Protective Effects of Radix Rosa laevigata against Propionibacterium acnes and Lipopolysaccharide-Induced Liver Injury

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We investigated the effects of an extract of Radix Rosa laevigata (R. R. laevigata) on Propionibacterium acnes (P. acnes) and lipopolysaccharide (LPS)-induced acute liver injury. The plasma alanine aminotransferase (ALT) activity was significantly elevated by an intravenous injection of heat-killed P. acnes at a dose of 0.4 mg/mouse and then with LPS at 0.1 μg/mouse after 5 d. However, the elevated ALT activity was significantly reduced by the administered of R. R. laevigata (125 and 500 mg/kg/d) for 7 d before the LPS injection. In addition, the extract treatment reduced the number of liver mononuclear cells (MNCs), and malondialdehyde (MDA) and nitric oxide (NO) contents, but improved the liver oxygen radical absorbance capacity (ORAC) and mitochondrial membrane potential. Moreover, the chemical profile of R. R. laevigata was performed by high-performance liquid chromatography (HPLC) and the main peaks were identified as a series of polyphenol compounds which had been confirmed as the significantly active components by their anti-oxidative and NO inhibitory effects. These results suggest that the extract of R. R. laevigata offered good efficacy for preventing liver injury.

Key words: liver injury; Radix Rosa laevigata; Propionibacterium acnes-lipopolysaccharide

Heat-killed Propionibacterium acnes (P. acnes) plus lipopolysaccharide (LPS)-induced liver injury has been used as an animal model for immunological liver injury1) which may reflect the clinical situation more accurately than the ordinarily used chemical-induced liver injury models.2) When LPS was intravenously injected into P. acnes-primed mice, massive hepatic cell necrosis usually occurred within 24 h.3) This injury was caused by the infiltration of liver mononuclear cells (MNCs) and Kupffer cells which produce inflammatory mediators including cytokines and free radicals.4–6) Among these inflammatory mediators, nitric oxide (NO) production at high level is thought to be an important causative factor for cellular injury and there-fore serves as an effective measure of the cell damage and dysfunction associated with inflammation.7)

Plant extracts are good sources of useful hepatoprotective agent, which can modulate the activities of free radicals, cytokines, NO and other inflammatory mediators.8) To find safer and more effective natural agents for treating liver injury, we have screened various herbal extracts using the P. acnes-LPS-induced liver injury model, and the water extract of Radix Rosa laevigata (R. R. laevigata) was selected. R. R. laevigata is the root of Rosa laevigata Michx. (Rosaceae) which is widely distributed in southern China and Japan. The fruit of Rosa laevigata, as a commonly used traditional Chinese medicine, has been used on the treatment of chronic urinary tract infections.9) R. R. laevigata has long been used as a Chinese folk medicine to cure pelvic inflammation, ascending infection, cervicitis and other types of inflammation.10) In addition, R. R. laevigata is an essential ingredient of several traditional Chinese formulations which are used for anti-stress and anti-inflammatory treatments.11,12) However, there is no report concerning its pharmacological mechanism and active components. We investigated in this study the hepatoprotective effects of R. R. laevigata on P. acnes-LPS-induced liver injury. The high-performance liquid chromatography (HPLC) fingerprint of the R. R. laevigata extract was also recorded and identified. In order to demonstrate the active components, the effects of those identified compounds on the oxygen radical absorbance capacity (ORAC) and inhibition of NO production in LPS-activated murine macrophages were further evaluated.

