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Chaiqin chengqi decoction alleviates severity of acute pancreatitis via inhibition of TLR4 and NLRP3 inflammasome: Identification of bioactive ingredients via pharmacological sub-network analysis and experimental validation

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ABSTRACT

Background: Chaiqin chengqi decoction (CQCQD) is a Chinese herbal formula derived from dachengqi decoction. CQCQD has been used for the management of acute pancreatitis (AP) in the West China Hospital for more than 30 years. Although CQCQD has a well-established clinical efficacy, little is known about its bioactive ingredients, how they interact with different therapeutic targets and the pathways to produce anti-inflammatory effects.

Purpose: Toll-like receptor 4 (TLR4) and the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome-mediated pro-inflammatory signaling pathways, play a central role in AP in determining the extent of pancreatic injury and systemic inflammation. In this study, we screened the bioactive ingredients using a pharmacological sub-network analysis based on the TLR4/NLRP3 signaling pathways followed by experimental validation.

Methods: The main CQCQD bioactive compounds were identified by UPLC-QTOF/MS. The TLR4/NLRP3 targets in AP for CQCQD active ingredients were confirmed through a pharmacological sub-network analysis. Mice received 7 intraperitoneal injections of cerulein (50 μg/kg; hourly) to induce AP (CER-AP), while oral gavage of CQCQD (5, 10, 15 and 20 g/kg; 3 doses, 2 hourly) was commenced at the 3rd injection of cerulein. Histopathology and biochemical indices were used for assessing AP severity, while polymerase chain reaction, Western blot and immunohistochemistry analyses were used to study the mechanisms. Identified active CQCQD compounds were further validated in freshly isolated mouse pancreatic acinar cells and cultured RAW264.7

Abbreviations: AP, acute pancreatitis; BA, baicalin; CCK, cholecystokinin; CER, cerulein; CH, chrysin; CI, combination index; CQCQD, chaiqin chengqi decoction; DAMPs, damage-associated molecular pattern molecules; DCQD, dachengqi decoction; DMEM, Dulbecco's modified Eagle medium; EM, emodin; ETCM, Encyclopedia of Traditional Chinese Medicine; IL-6, interleukin-6; IL-1β, interleukin-1β; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; MPO, myeloperoxidase; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor- kappaB; NLRP3, NOD-like receptor family pyrin domain containing 3; PI, propidium iodide; PPI, Protein-Protein Interaction; RH, rhein; RT-qPCR, real-time quantitative polymerase chain reaction; SITICH, Search Tool for Interactions of Chemicals; TCM, traditional Chinse medicine; TLCS, taurolithocholic acid 3-sulfate disodium salt; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-alpha

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macrophages.
Results: The main compounds from CQCQD belonged to flavonoids, iridoids, phenols, lignans, anthraquinones and corresponding glycosides. The sub-network analysis revealed that emodin, rhein, baicalin and chrysin were the compounds most relevant for directly regulating the TLR4/NLRP3-related proteins TLR4, RelA, NF-κB and TNF-α. *In vivo*, CQCQD attenuated the pancreatic injury and systemic inflammation of CER-AP and was associated with reduced expression of TLR4/NLRP3-related mRNAs and proteins. Emodin, rhein, baicalin and chrysin significantly diminished pancreatic acinar cell necrosis with varied effects on suppressing the expression of TLR4/NLRP3-related mRNAs. Emodin, rhein and chrysin also decreased nitric oxide production in macrophages and their combination had synergistic effects on alleviating cell death as well as expression of TLR4/NLRP3-related proteins.
Conclusions: CQCQD attenuated the severity of AP at least in part by inhibiting the TLR4/NLRP3 pro-inflammatory pathways. Its active ingredients, emodin, baicalin, rhein and chrysin contributed to these beneficial effects.

Introduction

Acute pancreatitis (AP) is an inflammatory digestive system disease with growing prevalence. It currently has an estimated global incidence rate of 33 cases per 100,000 person-years (Xiao et al., 2016). Although gallstones and excess alcohol consumption have been recognized as two leading causes of AP globally (Forsmark Ch et al., 2017), recent studies revealed that hypertriglyceridemia-associated AP is becoming the predominant etiology of AP cases in China (Ding et al., 2019; Mukherjee et al., 2019; Shi et al., 2020; Zhang et al., 2019c).

AP cases with local complications (i.e. acute necrotic and peripancreatic fluid collections) and persistent organ failure are defined as "moderately severe" or "severe" (Banks et al., 2013; Crockett et al., 2018). Due to an increased risk of developing sepsis, and needing invasive interventions, patients in these groups typically require intensive-care management (Schepers et al., 2019; Shi et al., 2020). The high morbidity and mortality rate associated with these patients results in significant socioeconomical burdens (Peery et al., 2019). Despite an intensive search of potential interventions, there remains no effective pharmacological treatment for managing these AP cases (Moggia et al., 2017).

Recent progress in translational research has deepened our understanding of the fundamental processes contributing to the initiation and aggravation of AP (Lee and Papachristou, 2019). Upon exposure to classic pancreatic toxins (i.e. bile acid), human pancreatic acinar cells release pro-inflammatory cytokines (interleukin [IL]-1β, IL-6 and tumor necrosis factor-alpha [TNF-α]), chemokines and chemokine receptors (Lugea et al., 2017) as well as damage-associated molecular pattern molecules (DAMPs; i.e. cell-free DNA, high mobility groupbox 1 and histones) (Hoque et al., 2011; Kang et al., 2014b; Liu et al., 2017; Merza et al., 2015) into extracellular milieu to initiate so-called sterile inflammation. Toll-like receptors (TLRs; TLR4/9) (Chen et al., 2014; Kang et al., 2014a; Vaz et al., 2013) and inflammasomes (Hoque et al., 2012; Hoque and Mehal, 2015) are proteins that are essential to the sensing of DAMPs and signal transduction, respectively, and are both needed to completely activate the systemic inflammation cascade after AP onset. The "TLR4" and "nucleotide-binding oligomerization domain-like receptor (NLR) family pyrin domain containing 3 (NLRP3)" inflammasome are the most well studied inflammatorysignaling cassettes in experimental AP. TLR4 is expressed in pancreatic acinar cells (Gu et al., 2013), immune cells, epithelium of pancreatic duct and pancreatic microcirculation (Vaz et al., 2013). Genetic knockout of TLR4 (or its co-receptor CD14) (Sharif et al., 2009), TLR4 downstream signaling cytosolic protein myeloid differentiation primary response 88 (MyD88) (Koike et al., 2012) and nuclear factor-kappaB (NF-κB) (Huang et al., 2013) or pharmacologic inhibition of TLR4 by lactate (Hoque et al., 2014) and TLR4 specific antagonist TAK242 (Awla et al., 2011) can all significantly reduce the severity of experimental AP in mouse models. The NLRP3 inflammasome is expressed by innate immune cells, stimulated pancreatic acinar cells and especially in macrophages (Hoque et al., 2012; Hoque and Mehal, 2015; 2011; Sendler et al., 2020). Genetic deletion or pharmacological antagonism of NLRP3 inflammasome elements (caspase-1, ASC, or NLRP3) also greatly diminished pancreatic injury and systemic inflammation in AP models (Hoque et al., 2011; Sendler et al., 2020). Since novel inhibitors are being developed for targeting TLR4 and NLRP3 inflammasome (Bhattacharyya et al., 2018; Coll et al., 2015), these strategies may hold substantial promise for treating human AP.

