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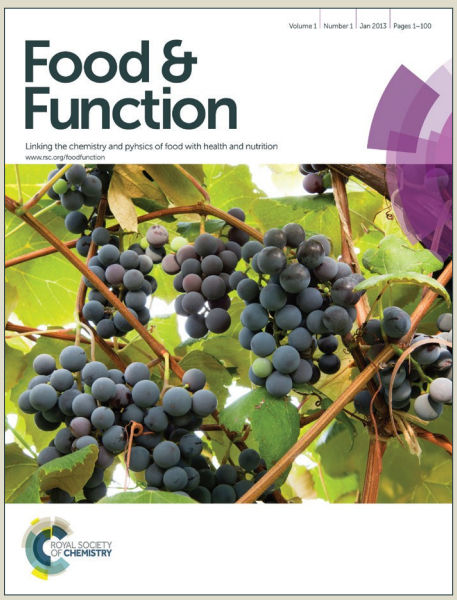

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Lonicera caerulea berry extract suppresses lipopolysaccharide-induced inflammation via Toll-like receptor and oxidative stress-associated mitogen-activated protein kinase signaling

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Abstract:

The protective effects of *Lonicera caerulea* berry extract (LCBE) against hepatic inflammation and the underlying mechanisms were investigated in a rat model of lipopolysaccharide (LPS)-induced chronic liver inflammation. Male Sprague-Dawley rats were injected with LPS (200 μg/kg bw) with or without LCBE co-administration (50, 100 and 200 mg/kg bw intragastrically once daily) for 4 weeks. We found that LCBE supplementation inhibited the increase in Toll-like receptor (TLR)2 and TLR4 expression induced by LPS, while preventing glutathione depletion and reactive oxidative species generation and abrogating increases in C-reactive protein and interleukin-6 levels, restoring alanine and aspartate aminotransferase activities, and blocking the phosphorylation of p38 and c-Jun N-terminal kinase mitogen-activated protein kinases (MAPKs). The protective effects of LCBE against liver damage caused by LPS were dose-dependent. These results demonstrate that LCBE suppresses liver inflammation caused by LPS via inhibition of TLR and MAPK signaling and oxidative stress pathways, and suggest that LCBE treatment can potentially prevent chronic liver injury.

Key words: *Lonicera caerulea*, liver inflammation, TLR, oxidative stress, MAPK signaling

Introduction

Liver inflammation is a response to the initiation and exacerbation of acute liver injury [1, 2] that can lead to hepatic fibrosis or cancer.[3,4] Previous studies have shown that inflammation is a basic response in chronic autoimmune, microbial, or metabolic diseases.[3] Lipopolysaccharide (LPS) is a major pro-inflammatory endotoxin of Gram-negative bacteria that consists of lipid A, core oligosaccharide, and O antigens; it is released upon cell death or lysis or multiplication.[5] LPS can induce an immune response in hosts that includes fever, inflammation, and even death,[6,7] and can increase the expression of pro-inflammatory factors such as interleukin (IL)-6, IL-1β, and tumor necrosis factor-α, which in turn stimulates reactive oxidative species (ROS) production, inhibits the activities of antioxidants such as glutathione (GSH), and alters normal cell morphology and division. ROS induce mitogen-activated protein kinases (MAPKs),[8,9] which play a critical role in inflammation;[10-12] in addition, persistent activation of MAPK family members such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 induces the expression of pro-inflammatory factors.[13,14] Phosphorylated (p-)ERK and p-p38 levels are attenuated by treatment with the isoflavone genistein or the saponin dioscin—two steroid-related molecules found in food products—in fulminant hepatic injury, [12,13] while *Terminalia arjuna* fruit extract or purple sweet potato consumption was shown to suppress inflammation via modulation of MAPK signaling.[6,15]

Lonicera caerulea is a fruit that is widely cultivated in China, Russia, and Japan [16] and is known for its high content of polyphenols, especially anthocyanins. *L. caerulea* berry extract (LCBE) has anti-inflammatory,[17,18] anti-radiation,[16] antioxidant,[19-22] and

antimicrobial properties.[23,24] However, there have been no studies to date investigating the effects of LCBE on liver inflammation and the involvement of MAPK signaling. This was addressed in the present study using an *in vivo* model of chronic liver inflammation. The results provide insight into the mechanisms underlying the protective effects of LCBE on LPS-induced liver inflammation.

Materials and methods

Preparation of LCBE

L. caerulea (600 g) was harvested in Hailin City, Heilongjiang Province, China and extracted with acidified methanol (0.1% HCl) in an ultrasonic bath (40°C) for 90 min. The primary extract was filtered, concentrated, and purified by passage through a glass column loaded with nonionic polystyrene–divinylbenzene resin (D101; Hushi Pharmaceutical Technology Co., Ltd., Shanghai, China) at 4°C. After concentration by rotary evaporation, the collected liquid was freeze-dried using a vacuum freeze dryer (LGO.2; Shenyang Aerospace Xinyang Quick Freezing Equipment Manufacturing Co., Shenyang, China), and the powder was stored at −20°C for subsequent experiments. The composition of the extracts has been described in our previous study.[25]

Animals and treatment

Male Sprague-Dawley rats (8 weeks old, weighting 200 ± 20 g) were purchased from Liaoning Immortality Biological Technology Co. (Liaoning, China) and maintained three per cage under standard conditions of temperature (20°C ± 2°C) and humidity (60%) on a 12:12-h light/dark cycle with free access to standard rat chow and water. All the experimental protocols were carried out in accordance with the guidelines of the

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experimental procedures were approved by the Institutional Animal Care and Use Committee at Shenyang Agricultural University, Shenyang, China.

