Anti-oxidative and hepatoprotective effects of lithospermic acid against carbon tetrachloride-induced liver oxidative damage \textit{in vitro} and \textit{in vivo}

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Abstract. Accumulation of an excess amount of reactive oxygen species (ROS) can cause hepatotoxicity that may result in liver damage. Therefore, development of anti-oxidative agents is needed for reducing liver toxicity. This study investigated the anti-oxidative and hepatoprotective activity of lithospermic acid, a plant-derived polycyclic phenolic carboxylic acid isolated from \textit{Salvia miltiorrhiza}, on carbon tetrachloride (CCl\textsubscript{4})-induced acute liver damage \textit{in vitro} and \textit{in vivo}. The results of the DPPH assay indicated that lithospermic acid was a good anti-oxidant. The CCl\textsubscript{4}-exposed Huh7 cell line exhibited decreased cell viability, increased necrosis and elevated ROS and caspase-3/7 activity. Lithospermic acid significantly attenuated the CCl\textsubscript{4}-induced oxidative damage in a concentration-dependent manner. The result of an \textit{in vivo} study with BALB/c mice corresponded with the anti-oxidative activity noted in the \textit{in vitro} study. Exposure of mice to CCl\textsubscript{4} resulted in a greater than 2-fold elevation in serum aspartate transaminase (AST) and alanine transaminase (ALT) levels. In addition, CCl\textsubscript{4}-intoxication led to an over 20% decrease in the level of intracellular hepatic enzymes including superoxide dismutase (SOD) and catalase (CAT) as well as increased lipid peroxidation. Upon histological examination of the CCl\textsubscript{4}-exposed mice, the mouse livers showed severe hepatic damage with a huge section of necrosis and structural destruction. Pretreatment of mice with lithospermic acid for six days significantly reduced CCl\textsubscript{4}-induced hepatic oxidative damage, serum AST and ALT. The pretreatment also increased SOD and CAT. The findings suggest that the health status of the liver was improved comparable to the control group after a high-dose treatment with lithospermic acid (100 mg/kg weight). The potential applicability of lithospermic acid as a hepatoprotective agent was demonstrated.

Introduction

The elevation of cellular reactive oxygen species (ROS) is believed to cause various types of human diseases such as diabetes, cardiovascular diseases, cancer and ageing (1,2). Increased oxidative stress leads to DNA damage, oxidation of proteins and intracellular components, which eventually cause apoptosis. Therefore, exploitation of potential anti-oxidants is beneficial to human health. Anti-oxidants can reduce cellular oxidative damage and be used for the prevention and treatment of oxidative stress. The cleavage of a carcinogen such as CCl\textsubscript{4} results in the formation of trichloromethyl free radicals, which subsequently increase ROS levels and cause hepatocyte injury. CCl\textsubscript{4} causes severe liver cellular damage via elevation of ROS, resulting in both necrosis and apoptosis of hepatocytes in acute liver injury. It is evident that direct reduction of ROS levels and inhibition of the CCl\textsubscript{4}-induced oxidative chain reaction are critical for the prevention and treatment of CCl\textsubscript{4}-induced acute liver damage (3,4). Compounds with anti-oxidant activity in reduction of the intracellular ROS level can be potential anti-oxidant agents for the prevention and treatment of oxidative damage (5).

\textit{Salvia miltiorrhiza}, a traditional Chinese medicinal herb, has been widely used in folk medicine to improve blood circulation and treat cardiovascular disease in humans (6). Many studies have illustrated the antitumor, anti-inflammatory and anticoagulant characteristics of \textit{Salvia miltiorrhiza} and its chemical constituents (6-10). Lithospermic acid, one of the major compounds present in \textit{Salvia miltiorrhiza}, is reported to have anti-oxidative activity as it shares a similar structure with salvianolic acid B, an anti-oxidant in \textit{Salvia miltiorrhiza}. The structure of lithospermic acid is shown in Fig. 1. A previous study showed that lithospermic acid was rapidly and widely distributed to tissues after intravenous administration in rats and it was also rapidly cleared (11). The rapid and high biliary excretion levels of lithospermic acid and its metabolites suggest that they undergo enterohepatic circulation in mice and are associated with the pharmacological effects of lithospermic acid (12,13). However, the chemical function of lithospermic acid is still under-studied, and its hypothesized anti-oxidative activity...
activity is yet to be investigated. In the present study, an Huh7 cell model and animal experiment were used to investigate the hepatoprotective and anti-oxidative effects of lithospermic acid against CCl4-induced acute liver damage.