In China, it is a common and well-established practice to use rhubarb-based Traditional Chinese Medicine (TCM) herbal formula such as dachengqi decoction (DCQD) for AP care. At our hospital, we have refined and successfully employed a DCQD-derived formula known as chaiqin chengqi decoction (CQCQD) to manage AP patients for over 30 years (Liu et al., 2004). Systematic review (Wang et al., 2005) and meta-analyses (Lu et al., 2014) of observation studies and trials with small sample size have demonstrated the effectiveness of TCM in improving AP's clinical outcomes, with higher quality randomized clinical trials planned for the near future. Existing work has shown that bioactive ingredients such as emodin, rhein, baicalin and honokiol in DCQD and CQCQD, can significantly reduce the severity of experimental AP by suppressing pro-inflammatory mediators (Lu et al., 2017; Zhou et al., 2016). However, the exact action of individual ingredients responsible for the various pharmacological responses and the mechanism underlying the synergistic protective effects remain to be clarified.

Since TLR4/NLRP3 pro-inflammatory pathways play a central role in determining the extent of pancreatic injury in AP, in this study we sought to: (1) screen CQCQD bioactive ingredients that had the main inhibitory effects on TLR4/NLRP3 pathways using a sub-network pharmacology analysis; (2) evaluate the efficacy of CQCQD on the severity of experimental AP and its pro-inflammatory signaling pathways *in vivo*; (3) validate the effects of these bioactive compounds on pancreatic acinar cells and macrophages.

Materials and methods

Ethics and animals

All animal experiments were approved by the Animal Ethics Committee of West China Hospital, Sichuan University (2017065A and 2019170A). Male C57BL/6J mice (22–25 g) were purchased from Beijing Huafukang Bioscience Co., Ltd. (Beijing, China). Animals were maintained at 22 ± 2 °C with a 12 h light-dark cycle and ad libitum feeding of standard laboratory chow and water throughout the experiment.

Materials and reagents

The detailed information of reagents, natural compound standards and quantitative reverse transcription polymerase chain reaction (RT-

qPCR) primer pairs are provided in the **Supplementary materials and methods** and Tables S1–2.

Preparation of CQCQD

The raw materia medica of CQCQD were purchased from Sichuan Hospital of Traditional Chinese Medicine (Chengdu, Sichuan, China). CQCQD formula consisted of Bupleurum marginatum Wall. ex DC, Scutellaria baicalensis Georgi, Rheum palmatum L., Sodium Sulfate, Magnolia officinalis Rehd. et Wils, Citrus aurantium L., Gardenia jasminoides Ellisand Artemisia capillaris Thunb. The detailed information of materia medica from CQCQD is provided in Table 1. All the dried herbs except Rheum palmatum L. and Sodium Sulfate were first soaked in 800 ml sterile filtered water for 30 min then boiled with 1000 ml sterile filtered water for 30 min and reduced to a concentrated solution (~200 ml). The concentrated liquid was then removed from the dregs and stored separately. Another 1000 ml water was added to the dregs then boiled and reduced again as described above. In the last 5 min of the boiling procedure, Rheum palmatum L. was supplemented, while Sodium Sulfate was mixed at the end of the procedure. The twice extracted solution was combined (~400 ml) and lyophilized into powder using an EYELA FDU-2110 lyophilizer (Tokyo Rikakikai Co., Ltd.; Tokyo, Japan) and stored at −20 °C.

UPLC-QTOF/MS analysis of CQCQD and standards

Lyophilized CQCQD extractions were dissolved in hot water and obtained as 0.5 mg/ml solution. Under optimized chromatography conditions, all ingredients were separated within 20 min. Solution for CQCQD and standards was analyzed on a UPLC H—Class coupled with a Waters SYNAPT G2-Si HDMS Q-TOF (Waters Corporation; Milford, MA, USA). Separation was performed on a BEH C18 column (2.1 × 100 mm, 1.7 μm) at 40 °C. Gradient elution of 0.1% formic acid solution (A) and methanol (B) at a flow rate of 0.4 ml/min was employed as follows: 0–1.0 min, 5%B; 1.0–5 min, 5–50%B; 5–12 min, 50–90% B; 12–15 min, 90% B; 15–16 min, 90–5%B; 16–20 min, 5% B. The injection volume was 1 μl. The Q-TOF system was operated using an electrospray ion source in negative ion modes. The mass range was set at *m/z* 50–1200. The capillary voltage was 1.0 KV. The source temperature was 120 °C, and the desolvation temperature was 400 °C. Cone gas flow and desolvation gas flow were 50 and 800 l/h, respectively. Data were acquired on MS^E mode and analyzed using a UNIFI 1.9.2 software (Waters Corporation).

Pharmacology sub-network construction and analysis

Main pharmacology network (compounds-targets-pathways) construction:

(1) Major identified compounds from CQCQD and their corresponding 149 targets were predicted using Search Tool for Interactions of Chemicals (SITICH; http://stitch.embl.de/) and Encyclopedia of Traditional Chinese Medicine (ETCM; http://www.nrc.ac.cn:9090/ETCM/) databases, based on structural and functional similarities (Tanimoto > 0.8);
(2) AP-associated 530 targets were identified from the following database: OMIM (https://www.omim.org/), DisGeNET (http://www.disgenet.org), NCBI, etc.;
(3) The Protein-Protein Interaction (PPI) network of con-targets (the overlapping targets from CQCQD major identified compounds and AP) was constructed based on STRING database (https://string-db.org/); then pathways enrichment analysis was conducted via Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling based on con-targets.

Sub-network establishment:

(1) TLR4/NLRP3-related pathways (TLR, NF-κB and NLR) were selected based on KEGG signaling pathways enrichment analysis;
(2) Subsequently, targets related to TLR4/NLRP3 pathways were obtained from PPI network of con-targets;
(3) A sub-network of TLR4/NLRP3 pathways-targets-compounds was extracted from the main network.

Induction of experimental AP and severity assessment

Mice received seven intraperitoneal injections of a cholecystokinin (CCK) analog cerulein (50 μg/kg) at hourly intervals, while controls received injections of normal saline. In the treatment groups, oral gavage of different doses of CQCQD (5, 10, 15 and 20 g/kg) begun at the 3rd injection of cerulein, and were given 3 times at 2 hourly intervals. Animals were humanely culled at 12 h from the first injection of cerulein/saline. Relevant organs were harvested via an abdominal midline incision for severity assessment. Pancreatic and lung histopathology assessment and detailed protocols for serum amylase, pancreatic trypsin and myeloperoxidase (MPO), lung MPO and serum IL-6 were as previously described (Huang et al., 2017).

RT-qPCR, Western blot and immunohistochemistry

Detailed protocols for these assays are demonstrated in **Supplementary materials and methods**.

Acinar cell isolation, RAW264.7 cell culture and cell injury assessment

Pancreatic acinar cells were freshly isolated using a collagenase IV digestion procedure as we previously described (Huang et al., 2014). Cells were maintained in HEPES buffer (pH 7.3; in mM: HEPES 10, D-glucose 10, NaCl 140, KCl 4.7, $MgCl_2$ 1.13 and $CaCl_2$ 1.2). Cells were pre-treated with compounds (12.5, 25 and 50 μM) for 30 min at designed concentrations, followed by co-incubation with CCK (500 nM) for 2 h, taurolithocholic acid 3-sulfate disodium salt (TLCS; 500 μM) for 30 min, or lipopolysaccharide (LPS; 1 μg/ml) for 30 min with gentle shake at 50 rpm under room temperature; control groups received HEPES buffer incubation. Then cells were viewed by epi-fluorescence microscopy ZEISS AX10 imager A2/AX10 cam HRC (Jena GmbH,

Table 1
The Ingredients list of CQCQD.