After acclimation for 1 week, rats were randomly divided into the following five groups (n = 6 each). Group 1 normal control rats were injected with the same volume of saline that was used for LPS injection, and were administered distilled water by gavage at the same volume as LCBE every day for 4 consecutive weeks. Group 2 (model group) rats were injected with LPS dissolved in saline (200 μg/kg body weight) and were administered distilled water by gavage every day for 4 weeks. Group 3 rats were injected with LPS and were administered LCBE (50 mg/kg body weight in distilled water) by gavage every day for 4 weeks. Group 4 rats were injected with LPS and were administered LCBE (100 mg/kg body weight in distilled water) by gavage every day for 4 weeks. Group 5 rats were injected with LPS and were administered LCBE (200 mg/kg body weight in distilled water) by gavage every day for 4 weeks. All animals were weighed daily.

After 4 weeks, animals were anesthetized by intraperitoneal infusion with 10% chloral hydrate (3.5 ml/kg body weight). Blood samples were collected from the inferior palpebral vein prior to sacrifice to determine the levels of the liver function indicators alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and of the inflammatory cytokines C-reactive protein (CRP) and IL-6. Liver tissue samples were weighed, and part of each sample was used for cell cycle analysis and determination of ROS and GSH levels; another part was frozen in liquid nitrogen and stored at −80°C for use in western blotting; and the remaining tissue was fixed in 4% paraformaldehyde for

89 histological analysis.

90 **Biochemical analysis**

91 **Determination of ROS levels**

92 To determine the ROS levels, liver tissue was homogenized in PBS. After three freeze-thaw
93 cycles, the homogenates were centrifuged at 12,000 rpm for 10 min. The amount of protein
94 in the supernatant was quantified using a bicinchoninic acid assay kit (Wanlei Biological
95 Technology Co., Shenyang, China). Samples were diluted to 2 μg/μL, and then ROS levels
96 were measured using an ROS assay kit (Nanjing Jiancheng Bioengineering Institute,
97 Nanjing, China) as previously described.[26] The results were expressed as fluorescence
98 intensity per mg protein.

99 **Determination of GSH levels**

100 For GSH level determination, protein was extracted and quantified as described above. The
101 samples were diluted with PBS to a final concentration of 0.1 mg/mL. GSH levels were
102 then determined using an assay kit (Nanjing Jiancheng Bioengineering Institute) according
103 to the manufacturer's instructions. The OD values were recorded at 405 nm using a
104 microplate spectrophotometer (ELX-800, Biotek Instrument Co., Ltd., USA). The GSH
105 levels, expressed as μmol/mg protein, were calculated as follows:

106 $$\text{GSH levels (μmol/mg protein)} = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{standard}} - OD_{\text{blank}}} \times \text{Standard concentration} \times \text{N} \div \text{Protein concentration}$$

107 where the standard concentration is 20 nmol/mL, protein concentration is 0.1 mg/mL,
108 and N is the dilution factor (dilution factor for supernatant preparation × dilution factor for
109 sample preparation).

110 **Measurement of AST and ALT activities**

To measure AST and ALT activities, serum samples were diluted 2 times with saline. The activities of AST and ALT were measured using commercial test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the manufacturer's instructions. OD values at 510 nm were measured by a microplate spectrophotometer. The results were expressed as U/L.

Measurement of CRP and IL-6 levels

CRP and IL-6 levels were evaluated using specific enzyme-linked immunosorbent assay kits (Lie Macro Biological Technology Co., Shanghai, China) according to the manufacturer's instructions. A standard curve was set up between the OD values (zeroed using the blank hole) and the standard concentrations. The results were expressed as ng/mL.

Cell cycle analysis

Liver tissue samples were washed with phosphate-buffered saline and then digested with trypsase; the reaction was terminated by adding complete medium. The mixture was filtered through a 200-mesh strainer, and the supernatant was used for cell cycle analysis by flow cytometry (Accuri C6; BD Biosciences, East Rutherford, NJ, USA) after treatment with an annexin V/propidium iodide labeling kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Histological analysis

Liver tissue samples were embedded in paraffin and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin and visualized by light microscopy (DP73; Olympus, Tokyo, Japan).

Western blotting

Liver tissue was homogenized with lysis buffer containing 1% proteinase inhibitors for 5 min. Homogenates were centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined with a bicinchoninic acid assay kit (Wanlei Biological Technology Co., Shenyang, China). Up to 40 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Danvers, MA, USA), which was blocked for 1 h with Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% nonfat milk. The membrane was then incubated overnight at 4°C with antibodies against p38, p-p38, JNK, p-JNK, Toll-like receptor (TLR)2, and TLR4. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence (Wanlei Biological Technology Co.). Protein band intensity was analyzed using Gel-Pro Analyzer v.4.0 software (Media Cybernetics, USA). All antibodies were purchased from Wanlei Biological Technology Co..

Statistical analysis

Data are expressed as mean ± SD and were analyzed with SPSS v.16.0 software (SPSS Inc., Chicago, IL, USA). Mean differences were evaluated by one-way analysis of variance followed by Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results and discussion

LCBE attenuates damage to the liver caused by LPS

In response to injury—including atrophy and fibrosis—the liver becomes enlarged, resulting in increased liver weight.[27] LPS treatment increased in liver weight and decreased total body weight in rats ($P < 0.05$; Table 1). In contrast, the LPS-induced liver enlargement was mitigated in LCBE-treated rats; moreover, the body weight of rats treated with LCBE was higher than that of rats in Group 2. These results suggest that LCBE restored the growth of LPS-treated mice to near normal.

