Protective effect of *Rosmarinus officinalis* L. on the expression of the glutamate transporter (GLT-1) and neuronal damage in the frontal cortex of CCl₄-induced hepatic damage

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*Rosmarinus officinalis* L. is one of the most commonly used plants in traditional medicine. It is characterised by hepatoprotective and antioxidant activities that can be attributed to its hydroxyphenolic components, including rosmarinic acid, carnosol and flavonoids. It has also been reported to have neuroprotective effects. The aim of this study was to determine the effect of *R. officinalis* L. treatment on the expression of the glutamate transporter (GLT-1) and on neuronal damage in the frontal cortex of rats with hepatic damage that was induced by carbon tetrachloride (CCl₄). The protective effect of *R. officinalis* L. against hepatic damage induced by CCl₄ was evaluated in Wistar rats that were treated with *R. officinalis* L. extract one week prior to, and then during chronic treatment with CCl₄. GLT-1 expression was determined using a reverse transcriptase-polymerase chain reaction (RT-PCR), and the morphological features of irreversibly damaged cells in the cerebral cortex were studied using light microscopy. The morphological evaluation of the frontal cortex showed that *R. officinalis* L. exerted a protective effect that was correlated with increased GLT-1 expression. The protective effect of *R. officinalis* L. against CCl₄-induced hepatic damage may be due to improved hepatocellular function. Moreover, the presence of antioxidant-containing flavonoids in the extract may contribute to a probable mechanism for this effect.

Key words: Hepatic damage, neuronal damage, frontal cortex, glutamate transporter (GLT-1), *Rosmarinus officinalis* L.

INTRODUCTION

Liver cirrhosis is a pathological condition that occurs as a result of widespread tissue scarring after repeated damage to that organ. The mechanisms underlying cirrhosis are not well understood; however, it is known
that oxidative stress and free radicals are profoundly involved (Clawson, 1989). Previous studies have reported that carbon tetrachloride (CCl₄)-induced hepatotoxicity and ischemia-induced neuronal death are similar in that they both involve N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, oxidative stress, lactic acidosis, and pro-inflammatory cytokines, as well as an increase in glutamate dehydrogenase (GDH) and a decrease in glutamine synthetase (GS) in the brain (Albrecht et al., 2010; Butterworth, 2007). These mechanisms have also been linked to ammonia intoxication due to liver failure (Norenberg, 1996). CCl₄ administration in rats increases astrocyte proliferation, but decreases the number of oligodendrocytes. In addition, astrocytic glutamate transporter expression decreases, resulting in increased extracellular glutamate (Chan and Butterworth, 1999; Knecht et al., 1997).

Despite advances in our knowledge of liver diseases, there is no effective treatment for these ailments and their effects on the brain. Current strategies are aimed at palliative treatment to delay the onset of symptoms associated with cirrhosis; these approaches consist primarily of a low-fat diet and vitamin supplementation. Till date, there is no effective treatment for liver diseases exists, but it has been suggested that medicinal plants may be a viable alternative for treating these diseases. Herbal medicines, also known as botanical medicines or phytomedicines include the medicinal products of plant roots, barks, seeds, berries or flowers that can be used to promote health and treat diseases. The medicinal use of plants has a long history around the world (Li et al., 2008). Ethnobotanists have reported a variety of medicinal plants with high levels of hydroxyphenolic compounds that can be used to treat gastrointestinal diseases and protect the liver (Calixto, 2005). For example, clinical studies and experimental models have shown that silymarin, a compound obtained from Silybum marianum (Asteraceae), has multiple hepatoprotective effects against liver damage (Shaker et al., 2010).

It has been proposed that the future of complementary and alternative medicine will involve combining plants to form mixtures, as the activity of such combinations is greater than that of any single plant (Mutalik et al., 2005). The traditional Chinese herbal medicine, Sho-saiko-to, is a mixture of seven herbal preparations that has long been used in the treatment of chronic liver disease. Various clinical trials have shown that Sho-saiko-to protects against the development of hepatocellular carcinoma in cirrhotic patients (Shimizu, 2000). Baicalin and baicalein are the most important components in the activity of Sho-saiko-to (Borchers et al., 2000). It has also been demonstrated in mice with liver injury that oral administration of Sho-saiko-to (TJ-9) significantly reduced collagen content by downregulating the mRNA expression of procollagen and of the metalloproteinase inhibitor (Sakaida et al., 2004). According to Pharmacognosy, the plants that are most often considered to possess hepatoprotective effects in traditional Mexican medicine have been shown to contain various types of polyhydroxyphenolic structures, which consist of terpenes and flavonoids, either free or as glycosides (Kuklinsky, 2000).