Ingredients	English name	Latin name	Plant name	Weight (g)
Zhuyechaihu	Chinese thorowax root	*Radix Bupleuri*	Bupleurum marginatum Wall. ex DC.	15
Huangqin	Baikal skullcap root	*Scutellaria baicalensis Georgi*	Scutellaria baicalensis Georgi	15
Dahuang	Rhubarb	*Radix et Rhizoma Rhei*	Rheum palmatum L.	20
Mangxiao	Sodium sulfate	Sodium Sulfate	$Na_2SO_4.10H_2O$	20
Houpu	Officinal magnolia bark	*Cortex Magnoliae Officinalis*	Magnolia officinalis Rehd. et Wils	15
Zhishi	Unripe bitter orange	*Fructus Aurantii Immaturus*	Citrus aurantium L.	15
Zhizi	Gardenia fruit	*Fructus Gardeniae*	Gardenia jasminoides Ellis	20
Yinchen	Capillary wormwood herb	*Herba Artemisiae Scopariae*	Artemisia capillaris Thunb	15

Germany) for determination of necrotic cell death pathway activation after staining with Hoechst 33342 (50 μg/ml) and propidium iodide (PI; 1 μM). Necrotic cells were calculated as PI positive cells divided by Hoechst 33342 stained cells and multiplied by 100%.

RAW264.7 mouse macrophages were cultured in Dulbecco's modified Eagle medium (DMEM) medium mixed with 10% fetal bovine serum in an incubator at 37 °C with saturated humidity and 5% CO2. Cell viability of RAW264.7 cells was assessed by Cell Counting Kit 8. Cells (approx. 8×10^3) were cultured overnight in a 96-well plate (100 μl medium per well) and were incubated with compounds at different concentrations (12.5, 25, 50, 100, 200 μM, final concentration) for 24 h. After changing the medium, Cell Counting Kit 8 solution (10 μl) was added into each well and incubated for 3 h away from light. Subsequently, the absorbance values of all the wells were measured by the BMG Labtech CLARIOstar Plate Reader (Ortenberg, Germany) at 450 nm. Nitric oxide production was monitored by measuring the nitrite levels in culture medium as previously described (Joo et al., 2014). Briefly, RAW264.7 cells were pre-treated with active compounds at various concentrations (12.5, 25, 50 μM) for 24 h and subsequently stimulated with LPS (1 μg/ml) for another 24 h. The supernatant was collected to detect the nitrite levels which were calculated with reference to a standard curve of sodium nitrite generated from known concentrations. Dose-effect curves of compounds on macrophage cell injury were analyzed using the median-effect method described previously (Chou, 2010). The combination index (CI) was calculated using 'CompuSyn' software (Cambridge, UK). Synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) represent combination effects, respectively. The suppression rate of nitric oxide production is expressed as fraction affected (Fa).

Statistical analysis

Data were analyzed using analysis of variance (Ordinary one-way ANOVA) and Tukey's post-hoc test for multiple comparisons with Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean ± S.E.M. A two-sided $p < 0.05$ was considered statistically significant.

Results

Analysis of CQCQD ingredients

The phytochemical analysis based on UPLC-QTOF/MS made a preliminary identification of 70 compounds from CQCQD by matching the molecular ions and fragment information using UNIFI 1.9.2 software. They belonged to flavonoids, iridoids, phenols, lignans, anthraquinones and corresponding glycosides, and those compounds with a high (> 10,000) intensity were listed in **Table S3**. Of 70 compounds initially

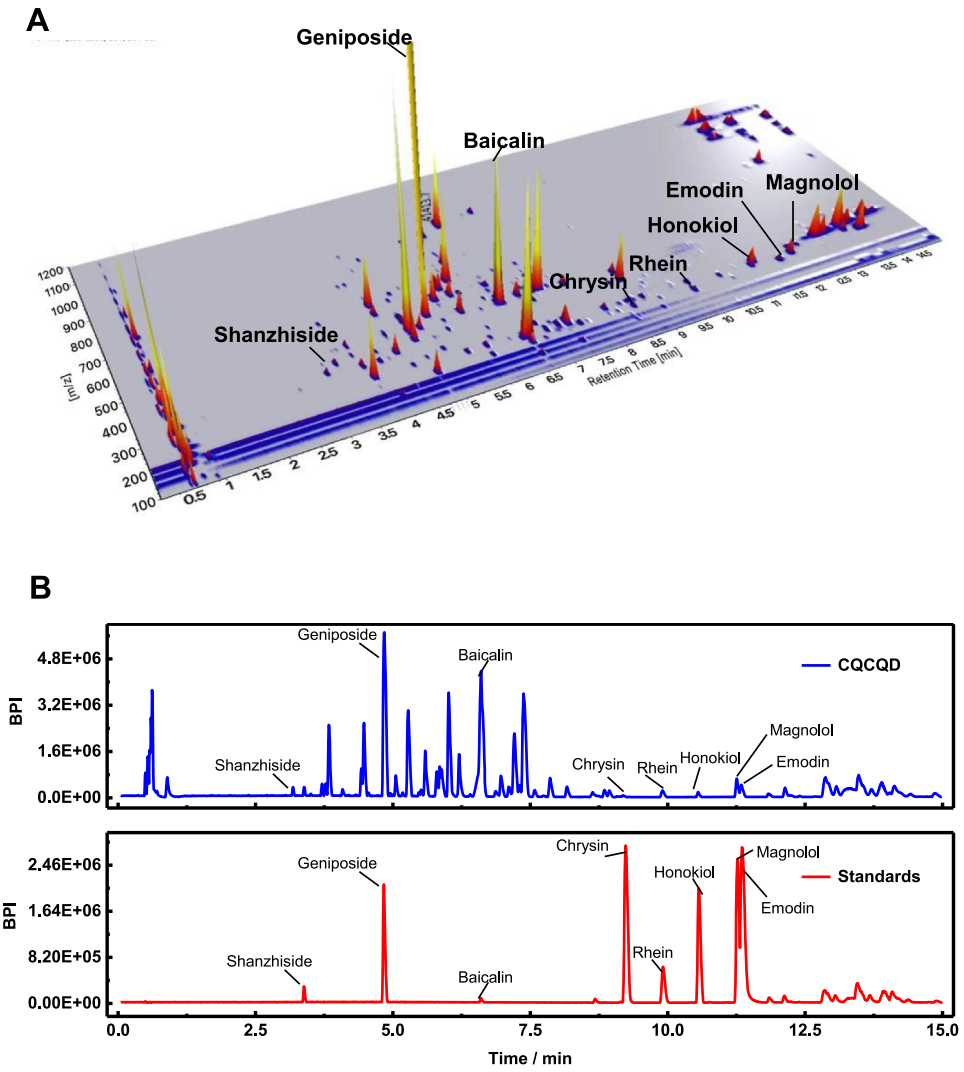


Fig. 1. Chemical ingredients analysis of CQCQD. (A) The three-dimensional UPLC-QTOF/MS of CQCQD. (B) Representative base peak intensity (BPI) chromatograms of CQCQD and standards.

identified in the CQCQD, 8 compounds (shanziside, geniposide, baicalin, chrysin, rhein, honokiol, magnolol and emodin) were listed as the representative active ingredients of monarch and minister drugs in CQCQD. They were confirmed by comparing retention time, molecular ions and fragmentions with corresponding standards in the mass spectrum (**Fig. S1** and **Table S5**). The representative 3D UPLC-MS picture of CQCQD is displayed in Fig. 1A. The representative chromatograms of CQCQD and 8 standards are shown in Fig. 1B.

Construction of TLR4/NLRP3 related sub-network and identification of targets-ingredients

The flowchart of pharmacology network construction was shown in **Fig. S2**. A total of 27 con-targets were obtained, among them, 24 con-targets were further filtered out by PPI network with the minimum required interaction score of 0.7 (high confidence) (Fig. 2A). Further KEGG pathway enrichment analysis based on con-targets indicated 62% of all signaling pathways were associated with pro-inflammatory process with AP, and a main "compounds-targets-pathways" network was constructed (**Fig. S3**). Of them, TLR, NF-κB and NLR are related to TLR4/NLRP3 pathways. In the TLR4/NLRP3 related sub-network, four cross-talk targets (TLR4, NF-κB1, RelA and TNF-α) are involved (Fig. 2B). PTGS2, TLR2 and IL-6 are targets related to NF-κB, TLR and NLR signaling pathway, respectively. Four active compounds (emodin, rhein, baicalin and chrysin) from CQCQD were predicted to have direct interactive function against cross-talking targets, while isosakuranetin-7-rutinoside, hesperidin, geniposide, (±)-catechin had indirect effect (Fig. 2C). The chemical structure of four compounds are depicted in (Fig. 2D).

