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The Protective Effect of Liquorice Plant Extract on CCl_4-Induced Hepatotoxicity in Common Carp *(Cyprinus carpio)*

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Abstract

The protective effect of liquorice plant extract (LPE) on CCl_4-induced hepatotoxicity in common carp was evaluated using fifty adult carps. The fish were cultured in a standard environment in terms of water flow rate, oxygen, pH, food and temperature. The fish were assigned into 5 groups (N = 10) as control, sham, and tests. The test groups were pre-treated for 3 h with various concentrations of LPE, 3 days before CCl_4 exposure. The control and sham groups received normal saline before and after CCl_4 exposure. To induce hepatotoxicity, animals in the sham and test groups were exposed against 100 µl L^{-1} CCl_4 for 45 min. The fish in all groups 1 h after CCl_4 exposure were anesthetized and the blood samples were collected. Immediately the liver specimens were dissected out and were stored in 10 % formalin for further pathological studies. Determination of serum level of ALP and SGOT revealed that acute form of CCl_4 exposure elevated significantly ($P < 0.05$) the serum level of either tested hepatic marker enzymes. While 3 days pretreatment with LPE prevented from ALP and SGOT enhancement. The pathological evaluation revealed that the CCl_4 exposure resulted in a minor pathologic manifestation such as slight congestion, which the LPE pretreated groups showed the remarkable improvement. The anti-oxidant capacity of LPE was assayed by FRAP and DPPH methods. Both provided techniques showed that LPE exerts an excellent anti-oxidant effect. This data suggest that LPE exerts protective effect against CCl_4-induced hepatotoxicity. Moreover, the hepatoprotective effect of LPE may attribute to its antioxidant capacity.

Key words: Antioxidant capacity, Biochemical changes, Hepatoprotective, Liquoric plant extract, CCl_4-induced hepatotoxicity

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Introduction

The worldwide use of persistent organic pollutants through disposal and spillage materials have resulted in both the contamination of fresh water and the accumulation of toxicants in aquatic organisms. Agricultural and industrial activities are the most important sources of chlorinated compounds. In addition, organochlorine pesticides (OCs) are atmospheric pollutants that are transported over long ranges and bioaccumulated in the food chain.[1] Piscivorous fish can bioaccumulate the organic pollutants to significantly higher concentrations than those found in the water, due to the lipid solubility and resistance of these compounds to numerous degenerative processes.[2,3]

Previous studies indicate that histopathological and biochemical analyses could provide a powerful tool for the assessment of biological effects of contaminants in freshwater ecosystems.[4] Moreover, the presence of contaminants interferes with molecular and cellular events, leading to some disorders in the energy production of the organisms.[5] Additionally, the chemical toxicity stress leads to a depletion of energy reserves resulting in reduced growth and/or reproduction as well.[6] A histopathological study of the target organs is a proven method mainly used in biomonitoring systems, which might indicate acute or chronic exposure to contaminants.[7] For example, analytical studies have been demonstrated that considerable amounts of persistent organic pollutants (POPs) in the liver and muscle of *Hoplias malabaricus* could be detected with the higher bioaccumulation in the liver than in the muscle. The higher bioaccumulation resulted to profound deleterious histopathological effects.[1]

Liquorice Plant Extract (LPE) from the dried roots of *Glycyrrhiza glabra* L. (Fabaceae) is one of the herbal medicines used commonly in different countries for various purposes including gastric ulcer treatment.[8] Extracted liquorice, containing glycyrrhizin is used as an additive for flavoring and sweetening tobacco, candies, chewing gum, toothpaste, and beverages all around the world.[9,10] Previous studies demonstrated that LPE exerts a potent antioxidant capacity as it possesses compounds such as the isoflavans hispaglabridin A, hispaglabridin B, glabridin, and 4'-O-methylglabridin, the two chalcones, isoprenylchalcone derivative and isoliquiritigenin, and the isoflavone, formononetin.[11]

This study was carried out to investigate the hepatoprotective effect of LPE in CCl_4-induced injuries in fish model. Moreover, the importance of histopathological and biochemical analyses in monitoring the impact of aquaculture pollutants were also highlighted.

Materials & Methods

Plant materials and preparation of plant extracts. Root samples of *Glycyrrhiza glabra* L. were collected in summer, 2009 from west Azerbaijan province (Iran) and authenticated by the experts in the botanic group of biology department, Faculty of Science, Urmia University. The plants were dried according to herbarium techniques. Plant samples were ground using a commercial blender. Chemical extraction was conducted in a suxhlet system. For each single extraction, 10 g of the sample were extracted in ethanol (Extra pure, Merck, Germany), with a sample to solvent ratio of 1:10 (w/v). Extracts were then dried under vacuum using a rotary evaporator to the volume of 3 ml and the final step of drying was conducted under the stream of N_2.