Rosmarinus officinal L. is one of the most important plants used in traditional Chinese medicine because of its high antioxidant activity and phenolic content. This suggests that extracts of this plant may contain compounds with strong radical-scavenging and antiradical-generating properties (Luo et al., 2010). The antioxidant efficiency of R. officinalis L. is due to its high content of phenolic compounds, including rosmarinic acid, flavonoids, diterpene phenols, carnosic acid, carnosol and rosmanol (Cheung and Tai, 2007). The antioxidant activity has been attributed specifically to carnosic acid, flavonoids and rosmarinic acid (Huang et al., 2005), which inhibit the production of nitric oxide (Lo et al., 2002) and protect dopaminergic neurons (Lee et al., 2008). Our previous studies have shown that R. officinalis L. extract has hepatoprotective effects against liver cirrhosis that is induced in a chronic CCl₄ model (Miranda-Beltrán, 2008). However, no information is available regarding the secondary effect of R. officinalis L. extract on the brain.

In this study, the protective effect of a 1.5 g/kg dose of R. officinalis L. extract against CCl₄-induced hepatic damage in Wistar rats was evaluated one week prior to, and then during chronic intoxication with CCl₄. GLT-1 expression was determined using a reverse transcriptase polymerase chain reaction (RT-PCR), and the morphological features of irreversibly damaged cells were examined under light microscopy.

**MATERIALS AND METHODS**

The plant was collected in July - August 2009 in Ejido (communal land) of San Javier, located on the road to the Barca Atotonilco on the México-Guadalajara. A voucher specimen (registration number IBUG-156 409) was deposited at the herbarium of the Institute of Botany from University of Guadalajara.

**Plant extraction**

Leaves were shade-dried at 22°C. In brief, 1 kg of leaves was extracted with 5 L of n-hexane at 60°C for 1 h. A second extraction was performed with 2 L of n-hexane. Afterward, the resulting liquid was extracted with 6 L of 60% ethanol at 60°C for 2 h. The extract was then filtered and the residue was washed with 2 L of ethanol.

Abbreviations: GLT-1, Glutamate transporter; CCl₄, carbon tetrachloride; RT-PCR, reverse transcriptase-polymerase chain reaction; GDH, glutamate dehydrogenase; GS, glutamine synthetase; RT, reverse transcription; ANOVA, analysis of variance.

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one week prior to administration and during administration of CCl

neurons.

either pyknosis/eosinophilia (red neurons) or a complete loss of

damaged cells by light microscopy. Necrotic neurons exhibited

(1965) to describe the morphological features of irreversibly

we applied the criteria developed by Farber (1982) and Trump et al.

series of graded ethanol and embedded in paraffin. Sections (8 µM

diluted in phosphate buffered saline (PBS), then dehydrated in a

liver, and immediately fixed by immersion in 10% paraformaldehyde

Tissues for the histological examination (approx. 0.5 cm²) were
taken randomly from the right, median and left lobes of each rat

Histopathological analysis

Tissues for the histological examination (approx. 0.5 cm²) were
taken randomly from the right, median and left lobes of each rat

Molecular biological studies

RNA extraction and quantification

Extraction and quantification of total RNA from the frontal cortex

Experimental animals

All experiments were carried out in accordance with the National

Preparation of animals

The rats were randomised into 5 groups of 9 rats, each group

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We standardised a semi-quantitative RT-PCR method based on the

specific pairs of groups that were significantly different. Data were presented as the means and standard deviations (SD). Differences among groups of data were considered statistically significant when \( p < 0.05 \).

RESULTS

Effects of *R. officinalis* L. treatment on liver morphology

Histological evaluations of the liver sections of animals from groups 1, 2 and 3 showed normal structure, architectural integrity and regenerative nodules. The animals from group 4 exhibited architectural distortion, with variations in the sizes of the lobules, each of which was surrounded by strands of proliferating fibrous connective tissue that appeared to resemble monolobular cirrhosis. Degenerative changes were characterised by vacuolation of the cytoplasm, ballooning (hydropic) and hepatocyte necrosis (Figure 1D). Collectively, these changes represent cirrhotic features. Group 5 showed preserved liver architecture, but some areas had thin walls of connective tissue. Furthermore, steatosis was moderately confluent. Portal tracts and the centrilobular zone were of normal appearance. Regenerative nodules were observed, similar in appearance to those of groups 1, 2 and 3, suggesting that *R. officinalis* L. treatment was hepatoprotective. Additionally, the fibrous connective tissue and bile duct proliferations appeared to be considerably restricted relative to group 4 (Figure 1E).

