenolic antioxidants attenuate the neurodegenerative alterations induced by oxidative stress (Antonio and Druse, 2008; Mancuso et al., 2007).

It is well known that, ethanol induces COX2 (cyclooxygenase-2) (Knapp and Crews, 1999), a pro inflammatory enzyme which is involved in alcohol-induced oxidative damage in brain (Crews and Nixon, 2009). Flavonoids such as quercetin inhibit the COX2 expression (Raso et al., 2001). Quercetin has been shown in a number of studies to be potent antioxidant, capable of scavenging free radicals. It can inhibit pericellular membrane lipid peroxidation, enhance the ability of the body's antioxidant defense systems and protect cells from the damage of peroxidation (Schmitt-Schillig et al., 2005). Quercetin and its derivatives are verified as the scavenger of superoxide anion free radicals. The mechanism of their scavenging free radicals and inhibiting lipid peroxidation is chelating transition metal ions to form inactive ion compounds, inhibiting biochemical reactions of ions in the formation of many free radicals, in which the iron ion compounds still maintain the free radical scavenger activity (Kuo et al., 1998). There are three periods in the inhibition of formation of free radicals in body: (1) inhibiting the induction of free radical reaction by means of the reaction with superoxide anion ions (O^{2-}), (2) inhibiting the formation of the hydroxyl radicals (OH^-) by chelating transition metal ions, and (3) inhibiting the lipid peroxidation process by means of reacting with lipid peroxidation radicals (ROO^-) (Diana et al., 1999). Fenton-type reactions between H_2O_2 and Fe (II) leads to the production of the harmful hydroxyl radicals, which are capable of initiating processes of lipid peroxidation (Brunk and Terman, 2002). On the other hand, it has been clearly indicated that the lipid peroxidation significantly increases by accumulation of H_2O_2 (which is not a free radical, but an ROS) in a concentration-dependent manner (Garcia et al., 2005). GPx can decompose H_2O_2 to water. Thus, it seems that the increase of GPx activity by Z. jujuba extract in this study causes more rapid conversion of H_2O_2 to H_2O and preventing of H_2O_2 accumulation and availability to shift for lipid peroxide production. This is further supported by the fact that treatment through Z. jujuba fruit extract decreased TBARS content in the hippocampus of rats received ethanol. In addition, it is believed that GPx functions in the detoxification of reactive lipid peroxides (Peltola et al., 1992) and therefore, the reduction in TBARS concentration in this work can be justified.

In line with previous studies, (Schulteis et al., 2008), we have demonstrated that chronic ethanol exposure impaired the spatial memory in the water maze task, as evidenced by increases in swim path length and latency. Progressive and severe anterograde learning deficits consistently occurs in chronic alcoholism, implicating

impairment in hippocampal circuits (Herrera et al., 2003). Hippocampus is a brain region shown to be necessary for normal learning and memory for the spatial water maze task (Berry et al., 2009). It has been suggested that ethanol preferentially impairs hippocampal-dependent learning and memory tasks (Acheson et al., 2001). On the other hand, it was shown that a decrease in hippocampal lipid peroxidation improves spatial cognition learning memory (Gamoh et al., 2001), and an increase in antioxidative activity in the hippocampus prevents (Hashimoto et al., 2002) or ameliorates (Hashimoto et al., 2005) the impairment of learning ability in rats. Given the role of the hippocampus in spatial learning and the known vulnerability of the hippocampus to alcohol-induced oxidative damage (Wen and Kim, 2004), it is obvious that the oxidative stress is involved in ethanol-induced cognitive impairments of the spatial water maze performance.

To our knowledge, there has been no report about effects of Z. jujuba fruit extract on spatial memory of ethanol-treated rats. In this study pre-treatment with Z. jujuba extract significantly decreased total path length and attenuated ethanol's effects on probe trial performance compare to the ethanol group. These results imply that co-treatment of Z. jujuba extract ameliorates ethanol-induced memory deficits not only in the acquisition process but also in the retrieval process of spatial memory performance in rats. It has been revealed that antioxidant constituents of plants could improve cognitive function (Renis et al., 1996; Bisson et al., 2008; Kumar el., 2009; Khalili et al., 2009; Farshchi et al., 2010; Divyaet al., 2010). This study confirms Jiancai et al. (2006) who showed that quercetin can enhance spatial memory of the mice through inhibiting the oxidative stress and increasing the GSH content in the hippocampus. Taken together, our findings suggest that improvement in spatial memory deficits induced by ethanol may be due to the antioxidant properties of flavonoids present in the Z. jujuba fruit extract.

ACKNOWLEDGEMENTS

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