Materials and methods

Materials. Preparation of lithospermic acid was carried out according to previously established methods (10). All aqueous solutions were prepared using double distilled water. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), carbon tetra-chloride (CCl4), and ethanol (99.9%) were purchased from Sigma-Aldrich. All other chemicals used were of highest commercial grade.

DPPH radical scavenging assay. The anti-oxidative ability of lithospermic acid was measured using DPPH assay. The reaction mixture contained 10 µl of DPPH, 89.6 µl of ethanol and 0.4 µl of lithospermic acid. The reaction mixture was kept in the dark for 30 min, prior to absorbance measurement at 517 nm. The scavenging activity was calculated and expressed as \( IC_{50} \).

Cell culture and treatment. The human liver carcinoma cell line Huh-7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Eagle's minimum essential medium (EMEM; Gibco) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 mg/ml) at 37°C in 5% CO2. Cells (1x10^5) were seeded onto 96-well plates and incubated overnight at 37°C in 5% CO2. The cells were treated with 0.5% CCl4 and different concentrations of lithospermic acid and incubated for 1 h. Cells were incubated with fresh medium as the control. After incubation, the medium was replaced with 100 µl of fresh medium; cells were used for further investigation.

Cell viability assay. Cell viability was measured with the MTT assay. The medium was replaced with 100 µl of fresh medium and 20 µl of MTT (5 mg/ml) and incubated for another hour. The MTT-containing medium was removed, and 150 µl of DMSO was added to each well. The plate was gently shaken, and the absorbance was measured at 540 nm on a microtiter plate reader (SpectraMax 190; Molecular Devices).

LDH assay. LDH activity was analyzed with the LDH cytoxicity assay kit (Promega). The level of LDH was measured according to the manufacturer's instructions. The absorbance at 490 nm was measured by a microtiter plate reader (SpectraMax 190; Molecular Devices).

Determination of caspase-3/7 activity. The activity of caspase-3/7 was determined using Caspase-Glo 3/7 assay kit (Promega). After incubation, the medium was replaced with 100 µl of caspase-3 reagent and incubated in room temperature in the dark for 4 h on a shaker. The fluorescence was recorded using a fluorescence microplate reader (Tecan infinite M200) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Alanine transaminase (ALT) activity assay. The alanine transaminase activity was determined with the alanine transaminase colorimetric activity assay kit (Cayman Biotech, Minneapolis, MN, USA). Cells (1x10^6) were seeded into culture dishes, incubated overnight, before incubation with 0.5% CCl4 and different concentrations of lithospermic acid for 1 h. Cells were collected and homogenized in the cold buffer. The level of ALT was measured according to the manufacturer's instructions.

Determination of ROS levels. The ROS level in the Huh7 cells was measured according to the manufacturer's protocol using the Image-iT™ LIVE green reactive oxygen species detection kit (I36007) (Invitrogen). Carboxy-H2DCFDA was used as a fluorescence probe. Fluorescence was recorded at an excitation wavelength of 495 nm and an emission wavelength of 529 nm. The images were captured with a fluorescence microscope (Eclipse 80i).

Tissue preparation. The mice were handled in accordance with the guidelines of the Animal (control of experiments) Regulations of the Special Health Services Division (Department of Health, Hong Kong). Male BALB/c mice (20±2 g) were bred and maintained in the Laboratory Animal Services Center at the Chinese University of Hong Kong. The mice were maintained in a 12-h light/dark cycle under standard conditions of constant temperature (25±2°C) and a humidity of 55±5%. The mice had free access to water and were fed rodent diet (Superstock Autoclavable Rodent Diet; Ridley Agriprouducts, Melbourne, Australia).

Mice were randomly assigned to five groups with five mice in each group as follows: Group N, control group, administered water only for six consecutive days; Group A, lipospermic acid group, administered a high dose of lipospermic acid (100 mg/kg body weight) by oral gavage for six consecutive days; Group B, low dose lipospermic acid and CCl4 group which was administered a low dose of lipospermic acid (50 mg/kg body weight) by oral gavage for six consecutive days, and CCl4 (10 ml/kg body weight, v/v=1:49 in corn oil) intraperitoneal injection 3 h after the last lipospermic acid administration; Group C, high dose lipospermic acid and CCl4 group which was administered a high dose of lipospermic acid (100 mg/kg body weight) by oral gavage for six consecutive days, and CCl4 (10 ml/kg body weight, v/v=1:49 in corn oil) intraperitoneal injection 3 h after the last lipospermic acid administration; Group D, CCl4 group which was administered water only and CCl4 (10 ml/kg body weight) intraperitoneal injection 3 h after the last water administration. The body weight of all mice was recorded daily. Twenty-four hours after CCl4 injection, the
mice were sacrificed. Blood was collected with cardiac puncture and was allowed to clot. Centrifugation of the clotted whole blood allowed serum collection, which was used for further experiments. The livers were excised, washed with saline and weighed. The livers were cut into one large piece and a few small pieces. The large piece was used for histopathological analysis and the small pieces for hepatic homogenized preparation. Other organs such as the thymus, heart, spleen, kidneys were also collected and weighed.