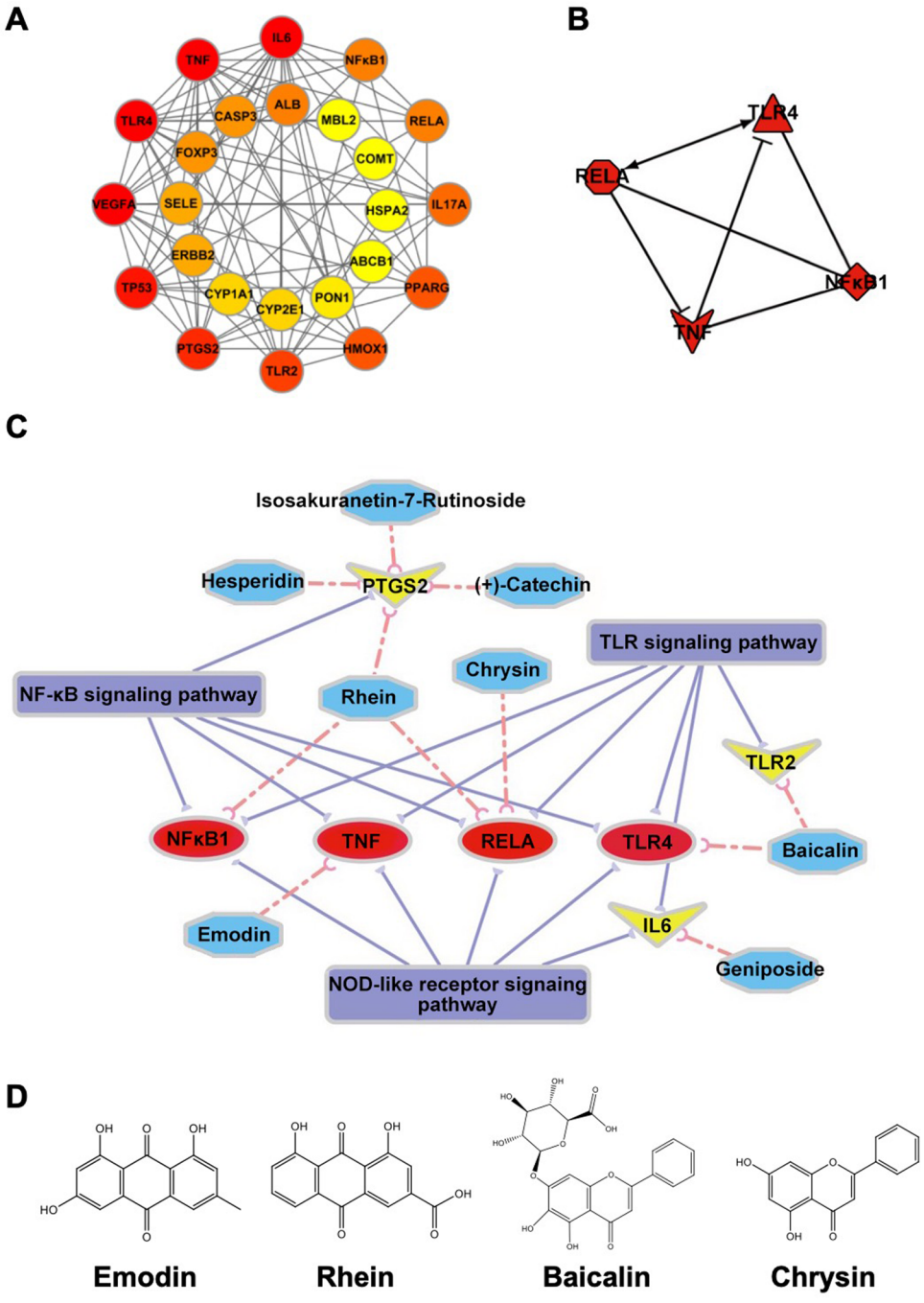


Fig. 2. Pharmacological sub-network analysis-assisted screening of individual anti-inflammatory active ingredients from CQCQD. (A) The PPI network of 24 con-targets related to CQCQD major identified compounds and AP. (B) Four cross-talk targets constructed by Protein-Protein Interactions Network. (C) Sub-network of four active compounds (emodin, rhein, baicalin and chrysin) predicted to target NF-κB1, TNF-α, RelA and TLR4 that are associated with NF-κB, TLR and NOD-like receptor (NLR) signaling pathways. Blue polygons denote ingredients from CQCQD, which could modulate targets in the 3 inflammatory pathways. Purple frames denote three signaling pathways involved. Red ovals denote four cross-talk targets. (D) Chemical structures of four compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

