Potential Hepatoprotective Effects of Licorice Root (Radix glycyrrhiza) Extract against Carbon Tetrachloride-Induced Hepatotoxicity in Isolated Rat Hepatocytes

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Abstract: Radix glycyrrhiza is one of the native Mediterranean plants. Licorice root is a popular soft drink in Egypt. Literature cited therapeutic effects of licorice. The present work is to evaluate the potential hepatoprotective effects of aqueous licorice root extract against the cytotoxic effects and the oxidative stress induced by carbon tetrachloride (CCl4) in isolated primary rat hepatocytes. Hepatocytes were isolated by collagenase perfusion technique. Cytotoxicity was determined by assessing cell viability and leakage of cytosolic enzymes, such as lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Oxidative stress was assessed by determining reduced glutathione (GSH) level and lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) production. Exposure of isolated rat hepatocytes to CCl4 (5mM) caused cytotoxicity and oxidative injury, manifested by loss of cell viability and significant increase in LDH, ALT and AST leakages. As well as, CCl4 caused progressive depletion of intracellular GSH content and significant enhancement of TBARS accumulation. Preincubation of hepatocytes with either licorice (25 μM/ml) or silymarin (0.5mM) which is a known hepatoprotective agent, ameliorated the hepatotoxicity and oxidative stress induced by CCl4, as indicated by significant improvement in cell viability, significant decrease in LDH, ALT and AST leakages, significant prevention of GSH depletion and significant decrease in TBARS formation as compared to CCl4 alone-treated cells. The present results indicate that CCl4 has a potential cytotoxic effect in isolated rat hepatocytes; and licorice extract possess a highly promising hepatoprotective effects against CCl4-induced hepatotoxicity.

Keywords: Licorice, hepatotoxicity, isolated rat hepatocytes

1.Introduction

Liver is the key organ of metabolism and excretion. It is often exposed to a variety xenobiotics and therapeutic agents. Until today, people have not yet found an actual curative therapeutic agent for liver disorder. In fact; most of the available remedies help the healing or regeneration of the liver (Huo et al., 2011).

Large number of xenobiotics is reported to be potentially hepatotoxic. Free radicals generated from the xenobiotic metabolism can induce lesions of the liver and react with the basic cellular constituents such as proteins, lipids, RNA and DNA (Ajith et al., 2007). CCl4 is a potent environmental hepatotoxin, has been served as a model compound for study of hepatotoxicity and the cellular mechanisms behind oxidative damage and further was used to evaluate the therapeutic potential of drugs and dietary antioxidants (Prasenjit et al., 2006).

Nowadays, many investigators have been focused for searching for the best approach in treatment of liver diseases using the effective herbal preparations. Natural products are gaining a revitalized attention in medical community and their therapeutic uses are gradually increasing. As many synthetic drugs have revealed serious side effects. Therefore, a better strategy is to look for natural substances with strong pharmacological action and less cytotoxicity. In the last few years much attention was directed to the potential health promoting properties of phenolic phytochemicals (Kartal, 2007).

Licorice has been used as a medicinal plant for thousands of years. The active component of licorice, glycyrrhizic acid, is hydrolyzed in vivo to glycyrrhetic acid, which is responsible for most of its pharmacological properties. In ancient Chinese medicine and during Roman times, licorice was also recommended to cure sterility in women (Davis and Morris, 1991; Armanini et al., 2002). Derivatives of licorice root have been used in Asia to treat children with biliary atresia, a cholestatic liver disease (Sokol et al., 2003). Licorice has held claim for therapeutic use for fevers, liver ailments, dyspepsia, gastric ulcers, sore throats, asthma, bronchitis, Addison’s disease and rheumatoid arthritis and has been used as a laxative, antitussive and expectorant (Schulz et al., 1998; Wang et al., 2000). Among its most consistent...
uses are as a demulcent for the digestive system, to
treat coughs, to soothe sore throats, and as a flavoring
agent.

The present study was undertaken to evaluate the
hepatoprotective effects of licorice aqueous extract against cytotoxicity and oxidative stress
induced by carbon tetrachloride in isolated primary
rat hepatocytes.

