1 **SUPPLEMENTARY MATERIAL**

2 **Hepatoprotective effect of total flavonoids from *Glycyrrhiza uralensis* Fisch in**
3 **liver injury mice**

4

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8

9 **Abstract**

10 This work aimed to investigate the hepatoprotective effect of total flavonoids from
11 *Glycyrrhiza uralensis*. The main compounds in licorice total flavonoids (LTF) were
12 isolated from *Glycyrrhiza uralensis* and their total content of were more than 60%.
13 Hepatoprotective effects of LTF were investigated in three kinds of hepatic injury
14 mice model induced by high-fat emulsion, Chinese liquor, and tetrachloromethane.
15 Serum ALT, AST, and ALP levels and hepatic MDA, TG, cholesterol, and
16 hydroxyproline of hepatic injury mice were reduced by LTF. Simultaneously, hepatic
17 SOD and glutathione were increased by LTF. These results suggested that LTF can
18 repair liver tissue and reduce hepatic injury via alleviating inflammation, improving
19 antioxidant enzyme activity, and reducing oxidative stress in liver tissue and it may be
20 a valuable natural source of hepatoprotective activity.

21 **Keywords***:* Alcoholic liver; Flavonoid; Glycyrrhiza; Hepatic fibrosis; Liver injury;
22 NAFLD

23

Experimental

Plant materials

Licorice is identified as *Glycyrrhiza uralensis* Fisch and harvested from *Glycyrrhiza* Planting Base in Jiuquan of China. The specimen number is LZUYJ-201506.

Experimental materials

PPC Capsules were produced by Sinofi Beijing Pharmaceutical Co., Ltd. Silybin Capsules were produced by Tasly Pharmaceutical Co., Ltd. Colchicine Tablets were produced by Xi-Shuang-Ban-Na Pharmaceutical Co., Ltd. 56° Er-Guo-Tou liquor was produced by Beijing Red Star Co., Ltd. Peanut oil was produced by Shandong Luhua Co., Ltd. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglyceride (TG), free fatty acid (FFA), total cholesterol (TC), superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), and hydroxyproline (Hyp) diagnostic kits were purchased from Nanjing Jiancheng Bioengineering.

Preparation of LTF and separation of main compounds in LTF

In our previous study, the 95% ethanol extracts from *G. uralensis* were subjected to polyamide columns (60-80 mesh, 100×1500mm), and the hydrochloric acid-magnesium reaction was used to detect the flavonoids in the fractions. The lyophilized and collected licorice flavonoids powder were LTF. Sephadex LH-20 and silica gel column chromatography were used to separate LTF. Six major flavonoids monomers and one flavonoid-like coumarin compound were successfully isolated and their content in LTF was determined by reversed-phase HPLC.

Establishment of liver injury mice models and treatment approaches

All experiments involving animals were performed according to protocols

49   approved by the Animal Care and Use Committee of Lanzhou University. Animals

50   were maintained in a temperature- and light-controlled facility and fed normal chow.

51   Male Kunming mice aged 8 weeks and weighted 20 ± 2 g were purchased from the

52   Lanzhou University (laboratory animal certificate: scxk2013-0002). 180 mice were

53   fed ad libitum for 5 days and randomly divided into 3 big groups (60 mice were in

54   each big group), which were high-fat emulsion-induced fatty liver group, Chinese

55   liquor-induced alcohol liver group, and CCl_4-induced liver fibrosis group, respectively.

56   Each big group was divided into 6 small groups, which were normal control group,

57   model control group, positive control group and low (87.5 mg/kg), medium (175

58   mg/kg), and high (350 mg/kg) dose of LTF treatment group, respectively. Mice were

59   treated with interventional LTF for 4 weeks after 6 weeks of modeling. The specific

60   modeling methods of the three liver injury models are as follows:

61   1) NAFLD of mice was induced by orally administered with high-fat emulsion (1 g

62   of propylthiouracil, 10 g of cholesterol, 2 g of sodium cholate, 20 g of lard, 10 mL of

63   peanut oil, 5 mL of Tween-80 in 100 mL Water solution) at a dose of 10 mL/kg body

64   weight. After 6 weeks of modeling, the mice were treated with interventional LTF for

65   4 weeks. PPC (100 mg/kg) was used to control positive drug.