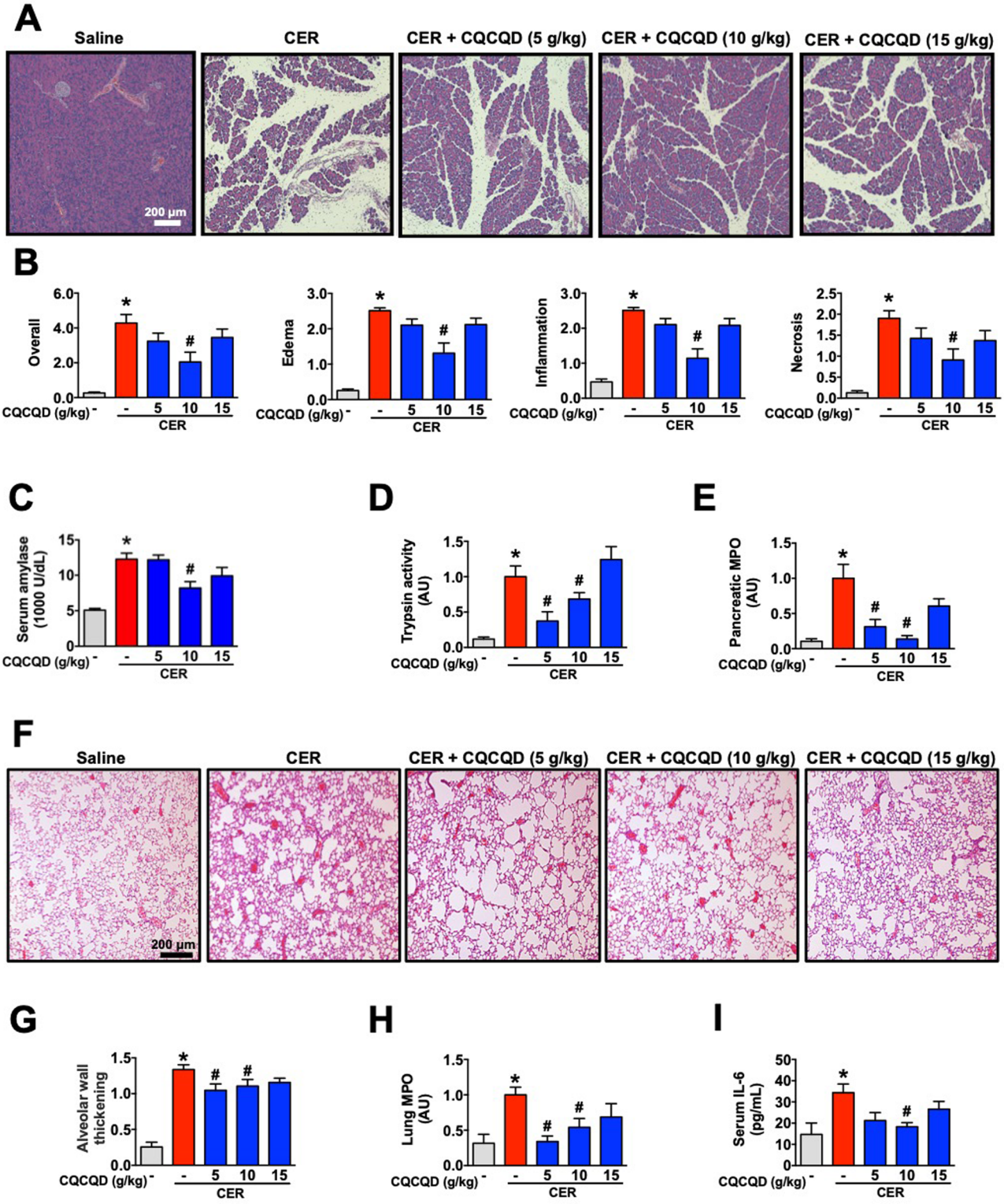


Fig. 3. Effects of CQCQD on severity indices of cerulein-induced AP in mice. Mice received 7 intraperitoneal injections of cerulein (CER; 50 μg/kg) at hourly interval to induce AP, while controls received normal saline at the same regimen. In the treatment groups, oral gavage of CQCQD (5, 10 and 15 g/kg) was implemented at 3, 5 and 7 h from the first injection of cerulein. Animals were sacrificed at 12 h after the first CER/saline injection. (A) Representative H&E images of pancreatic sections (magnification 200 ×). (B) Pancreatic histopathology scores were exhibited as y axis. (C) Serum amylase. (D) Pancreatic trypsin activity. (E) Pancreatic myeloperoxidase (MPO) activity. (F) Representative H&E images of lung sections (magnification 200 ×). (G) Lung histopathology score. (H) Lung MPO activity. (I) Serum interlukin-6 (IL-6). Data are expressed as mean ± SEM of 6–8 animals per group. * $p < 0.05$ *vs.* control group, # $p < 0.05$ *vs.* CER group. In the histogram: *gray* = control, *Red* = CER, and *Blue* = CER with CQCQD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CQCQD attenuates pancreatic injury and systemic inflammation of CER-AP

The representative hematoxylin and eosin (H&E)-stained pancreatic images are shown in Fig. 3A. Cerulein induced typical histopathological changes of AP manifesting as diffused edema, marked periductal and parenchymal neutrophil filtration (indicating inflammation) as well as scattered acinar cell necrosis that were reflected by increased histopathological scores (Fig. 3B). These changes were associated with significantly increased serum amylase (Fig. 3C), pancreatic trypsin activity (Fig. 3D), pancreatic MPO activity (Fig. 3E). The representative H&E lung images are shown in Fig. 3F. Cerulein injections caused significantly increased alveolar septate thickening score (Fig. 3G), lung MPO activity (Fig. 3H) and serum IL-6 levels (Fig. 3I) compared with normal saline controls. Among 3 doses (5, 10, and 15 g/kg) tested, CQCQD at 10 g/kg significantly and consistently reduced all histopathological and biochemical severity indices, while 5 and 15 g/kg were ineffective or only had partial protective effects (Fig. 3A-I). CQCQD at 20 g/kg had no significant impact on pancreatic histopathology but increased pancreatic trypsin activity, lung histopathology score, lung MPO activity and serum IL-6 levels (**Fig. S4**), indicating toxic effects caused by this dose.

CQCQD down-regulates expression of TLR4/NLRP3-related mRNAs and proteins

The results from semi-quantitative qRT-PCR are summarized in

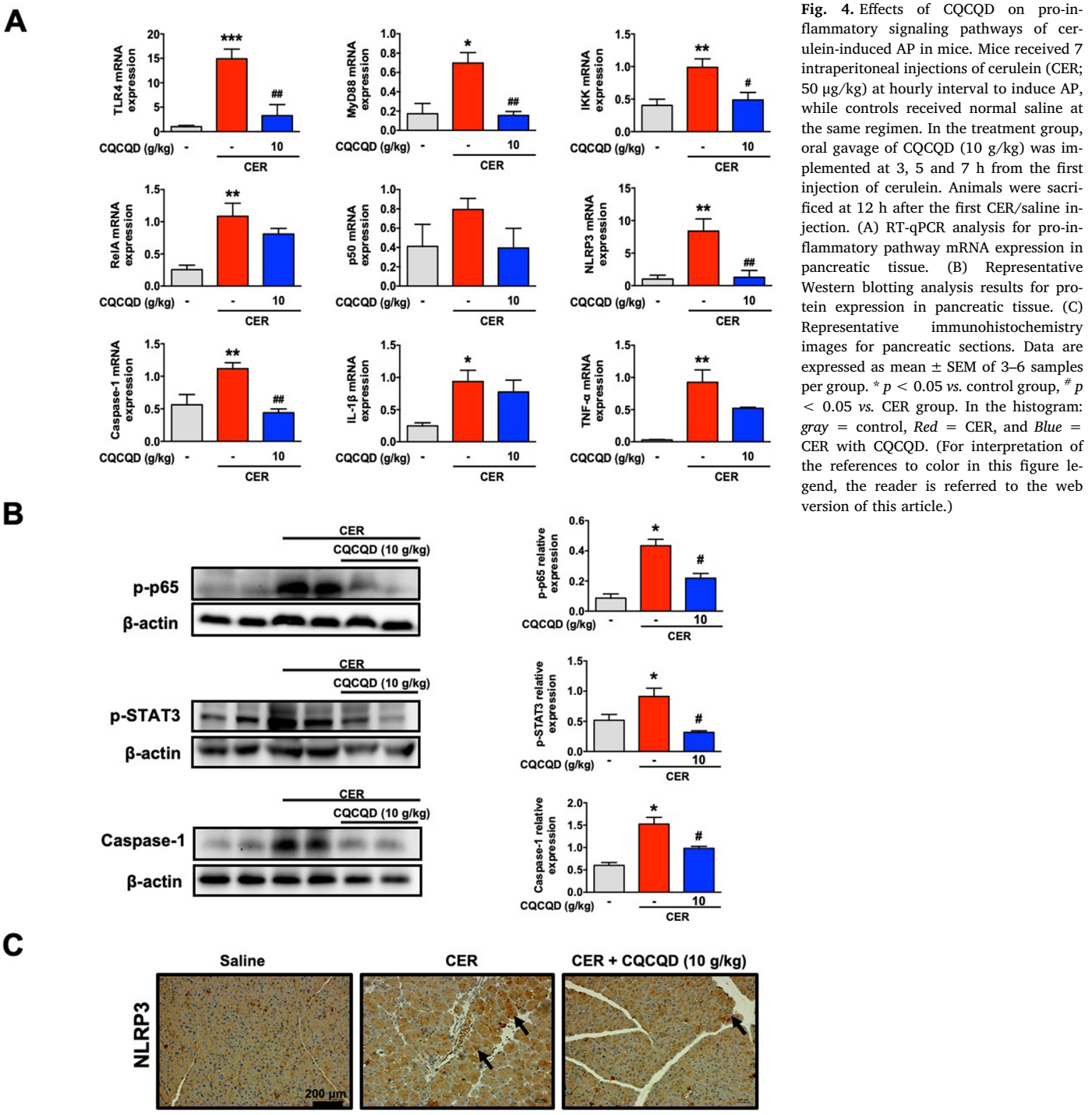


Fig. 4. Effects of CQCQD on pro-inflammatory signaling pathways of cerulein-induced AP in mice. Mice received 7 intraperitoneal injections of cerulein (CER; 50 μg/kg) at hourly interval to induce AP, while controls received normal saline at the same regimen. In the treatment group, oral gavage of CQCQD (10 g/kg) was implemented at 3, 5 and 7 h from the first injection of cerulein. Animals were sacrificed at 12 h after the first CER/saline injection. (A) RT-qPCR analysis for pro-inflammatory pathway mRNA expression in pancreatic tissue. (B) Representative Western blotting analysis results for protein expression in pancreatic tissue. (C) Representative immunohistochemistry images for pancreatic sections. Data are expressed as mean ± SEM of 3–6 samples per group. * $p < 0.05$ *vs.* control group, # $p < 0.05$ *vs.* CER group. In the histogram: *gray* = control, *Red* = CER, and *Blue* = CER with CQCQD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