Effects of *R. officinalis* L. treatment on frontal cortex morphology

The frontal cortex from groups 1, 2 and 3 showed normal structural and architectural integrity (Figure 2A to C). Sections of frontal cortex from group 4 exhibited lesions characterised by an increased numbers of cytoplasmic astrocytes with swelling. The neuronal analysis of group 4 showed cell shrinkage, cell cavitation and cytoplasmic vacuolation. Many neurons in the frontal cortex became fusiform; their cytoplasm was retracted and displayed an intense eosinophilic reaction, and their nuclei were pyknotic with condensed chromatin and no apparent nucleolus (Figure 2D). The frontal cortex from group 5 showed normal tissue integrity with minimal changes (Figure 2E).

Quantitative neuronal analysis in frontal cortex

The effects of *R. officinalis* L. on the frontal cortex...
histology of the rats in the 5 treatment groups are shown in Figure 3. Group 4 showed altered morphological characteristics, including cell shrinkage, cell cavitation and cytoplasmic vacuolation. Additionally, the quantitative analysis indicated a decreased number of normal neurons in the frontal cortex (p< 0.001 when compared to groups 1, 2 and 3). Group 1 had low numbers of neurons compared to groups 2 and 3. However, there were significant differences between group 1 and groups 4 and 5, perhaps because perfusion fixation fluids were equilibrated to room temperature. This may be the reason for the low number of normal neurons in the group 1. Also, the frontal cortex in the animals of group 5 showed relatively normal cells and less morphological damage, as well as an increase in the number of neurons compared to group 4 and control rats (p< 0.001).

**Effects of *R. officinalis* L. treatment on the expression of GLT-1 in the frontal cortex**

Since groups 1, 2 and 3 showed no significant histological changes, we limited our analysis of GLT-1 expression to groups 1, 4 and 5. Compared to control group 1, there was a significant reduction in GLT-1 mRNA in the frontal cortex of rats with liver failure (p= 0.022). However, in group 5, the changes in GLT-1 expression were minimal (Figure 4). Treatment with the extract of *R. officinalis* L. normalised the expression of GLT-1 when compared to group 4 (p= 0.034).

**DISCUSSION**

The results obtained in this study showed that animals treated with CCl₄ exhibited symptoms that are characteristic of liver damage (Butterworth, 2003; Miranda-Beltrán, 2008; Muñoz Torres et al., 1988), concomitant with changes in the mRNA levels of the astrocytic transporter GLT-1 in the prefrontal cortex. Moreover, our findings suggested that treatment with *R. officinalis* L. may protect liver function. Other studies have reported that *R. officinalis* L. extract has antioxidant activity with hepatoprotective effects against CCl₄-induced damage (Farber, 1982; Sotelo-Felix et al., 2002). In addition, the histological sections of livers from rats treated with *R. officinalis* L. and CCl₄ (Figure 1E) showed a reduced incidence of liver injury, hepatocyte swelling, leukocyte infiltration, fibrous connective tissue proliferation and necrosis. These observations suggest that *R. officinalis* L. is effective in ameliorating liver damage caused by CCl₄ (Figure 1D).

It is generally assumed that neuronal death is minimal in liver failure (Butterworth, 2007); however, neuronal
damage and death have been well documented in cases of liver failure patients (Butterworth, 2003), and cirrhotic patients often manifest Alzheimer type II astrocytes in the cerebral cortex (Schafer and Jones, 1982). In this study, the induction of liver damage with CCl₄ resulted in histological changes in the frontal cortex. *R. officinalis* L. treatment markedly reduced these histological changes (number of astrocytes, absence of hypertrophy) that were linked to the functional status of the liver, as evidenced by the liver histology. Park et al. (2010) showed that *R. officinalis* L. extract protects neurons against H₂O₂-induced injury, and this study revealed that astrocytic GLT-1 transporter mRNA was decreased in cirrhotic rats. These results are consistent with those reported previously (Chan and Butterworth, 1999; Knecht et al., 1997) and suggest that liver damage alters glutamatergic synaptic transmission in the brain. Studies using cerebral microdialysis have shown that the extracellular concentration of glutamate in the prefrontal cortex increases (Bosman et al., 1992) due to a decrease in astrocyte-mediated glutamate re-uptake. It has also been reported that increased glutamate concentrations and decreased expression of GLT-1 in the brain may cause alterations in glutamate receptor expression (Soria Fregozo et al., 2012). Furthermore, Michalak and Butterworth (1997) showed that the density of binding sites for 2-(aminomethyl) phenylacetic acid/kainate (AMPA/kainate) glutamate receptors in the brain decreased in an animal model of liver damage.