66   2) The mice alcoholic liver was induced by 56° red star Er-Guo-Tou (a Chinese

67   liquor). The mice were orally administered with starting dose from 5 mL/kg body

68   weight at am 9:00 daily, subsequently, increasing dose 1 mL/kg every two days until

69   to 10 mL/kg. After 6 weeks of modeling, the mice were treated with interventional

70   LTF for 4 weeks. Silybin (100 mg/kg) was used to positive control drug.

71   3) 15% CCl_4/ peanut oil solution (v/v, 10 mL/kg body weight, every 5 days) were

72   subcutaneously injected into the back (We did not choose intraperitoneal injection

73   because the mouse will scratch the abdomen and cause its inflammation and

74   ulceration) to induce mice liver fibrosis. After 6 weeks of modeling, the mice were

75   treated with interventional LTF for 4 weeks. Colchicine (45 mg/kg) was used to

76   positive drug.

77   After 10 weeks, overnight fasting plasma samples, organs and tissues were
78   collected.

Calculation of liver weight index

80   After all the mice sacrificed, the liver tissues were taken out and washed with saline.
81   The surface water of liver was dried with a filter paper, and then livers were weighed.
82   The liver index is calculated as liver weight index = liver weight/ body weight $\times$
83   100%.

Determination of indicators of liver injury in serum

85   Serum samples were prepared to measure biochemical parameters. Briefly, the
86   blood samples were allowed to clot at 4 ℃ and centrifuged at 5,000 $\times$ g for 10 min.
87   The serum samples were collected and stored at -20 ℃ until assayed. The kits for the
88   analyses of serum ALT, AST, ALP, TG, FFA, and TC were conducted according to the
89   manufacturer's instructions using a spectrophotometric plate reader.

Determination of indicators of liver injury in the liver

91   Liver tissue homogenate in saline were prepared to measure biochemical
92   parameters. 1 g of mouse liver tissue was placed into tube 4 mL of pre-cooled saline
93   and smashed by homogenizer in an ice bath. After diluting liver tissue suspension to
94   0.1 g/mL of concentration, liver tissue suspensions were centrifuge at 3000 rpm for 15
95   min at 4 ℃ to obtain liver tissue homogenate. The samples were collected and stored
96   at -80 ℃ until assayed. The kits for the analyses of liver tissue SOD, GSH, MDA,
97   Hyp, TG, and TC were conducted according to the manufacturer's instructions using a
98   spectrophotometric plate reader.

Histopathological examination of the liver

100 The mouse liver tissues were fixed in 10% neutral formalin solution. Then, liver
101 tissues were dehydrated and embedded in paraffin, which was sectioned 4-6 μm and
102 stained with hematoxylin-eosin (HE). Fibrosis and inflammation of liver tissue were

103   observed under a light microscope (Olympus CX21, Takachiho, Japan). The relative

104   area of hepatic fat vacuoles of mice induced by high-fat emulsion, the relative area of

105   hepatic steatosis and edema of mice induced by Chinese liquor, and relative area of

106   liver fibrosis induced by CCl_4 were calculated using Adobe Photoshop CC 2017

107   software.

108   *Statistical analysis*

109   All the analyses were carried out in triplicate, and the results were expressed as

110   mean ± standard deviation (SD). The quantitative data were analyzed using the

111   one-way analysis of variance (ANOVA) and the GraphPad Prism 5.0 program. The

112   significance level was set at $P < 0.05$ and $P < 0.01$.

113   **Results and discussion**

114   *Liver biochemical indicators*

115   As shown in Table S3, hepatic MDA, TC, and TG in NAFLD mice were

116   significantly higher than normal mice. 175 and 350 mg/kg of LTF could effectively

117   reduce hepatic MDA and TC in NAFLD mice. In the LTF treatment group, hepatic

118   TG was significantly decreased. Hepatic MDA and TG in NAFLD mice can be

119   reduced more greatly by LTF than PPC. LTF also can significantly reduce hepatic

120   cholesterol and TG in NAFLD mice induced by high-fat emulsion.