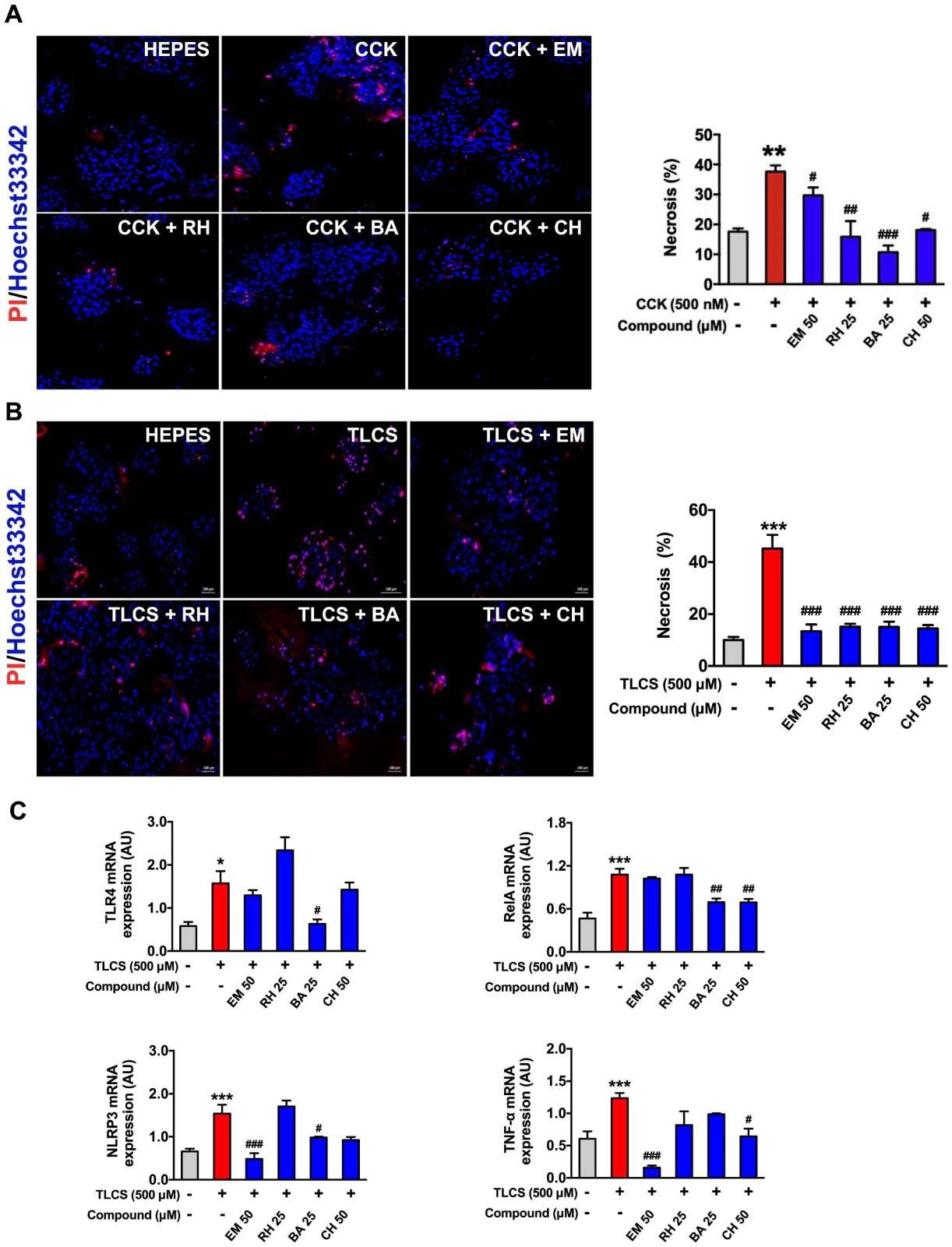
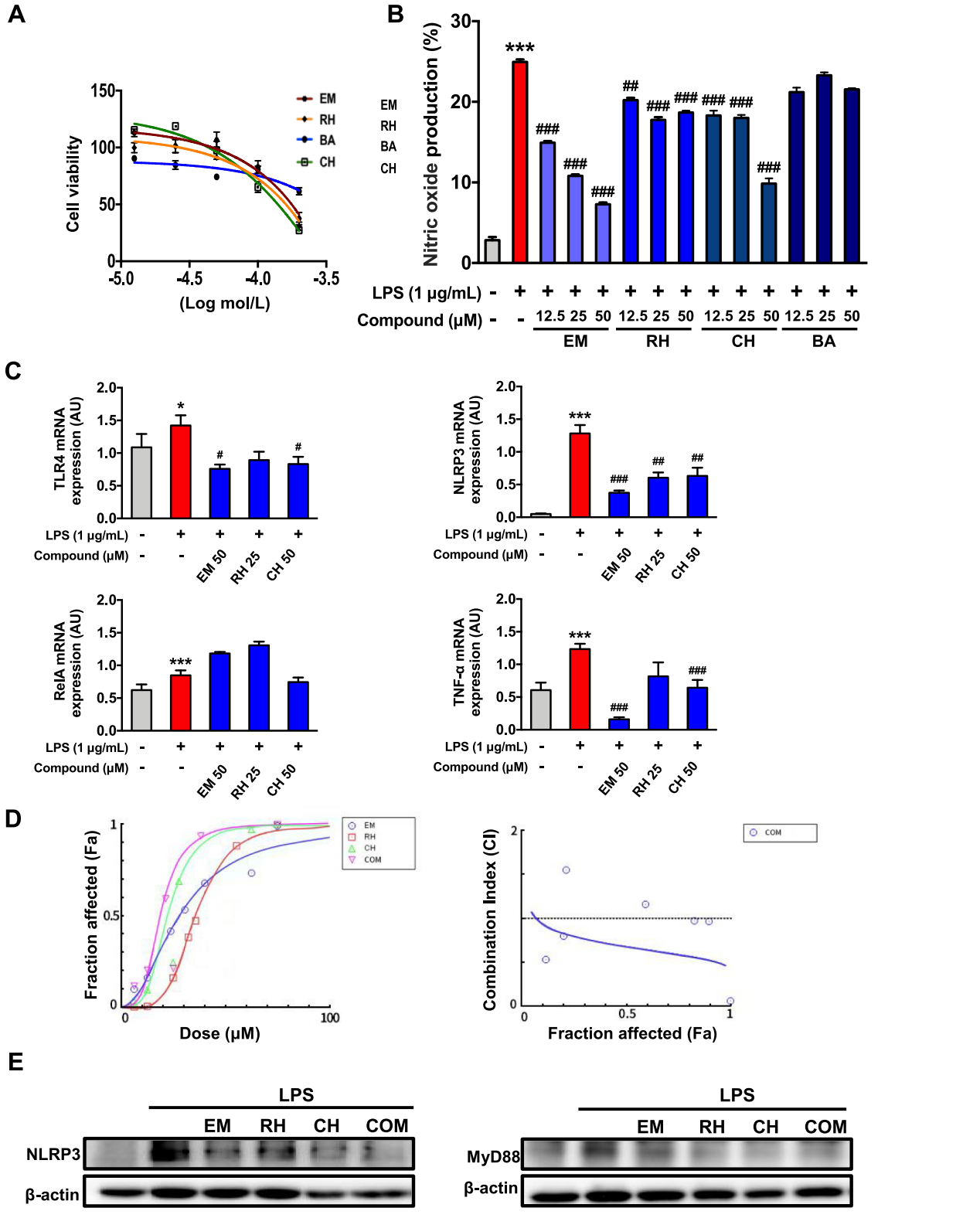