**Biochemical parameters of liver function.** Serum AST and ALT levels were measured according to the manufacturer's protocols with the use of AST/GOT (Liqui-UV) and ALT/GPT (Liqui-UV) assay kit (Stanbio), respectively. The absorbance was measured with a microtiter plate reader (SpectraMax 190; Molecular Devices). The average absorbance per minute was used to calculate the relative AST/ALT levels.

**Histopathological examination.** The larger piece of liver was fixed in 10% (v/v) phosphate-buffered formalin, embedded with paraffin, sectioned at a 5-µm thickness. Hematoxylin and eosin (H&E) staining was completed, and images were captured by a fluorescence microscope (Eclipse 80i) according to previously established methods (14).

**Determination of lipid peroxidation.** The TBRAS level was determined using the TBRAS assay kit (Cayman). The absorbance was measured at 530 nm with a microtiter plate reader (SpectraMax 190; Molecular Devices). The level of lipid peroxidation was determined by comparison with the MDA standard according to the manufacturer's protocols.

**Determination of anti-oxidants.** The mouse liver was cleansed with cold saline and homogenized in cold Tris-HCl buffer (0.01 M, pH 7.4). Homogenates were centrifuged and supernatants were collected for catalase (CAT) and superoxide dismutase (SOD) level measurement according to the manufacturer's protocols. CAT and SOD activities were measured using the catalase assay kit (Cayman) and SOD determination kit (Sigma), respectively. The absorbance was measured with a microtiter plate reader (SpectraMax 190; Molecular Devices) at 540 nm (CAT) and 450 nm (SOD).

**Results**

**DPPH radical scavenging assay.** Fig. 2 shows the DPPH free radical-scavenging activity of lithospermic acid. The scavenging activity was concentration-dependent with an IC₅₀ value of 23.2 µg/ml, and reached a maximal level at 160 µg/ml.

**CCl₄ cytotoxicity in the Huh7 cells.** Fig. 3 shows the hepatotoxic activity of CCl₄ in the human hepatocellular carcinoma Huh7 cells. The level of hepatotoxicity was elevated with an increase in CCl₄ concentration in the medium. At 0.1% of CCl₄ in medium, a significant decrease in cell viability to ~20% was noted, and the IC₅₀ value was calculated to be 0.069%.

**Effect of lithospermic acid on Huh7 cell viability.** The protective role of lithospermic acid was determined in the Huh7 cells by MTT assay (Fig. 4). The MTT assay showed that CCl₄ caused a significant reduction in cell viability (33.8%), as compared with the control group (P<0.01). However, after treatment with lithospermic acid, the effect of CCl₄ cytotoxicity was significantly reduced. The cell viability was increased in concentration-dependent manner in the cells. The lowest concentration of lithospermic acid (5 µg/ml) significantly
elevated the cell viability to 60.9%, while the cell viability was increased to ~75% following treatment with 30 µg/ml of lithospermic acid.

**Effect of lithospermic acid on LDH leakage in Huh7 cells.** The level of lactate dehydrogenase (LDH) leakage is shown in Fig. 5. Addition of 0.5% CCl₄ in the medium caused a significant LDH leakage of 153.3% as compared to the control group (100%). A reduction in LDH leakage was shown after incubation with lithospermic acid. Lithospermic acid (20 µg/ml) significantly decreased LDH leakage to <115% compared with the control group.

**Effect of lithospermic acid on apoptotic activity in the Huh7 cells.** Caspase-3/7 are executive enzymes in the apoptotic pathway. As shown in Fig. 6, the level of caspase-3/7 activity was doubled in the CCl₄-exposed group compared to the control group (100%). A reduction in caspase activity was shown after incubation with lithospermic acid. Lithospermic acid (20 µg/ml) significantly decreased caspase activity to <115% compared with the control group.