121   As shown in Table S4, in Chinese liquor-induced alcoholic liver experiment,

122   compared with normal mice, hepatic MDA and TG were significantly increased ($P <$

123   0.01), and hepatic GSH and SOD activities were significantly decreased ($P < 0.01$) in

124   Chinese liquor-induced alcoholic liver mice. The 87.5 mg/kg of LTF had no

125   significant effect on hepatic MDA ($P > 0.05$), while 175 and 350 mg/kg of LTF can

126   significantly reduce the hepatic MDA in alcoholic liver mice ($P < 0.01$). Hepatic TG

127   was decreased by LTF while hepatic SOD and GSH were increased in alcoholic liver

128   mice. LTF had a more markedly up-regulation effect on these biochemical factors

129   than Silybin in alcoholic liver mice.

As shown in Table S5, compared with normal mice, hepatic GSH and SOD in hepatic fibrosis mice were significantly decreased ($P < 0.01$). Hepatic Hyp, TG, and MDA were significantly after CCl_4-induced. After treatment by LTF. Hepatic Hyp and TG were dramatically reduced by LTF. However, only 175 and 350 mg/kg of LTF exhibited a certain moderating effect on hepatic MDA, SOD, and GSH.

Morphological and histopathological examination of the liver

Hepatocytes radially arranged and their nuclear structure was clear, and hepatic tissue was not infiltrated by inflammatory cells and edema cells in normal mice (Figure S5-5A).

After 10 weeks of inducing with high-fat emulsion, the hepatocytes of mice exhibited obvious fatty degeneration and disorderly arranged. However, they did not have obvious necrosis cells and pyknosis or disappearance of the partial nucleus (Figure S5B). After treated by PPC, the hepatic nucleus was stained dark and the cell gap was obviously enlarged (Figure S5C). The low-dose LTF treated-mice had no obvious hepatic steatosis, but they still exhibited intracellular edema (Figure S5D). the medium-dose LTF treated-mice showed no obvious fatty degeneration and their hepatocytes edemas were improved significantly (Figure S5E). The hepatic tissue morphology of high-dose LTF treated-mice was significantly approached to normal. Their cells were close to the hepatocytes and had no obvious cell edema and fatty degeneration (Figure S5F). 100 mg/kg of PPC and 87.5, 175, and 350 mg/kg of LTF can reduce relative areas of hepatic fat vacuoles by 82.32, 73.68, 79.53, and 85.14%, respectively (Figure S5G).

After 10 weeks of inducing with Chinese liquor, liver tissue of mice shown marked edema and fatty degeneration, while hepatocytes lines disorderly arranged and cell nucleus with inflammatory infiltration were disappeared (Figure S5B). Liver tissue morphology of mice treated with silybin approached to normal (Figure S5C). The hepatic steatosis was significantly reduced by low-dose LTF, but some steatosis was still shown (Figure S5D). Medium-dose LTF treated-mice did not exhibit obvious

158 steatosis. Hepatic cords orderly arranged and hepatocyte shapes were normal (Figure

159 S5E). Hepatocyte morphology of high-dose LTF treated-mice was better than the

160 untreated model mice. 100 mg/kg of silybin and 87.5, 175, and 350 mg/kg of LTF can

161 reduce relative area of hepatic steatosis and edema by 82.65, 73.66, 82.72, and

162 79.89%, respectively (Figure S5G).

163 After 10 weeks of inducing with CCl_4, the hepatic portal area of model mice had

164 massive fibrotic hyperplasia and the hepatic cords disorderly arranged. Hepatocytes

165 had obvious fibrosis, steatosis, and inflammatory cell infiltration (Figure S6B). The

166 liver nucleus of mice treated with colchicine became larger and the cell boundaries

167 were blurred compared with normal mice, but the cells were not shown obvious

168 fibrosis (Figure S6C). Liver pathology was apparently improved after low-dose LTF

169 treatment, but the fibrosis near the central vein was still observed (Figure S6D).

170 Medium-dose LTF treated-mice had no obvious steatosis, and the hepatic cords

171 orderly arranged. However, some small ranges of fibrosis were observed in the central

172 venous area (Figure S6E). High-dose LTF visibly suppressed the steatosis, and hepatic

173 tissue morphologies were closed to the normal group mice, but some cells edemas

174 were still observed (Figure S6F). 45 mg/kg of colchicine and 87.5, 175, and 350

175 mg/kg of LTF can reduce the relative area of liver fibrosis by 77.24, 68.7, 58.02, and

176 81.12%, respectively (Figure S6G).