Maintenanceof fish. Fifty fish (common carp, average wt. 200 ± 20 g) were obtained from a local carp farm and transported to the aquatic animals laboratory, in oxygenated tank. They were then stocked in a concrete pond containing

underground freshwater. The fish were cultured in a flow-through system with a flow rate of 50 L min^{-1}. Dissolved oxygen was maintained above 7.5 mg L^{-1} using constant aeration and fish were exposed to a natural photoperiod of approximately 12:12 L: D. The water temperature was 20 - 23 °C, water pH 8.37. Food was given *ad libitum*. The fish were fed on commercially formulated carp diet (Milad Mahabad CO., West Azerbaijan, Iran) throughout the culture and experiment period. The fish were kept in accordance with the guidelines of the Local Ethical Committee applying to principles of Laboratory Animal Care, NIH publication No. 86 - 23, revised in 1985 (NIH, 1985).

Exposure to CCL_4 and treatment with LPE. All animals were initially assigned into three groups of control, sham and test. The test group was sub-divided into three groups with ten fish in each based on different concentrations of protective agent. The control and sham groups received only saline normal and CCl_4, respectively without any further treatment.

Sham and test groups were received CCl_4 at 100 µl L^{-1} for 45 min. Animals in the test groups prior the CCL_4 exposure were treated with various concentrations of LPE as a protective substance 3 hours for 3 days. LPE was dissolved in 1ml DMSO and added to1.5 litter water.

Fish serum preparation and tissue collection. Immediately after CCl_4 exposure the animals were anesthetized by immersion in eugenol solution (20 mg L^{-1}) and the blood samples were collected from caudal vein and left to stand at room temperature for 1 h, and then centrifuged at 3000 rpm for 10 min to obtain the serum. The liver specimens were dissected out and stored in formaldehyde (10 %) for further histopatholgical examinations.

Biochemical analyses

Determination of the serum biochemical parameters. Serum levels of alkaline phosphatase (ALP, 744, Man Inc. Tehran, Iran) and glutamic oxalacetic transaminase (SGOT, 10-513, Zist Shimi, Tehran, Iran) were measured using commercially available standard kits according to the manufacturer's instructions.

Assessment of total antioxidant power (TAOP). To determine the effect of CCl_4 exposure on oxidative stress system and consequently potential therapeutic effect of LPE on disturbed system by CCl_4, total antioxidant capacity of control, sham and test groups were measured. The assessment carried out based on ferric reduction antioxidant power (FRAP) assay.[12] Briefly, at low pH which was provided using acetate buffer (300 mM, pH 3.6), reduction of Fe^{III}-TPTZ (2, 4, 6-tri-2-pyridyl-1,3,5-triazin, Merck, Germany) complex to the ferrous form produces an intensive blue color that could be measured at 593 nm. The intensity of the complex following adding the appropriate volume of the serum to reducible solution of Fe^{III}-TPTZ is directly related to total reducing power of the electron donating antioxidant. Aqueous solution of Fe^{II} ($FeSO_4.7H_2O$) and appropriate concentration of freshly prepared ascorbic acid were used as blank and standard solutions, respectively.

Determination of radical scavenging activity of LPE. To determine the radical scavenging activity of various concentrations of LPE, the DPPH assay was conducted as described previously.[13] 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, Germany) in form of radical is dark purple-colored and absorbs at 515 nm its maximum absorption, however when it is reduced by antioxidant agents, the purple color changes to yellow and absorption disappears. The change in absorption is proportional to the radical scavenging power of the test compound. The average of three times measurements was expressed as percentage of the antioxidant activity (AA) and calculated by using the following formula:

% $DPPH^{\cdot}$= (Ab_{515} control –Ab_{515} sample)/ Ab_{515} control

Histopathological studies. The collected liver samples were processed through paraffin embedding and cut equally with rotary microtome (6µ) and were stained with H&E technique and ultimately examined under light microscope.

Statistical analyses. For all results, numerical mean and standard deviation of the measured parameters were calculated. The results were analyzed using Graph Pad Prism software (version 2.01. Graph Pad software Inc. San Diego, California). The comparisons between groups were made by analysis of variance (ANOVA) followed by Bonferoni post test. A value of $P < 0.05$ was considered as significant.

Results

The results of biochemical analyses showed that the serum level of ALP and SGOT in CCl_4 exposed animals elevated significantly in comparison with the control group significantly ($P < 0.05$). Interestingly, the concentration of both measured enzymes in serum in test groups which pre-treated with various concentrations of LPE, showed a dramatic and significant reduction ($P < 0.05$). Although the mentioned reduction in the serum level of SGOT was concentration-dependent, ALP level did not reduce in concentration-dependent fashion (Fig. 1-A and 1-B).