A histological analysis revealed that the livers of rats in the control group had an intact lobular structure with clear central veins and normal cellular architecture, whereas those of LPS-treated rats exhibited broad hemorrhagic necrosis, lipid deposition, and massive inflammatory cell infiltration (Fig. 1). These effects were attenuated by LCBE treatment in a concentration-dependent manner.

Previous studies have shown that exposure to CCl_4, thioacetamide, or D-galactosamine increases lipid accumulation and fibrosis in rat liver.[27-29] Our observation that LPS caused serious liver damage in rats is consistent with the findings of a previous study.[30] Liver damage is typically caused by environmental toxins; extracts of *Solanum xanthocarpum* and *Juniperus communis* have shown synergistic protective effects against liver injury induced by paracetamol and azithromycin, respectively,[31] while chokeberry can attenuate fat deposition in rat liver.[32] The present results are the first demonstration that LCBE can mitigate lipid deposition and damage to the liver resulting from LPS-induced inflammation.

LCBE suppresses ROS production and GSH depletion

To assess the effects of LCBE on LPS-induced oxidative stress, we measured ROS levels in

liver tissues. As expected, LPS increased ROS levels in the liver ($P < 0.05$; Fig. 2A). However, LCBE abrogated this increase in a concentration-dependent manner, although the differences among the various LCBE concentrations were not statistically significant. Nonetheless, 200 mg/kg LCBE inhibited LPS-induced ROS productive relative to LPS-treated rats without LCBE supplementation ($P < 0.05$).

GSH is a low-molecular weight thiol and non-enzyme antioxidant that inhibits ROS and oxidative stress in cells.[26] The GSH content in the liver of rats treated with LPS alone was lower than in the control group ($P < 0.01$; Fig. 2B). This decrease was abrogated by LCBE treatment; in particular, liver GSH content in rats receiving 200 mg/kg LCBE was higher than that in rats treated with LPS only ($P < 0.05$) and was equivalent to that in control rats ($P > 0.05$).

Oxidative stress resulting from accumulation of ROS promotes hepatic inflammation.[33] GSH depletion caused by chronic hepatic damage enhances oxidative stress and induces the expression of pro-inflammatory factors.[26] We found that LCBE had an antioxidant effect and suppressed ROS generation in a concentration-dependent manner, which may be ascribed to the attenuation of GSH depletion. These findings are in accordance with a previous study demonstrating that ROS level was reduced and GSH content increased in acrylamide-stimulated rats supplemented with blueberry extract.[34]

LCBE treatment improves liver function

Increased levels of liver enzymes such as AST and ALT in serum are a marker of liver damage.[35] To investigate the effect of LCBE on liver dysfunction induced by LPS, we measured serum AST and ALT levels. As shown in Fig. 3, rats injected with LPS showed

higher levels of AST and ALT relative to the control group ($P < 0.01$; Fig. 3). However, administration of LCBE (particularly 200 mg/kg) reversed this increase, restoring baseline AST and ALT levels ($P > 0.05$, 200 mg/kg LCBE vs. control), indicating that LCBE plays an important role in modulating liver function. The effects of LCBE may be attributable to inhibition of GSH depletion and ROS generation; indeed, mulberry extract has been shown to suppress increases in ALT and AST levels via attenuation of oxidative stress. [27,36]

LCBE abrogates the LPS-induced increase in inflammatory cytokine levels

Previous studies have shown that hepatitis is triggered by hepatocyte injury. In the present study, CRP and IL-6 levels were determined to assess whether LCBE suppresses liver inflammation. The results showed that LPS injection significantly increased serum CRP concentration ($P < 0.01$; Fig.4A); however, the level was reduced in LPS-treated rats that received LCBE supplementation, with the most robust effects observed for 200 mg/kg LCBE ($P < 0.05$). Similarly, LPS induced a significant increase in IL-6 level relative to the control group ($P < 0.01$; Fig. 4B), but this was attenuated by LCBE treatment, particularly at a concentration of 200 mg/kg ($P < 0.05$), which reduced the IL-6 level to that in control rats ($P > 0.05$).

LPS stimulates oxidative stress in tissue, which induces the production and release of various pro-inflammatory cytokines, including IL-6;[33,37] and an elevation in these factors is highly associated with chronic liver diseases. [38] CRP is a highly sensitive marker that has been used to detect low levels of chronic inflammation.[39] Our results suggest that LCBE exhibits anti-inflammatory activity, which was evidenced by the decrease in CRP and IL-6 levels in the serum of LCBE-supplemented rats. The inhibitory effects of LCBE against

LPS-induced liver inflammation may be associated with reduction of ROS levels and GSH depletion, which is consistent with a previous report. [40] Thus, the analysis of pro-inflammatory cytokines in the present study also substantiated that LCBE could inhibit the inflammatory response induced by LPS in the liver. Additionally, our findings introduce the idea of evaluating the anti-inflammatory potential of fruits and vegetables that are rich in phenolics (particularly anthocyanins) having strong antioxidant capacity.[41]

LCBE restores normal cell cycling dysregulated by LPS

Cell division is regulated by a complex signal transduction process, and cell cycling can be altered by external stimuli. In vitro studies have shown that berry extract can induce cell cycle arrest and apoptosis of tumor cells.[42,43] We evaluated the effect of LCBE on cell cycling in LPS-treated rats. As shown in Fig. 5, compared to controls, exposure to LPS altered cell cycle distribution in the liver: the percentage of cells in S-phase decreased from 19.3% to 9.6%, whereas the percentage of G1-phase cells increased from 59.8% to 79%, which is indicative of apoptosis. However, the increase in the G1 fraction was abrogated by LCBE supplementation; this was accompanied by increases in the G2- and S-phase populations. The effect was strongest at 200 mg/kg LCBE, which may be attributed to the high concentration of anthocyanins decreasing the proportion of cells in the G1 phase.[44] These findings demonstrate that LCBE can restore normal cell cycling dysregulated by LPS.