177 **Table S1. Monomeric compounds isolated from total flavonoids of licorice and their content.**

No.	Compound	Content
1	Neoliquiritin	15.74%
2	Liquiritin	22.55%
3	Liquiritigenin	3.33%
4	Glycycoumarin	11.74%
5	Isolicoflavonol	2.41%
6	Licochalcone A	4.23%
7	Medicarpin	1.52%

178

179 **Table S2. Effects of LTF on hepatic MDA, Cholesterol and TG of mice induced by high-fat**

180 **emulsion.**

Group	MDA (nmol/g)	Cholesterol (mmol/g)	TG (µmol/g)
NC	1.76±0.20	0.091±0.008	50.13 ±8.24
MC	2.38±0.22[**]	0.115±0.020[**]	188.21±16.13[**]
PC	2.04±0.13[††]	0.080±0.039[†]	131.43±14.45[††]
L-LTF	2.25±0.16	0.113±0.012	166.22±17.32[††]
M-LTF	2.12±0.16[††]	0.100±0.006[†]	147.33±10.78[††]
H-LTF	1.99±0.05[††]	0.096±0.016[†]	128.45±12.35[††]

181 **Notes**: The data was indicated by mean ± SD, n=10, [*]$P < 0.05$ and [**]$P < 0.01$, compared with

182 normal control group. [†]$P < 0.05$ and [††]$P < 0.01$, compared with model control group. MDA,

183 malondialdehyde. TG, triglyceride. NC, Normal control group. MC, Model control group. PC,

184 Positive drugs (Polyene phosphatidylcholine, 100 mg/kg) treatment group. L, M and H-LTF, low

185 (87.5 mg/kg), medium (175 mg/kg) and high (350 mg/kg) dose LTF treatment groups.

186 **Table S3. Effects of LTF on hepatic MDA, SOD, GSH and TG of mice induced by Chinese**

187 **liquor.**

Group	MDA (nmol/g)	SOD (U/mg)	GSH (μmol/g)	TG (μmol/g)
NC	1.26±0.13	238.06 ± 12.15	14.66 ± 3.20	47.20 ±6.99
MC	3.82±1.25[**]	133.53 ± 3.98[**]	7.92 ± 1.04[**]	154.97 ± 25.72[**]
PC	1.96±0.32[††]	214.74 ± 10.84[††]	12.89 ± 0.98[††]	106.37 ± 17.11[†]
L-LTF	3.52 ±1.29	183.58 ± 17.13[††]	9.69 ± 0.81[†]	124.56 ± 11.3[†]
M-LTF	2.44±0.82[††]	183.71 ± 14.43[††]	10.92 ± 1.16[††]	92.60 ± 13.76[††]
H-LTF	1.94±0.79[††]	195.01 ± 19.71[††]	12.94 ±1.04[††]	92.22 ± 8.50[††]

188 **Notes**: The data was indicated by mean ± SD, n=10, [*]$P < 0.05$ and [**]$P < 0.01$, compared with

189 normal control group. [†]$P < 0.05$ and [††]$P < 0.01$, compared with model control group. MDA,

190 malondialdehyde. SOD, superoxide dismutase. GSH, glutathione. TG, triglyceride. NC, Normal

191 control group. MC, Model control group. PC, Positive drugs (Silybin, 100 mg/kg) treatment group.

192 L, M and H-LTF, Low (87.5 mg/kg), medium (175 mg/kg) and high (350 mg/kg) dose LTF

193 treatment groups.

194 **Table S4. Effects of LTF on hepatic Hyp, MDA, GSH, SOD and TG of mice induced by CCl$_4$.**

Group	Hyp (mg/g)	MDA (nmol/g)	SOD (U/mg)	GSH (μmol/g)	TG (μmol/g)
NC	10.28±3.74	1.56±0.48	148.69±57.18	7.91±3.95	48.23±14.84
MC	17.84±1.96[**]	3.45±1.09[**]	98.94±19.19[*]	4.02±1.66[*]	106.67±33.70[**]
PC	12.47±2.43[††]	1.68±0.46[††]	146.44±21.57[††]	7.29±3.00[†]	51.64±14.22[††]
L-LTF	14.91±2.45[†]	2.85±0.79	109.00±30.85	5.30±1.79	77.61±12.99[†]
M-LTF	13.84±4.73[†]	1.72±0.37[††]	136.22±45.91[†]	6.29±2.44[†]	53.18±11.44[††]
H-LTF	13.22±2.91[††]	1.58±0.71[††]	139.44±24.35[††]	6.34±2.21[†]	49.47±22.57[††]