2.Materials and methods
Animals and Chemicals
Animals:
Male Sprague - Dawley rats of locally bred
strains (225 - 250 g) were supplied from faculty of
Veterinary Medicine, Cairo University. They were
kept under good ventilation and standard hygienic
conditions and allowed free access to balanced
standard laboratory chow (El-Nasr Co., Abo-Zaable,
Egypt) and tap water ad libitum.

Isolation of Hepatocytes
The hepatocytes were isolated by a collagenase
two-step perfusion technique (Berry and Friend,
1969) with slight modifications as published by
El-Tawil and Abdel-Rahman (1997). The rat was
anaesthetized with 100 mg ketamine/ kg, restrained,
and an incision was made in the abdominal cavity to
expose the portal vein. A polyethylene cannula was
inserted into the portal vein and the liver was
perfused in situ for 8 min with calcium-free Hank's
bicarbonate buffer maintained at 37oC. The liver was
then mechanically dislocated from the abdomen with
the cannula in place and recirculated for 10 min in
phosphate buffered saline. At the specified time
points and centrifuged with phosphate
buffer saline (PBS) at 3000 g for 5 min. The obtained
collagenase (0.67 mg/ ml) containing 5 mM calcium
chloride. The isolated liver cells were filtered through
bicarbonate buffer (pH 7.4) and incubated at 37oC in a shaking water bath at
four layers of cotton gauze and centrifuged for 2 min
600 rpm. The cells were washed twice and suspended in HEPES-bicarbonate buffer (pH 7.4)
containing 0.5 % bovine albumin. The isolated hepatocytes were counted in a hemocytometer, while
the viability of the cells was assessed by 0.4% Trypan
Blue in Krebs Hanseliet buffer without albumin
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Incubation and treatment of hepatocytes
Freshly isolated hepatocytes (5 X 10^6 cells/ ml)
were suspended in a HEPES-bicarbonate buffer (pH 7.4) and incubated at 37oC in a shaking water bath at
30 oscillations per minute. Hepatocytes were
incubated in plastic vials equipped with covers and
were used for determination of CCL4 cytotoxicity and the possible protection with Licorice extract and compared with silymarin as a known
hepatoprotective agent at different incubation time
intervals (30, 60, 120 min).

The concentrations of CCL4, Licorice extract
and silymarin in the incubation medium were
adjusted to reach a final concentration of 5mM CCL4
(Dvorak et al., 2003), 25 μM Licorice (Gumpricht
et al., 2005) and 0.5mM silymarin (Farghali et al.,
2000). Twelve replicates were used for each group.
Cytotoxicity was determined by assessing of cell
viability using trypan blue exclusion method,
cytosolic enzymes leakage percent [lactate
oxidation, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], GSH
dehydrogenase (LDH), alanine aminotransferase
content and thiobarbituric acid reactive substances
(TBARS) accumulation. Control replicates were
carried out simultaneously under the same conditions and at the same time intervals.

Sample preparation for enzyme leakage:
Enzymes activity (ALT), (AST) and (LDH) was
monitored using Sigma–Aldrich ready made kits
(Sigma-Aldrich Chemical Co., St. Louis, MO, USA)
in an aliquot of cell-free medium and compared to the
total activity achieved after lysis of the cells
(Moldeus et al., 1978). The cell-free medium was
obtained by centrifugation of the aliquots at 2200
rpm’s for 15 min. Lysate was obtained by addition of
1% triton X-100 and shaking for 15 min followed by
centrifugation at 2200 rpm’s. The leakage was
expressed as percentage of total lysate activity at each
time point.

Glutathione (GSH) assay
Reduced GSH levels in hepatocytes were
determined by measuring total soluble-reduced
sulphhydryl content. Aliquots were collected at
specified time points and centrifuged with phosphate
buffer saline (PBS) at 3000 g for 5 min. The obtained
precipitate was mixed with 0.7 ml of 0.2% triton X-100
and 2.5% sulfosalicylic acid. Solutions were
centrifuged at 3000 g for 5 min. A 0.5 ml aliquot of the
acid-soluble supernatant medium was then added
to 1.0 ml of 0.3 M Na2HPO4 solution. Spectrophotometric determinations were performed at
412 nm immediately after the addition of 0.125 ml of
5,5′-dithiobis-(2-nitrobenzoic acid) (Beutler et al.,
1963).