LCBE suppresses the LPS-induced upregulation of TLR2 and TLR4 expression

TLRs are critical mediators of the inflammatory response;[45] activated TLR2 and TLR4 stimulate MAPK and nuclear factor-κB signaling, thereby stimulating the release of

inflammatory cytokines such as IL-6. TLR2 and TLR4 signals are also involved in hepatic inflammation induced by CCl_4.[45,46] We observed in this study that relative to the control group, LPS stimulation induced the upregulation of TLR2 and TLR4 expression, as determined by western blotting ($P < 0.01$; Fig. 6A, B). However, this effect was abolished in rats treated with LCBE ($P < 0.01$). Our results are consistent with those reported in a previous study,[47] and further confirm that LCBE treatment mitigates hepatic inflammation induced by LPS.

LCBE suppresses inflammation by negatively regulating MAPK signaling

MAPK signaling regulates the inflammatory response and is a target of anti-inflammatory drugs; persistent activation of JNK and p38 leads to inflammation.[14] Anthocyanins, the major phenolics present in LCBE, have been suggested to inhibit MAPK phosphorylation.[48] In the present study, we found that p-JNK and p-p38 levels were upregulated by LPS treatment ($P < 0.01$; Fig. 6C–F). This may be due to increased ROS production and TLR2 and TLR4 levels, since previous studies have shown that ROS activate p-38 MAPK signaling.[49] However, JNK and p38 phosphorylation induced by LPS was suppressed by LCBE in a dose-dependent manner ($P < 0.01$), and total JNK and p38 levels were not obviously changed ($P > 0.05$); this inhibitory effect may be mainly ascribed to the high anthocyanin content in LCBE. These results indicate that LCBE reduces liver inflammation via suppression of MAPK signaling.

Oxidative stress and GSH act upstream of p38 MAPK.[26] Based on our findings, we propose the following model to explain the anti-inflammatory effect of LCBE in the liver (Fig.7). LCBE inhibits TLR expression, and mitigates GSH depletion and oxidative stress

induced by LPS; this suppresses the MAPK pathway, which in turn suppresses liver inflammation, as evidenced by the decrease in inflammatory cytokine levels (i.e., CRP and IL-6) and transaminase (ALT and AST) activities.

Conclusions

The results from this study demonstrate for the first time that LCBE prevents MAPK activation by suppressing oxidative stress caused by inflammation. In addition, cell cycle analysis revealed that LCBE blocked LPS-induced apoptosis of hepatocytes. These findings provide insight into the mechanism underlying the anti-inflammatory effects of LCBE and provide a basis for its use as a supplement or drug to treat liver inflammation and prevent chronic liver injury.

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Figure 1: Histological changes in liver tissue (stained with haematoxylin and eosin dye). Control group (Group 1): rats were injected with the same volume of saline that was used for LPS injection, and were administered distilled water by gavage at the same volume used for LCBE every day for 4 consecutive weeks; model group (Group 2): rats were injected with LPS dissolved in saline (200 μg/kg bw) and were administered distilled water by gavage every day for 4 weeks; LPS + LCBE-50, -100 and -200 mg/kg bw (Groups 3, 4 and 5, respectively): rats were injected with LPS dissolved in saline (200 μg/kg bw) and were administered LCBE at a dose of 50, 100 and 200 mg/kg bw, respectively, through daily gavage for 4 weeks. (HE × 200).

Figure 2: Suppressive effects of LCBE against LPS-induced ROS increase and GSH decrease. One-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison analysis was performed to analyze the statistical differences among means. Results are presented as the mean ± SD (n=6). 'NS' indicates not significant. *p<0.05 or **p<0.01, compared with Group 1; $^{\#}p$<0.05, compared with Group 2.

Figure 3: Suppressive effects of LCBE against LPS-induced AST and ALT increase. One-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison analysis was performed to analyze the statistic differences amongst means. Results are presented as the mean ± SD (n=6). 'NS' indicates not significant. *p<0.05 or **p<0.01, compared with Group 1; $^{\#}p$<0.05 or $^{\#\#}p$<0.01, compared with Group 2.

Figure 4: Inhibitory effects of LCBE against LPS-induced CRP and IL-6 increase. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison analysis was performed to analyze the statistical differences amongst means. Results are presented as mean ± SD (n=6). 'NS' indicates not significant. *p<0.05 or **p<0.01, compared with Group 1; $^{\#}p$<0.05, compared with Group 2.