**Effect of lithospermic acid on medium ALT in Huh7 cells.** The medium ALT activity of the Huh7 cell line is shown in Table I. ALT levels were measured in the Huh7 cells after treatment with lithospermic acid.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>ALT (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Control</td>
<td>1.25±0.078a</td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>CCl₄ only</td>
<td>2.02±0.16b</td>
</tr>
<tr>
<td>5</td>
<td>CCl₄ and 5 µg/ml LA</td>
<td>1.82±0.108cd</td>
</tr>
<tr>
<td>10</td>
<td>CCl₄ and 10 µg/ml LA</td>
<td>1.72±0.11e</td>
</tr>
<tr>
<td>20</td>
<td>CCl₄ and 20 µg/ml LA</td>
<td>1.39±0.085f</td>
</tr>
</tbody>
</table>

Significant differences are indicated by "P<0.001, and "P<0.05 as compared with the control group; "P<0.01 as compared to the CCl₄-exposed group. LA, lithospermic acid.

**Effect of lithospermic acid on ROS generation in the Huh7 cells.** In Fig. 7, changes in the reactive oxygen species (ROS) level were recorded with the carboxy-H₂DCFDA fluorescent probe (Promega). CCl₄ intoxicates cells by producing an excessive amount of ROS. A high level of fluorescence was detected in the CCl₄-exposed cells. After the addition of lithospermic acid, the ROS level was significantly reduced in a concentration-dependent manner. At 20 µg/ml, the level of ROS was attenuated, and was comparable to the control group.

**Effect of lithospermic acid on serum AST and ALT levels in mice.** Table II shows the change in serum AST and ALT levels in mice after pretreatment with lithospermic acid. In the control group, the serum levels of AST and ALT were 66.6±10.54 and 15.58±2.86 U/l, respectively. The levels of AST and ALT in group A were improved when compared to these levels in the control group, while the serum AST and ALT levels were doubled in group D. Treatment with lithospermic acid significantly reduced the serum AST and ALT levels in a dose-dependent manner, as shown in group B and C.

**Effect of lithospermic acid on lipid peroxidation in mice.** Cellular oxidative damage often induces lipid peroxidation, and thiobarbituric acid reactive substances (TBRAS) are formed as a by-product. Fig. 8 shows that the level of TBRAS was considerably elevated in group D compared to the control group, suggesting a high level of lipid peroxidation. After treatment with lithospermic acid, the level of TBRAS was reduced to 34.11 and 32.07 µM in group B and C, respectively.

**Effect of lithospermic acid on hepatic anti-oxidants in mice.** The superoxide dismutase (SOD) and catalase (CAT) levels in group D were significantly reduced in a concentration-dependent manner. At 30 µg/ml, the level of SOD was attenuated, and was comparable to the control group.
decreased, compared to these levels in the control group (P<0.01, Fig. 9). Pretreatment of lithospermic acid significantly elevated the levels of SOD and CAT in a concentration-dependent manner, when compared to levels in group D.

Histological examination of liver sections. CCl₄-induced hepatic histopathological damage was examined. In group D, considerable necrotic damage across wide areas of the liver section, and hepatic structural change around vessels were observed. Pretreatment of mice with lithospermic acid resulted in reduced areas of necrotic tissue and restored liver architecture (Fig. 10).

Discussion

In the present study, we demonstrated the anti-oxidative and hepatoprotective properties of lithospermic acid. In both the in vitro and in vivo studies, lithospermic acid was shown to significantly reduce the extent of CCl₄-induced hepatic injury by scavenging free radicals and elevating the amount of endogenous anti-oxidants in a dose-dependent manner. Furthermore,
Lithospermic acid significantly attenuated the level of necrotic and apoptotic liver injury. The activity of apoptotic executive caspases was considerably reduced. The results suggest an inhibition of apoptotic activity.

The Huh7 cell line was used to evaluate the hepatoprotective effect of lithospermic acid against CCl4-induced hepatic damage. The results of the cell viability assay indicated the protective effects of lithospermic acid on the Huh7 cell line by reducing the percentage of cell death caused by CCl4 (Fig. 4). Lactate dehydrogenase (LDH) leakage is a marker of necrotic cellular death and it provides supportive evidence for the protective effects of lithospermic acid. After incubation of cells with lithospermic acid, LDH leakage was reduced in a concentration-dependent manner (Fig. 5). The results suggest a protective effect of lithospermic acid against CCl4 cytotoxicity in the Huh7 cell line. Furthermore, the increased level of medium of ALT caused by CCl4-exposure was reduced by the presence of lithospermic acid (Table I).