Lipid peroxidation assay
Lipid peroxidation was assessed by determining
thiobarbituric acid reactive substances (TBARS) in
hepatocyte culture media by the method of
Uchiyama and Mihara (1978).

Data Analysis
The GRAPHPAD (ISI Software, Philadelphia,
PA, USA) computer program was used to conduct
regression analysis and to plot collected data. Data
were expressed as means ± standard error of means
(S.E). Assessment of the results was performed using
one-way analysis of variance (ANOVA) procedure
followed by Tukey-Kramer multiple comparison
post-tests. Statistical analyses were performed using Software GRAPHPAD INSTAT (Version 2). The 0.05 level of probability was used as the criterion for significance.

3. Results

Effects of Carbon tetrachloride (CCL\textsubscript{4}) and Licorice on viability of isolated rat hepatocytes

Cell survival was assessed by trypan blue exclusion method after exposing isolated rat hepatocytes to CCL\textsubscript{4} (5mM), licorice extract (25 μM/ml), and silymarin (0.5mM). A significant progressive time dependent decrease in cell viability was observed as early as after exposure to CCL\textsubscript{4} compared to control cells. Prior incubation of hepatocytes with licorice (25 μM/ml) offered a significant protection against CCL\textsubscript{4}. A marked protection was shown after 30, 60, and 120 min. On the other hand, concomitant incubation of the cells with silymarin and CCL\textsubscript{4} inhibited the decrease in the cell viability caused by CCL\textsubscript{4} alone (Fig.1).

![Fig (1): Effects of CCL\textsubscript{4} and Licorice Extract on Viability % of isolated rat hepatocytes](image)

**Fig (1): Effects of CCL\textsubscript{4} and Licorice Extract on Viability % of isolated rat hepatocytes**

(a) Significantly different from control group
(b) Significantly different from CCL\textsubscript{4} group

The time course of AST leakage from isolated hepatocytes is demonstrated in (Fig.4). CCL\textsubscript{4} caused significant time dependent increase in AST leakage in comparison to control. This increase of AST leakage% was elevated by time after 30, 60 min, and showed a significant increase at 120 min as compared to control group. Pretreatment with licorice (25 μM/ml) for 30 min before CCL\textsubscript{4} addition induced a significant protection against CCL\textsubscript{4}-induced AST leakage after 60 and 120 min as compared to CCL\textsubscript{4} group. Also, pretreatment with silymarin showed protective effect similar to licorice extract as compared to CCL\textsubscript{4} group.

**Effects of Carbon tetrachloride (CCL\textsubscript{4}) and Licorice on reduced glutathione (GSH) depletion of isolated rat hepatocytes**

Assessment of oxidative stress-induced by CCL\textsubscript{4} in isolated hepatocytes was done by measuring cellular GSH level. Figure (5) depicts the time-course effects of CCL\textsubscript{4} on hepatocytes glutathione content and its possible protection by either licorice extract or silymarin. CCL\textsubscript{4} caused significant depletion of glutathione content from isolated rat hepatocytes compared to control during the 2-h incubation period. Concomitant incubation of cells with silymarin and
CCL$_4$ or licorice extract and CCL$_4$ prevented the depletion of glutathione induced by CCL$_4$ exposure alone. The protection against glutathione depletion by silymarin or licorice extract in the presence of CCL$_4$ was almost the same at the incubation time studied.

**Effects of Carbon tetrachloride (CCL$_4$) and Licorice on lipid peroxide formation of isolated rat hepatocytes**

Assessment of oxidative stress-induced by CCL$_4$ in isolated hepatocytes was also demonstrated by measuring lipid peroxidation. The effect of CCL$_4$, licorice extract and silymarin on lipid peroxidation, as indicated by TBARS formation, was estimated (Fig.6) The hepatotoxin CCL$_4$ (5mM) induced a significant increase in the level of lipid peroxidation starting at 30 min after addition of CCL$_4$ as indicated by elevation in TBARS level. CCL$_4$ treated group showed a significant increase in TBARS level within the incubation period as compared to the control group. Both licorice extract and silymarin significantly decreased the TBARS formation induced by CCL$_4$. This protective effect was a time dependent, reached the maximum at 120 min as compared to CCL$_4$ group.
4. Discussion
Acute and chronic liver diseases constitute a global concern, and the medical treatments for these diseases are often difficult to handle and have limited efficacy. Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases (Seeff et al., 2001). Developing therapeutically effective...
agents from natural products may reduce the risk of toxicity when the drug is used clinically (Lee et al., 2007).