Liver disease causes ammonia levels in the bloodstream to rise, and higher levels of ammonia could be responsible for the morphological alterations observed in the brain, such as changes in astrocyte number (Suarez et al., 2002). Moreover, liver damage is characterised by increased extracellular glutamate in the brain that can lead to neuronal death, most likely due to NMDA receptor-mediated excitotoxicity. Treatment with *R. officinalis* L. extract in animals with CCl₄-induced liver injury normalised astrocytic transporter (GLT-1) mRNA levels, which correlated with the results of the structural and functional liver analyses. This is likely due to a hepatic effect that may be caused by the extract's high polyphenol content and antioxidant activity, as demonstrated in previous studies (Huang et al., 2005; Miranda-Beltrán, 2008). It has also been reported that the administration of a plant mixture called HE-03 to rats with liver damage reduces the number of astrocytes and regulates ammonia metabolism (Mitra et al., 2000).

### Figure 3
Percentage of normal neurons in the frontal cortex. Group: 1) Normal rats; 2) rats treated with mineral oil only; 3) rats treated with extract of *R. officinalis* L; 4) rats treated with CCl₄ only; 5) CCl₄-treated rat pre-treated with extract of *R. officinalis* L at 1.5 g/kg 3 times a week for 8 weeks. Data represent mean ± SD expressed as a percentage of the normal neuronal count in five fields per area in four slides per rat. Statistical significance: *p < 0.001, 4 vs. 1, 2 and 3. **p < 0.001, 5 vs. 1, 2 and 3. ***p < 0.001, 5 vs. 4.
The loss of GLT-1 expression in the brain in liver failure is hypothesised to be a consequence of ammonia toxicity. This is supported by the following in vitro and in vivo data: 1) exposures of cultured astrocytes to millimolar concentration of ammonia results in a loss of GLT-1 gene expression; and 2) reduced GLT-1 expression correlates with increased extracellular glutamate concentrations and arterial ammonia levels (Albrecht et al., 2010; Chan and Butterworth, 1999; Norenberg, 1996). It is possible that under certain circumstances, the reduced capacity for astrocytic glutamate uptake as a result of decreased GLT-1 expression could impair ammonia removal. Glutamine synthetase, the enzyme primarily responsible for ammonia detoxification in the brain is predominantly localised to astrocytes, and a recent study suggests that the metabolic rate of glutamate in astrocytes is regulated by extracellular glutamate concentrations (Knecht et al., 1997). In the present work, the compounds of R. officinalis L. extract might have regulated the concentration of ammonia in animals with liver damage and normalised the expression of GLT-1. However, the mechanism of this action is unknown. Further studies are required to measure brain ammonia levels and the activity of enzymes involved in brain aromatic amino acid metabolism.

Although the mechanism involved in the neuroprotective effects of R. officinalis L. extract is not fully understood, the presence of antioxidants or phenolic compounds, including rosmarinic acid, flavonoids, diterpene phenols, carnosic acid, carnosol and rosmanol may contribute to this process. Satoh et al. (2008) reported that carnosic acid (CA), which is found in R.
officinalis L., has neuroprotective effects and is able to penetrate the blood-brain barrier. CA activates the Keap1/Nrf2 transcriptional pathway by binding to specific Keap1 cysteine residues, protecting neurons from oxidative stress and excitotoxicity. This suggests that CA may represent a new type of neuroprotective compound. Our histological results demonstrate the hepatoprotective effects of R. officinalis L. against CCl₄-induced liver damage. Protection of the brain may be caused by the preservation of hepatocellular function or it may be that the active components of the extract of R. officinalis L., such as CA, act directly on the neurons. Meanwhile, the elucidation of the mechanism(s) of action of R. officinalis L. requires further studies that measure urea cycle enzyme activity and levels of brain aromatic amino acids.

Conclusion
This research reveals that R. officinalis L. extract is hepatoprotective and preserves neuronal morphology in the frontal cortex of cirrhotic rats. These findings are supported by histological changes in the liver and brain and by observed altered levels of GLT-1 mRNA. Scientific research of plants requires a deep knowledge of their chemical and physicochemical characteristics to better understand their molecular mechanisms in biological systems. R. officinalis L. extract is a folk remedy used to treat liver damage, and it may protect hepatocellular function or it might directly affect neurons. Therefore, this extract should be considered as a possible therapeutic treatment for patients with liver disease.

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REFERENCES


protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1.

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