195 **Notes**: The data was indicated by mean ± SD, n=10, [*]$P < 0.05$ and [**]$P < 0.01$, compared with

196 normal control group. [†]$P < 0.05$ and [††]$P < 0.01$, compared with model control group. Hyp,

197 hydroxyproline. MDA, malondialdehyde. SOD, superoxide dismutase. GSH, glutathione. TG,

198 triglyceride. NC, Normal control group. MC, Model control group. PC, Positive drugs (Colchicine

199 45 mg/kg) treatment group. L, M and H-LTF, Low (87.5 mg/kg), medium (175 mg/kg) and high
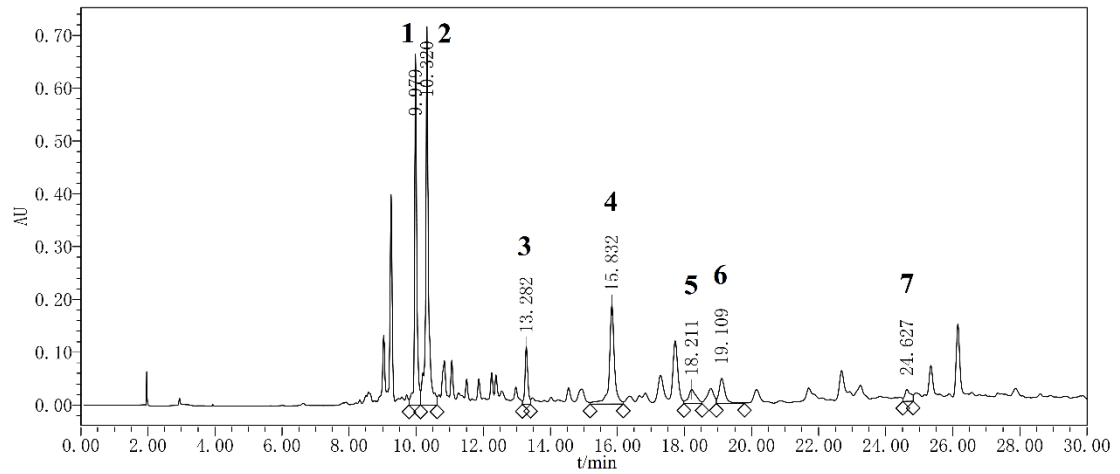
200 (350 mg/kg) dose LTF treatment groups.