Materials and Methods

R. R. laevigata and preparation of the R. R. laevigata extract. R. R. laevigata was generously provided by Guangzhou Wanglaoji Pharmaceutical Co. (Guangzhou, China). A voucher specimen (2006WZY0044) is maintained at Institute of Traditional Chinese Medicine & Natural Products, Jinan University. Guangzhou 510632, China. 2.0 kg of dry R. R. laevigata was decocted with 2,000 ml of boiling water for 1.5 h to half its volume. The extracted solution was...
Preparation of P. acnes and Reagents. P. acnes (ATCC6919) was cultured with a brain heart infusion medium (Difco Laboratories, USA) supplemented with L-cysteine (0.03%) and Tween 80 (0.03%) under anaerobic conditions, using the Anaerobic System (Forma Scientific, USA) for 48 h at 37 °C. The cultured cells were centrifuged at 10,000 rpm for 15 min at 4 °C and washed with phosphate-buffered saline (PBS). The bacterial pellet was re-suspended with PBS and the cells were killed by a heat treatment at 80 °C for 30 min, before being lyophilized to prepare heat-killed P. acnes powder. LPS from Escherichia coli 055:B5 was purchased from Difco Laboratories. These samples were dissolved in a PBS solution just before use. Sodium fluorescein (FL), 2',7'-azobis (2-amidinopropane)-dihydrochloride (AAPH), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) for the ORAC assay were purchased from Wako Pure Chemical Industries (Osaka, Japan). The commercial kits used for determining alamine aminotransferase (ALT) activity and malondialdehyde (MDA) were purchased from Jiancheng Institute of Biotechnology (Nanjing, China).

Animals and experimental protocol. Seven-week-old male BALB/c mice, which were purchased from the Center of Laboratory Animal Science Research of Southern Medical University (Guangdong, China), were kept in a specific pathogen-free animal room at 23 ± 1 °C with a 12-h dark/light cycle and were fed with a standard laboratory diet and water. The animals were allowed to acclimatize to the environment for 1 week before the experiment. The experimental groups received an oral administration of the R. laevigata extract dissolved in drinking water at a final concentration of 25.0 mg/ml and 50.0 mg/ml respectively, while the saline control group and liver injury control group mice received only water. The intake of the R. laevigata water solution was 0.1 ml/10 g weight for 7 d. Liver injury was induced by injecting 0.2 mg/kg weight of P. acnes in saline via the lateral tail vein. 5 d later, the mice were given 5 μg/kg weight of LPS by lateral tail vein injection. 5 h following the LPS injection, the mice were sacrificed to collect blood and liver samples. The care and treatment of the animals in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. All the experiments in this study were approved by the Research Commission on Ethics of Jinan University.

Preparation of MNCs and analysis of T lymphocyte subsets. Mice livers were removed, passed through a 200-gauge stainless steel mesh and then suspended in a Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% fetal bovine serum (FBS; Invitrogen, USA). After washing once, the cells were resuspended in a 40% Percoll solution containing 100 U/ml of heparin and then centrifuged at 500 g for 10 min at room temperature. The pellet was resuspended in an ammonium chloride reagent to lyse the erythrocytes. The cells were washed twice with PBS and suspended in 1 ml of a cold (10% PBS)-RPMI-1640 medium. The mononuclear cell viability was determined by trypan blue exclusion, and total MNCs were counted. Samples containing 2 x 10⁵ mononuclear cells in (10% PBS)-RPMI-1640 were treated with 5 μl of selected monoclonal antibodies conjugated with fluorescein-isothiocyanate (FITC) or phycoerythrin (PE) (Beckman, USA). We used the following double-staining combinations: anti-CD3 (FITC)/anti-CD4 (PE), and anti-CD3 (FITC)/anti-CD8 (PE). Mouse immunoglobulin G1-FITC and -PE were used for control staining. After 15 min of incubation at room temperature in the dark, the cells were washed with PBS, re-suspended in 0.5 ml of cold PBS and analyzed by using FACS Epics XL (Beckman, USA). 10,000 number of cells were scanned for each sample, and the findings are expressed as the percentage of cells yielding specific fluorescence in a gated lymphocyte region.

Measurement of the plasma ALT activity, and liver homogenate MDA and ORAC contents. The plasma ALT activity and liver homogenate content of MDA were determined by using commercial kits. The procedure for the ORAC assay of the liver homogenate was modified from the previously described method. The ORAC assay on the R. laevigata water extract and its compounds was performed in vitro.