Recently, there is a growing interest in the interaction between pharmacology and nutrition science. Pharmaceuticals are generally developed to treat, cure or prevent disease and the primary goal of nutrition is to maintain or even improve health. This does not imply that there is no role for nutrition in preventing or curing disease (Georgiou et al., 2001).

Many naturally occurring plant constituents have been reported to protect against liver disease in experimental animals. Examples of these plants are *Silybum marianum* (milk thistle), *Allium sativa* (garlic), and *Picrohiza kurroa* (Luper, 1998). In view of this, the present study was undertaken to evaluate the hepatoprotective effects of licorice aqueous extract against cytotoxicity and oxidative stress induced by carbon tetrachloride in isolated primary rat hepatocytes.

Licorice, the root of the *Glycyrrhiza glabra* L. (Fabaceae) plant species, has been used medicinally for more than 4000 years (Aoki et al., 2005). The genus glycyrrhiza consists of approximately 30 species, of which six species produce a sweet saponin glycyrrhizic acid (GA) (Fukai et al., 2003). Licorice is one of the most widely used herbal drugs around the world, being present in most pharmacopoeias of eastern and western countries (Biondi et al., 2005). These medicinal plants are used as flavorings, sweeteners and herbal medicine, and also for improving health, detoxification and cures for injury (Cherng et al., 2006). They have been traditionally used for respiratory, gastrointestinal, cardiovascular, genitourinary, eye, and skin disorders, and for their antiviral effects (Zhang and Ye, 2009). GA, the most studied active constituent of Licorice, is a sweet-tasting material. The constituent is 50 times sweeter than sugar, and is widely used as a sweetening additive in the food industry, baked goods, ice cream and soft drinks (Acharya et al., 1993). In many countries, GA is used as a major therapeutic agent to treat chronic viral hepatitis and allergic dermatitis (Tanahashi et al., 2002). It is also known to have antiinflammation (Fujisawa et al., 2000), antiulcer, antihypertensive (Ito et al., 1997), and antivirus activities (Cinatli et al., 2003; Fu et al., 2005).

Oxidative stress plays an important role in hepatic injury and in initiating liver fibrogenesis through production of ROS. Hepatocytes' necrosis and apoptosis appear following lipids, proteins and DNA oxidation, followed by amplifying inflammatory response and initiating fibrogenesis. ROS stimulates both Kupffer and inflammatory cells in releasing profibrogenic mediators, which in turn stimulates HSCs proliferation which amplifies the production of ECM (Galli et al., 2000).

Carbon tetrachloride (CCl₄) is frequently used to induce liver fibrosis in animal models (Neubauer et al., 1998). Treatment with CCl₄ generates free radicals that trigger a cascade of events that result in hepatic fibrosis, mimicking the oxidative stress that has a fibrogenic effect on HSC (Poli, 2000; Reeves and Friedman, 2002; Huang et al., 2003). In fact, reactive oxygen species may cause tissue injury through activation of the precursors of MMPs (proMMPs) (Okamoto et al., 2001; Huang et al., 2003). Although no successful therapeutic approach to this pathogenetic mechanism in liver disease has been developed, antioxidants therapies have shown to achieve some positive effects (Wasser et al., 2001; Guo et al., 2002; de Freitas et al., 2003).

In the present study, CCl₄ induced its toxicity which was indicated by a significant decrease in the viability of isolated rat hepatocytes, and a significant increase in the leakage of intracellular enzymes (LDH, ALT, and AST) into the incubation medium as compared with the control group, reflecting the cell membrane integrity. These results are in agreement with many reports of Kim, (1995), Mahran et al. (1996), Wu et al. (1997), Du et al. (2000), and Dvorak et al. (2003).

The decrease in hepatocytes viability% and the increase in leakage% of intracellular enzymes after CCl₄ exposure was a time dependent Berger et al. (1986) and Farrel (1994).