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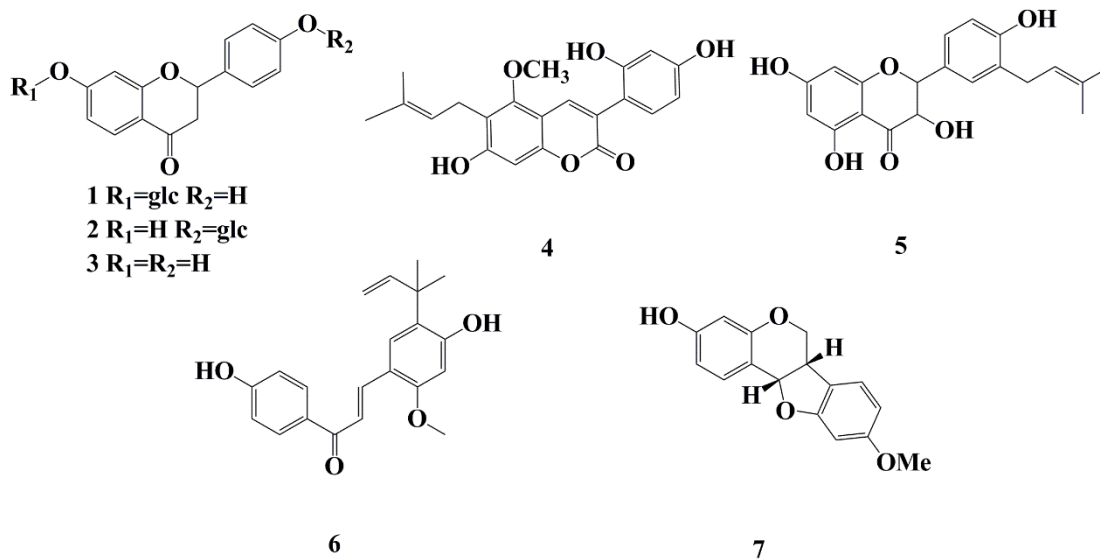
Figure S1: Monomeric compounds in licorice total flavonoids. **1**. Neoliquiritin. **2**. Liquiritin. **3**. Liquiritigenin. **4**. Glycycoumarin. **5**. Isolicoflavonol. **6**. Licochalcone A. **7**. Medicarpin.
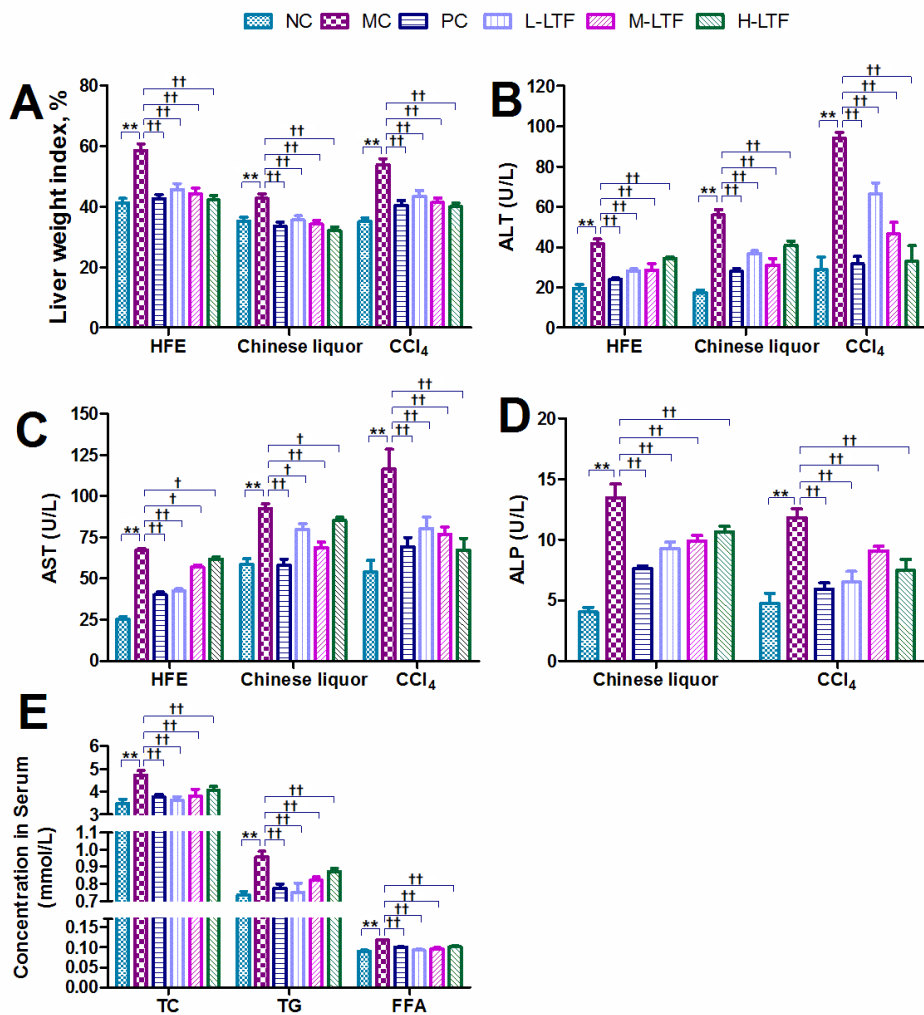
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206 **Figure S2:** The HPLC peaks positions of monomeric compounds isolated from in

207 licorice total flavonoids. **1**. Neoliquiritin. **2**. Liquiritin. **3**. Liquiritigenin. **4**.