Fig. 5. Effects of screened individual anti-inflammatory compound on necrotic cell death and TLR4/NLRP3 pro-inflammatory signaling pathway in acinar cells. Freshly isolated mouse pancreatic acinar cells received prior incubation with emodin (EM; 50 μM), rhein (RH; 25 μM), baicalin (BA; 25 μM) and chrysin (CH; 50 μM), or HEPES only for 30 min. Then cells were incubated with cholecystokinin (CCK; 500 nM) for 2 h or taurolithocholic acid 3-sulfate disodium salt (TLCS; 500 μM) for 30 min, respectively. Cells were further stained with Hoechst 33342 and propidium iodide (PI; 1 μM) for determination of cell death by epifluorescence microscopy. Representative images and summary necrotic scores (%) for CQCQD active ingredients on (A) CCK- and (B) TLCS-induced necrotic cell death. (C) RT-qPCR analysis for pro-inflammatory pathway mRNA expression in acinar cells. Data are expressed as mean ± SEM from 3 or more independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *vs.* control group, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ *vs.* CCK or TLCS group. In the histogram: *gray* = control, *Red* = CCK or TLCS, and *Blue* = CCK or TLCS with different compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4A. The mRNA expression related to TLR4/NLRP3 pro-inflammatory signaling pathway (TLR4, MyD88, IKK, RelA, p50, NLRP3, caspase-1, IL-β and TNF-α) was significantly up-regulated in the CER-AP compared with normal saline controls. CQCQD at 10 g/kg significantly reduced the mRNA expression of TLR4, MyD88, IKK, NLRP3 and caspase-1 and with a tendency to reduce expression of other mRNAs. CQCQD also suppressed cerulein-induced up-regulation of protein expressions for p-p65, p-STAT3, caspase-1 (Western blot; Fig. 4B) and NLRP3 (immunohistochemistry; Fig. 4C).

Validation of screened ingredients for necrotic cell death and TLR4/NLRP3-related pathways in acinar cells

Next, we sought to validate the anti-inflammatory effects of the four



(caption on next page)

compounds in both CCK- and TLCS-induced necrotic acinar cell death. The optimal concentration of corresponding isolated authentic compounds was selected from various concentrations (12.5, 25 and 50 μM) tested with TLCS (500 μM) incubation (**Fig. S5**). All compounds at their respective optimal concentration significantly decreased CCK-induced acinar cell death with baicalin had the best protective effects followed by rhein, chrysin and emodin (Fig. 5A). All compounds significantly and equivalently reduced TLCS-induced acinar cell death (Fig. 5B). Individual compound had different inhibitory effects on the expression of TLR4, RelA, NLRP3 and TNF-α mRNAs that were up-regulated after TLCS stimulation: baicalin significantly reduced expression of TLR4 mRNA; baicalin and chrysin significantly reduced expression of RelA mRNA; emodin and baicalin significantly reduced expression of NLRP3 mRNA; emodin and chrysin significantly reduced expression of TNF-α mRNA (Fig. 5C). While in the presence of LPS, only emodin significantly decreased TLCS-induced over-expression of TLR4 and NLRP3 mRNAs (**Fig. S6**).

Validation of screened ingredients for cell viability and TLR4/NLRP3-related pathways in RAW 264.7 macrophages

The cell viability showed that the four compounds had no cytotoxicity to RAW264.7 cells up to 50 μM (Fig. 6A). Appropriate timing for LPS stimulation and compound pre-incubation was assessed first (**Fig. S7**). Then a strategy of 24 h pre-incubation of individual compounds followed by another 24 h LPS co-incubation was employed. Cell injury of RAW264.7 cells was assessed by measuring nitric oxide production shown in histogram (Fig. 6B). Compared with culture medium controls, LPS caused dramatic nitric oxide release that was suppressed by emodin in a dose-dependent manner and with the maximal effect obtained at 50 μM. While both rhein and chrysin significantly reduced nitric oxide production at all concentrations tested, the same dose-dependent effect was not observed. Baicalin had no significant effect on LPS-induced nitric oxide production.

The effects of emodin, rhein and chrysin on the mRNA expression profile of the TLR4/NLRP3 pathways are shown in (Fig. 6C). LPS stimulation significantly up-regulated expression of TLR4, NLRP3, RelA and TNF-α mRNAs. Both emodin and chrysin significantly reduced expression of TLR4, NLRP3 and TNF-α mRNAs. Rhein significantly reduced expression of NLRP3 mRNA but was not effective for other mRNAs. None of the three compounds significantly altered expression of RelA mRNA. Dose-effect curves of individual compound and combination of them were plotted for nitric oxide suppression activity in RAW264.7 cells (Fig. 6D). A synergistic suppression effect was observed on LPS-induced nitric oxide production with Fa values (Fig. 6D). Combination showed synergistic effects both at low dose (Fa < 0.3) and high dose (Fa > 0.8). However, an antagonistic interaction was observed with CI values ranging from 1.16 to1.55. Emodin, rhein and chrysin respectively inhibited the expression of MyD88 and NLRP3 proteins (Fig. 6E). Further, their combination had even stronger inhibitory effects compared with individual compounds alone, highlighting a synergistic effect.

Discussion

For the first time, the anti-inflammatory effects of CQCQD against AP were studied using the “multiple ingredients-targets-pathways” approach through a network pharmacology analysis. The main findings from this study were: (1) A pharmacological sub-network of “3 inflammatory pathways-4 cross talk targets-4 compounds” was built. Four active compounds: emodin, rhein, baicalin and chrysin from CQCQD were identified. These molecules act on the TLR4/NF-κB/NLRP3-related proteins, TLR4, RelA, NF-κB and TNF-α; (2) CQCQD significantly reduced the severity of CER-AP and this was associated with suppression of TLR4/NLRP3 inflammasome-mediated pro-inflammatory signaling pathway-related mRNAs and proteins; (3) *In vitro* experiments validated the protective effects of these four compounds on acinar cells via inhibition of TLR4/NLRP3 pathways. While baicalin was the most effective in preventing pancreatic acinar cell injury, the other three compounds (emodin, rhein and chrysin) reduced LPS-induced cell death and TLR4/NLRP3 pathway activation in macrophages. The combination of these compounds representing CQCQD bioactive ingredients produced a synergistic protective effect when compared to individual compound acting on their own. These data support the potential for developing a range of CQCQD-based pharmacological treatments for AP.