Flow-cytometric analysis of the liver intracellular NO level and mitochondrial membrane potential (ΔΨm). Liver intracellular NO production was determined by using diaminofluorescein-DMAC diacetate (DAF-FM DA; Beyotime, China). DAF-FM DA passively diffuses across the cell membranes and is then deacetylated into DAF-FM by intracellular esterases and caged. DAF-FM is nonfluorescent until it reacts with NO to form a fluorescent benzotriazole. The Mitochondrial membrane potential was determined by using rhodamine-123 (Sigma, USA), a lipophilic cation, which accumulates in the mitochondrial matrix in proportion to mitochondrial membrane potential. A liver cell suspension (5 x 10⁶ cells) was incubated with 10 μM of DAF-FM or rhodamine-123 at 37 °C for 30 min and then thoroughly washed three times with PBS. The fluorescence was excited at 488 nm and analyzed by using a 525 nm band-pass optical filter.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the liver samples by using Trizol (Invitrogen, USA). A 3-μg amount of total RNA was reverse-transcribed at 42 °C for 1 h in a 20 μl reaction mixture containing mouse Moloney leukemia virus reverse transcriptase (Tiangen, Germany) with oligo (dT) primers (Tiangen, Germany) followed by PCR amplification. Thereafter, cDNA was amplified together with Taq polymerase (Tiangen, Germany), using specific primers with 35 cycles at 94 °C for 30 s, an annealing temperature of 55 °C for 30 s, and then 72 °C for 1 min, with final incubation at 72 °C for 7 min. The PCR primers for mouse iNOS mRNA were (F): 5'-CCCAAGGTCTCACGTCGTAGGACAA-3' and (R): 5'-GGGAAGATCTGCAACGGAGAATCTT-3', and the product size is 246 bp. The primers for the mouse housekeeping gene beta-actin (β-actin) mRNA were (F) 5'-AGGGGAAATCGTGCGTGAC3' and (R) 5'-GCTGGAAGTTGACAGT-3', and the product size was 446 bp. The PCR products were fractionated on 1% agarose gel and visualized by ethidium bromide staining. The band intensity of ethidium bromide fluorescence was measured by using an image analysis system (Bio-Rad, USA), quantified by a Bandscan (Glyko, USA) and expressed as the ratio to β-actin.

Determination of the effects of the R. laevigata compounds on NO production in vitro. The effects of the R. laevigata compounds on NO production were determined in LPS-activated murine macrophages. The macrophages were isolated and purified from peritoneal cells by using the method previously described. All R. laevigata compounds were dissolved in dimethyl sulfoxide (DMSO). Peritoneal macrophages were seeded at a concentration of 5 x 10⁶ cells/well in 96-well culture plates and treated with R. laevigata compounds at final concentrations of 10 μM, 5 μM and 2.5 μM (containing 0.2% DMSO) for 1 h, before stimulation with LPS (1 μg/ml). Cell viability and NO production were measured by quantitative colorimetric assay with MTT and the Griess reagent, respectively.

Statistical analyses. The data are presented as the mean ± SE. A one-way analysis of variance (ANOVA) was applied to analyze for differences in data for the biochemical parameters among the experimental groups, this being followed by Dunnett’s test for pairwise multiple comparisons. Differences are considered as statistically significant at P < 0.05.

Results

Chemical profile of R. laevigata analyzed by reverse-phase HPLC

The chemical profile of R. laevigata was analyzed by HPLC. As illustrated in Fig. 1, the compounds of the main peaks of the HPLC map were identified as gallic...
acid (peak 1, \( t_R \) 6.7 min), isostrictinin (peak 2, \( t_R \) 13.7 min), (+)-catechin (peak 3, \( t_R \) 17.4 min), 3-hydroxyl-4-O-[\( 6^0 \)-O-galloyl]-glucopyranoside] phenethyl alcohol (peak 4, \( t_R \) 23.8 min), (+)-epicatechin (peak 5, \( t_R \) 24.9 min), 4'-O-arabinofuranosyl ellagic acid and 4'-O-xlyopyranosyl ellagic acid (peak 6, \( t_R \) 38.6 min), and ellagic acid (peak 7, \( t_R \) 40.1 min).

**Protective effects of R. R. laevigata on P. acnes and LPS-induced liver injury**

As shown in Fig. 2, liver injury was induced by lateral tail vein injection of LPS into P. acnes-pretreated mice, with the ALT level significantly increased from 22.8 ± 5.1 IU/l to 192.3 ± 7.2 IU/l (\( P < 0.01 \)). When R. R. laevigata (125 and 500 mg/kg/d) was administered orally to mice for consecutively 7 d before LPS injection, the elevated plasma ALT activity was significantly reduced to 99.1 ± 6.1 IU/L and 75.1 ± 5.4 IU/L (\( P < 0.01 \)) respectively.