208 Glycycoumarin. **5**. Isolicoflavonol. **6**. Licochalcone A**. 7**. Medicarpin.

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Figure S3: Effects of LTF on liver weight index and serum biochemical indicators in mice for 4 weeks. (A) Liver weight index= liver weight/body weight. (B) Serum ALT levels of mice. (C) Serum AST levels of mice. (D) Serum ALP levels of mice. (E) Serum TC, TG, and FFA levels of mice induced by high-fat emulsion. Data is represented by mean ± SD, n=10, $*P < 0.05$ and $**P < 0.01$, compared with normal control group. $†P < 0.05$ and $††P < 0.01$, compared with model control group. ALT, Alanine aminotransferase. AST, Aspartate aminotransferase. ALP, Alkaline phosphatase. TC, Total Cholesterol. TG, Triglyceride. FFA, Free fatty acid. HFE, High-fat emulsion. NC, Normal control group. MC, Model control group. PC, Positive drugs (NAFLD mice induced by high-fat emulsion were used polyene phosphatidylcholine, Alcoholic liver mice induced by Chinese liquor were used

222   silybin and liver fibrosis mice induced by CCl_4 were used colchicine) treatment group.

223   L, M and H-LTF, low (87.5 mg/kg), medium (175 mg/kg) and high (350 mg/kg) dose
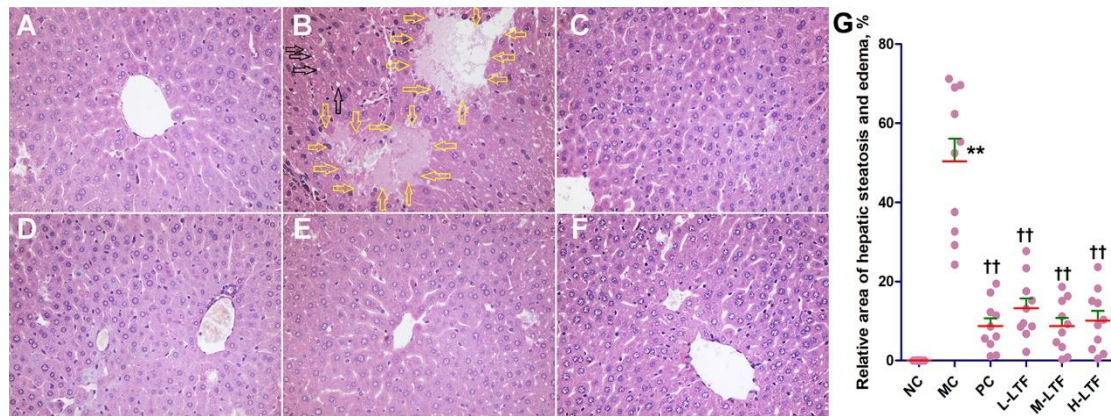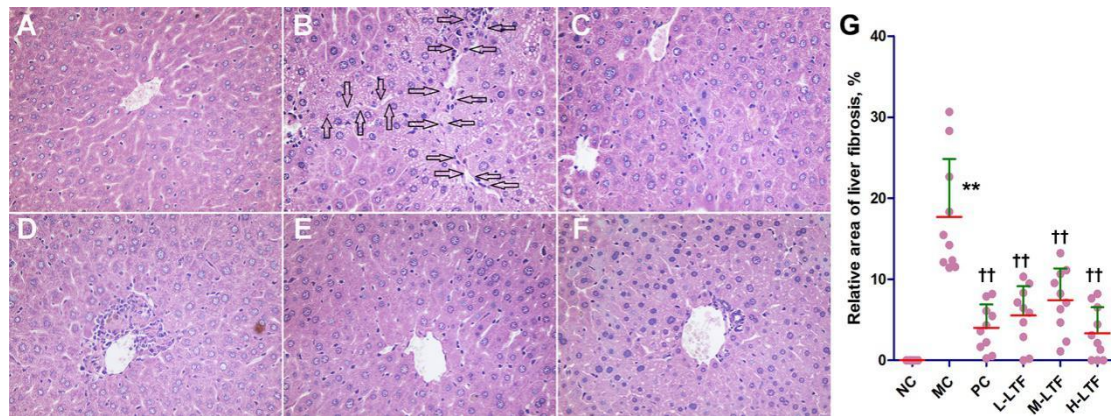
224   LTF treatment groups.