The total antioxidant capacity of animals after CCl_4 exposure was measured. The results indicate that the CCl_4 exposure slightly but significantly ($P < 0.05$) declined the total antioxidant power, while LPE pre-treatment resulted in remarkable elevation of the antioxidant capacity. LPE exerted the highest protective effect in terms of total antioxidant capacity recovering at 100 mg ml^{-1} concentration (Fig. 2).

To show the antioxidant power of LPE and its free radical scavenging potency, the DPPH assay was performed and the results showed that LPE even at lower concentration also exerts a potent radical scavenging activity (Fig. 3).

Comparing the hepatic microphotograms from CCL_4 exposed animals with the control group (Fig. 4-A) indicated that a slight congestion and minor hemorrhages occurred in CCL_4 exposed animals (Fig. 4-B). In the pre-treated group with 100 mg L^{-1} of LPE, a remarkably improved feature of hepatic histology was observed (Fig. 4-C).

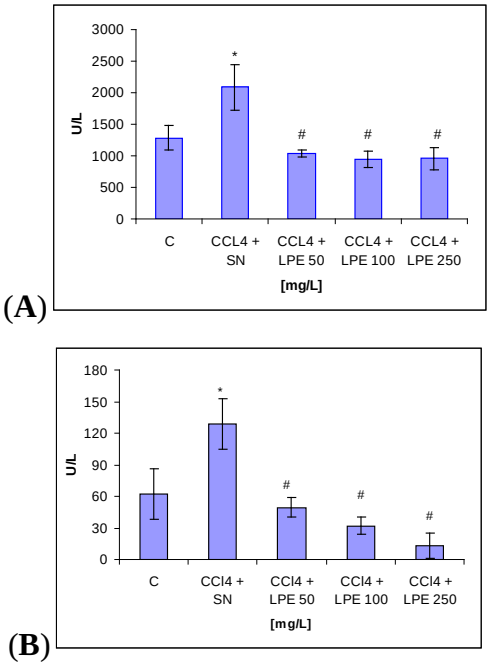


Fig 1. Effect of LPE on CCl_4-enhanced level of **(A)** alkaline phosphatase and **(B)** SGOT.

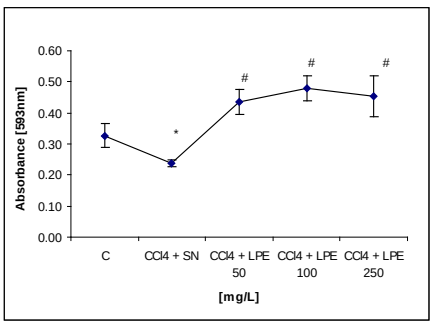


Fig 2. Effect of LPE on CCl_4-reduced total antioxidant capacity.

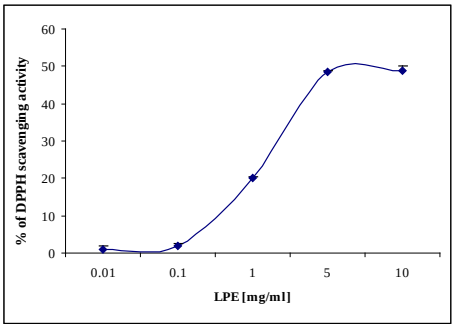
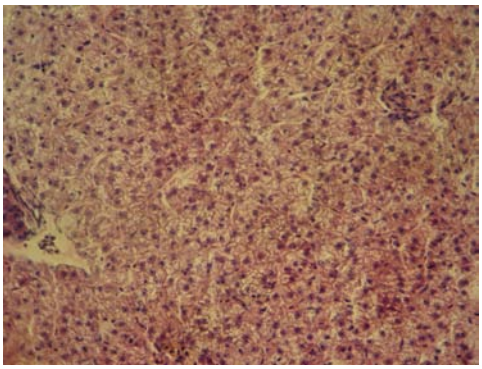
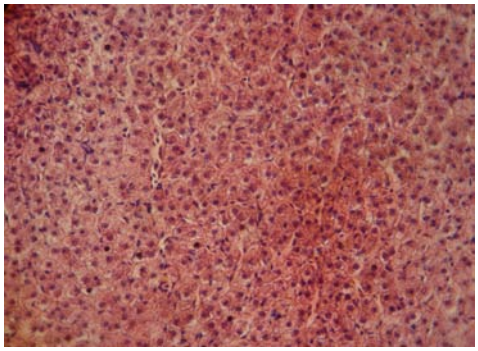


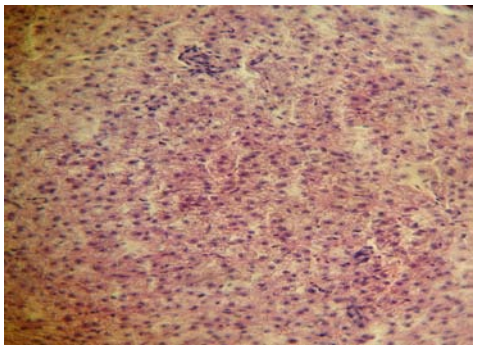
Fig 3. DPPH scavenging activity of LPE.