In addition, the data of the present study showed a marked depletion of GSH, which reflected the redox status of hepatocytes, and a significant increase in lipid peroxidation which reflected the extent of lipid peroxidation of the isolated rat hepatocytes after CCl₄ exposure. CCl₄ is a well-known hepatotoxic agent. CCl₄ toxicity was reported to be associated with depletion of GSH where the trichloromethyl free radicals can react with compounds containing sulphydryl groups such as GSH and protein thiols leading to membrane lipid peroxidation and finally cell necrosis (Recknagel et al., 1989). Moreover, hepatic GSH level was decreased after CCl₄ addition due to the reaction of GSH with CCl₄ derived free radicals in the hepatocytes (Connor et al., 1990; Nishida et al., 1998). The depletion of GSH in liver is a well-known concomitant of CCl₄ toxicity (Kim et al., 1999, Dvorak et al., 2003).

Also, increased lipid peroxidation, as evidenced by elevated levels of thiobarbituric acid reactive substances (TBARS) in isolated rat hepatocytes, was demonstrated in the present study after CCl₄ exposure. These results are in harmony with those of other investigators who reported the association between CCl₄ toxicity and lipid peroxidation (Kim, 2007).
The membrane lipids are susceptible to oxidation because of their association in the cell membrane with enzymatic and non-enzymatic systems capable of generating free-radical species. The oxidation of unsaturated fatty acids in biological membranes leads to a reduction in membrane fluidity and disruption of membrane structure and function (Slater and Cheesman, 1987).

The increase in the leakage of intracellular enzymes (LDH, ALT, and AST), loss of cell viability, depletion of GSH content and lipid peroxidation indicated that the model had been successfully built. It was more important to confirm whether there is any difference between the treatment with or without licorice extract under the damage of whether there is any difference between the treatment with or without licorice extract under the damage of CCl4. Since free radicals play such an important role in CCl4-induced hepatotoxicity, it seems logical that compounds that neutralize such radicals may have an hepatoprotective effect. Indeed, various natural products have been reported to protect against CCl4-induced hepatotoxicity (Hsiao et al., 2003).

Silymarin (0.5mM) exerted marked protective effect against CCl4 toxicity which was indicated by increasing the viability of hepatocytes when compared to CCl4 treated group. Also, silymarin significantly decreased the leakage of intracellular enzymes to the medium, the depletion of GSH, and decreased lipid peroxidation as it was previously reported by others Chrunigoo et al. (1997) and Tasaduq et al. (2003).

The results showed that Pre-incubation of isolated rat hepatocytes with licorice extract (25μM/ml) afforded a protection against CCl4-induced hepatocyte toxicity, as evidenced by an increase in the viability%, decrease in the leakage% of LDH, ALT and AST, suppression of lipid peroxidation as well as by maintenance of intracellular level of GSH. The hepatoprotective effect of licorice extract against oxidative stress induced by CCl4 attributed to its antioxidant and free radical scavenging properties which have been demonstrated in various studies using licorice extract itself and the main active constituents of licorice extract.

The main components of licorice root are the triterpene, saponins, glycyrrhizin/glycyrrhetic acid and glycyrrhetic acid. Glycyrrhetic acid (GA) or Glycyrrhizin (GL) exhibits a number of pharmacological effects including anti-inflammatory and is used in hepatoprotective formulations. Pretreatment with GA has been reported to show protective action against carbon tetrachloride (CCl4)-induced liver injury in rats (Wang et al., 1993). Glycyrrhizin is a major active constituent isolated from licorice that scavenges reactive oxygen species (ROS) and has an anti-inflammatory action (Gumpricht et al., 2005; Yoshida et al., 2006).

Suzuki et al. (1977) reported that the principal triterpene component of licorice root, glycyrrhizin (GL), benefits patients with chronic hepatitis C infection. Derivatives of licorice root have been used in Asia to treat children with biliary atresia (Sokol et al., 2003), a choledacolic liver disease, although no clinical trials have been reported. Increasing evidence supports the hypothesis that GL, or its hydrolyzed metabolite 18β-glycyrrhetic acid, protects against several models of oxidant-mediated toxicity, including exposure to CCl4 (Jeong et al., 2002), t-butyl hydroperoxide (Kinjo et al., 2003), and ischemia-reperfusion injury (Nagai et al., 1991).