Figure 5: Modulatory effects of LCBE on LPS-induced hepatocyte cell-cycle redistribution. (A) Representative histogram of hepatocyte cell-cycle distribution. (B) Percentage of cells in G1, S and G2 phase. Control group (Group 1): rats were injected with the same volume of saline that was used for LPS injection and were administered distilled water by gavage at the same volume used for LCBE every day for 4 consecutive weeks; model group (Group 2): rats were injected with LPS dissolved in saline (200 μg/kg bw) and were administered distilled water by gavage every day for 4 weeks; LPS + LCBE-50, -100 and -200 mg/kg bw (Groups 3, 4 and 5, respectively): rats were injected with LPS dissolved in saline (200 μg/kg bw) and were administered LCBE at a dose of 50, 100 and 200 mg/kg bw, respectively, through daily gavage for 4 weeks. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison analysis was performed to analyze the statistical differences among means. Results are presented as the mean ± SD (n=6). *p<0.05 or **p<0.01, compared with Group 1; $^{\#}p$<0.05 or $^{\#\#}p$<0.01, compared with Group 2.

Figure 6: Western blot analysis of TLR2, TLR4 and MAPKs (P38 and JNK) in liver tissue.

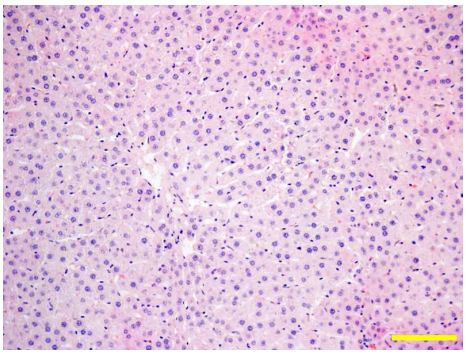
One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison analysis was performed to analyze the statistical differences among means. Values are presented as mean ± SD (n=3). 'NS' indicates not significant. **p<0.01, compared with Group 1; $^{\#\#}p$<0.01, compared with Group 2.

Figure 7: Schematic diagram of the role of LCBE in inhibiting LPS-induced liver inflammation.

Table 1: Effects of LCBE on liver weight and body weight of rats treated with LPS. Results are presented as the mean ± SD (n=6). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison analysis was performed to analyze the statistical differences among the means. $p^a < 0.05$ vs. control group (Group 1); $p^b < 0.05$ vs. model group (Group 2).

Groups	Liver weight	Body weight	Liver index (%)
Group 1	10.8 ± 1.21	268.8 ± 12.27	4 ± 0.6
Group 2	13.3 ± 2.19	233 ± 11.57[a]	5.7 ± 1.21
Group 3	12.4 ± 2.54	239 ± 5.91[a]	5.2 ± 1.13
Group 4	12 ± 3.08	252.8 ± 12.63[b]	4.7 ± 1.12
Group 5	10.9 ± 1.82	259.8 ± 15.31[b]	4.2 ± 0.69

Figure 1



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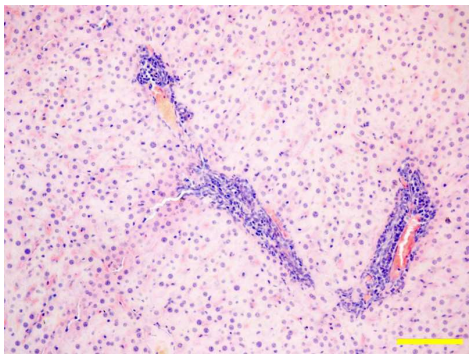
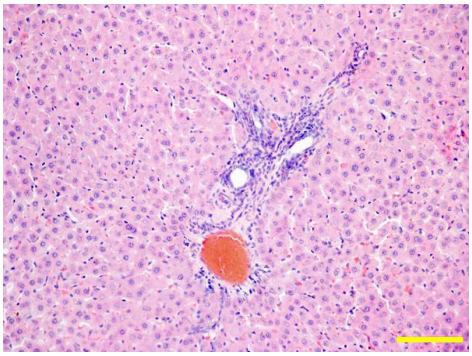
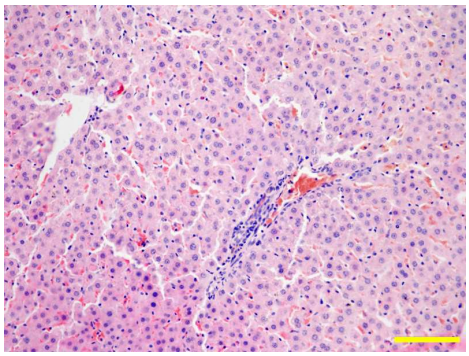


Model group



LPS + LCBE-50 mg/kg bw



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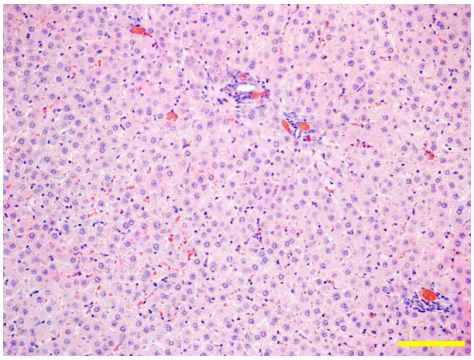