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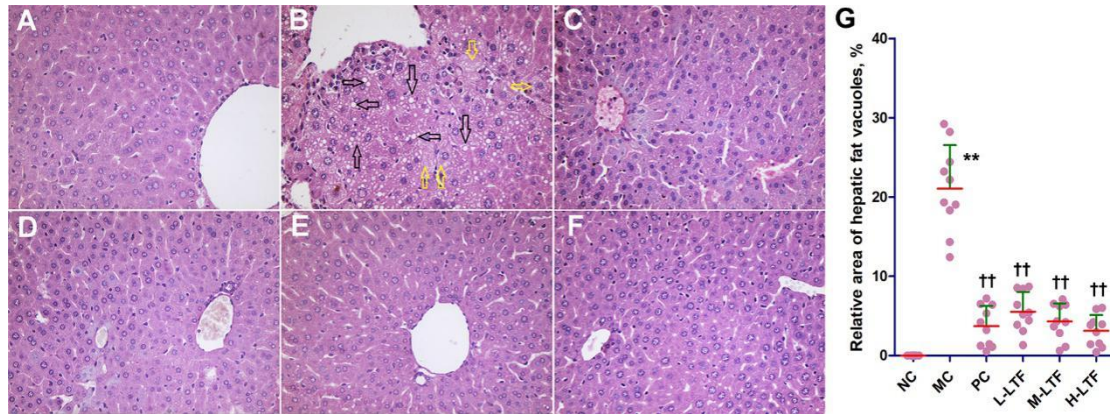
Figure S4: Liver histopathological section of NAFLD mice induced High-fat emulsion. (stained with HE, 200 ×). (**A**) Normal control group (n=10). (**B**) High-fat emulsion-induced model control group (n=10). (**C**) Polyene phosphatidylcholine (100 mg/kg) treatment group (n=10). (**D**) Low-dose (87.5 mg/kg) LTF treatment group (n=10). (**E**) Medium-dose (175 mg/kg) LTF treatment group (n=10). (**F**) High-dose (350 mg/kg) LTF treatment groups (n=10). (**G**) Relative area of hepatic fat vacuoles of mice induced by high-fat emulsion. Relative area of hepatic fat vacuoles % = area of hepatic fat vacuoles in every random field of liver section/ area of corresponding field of liver section. Data is represented by mean ± SD, n=10, $^*P < 0.05$ and $^{**}P < 0.01$, compared with normal control group. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, compared with model control group. Black arrows refer to large fat between cells. Yellow arrows refer to small intracellular fat.

Figure S5: Liver histopathological section of alcoholic liver mice induced by Chinese liquor (stained with HE, 200 ×). (**A**) Normal control group (n=10). (**B**) Chinese liquor-induced model control group (n=10). (**C**) Silybin (100 mg/kg) treatment group (n=10). (**D**) Low-dose (87.5 mg/kg) LTF treatment group (n=10). (**E**) Medium-dose (175 mg/kg) LTF treatment group (n=10). (**F**) High-dose (350 mg/kg) LTF treatment groups (n=10). (**G**) Relative area of hepatic steatosis and edema of mice induced by Chinese liquor. Relative area of hepatic steatosis and edema % = area of hepatic steatosis and edema in every random field of liver section/ area of corresponding field of liver section. Data is represented by mean ± SD, n=10, $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with normal control group. $^{†}P < 0.05$ and $^{††}P < 0.01$, compared with model control group. Black arrows refer to fat vacuoles. Yellow arrows refer to edema tissues.

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Figure S6: Liver histopathological section of liver fibrosis mice induced by CCl₄ (stained with HE, 200 ×). (**A**) Normal control group (n=10). (**B**) CCl₄-induced Model control group (n=10). (**C**) Colchicine (45 mg/kg) treatment group (n=10). (**D**) Low-dose (87.5 mg/kg) LTF treatment group (n=10). (**E**) Medium-dose (175 mg/kg) LTF treatment group (n=10). (**F**) High-dose (350 mg/kg) LTF treatment groups (n=10). (**G**) Relative area of liver fibrosis induced by CCl₄. Relative area of liver fibrosis % = area of liver fibrosis in every random field of liver section/ area of corresponding field of liver section. Data is represented by mean ± SD, n=10, *P < 0.05 and **P < 0.01, compared with normal control group. †P < 0.05 and ††P < 0.01, compared with model control group. Black arrows refer to fibrosis and cell shrinkage.