Effects of R. R. laevigata on liver MNCs and T lymphocyte subsets in liver-injured mice

The number of total liver MNCs was 2.26 × 10⁵ in the saline control mice. After LPS had been intravenously injected into P. acnes-primed BALB/c mice, the number of total liver MNCs was significantly increased to 14.41 × 10⁵ (\( P < 0.01 \)). However, the elevated liver MNCs were reduced to 8.09 × 10⁵ and 5.65 × 10⁵ (\( P < 0.05 \)), respectively, by the daily treatment of R. R. laevigata (125 and 500 mg/kg/d, for 7 d) (Fig. 3A). As shown in Fig. 4, the percentage of each type of T lymphocyte was obtained by double fluorescent staining. Compared with the saline control mice, the percentage of the Th type of lymphocyte decreased by 50% in the liver-injured mice. In the lymphocyte subpopulations, as shown in Fig. 3B, the ratios of Th to Tc cells in the normal control group and liver-injured group were 2.00 ± 0.36 and 0.93 ± 0.25, and those of oral administration of R. R. laevigata (125 and 500 mg/kg/d, for 7 d) were 1.50 ± 0.19 and 1.72 ± 0.29, respectively.

Effects of R. R. laevigata on liver homogenate MDA and ORAC in liver-injured mice

Liver homogenate MDA and ORAC respectively indicate the oxidative stress and anti-oxidative defense in the liver. Compared to the saline control mice, liver injury induced by P.acnes-LPS provoked a significant...
increment of MDA (from 11.6 ± 1.8 to 26.5 ± 3.3 μmol/mg of protein; \( P < 0.01 \)), and decrease of ORAC level (from 4288.6 ± 950.1 to 3068.5 ± 222.9 U/ml; \( P < 0.01 \)). As show in Fig. 5, pretreatment with \( R. \ R. \ laevigata \) (125 and 500 mg/kg/d, for 7 d) reduced the MDA content to 18.6 ± 2.6 and 13.6 ± 2.0 μmol/mg of protein, respectively (\( P < 0.01 \)), and restored the ORAC level significantly to 3550.3 ± 176.6 and 4033.4 ± 785.2 U/ml, respectively (\( P < 0.01 \)).

Effects of \( R. \ R. \ laevigata \) on liver intracellular NO and mitochondrial membrane potential (\( \Delta \Psi_m \)) in liver-injured mice

Compared with the saline control mice, the NO fluorescence signal was markedly intensified in the \( P. \ acnes \)-LPS induced liver-injured mice (Fig. 6A). However, as shown in Fig. 6B, the intensified liver intracellular NO fluorescence signals were weakened by the administration of \( R. \ R. \ laevigata \) (\( P < 0.01 \)). The mitochondrial \( \Delta \Psi_m \) level was determined by measuring the accumulation of cationic fluorescent dye Rh123 in the liver cells. As shown in Fig. 7A, the liver cell mitochondrial \( \Delta \Psi_m \) level was markedly depolarized in the liver-injured mice, indicated by the weaker Rh123 fluorescence intensity. However the depolarized mitochondrial \( \Delta \Psi_m \) level in the liver-injured mice was recovered by administering \( R. \ R. \ laevigata \) (\( P < 0.01 \)) for consecutive 7 d (Fig. 7B).

Effects of \( R. \ R. \ laevigata \) on liver iNOS gene expression in liver-injured mice

As shown in Fig. 8A, the iNOS mRNA level was almost undetectable in the saline control mice liver. In
response to *P. acnes* and LPS, the amount of iNOS mRNA was markedly up-regulated without affecting housekeeping gene expression (Fig. 8A). However, as shown in Fig. 8B, *R. laevigata* administration significantly suppressed the liver iNOS mRNA expression in the *P. acnes* and LPS-induced liver-injured mice (*P* < 0.01).