Treatment of concanavalin A (Con A)-treated mice with glycyrrhizin suppressed the increases in AST and ALT, cell infiltration and the degeneration of hepatocytes in the liver of these mice, which is due partly to the modulation of hepatic iNOS induction and of degeneration of hepatocytes (Tsuruoka et al., 2009).

Prophylactic administration of aqueous suspension of powdered Glycyrrhiza glabra roots at three different doses for 7 days to mice could provide appreciable protection against acetaminophen challenge on 8th day in sublethal experiments (Sharma and Rathore, 2011). Licorice constituents stabilize integrity of hepatic lysosomes and mitochondria (Gumpricht et al., 2005; Wu et al., 2008).

Glycyrrhiza glabra could attenuate peroxynitrite induced renal oxidative damage through inhibition of protein nitration (Yokozawa et al., 2005). Antioxidant capacity of licorice is used to treat kidney or urinary system based on oxygen radical absorbance capacity method (Wajcikowski et al., 2007).

Moreover, Glycyrrhiza glabra and lipoic acid could prevent gentamycin induced nephrotoxicity (Desai et al., 2004). Glycyrrhizin could prevent lead acetate induced hepatic oxidative stress and hyperproliferative activity in wistar rats. Pretreatment of rats orally with glycyrrhizin decreased hepatic microsomal lipid peroxidation and increase in the level of GSH content and lowered DNA synthesis (Rahman et al., 2004).

In addition, 18-betagallicyrrhethic acid (the major active metabolite of licorice) could prevent CCl4-induced liver injury in mice by inhibiting depletion of hepatic GSH. This component of licorice also showed antioxidant effect upon FeCl2-ascorbate induced lipid peroxidation in mice liver homogenate and upon superoxide radical scavenging activity (Jeong et al., 2002).
Glycyrrhizin was reported to have protective effect against CCl₄-induced liver injury by diminishing free radical toxic properties and inducing heme oxygenase-1 and down regulating proinflammatory mediators in mice (Lee et al., 2007).

Lin et al. reported that a three-day pretreatment with either glycyrrhizin or glycyrrhetinic acid exhibited protective effect on retorsine-induced liver damage in rats (Lin et al., 1999).

Several hypotheses have been put forward to account for the hepatic protection offered by these compounds including stimulation of cytochrome P-450 and glutathione S-transferase activities (Chan et al., 2003) or their activity as an antioxidant through glutathione preservation (Jeong et al., 2002).

Nose et al reported that the oral administration of 18 beta-GA at 1, 24, and 48 h before D-galactosamine treatment significantly reduced the increase of serum transaminase activities 24 h after galactosamine treatment (Nose et al., 1994). Glycyrrhizin can reduce the mortality of acetaminophen overdosed mice, attenuate the development of acetaminophen-induced hepatotoxicity in mice, and reduce the number and area of γ-GT positive foci, thus protecting liver function and preventing hepatocellular carcinoma from occurring (Wan et al., 2009).

In addition, Glycyrrhizin produced by the licorice plant is an anti-inflammatory that has been used in the treatment of patients with chronic hepatitis B and C (Iino et al., 2001; Miyake et al., 2002; Matsui et al., 2006; Yoshida et al., 2007). Furthermore, glycyrrhizin significantly prevented increased serum ALT levels and I/R-induced liver injury in rats (Nagai et al., 1992; Mabuchi et al., 2009; Ogiku et al., 2011).

The components of licorice extract might be responsible for the hepatoprotective effect licorice extract (Huo et al., 2011). Based on the experimental results reported here, we hypothesize that licorice extract may play an important role in medicine by scavenging free radicals, stimulating activities of antioxidant enzymes, subsequently protecting the liver against CCl₄-induced damage. We supposed that the components (triterpene, saponins, glycyrrhizic acid) in single or in combination with other components present in the licorice extract might be responsible for its hepatoprotective properties. In conclusion, CCL4 has a potential cytotoxic effect in isolated rat hepatocytes and exposing hepatocytes to licorice extract possess a highly promising hepatoprotective effects against CCL4-induced hepatotoxicity. Licorice extracts significantly improved cell survival and played an essential role to maintain the cellular membranes integrity against CCL4 Hepatotoxicity.

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