LPS + LCBE-200 mg/kg bw

Figure 2

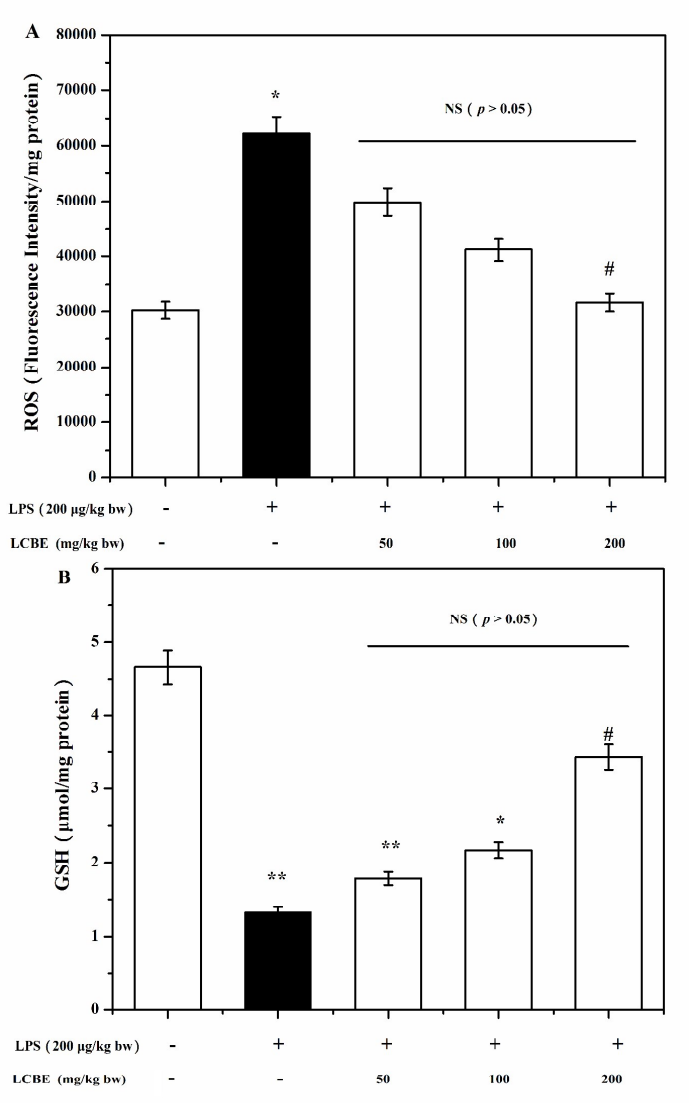


Figure 3

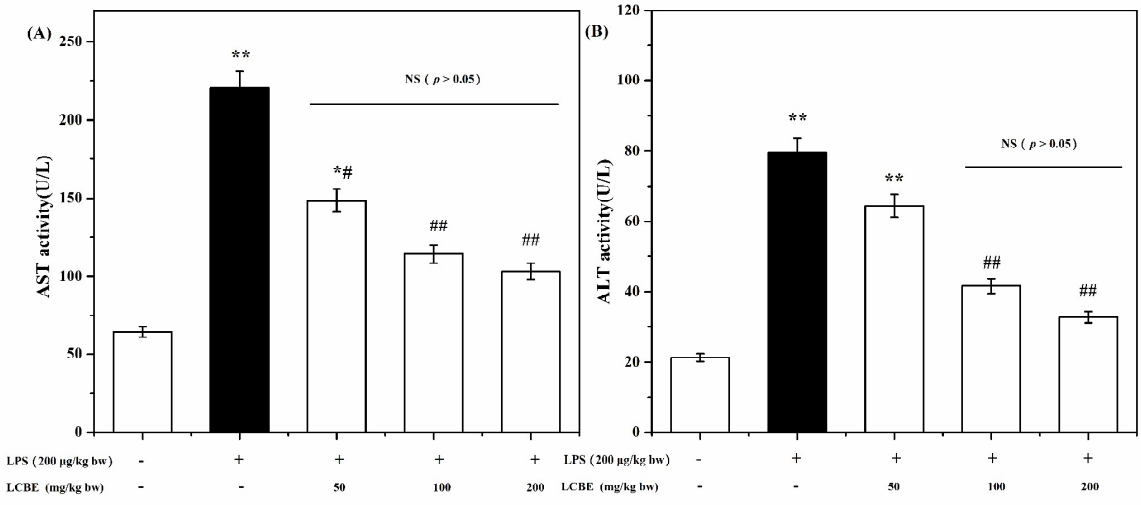


Figure 4