The anti-oxidative activity of lithospermic acid was measured by the DPPH radical scavenging assay (Fig. 2). The free radical scavenging activity of lithospermic acid reduced the level of free radicals produced by CCl4 toxicity. The results suggest the protective effect of lithospermic acid against liver damage. This is supported by the fluorescent detection of ROS levels (Fig. 7). The intracellular ROS level was significantly elevated when cells were exposed to CCl4. The protective effect of lithospermic acid against CCl4 was shown by attenuating the level of ROS in a concentration-dependent manner. CCl4 intoxicates cells by producing excessive amount of reactive oxygen species (ROS). ROS cause DNA damage, lipid peroxidation as well as oxidation of amino acids and co-factors (15,w). Therefore, the elimination of excess ROS confirms the anti-oxidative properties of lithospermic acid and suggests its use as a potential agent for the prevention of oxidative-related diseases (1,3).

In addition to its anti-oxidative ability, lithospermic acid was shown to be capable of inhibiting key enzymes involved in apoptosis. Activation of caspase-3 and -7 plays a major role in the cleavage events that dissemble various enzymes and structures of liver cells, resulting in apoptotic cell death. In Fig. 6, CCl4 exposure increased the caspase activity, indicating an elevation of apoptotic events in the Huh7 cell line. However, a high dose of lithospermic acid was shown to reduce the activity of caspases to the levels comparable to the control group. These findings suggest that lithospermic acid modulates apoptotic activity via inhibition of key executive caspases.

Figure 9. Effect of lithospermic acid on the hepatic SOD and CAT levels in CCl4-intoxicated mice. N, control mice; A, mice treated with lithospermic acid at 100 mg/kg; B, CCl4-intoxicated mice treated with lithospermic acid at 50 mg/kg; C, CCl4-intoxicated mice treated with lithospermic acid at 100 mg/kg and D, CCl4-intoxicated mice. The data represent the mean ± SD, n=3. Significant differences are indicated by ****P<0.0001, ***P<0.001 and **P<0.01 as compared to the control group; ###P<0.001, ##P<0.01 and #P<0.05 as compared to the CCl4 group.
the development of many diseases is associated with oxidative stress (3,4), lithospermic acid could modulate oxidative stress associated with apoptotic cell death.

*An in vivo* study with mice showed the protective effect of lithospermic acid against CCl₄ toxicity. Serum AST and ALT are biomarkers of liver function. An increase in these markers suggests liver damage such as necrosis, cirrhosis and liver metastasis. The study showed that serum AST and ALT levels in mice were significantly elevated after treatment with CCl₄, as compared with the control group. Pretreatment with lithospermic acid reduced serum AST/ALT levels, suggesting the restoration of liver functions (Table II). Our study suggests the potential hepatoprotective effect of lithospermic acid.

Thiobarbituric acid reactive substances (TBRAS) are formed as a by-product of lipid peroxidation, and are used to measure oxidative damage. After CCl₄ exposure, the TBRAS level was increased, whereas pretreatment with lithospermic acid resulted in a reduction in the TBRAS level compared with the control group (Fig. 8). Moreover, the level of superoxide dismutase (SOD) and catalase (CAT) anti-oxidant enzymes which are essential in detoxification were analyzed. Consistent with the TBRAS measurement, the reduction in anti-oxidant enzyme activities caused by CCl₄ injection were significantly noted after lithospermic acid pretreatment. The results suggest that lithospermic acid was able to reduce oxidative damage caused by CCl₄ toxicity.
The effect of lithospermic acid on CCl₄-induced hepatic histopathological damage is shown in Fig. 10. CCl₄ caused significant necrosis as well as loss of hepatic structures around vessels. Lithospermic acid pretreatment was shown to reduce the area of necrotic tissue, along with restoration of liver morphology.

In summary, both in vitro and in vivo studies provide supportive evidence for the hepatoprotective effects of lithospermic acid against CCl₄ toxicity through reduction of free radicals, restoration of liver functions and inhibition of caspase activity associated with apoptosis. However, the detailed mechanisms that contribute to its protective effect remain to be investigated. Inflammatory signaling may also play an important role in the attenuation of cellular damage. Collectively, the present study provides substantial evidence that lithospermic acid is a good candidate for use as an antioxidant agent.

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References