**Effects of *R. laevigata* compounds on ORAC and NO production in vitro**

Working curves for fluorescent oxidation were used as an index of resistance time for the oxidative reaction. Figure 9 shows that the curves for the fluorescence induced by AAPH were amended in the presence of *R. laevigata* at different concentrations. The ORAC value was calculated by using the ratio of the area under the fluorescence decay curve to that of 10 μg/ml of trolox which served as the standard. As shown in Fig. 10, the *R. laevigata* compounds increased the level of scavenging activity for the oxidation of fluorescein in a dose-dependent manner, the antioxidative capacity being stronger than that for vitamin C. Furthermore, the effect of the pretreatment with the *R. laevigata* compounds on LPS-induced NO production in murine peritoneal macrophages was also determined. Pretreatment of the cells with various concentrations of the *R. laevigata* compounds before stimulation with LPS (1 μg/ml) resulted in reduced NO production in a concentration-dependent manner (Fig. 11). All these compounds had light impact on cell growth as measured by the MTT test.

**Discussion**

An intravenous injection of *P. acnes* followed by LPS caused significant acute liver inflammation indicated by elevation of the plasma level of ALT in mice. When the mice had been pretreated with 125 and 500 mg/kg/d of *R. laevigata* once a day for 7 d before the LPS injection, the elevated plasma ALT activity was signifi-
Fig. 7. Effects of *R. laevigata* on the Liver Cell Mitochondrial Membrane Potential.
A. Representative flow cytometry histograms indicating mean channel of Rh123 fluorescence (FL-1). The histogram of the saline control mice cells (unshaded curve) is overlaid on the liver-injured control cell (shaded curve). B. Mean channel values for Rh123 fluorescence. The results represent the mean ± SE of the values obtained from 7 animals in each group. **Significantly different from the liver injury control mice at *P* < 0.01.

Fig. 8. Effects of *R. laevigata* on iNOS mRNA Expression in Liver-Injured Mice.
A. Representative results of the RT-PCR analysis of iNOS mRNA expression in mice liver. B. Densitometric analysis of PCR products of iNOS in mice liver. Results were generated as relative intensity units by densitometry and are expressed as the ratio of iNOS to β-actin. Data represent the mean ± SE of the values obtained from 7 animals in each group. **Significantly different from the liver injury control mice at *P* < 0.01.

Fig. 9. Curves of Fluorescence Decay Induced by AAPH in the Presence of *R. laevigata* at Different Concentrations.
Trolox, a water-soluble vitamin E analogue, was used as the control standard. The anti-oxidative activity of a sample is expressed as the net area under the curve. One ORAC unit was calculated as 

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U = K(S_{\text{Sample}} - S_{\text{AAPH}})/(S_{\text{Trolox}} - S_{\text{AAPH}}),
\]

where *K*, sample multiple of dilution; *S*, area below the fluorescence decay curve. Data are expressed as the mean of three experiments.
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Fig. 10. Effects of Compounds from *R. laevigata* on ORAC at Different Concentrations.

Vitamin C was used as a positive control. Data are expressed as the mean ± SE of quintuplicate experiments.

![Graph showing effects of compounds on ORAC at different concentrations.]

Fig. 11. Effects of *R. laevigata* Compounds on the LPS-Induced NO Production in Macrophages.

The cells were incubated with a medium containing various concentrations of *R. laevigata* compounds for 1 h before the LPS treatment (1 μg/ml) and the amount of nitrite in the medium was detected after 24 h. NO inhibition ratio (%) = ([NO₂⁻]LPS−[NO₂⁻]LPS+sample)/([NO₂⁻]LPS−[NO₂⁻]LPS+sample). Data are expressed as the mean ± SE of quintuplicate experiments.