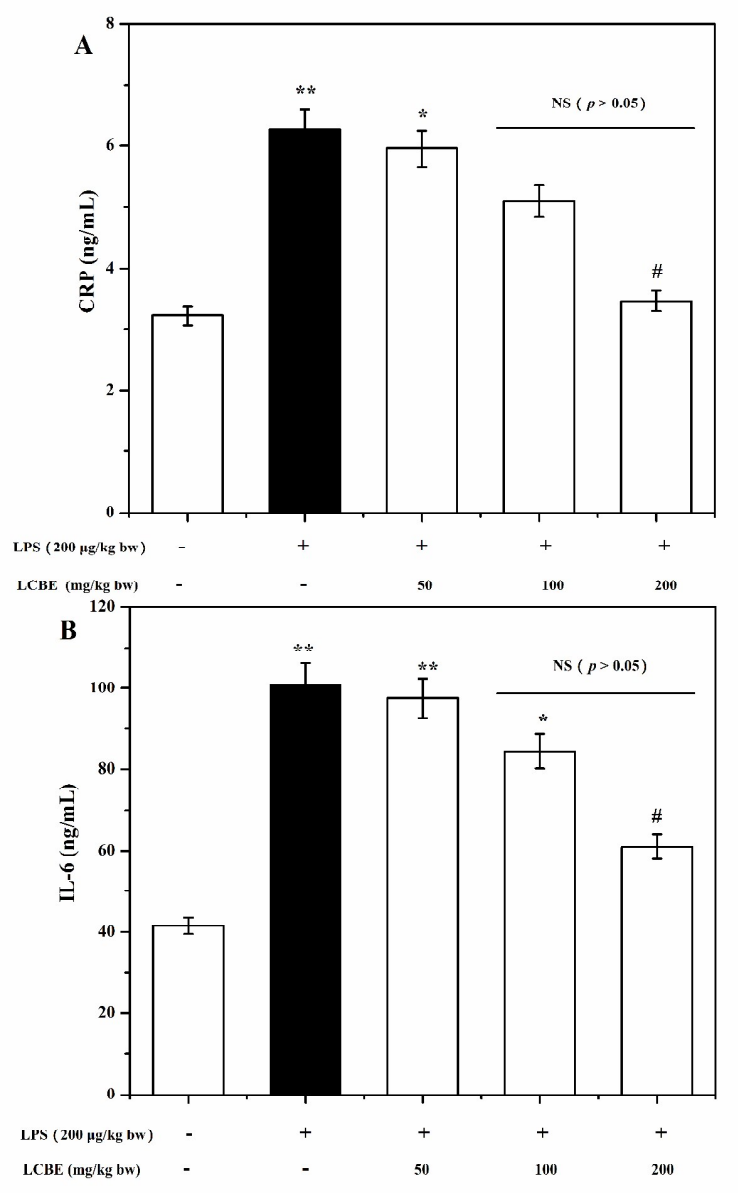


Figure 5

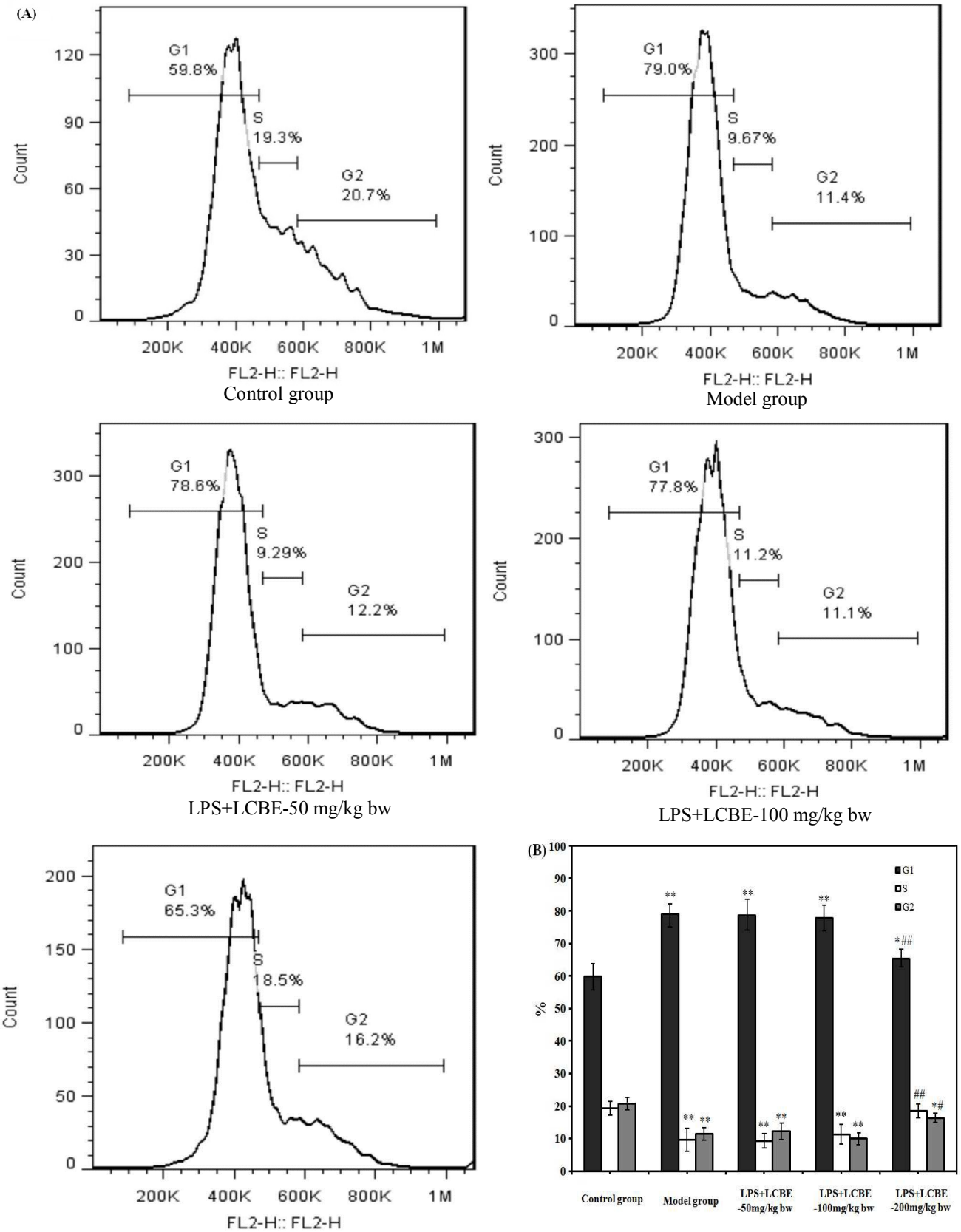
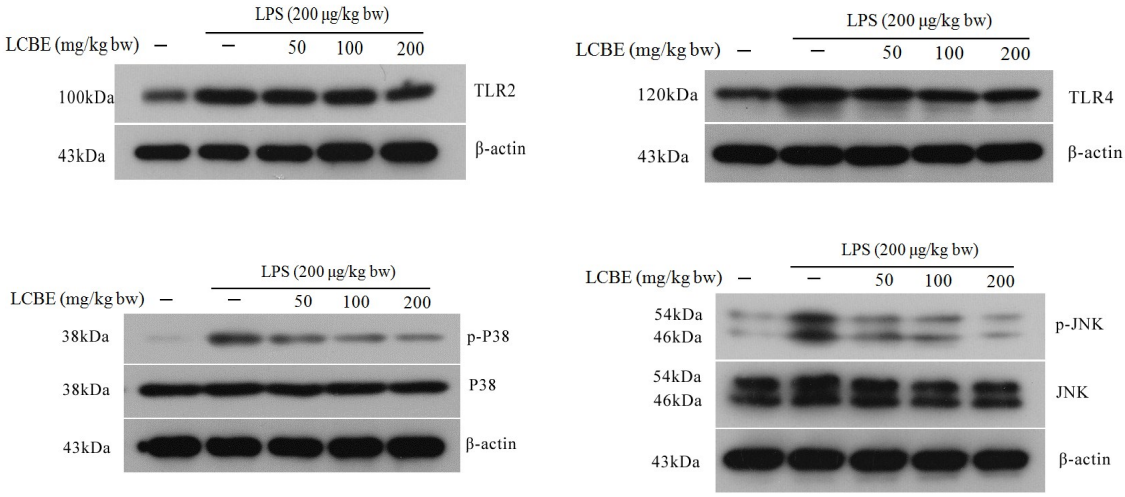