(**A**)



(**B**)



(**C**)

Fig 4. Microphotograph of the fish liver: **(A)** control group, **(B)** CCl_4-exposed group and **(C)** CCl_4-exposed group which were pretreated with 100 mg L^{-1} LPE. A minor congestion is seen in the liver of CCl_4 exposed group, which remarkably improved in the LPE-treated group. (H & E, 100x)

Discussion

This study reports the protective effect of LPE on the CCl_4-induced hepatotoxicity. Along with *in vivo* protective effects of LPE which reflected in the normalizing of CCl_4-elevated level of ALP and SGOT in serum and improving the total antioxidant capacity, the radical scavenging potency of the LPE was evaluated in *in vitro* condition.

Although the use of halogenated alkanes such as CCl_4, $CHCl_3$ or CHI_3 has been prohibited due to their severe toxicity, CCl_4 however continues to be used as a model substance to elucidate the mechanisms of action of hepatotoxic effects such as fatty degeneration, fibrosis, hepatocellular death, and carcinogenicity.[14] There are several isoenzymes of cytochrome P450 such as CYP2E1, CYP2B1 or CYP2B2, and possibly CYP3A that are involved in the activation of CCl_4 and forming the trichloromethyl radical, $CCl_3^{\cdot}$. This radical can bind to cellular molecules (nucleic acid, protein, lipid), and ultimately impair the crucial processes in cells. At the molecular level CCl_4 pushes the affected cells primarily toward (self-) destruction or fibrosis.[15]

Despite the short period of exposure time to CCl_4 in this study, which resulted in minor pathological impact on the liver, the serum level of ALP and SGOT as marker enzymes both markedly elevated. Measurement of serum enzyme levels has provided a powerful tool for studies of hepatotoxicity. There is accumulated evidence in rodent model indicating that CCl_4-induced hepatotoxicity leads to remarkable elevation of serum level of marker enzymes such as ALP and SGOT.[16] We, in this study, confirmed and extended the biochemical changes due to CCl_4 intoxication in a fish model. Thus monitoring of the serum marker enzymes could be useful tool to early diagnosis of CCl_4 intoxication in aquatic animals and equally a good marker for early evaluation

of water safety. We also found that the pretreatment of fish with various concentrations of LPE resulted in a preventive effect against CCl_4-induced enzymes elevation in serum indicating that LPE managed to minimize the CCl_4-induced hepatic damage. The possible explanation by which LPE pretreatment prevented from the elevation of serum enzymes in CCl_4-induced liver damage may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is rather accepted that serum levels of transaminases remain unchanged with normal hepatic parenchyma and intact or regenerated hepatocytes.[17] Effective controls of ALP and SGOT in the animals which received LPE suggests an early improvement in the secretary mechanism of the hepatic cells. The efficacy of hepatoprotective compounds is largely dependent on their ability in preventing or reducing the detrimental effects of a hepatotoxin on hepatic physiology. It is interesting to be noted that LPE decreased CCl_4-induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane.

To explain how LPE could prevent from CCL_4 hepatic injuries, one should note that free radical mediated process has been implicated in pathogenesis of most of the diseases including CCl_4-induced hepatic damages. As mentioned earlier the CCl_3^- free radical production is the key step in CCl_4 intoxication. Therefore, it would be logic to hypothesis that any efforts that support the antioxidant capacity may minimize the free radicals damages in CCl_4 intoxication. In this study our results demonstrated that in both *in vivo* and *in vitro* situation, LPE exerts remarkable antioxidant effects. As the serum level of total antioxidant capacity was increased in LPE-treated groups and at the same time LPE exerted an excellent DPPH scavenging power, therefore it would be acceptable if the LPE pretreatment could prevent from CCl_4 hepatotoxicity in fish. Previous studies demonstrated that the potent antioxidant power of LPE is related to compounds including flavonoides.[11]

Taken together, our results indicate that the acute form of CCl_4 exposure results in early biochemical alterations in serum such as hepatic marker enzymes level. These changes in biochemical factors might be used as useful diagnostic tools to monitor the CCl_4 and other halogenated alkanes contaminations. Moreover, it is also shown that the biochemical changes may partly be attributed to CCl_4-induced imbalance in oxidative system of fish which was normalized by pretreatment of test group with LPE. The antioxidant potency of LPE was demonstrated both *in vitro* and *in vivo* systems.

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