significantly decreased. We also found that the *P. acnes* plus LPS injection increased the number of liver MNCs and T lymphocytes, and decreased the ratio of Th to Tc cells by significantly reducing the percentage of Th-type lymphocytes. As has been previously reported, the increase of leukocytes and infiltration of inflammatory cells in the liver caused massive hepatic cell necrosis, which could be prevented by a treatment with such immunosuppressants as FK506 and cyclosporin A. In our study, treatment with the natural plant extract of *R. laevigata* also reduced the elevated liver MNCs and T cell number, and attenuated the disorder of the T lymphocyte subset balance. HPLC-guided separation was used to isolated the main peaks of the *R. laevigata* HPLC fingerprint which were identified as gallic acid, isostrictinin, (+)-catechin, 3-hydroxy-4-O-[6'-O-galloyl]-glucopyranoside phenethyl alcohol, (+)-epicatechin, 4'-O-arabinofuranosyl ellagic acid and 4'-O-xylopyranosyl ellagic acid, and ellagic acid. All these data suggest that this active extract contained plenty of polyphenol compounds which can be absorbed through stomach and intestines by their affinity for the monocarboxylic acid transporter. It has been proposed in recent years that polyphenol compounds can exert an anti-inflammatory effect through both the NF-κB-dependent and NF-κB-independent pathways.

It has also been reported that polyphenol compounds have the effect of immunomodulation by reversing the changes in IL-2 production and protein tyrosine phosphorylation, and increasing intracellular calcium mobilization, low-DNA binding activity of nuclear transcription factors, and nuclear factor of activated T-cells (NFAT), and reducing the binding activity of activating protein-1 (AP-1). These results are the same as ours indicating that *R. laevigata* had a protective effect on *P. acne* plus LPS-induced liver injury via an anti-inflammatory effect and proper immune regulation in the liver.

Liver injury induced by *P. acnes*-LPS caused a significant decrease of ORAC level and promoted lipid peroxidation in the mouse liver, suggesting a correlation between oxidative stress and inflammatory response in the liver. Oxidative stress may generally be caused by reactive oxygen species (ROS) which can promote the production of TNF-α and other pro-inflammatory mediators and consequently cause liver damage. Our further studies indicate that the injection of LPS into *P. acnes*-infected mice caused a massive release of NO in the liver. This increment of NO production might have been due to the markedly up-regulated iNOS mRNA expression in response to *P. acnes* plus LPS. Excess NO causes hepatotoxicity, directly or indirectly, by the formation of cytotoxic peroxynitrite or the activation of cytokines such as TNF-α which is essential for the process of liver injury. The integrative factors just mentioned regulated each other to cause liver mitochondrial toxicity, as indicated by the liver cell mitochondrial potential depolarization which is involved in a large variety of cell functional damage, and were found to be the initial characterization of necrosis or apoptosis.

Our study has demonstrated that an oral administration of *R. laevigata* significantly increased the diminished ORAC level and also reduced the elevated content of liver MDA, suggesting that the hepatoprotective effect of *R. laevigata* might be through blocking of oxidative stress-induced liver cell damage. *R. laevigata* administration also markedly suppressed the NO production and down-regulated iNOS mRNA expression in the liver cells. It is likely that the hepatoprotective effect of *R. laevigata* may be related to the inhibition of NO release, at least in part, by suppression of iNOS mRNA expression. This hepatoprotective effect was further confirmed by the recovery of the liver depolarized mitochondrial potential in liver-injured mice after the administration of *R. laevigata*.

The results of the chemical component study demonstrate that the *R. laevigata* extract contained plenty of polyphenols such as gallic acids and catechins which have anti-oxidative and NO production inhibitory activities. In our present study, the ORAC assay of these compounds in vitro also indicated that they had strong free radical scavenging action. Furthermore, the anti-inflammatory effects of these compounds prepared pure were confirmed by elucidating the ability of these...
compounds in inhibiting LPS-induced NO in murine peritoneal macrophages without affecting the cellular viability. In particular, Papoutsi et al. have recently reported that gallic acid and its dimer, ellagic acid, both in the free form and esterified with glucose, exhibited a significant anti-inflammatory effect in vitro. Devipriya’s studies have demonstrated that ellagic acid could protect against alcohol-induced hepatotoxicity, and could be developed as an antifibrotic compound in the near future. Catechins have been found effective in suppressing the collapse of liver mitochondrial membrane potential induced by Tamoxifen and preventing the liver injury induced by LPS. These studies have suggested that these polyphenols should be the significant active components in the extract.

Conclusions

The total water extract of R. laevigata, which contained mainly polyphenol compounds, was identified to possess a protective effect against P. acnes and LPS-induced liver injury by a complex mechanism. This study has provided evidence for the extract of R. laevigata possessing good efficacy for preventing liver injury.

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