Figure 6



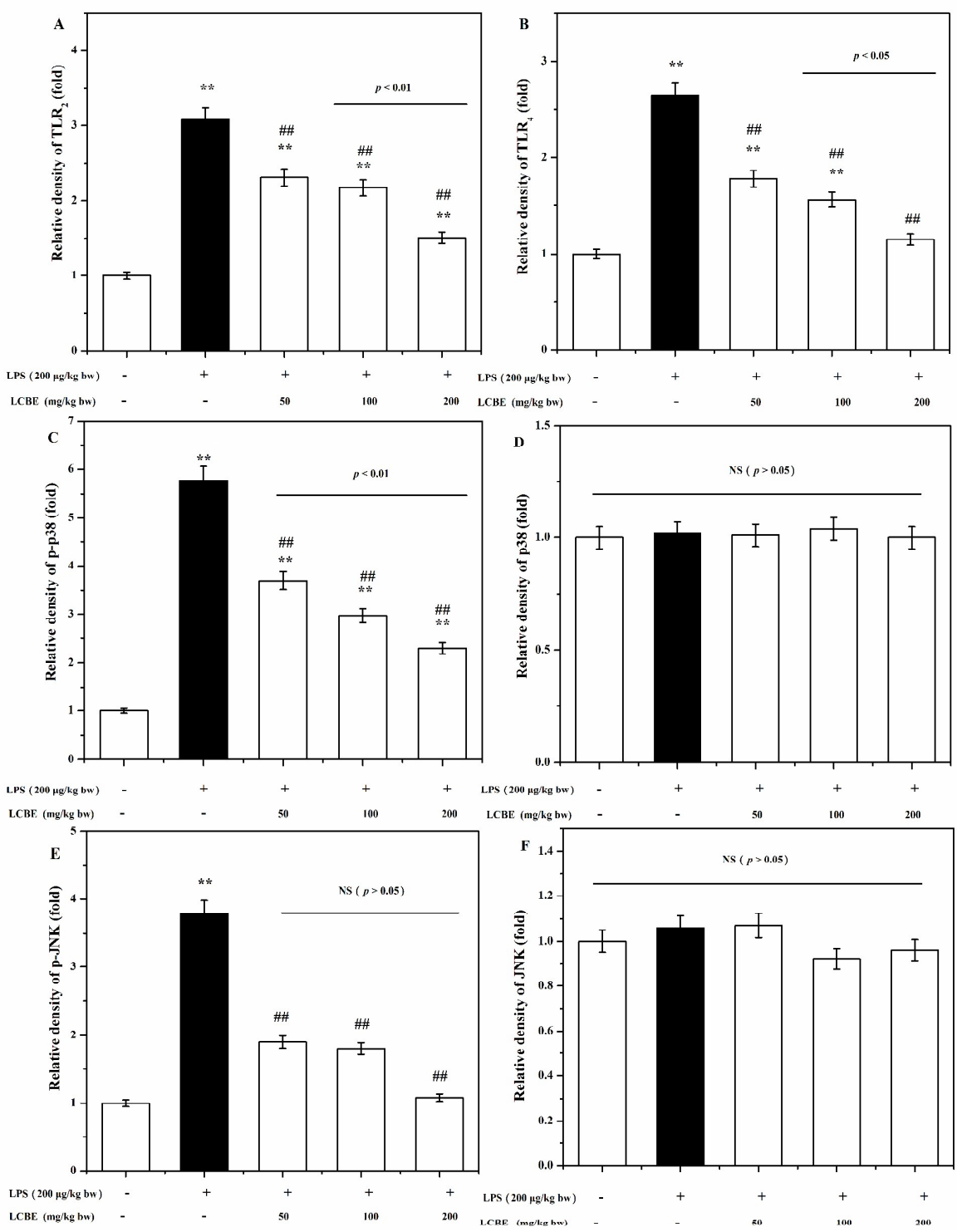


Figure 7