Severe AP is characterized by the early development of persistent the systemic inflammatory response syndrome, followed by irreversible organ failure. It is often complicated by pancreatic necrosis, sepsis, and death (Guo et al., 2014; Schepers et al., 2019; Shi et al., 2020). Correspondingly, according to TCM theory, the early stage of severe AP is defined as “Yangming viscera syndrome”, manifested as abdominal distention, fullness and pain. DCQD is a classical prescription for the treatment of this syndrome and has been shown to improve gut dysfunction and reduce severity of AP in animal models (Lu et al., 2017) and human patients (Wan et al., 2012). This is proposed to be largely due to its anti-inflammatory and anti-oxidative stress effects. CQCQD is derived from DCQD with the original intention to enhance its therapeutic efficacy against biliary AP. The addition of Bupleurum marginatum Wall. ex DC., Scutellaria baicalensis Georgi, Gardenia jasminoides Ellis and Artemisia capillaris Thunb in CQCQD was designed to increase the anti-inflammatory effects of DCQD (Jang et al., 2015; Jung et al., 2008; Zhao et al., 2015). These anti-inflammatory effects of CQCQD observed in this current study were consistent with previous reports showing that in rats CQCQD significantly reduced lung and intestinal injuries in sodium taurocholate-induced experimental AP (NaT-AP) (Wu et al., 2016) and L-arginine-induced AP (Zhang et al., 2017) respectively. In recent years, the incidence of hypertriglyceridemia-associated AP has rapidly increased in China (Ding et al., 2019; Mukherjee et al., 2019; Shi et al., 2020; Zhang et al., 2019c), attributing to a high prevalence of central obesity (Zhang et al., 2019a) or metabolic syndrome (Ding et al., 2016). In addition, hypertriglyceridemia-associated AP is often more severe when compared with other etiologies due to its more pronounced pro-inflammatory effects and worse clinical outcomes (Ding et al., 2019; Zhang et al., 2019c). Recent studies have demonstrated that components in CQCQD such as

Fig. 6. Effects of screened compounds for cell viability, cell injury and TLR4/NLRP3 pro-inflammatory signaling pathway in RAW 264.7 cells. RAW 264.7 macrophage cells received prior incubation with emodin (EM; 50 μM), rhein (RH; 25 μM), baicalin (BA; 25 μM) and chrysin (CH; 50 μM), or culture medium only for 24 h followed by co-incubation with lipopolysaccharide (LPS; 1 μg/ml) for another 24 h. (A) Effects of four compounds representing screened ingredients on cell viability (Cell Counting Kit 8). Solid hexagon (EM), solid rhombus (RH), solid round (BA), square (CH) (B) Dose effects of each compound on nitric oxide production after LPS stimulation. Three concentrations (12.5, 25 and 50 μM) were tested for each compound. (C) Effects of each compound at their respective optimal concentration on expression of TLR4, NLRP3, RelA and TNF-α mRNAs after LPS stimulation. (D) Does-effect curve of EM, RH and CH at respective optimal concentration and their combination index (CI) values were plotted according to suppression rate of nitric oxide production (Fa). In dose-effect curve, round (EM), square (RH), regular triangle (CH), inverted triangle (COM). In CI curve, dotted line is the reference line, where CI value equals to 1; solidline (*Blue*) represents CI values at different Fa values. (E) Effects of optimal concentration of EM, RH, CH and their combination ‘COM’ (EM:RH:CH = 4:1:1) on expression of NLRP3 and MyD88 proteins (Western blot). Data are expressed as mean ± SEM from 3 or more independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *vs*. control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs*. LPS group. In the histogram: *gray* = control, *Red* = LPS, and *Blue* = LPS with different compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of thisarticle.)

Gardenia jasminoides Ellis (Ahmed et al., 2018) can antagonize pancreatic triglyceride lipase, a key factor for lipolysis and lipotoxicity (de Oliveira et al., 2020) .

TLR4/NLRP3 pro-inflammatory signaling pathways play a critical role for primary pancreatic toxin- and DAMP-induced pancreatic injury and systemic inflammation (Chen et al., 2014; Kang et al., 2014a; Szatmary et al., 2018). However, the specified and precise active ingredients of CQCQD and their synergistic protective effect on inhibiting key pro-inflammatory pathways (i.e. TLR4/NLRP3) have remained elusive. Through RT-qPCR, Western blot and immunohistochemistry, we confirmed that CQCQD and its active ingredients alleviated severity of AP at least in part through suppressing TLR4/NLRP3 related pathways. Natural TLR4 antagonists were mainly obtained from gram-negative bacteria, cyanobacteria, or from plants. Curcumin, sulforaphane, iberin, xanthohumol, celastrol, berberine, atractylenolide I and zhankuic acid A have been described as natural molecules for TLR4 antagonism (Molteni et al., 2018). A study by Li et al., (2009) showed that combination of emodin and baicalin suppressed severity and reduced TLR4 expression in pancreas and lung in NaT-AP in rats. Recently, a mechanistic study revealed that about 34 phytochemicals could alter the NLRP3 inflammasome activity in acute and chronic inflammatory diseases (Jahan et al., 2017). Among them, emodin and curcumin were found to be involved in modulation of NLRP3 activation in septic shock models. One recent study indicated that emodin could attenuate the severity of AP via the pancreatic P2X7/NLRP3 signaling pathway in NaTC-AP in rats (Zhang et al., 2019b). Furthermore, some natural herbal medicines can neutralize the toxicity of extracellular histones (Isobe et al., 2016). In according with this, our team has recently found some active ingredients (e.g. baicalin) from CQCQD demonstrated high binding affinity to histone H3, implying a more potent detoxifying capability of CQCQD. This research topic, therefore, warrants further in-depth investigation to establish a potential pathway for the discovery of novel drugs.

Pharmacological network analysis and mapping are essential tools for exploring the dynamic ingredient-target-pathway relationship between TCM herbal formulae and diseases (Sun et al., 2020). Using these tools, the team identified correlations between the bioactive ingredients of CQCQD and AP targets using established disease and target databases. In addition, KEGG was used to search for 21 known AP-related pathways, including MAPK, PI3K-Akt, IL-17, TNF, TLR, NF-κB, NLR, sphingolipid, adipocytokine, RIG-I-like receptor, T cell receptor, P53 and etc.Through analysis, we found that inflammatory pathways accounted for 62% of all mechanisms involved in AP, and TLR4/NF-κB/NLRP3 cassette plays a central role in all inflammatory pathways. Therefore, we subsequently selected four common targets related to TLR4/NLRP3 inflammasome-mediated pro-inflammatory pathways and constructed a pharmacological sub-network for CQCQD. The sub-network revealed that RelA, TLR4, NF-κB1 and TNF-α were crucial cross-talk targets for TLR, NF-κB and NLR signaling pathways. Further sub-network analysis enabled the team to filter out four active compounds of CQCQD (emodin, rhein, baicalin and chrysin) that directly act on these targets by using relevant TCM databases.

Among these four compounds, emodin and rhein were from Rheum palmatum L., the principle drug in both DCQD and CQCQD. Baicalin and chrysin were from Scutellaria baicalensis Georgi. In this study, no protective effect of baicalin on reducing cell injury was observed in cultured macrophages. Baicalin was the main metabolite of baicalein after its administration in animals and human (Tian et al., 2012). However, there is no clear evidence showing this conversion occurred in cell culture systems (Chen et al., 2001), thus it is difficult to rule out the possibility that baicalein may actually be responsible for the protective effects. While various studies have investigated the role of emodin, rhein and baicalin or its aglycone baicalein in experimental AP, the role of chrysin has not been appraised in AP settings yet. Chrysin (5,7-dihydroxy-2-phenyl-4H-chromen-4-one) is a natural flavone found in several plants, mushroom, and honeycomb. It has been reported to be protective in inflammatory diseases such as sclerosis and diabetes (Ramirez-Espinosa et al., 2017). Here we have now shown that chrysin had consistent effects in reducing cell death in pancreatic acinar cells and macrophages by down-regulating the expression of TLR4/NLRP3 pathways related mRNAs and proteins.

Importantly in this study, in order to evaluate the synergistic effects of these compounds that representing active ingredients in CQCQD, all three compounds were combined at their relative ratios according to the HPLC results. It was observed that their synergistic protective effect was positively correlated with an increase in dosage until the maximum safety dose was reached. This finding highlighted the existence of a potent dose-effect relationship, but also the need for robust quality control of raw TCM herbal ingredients and the extraction process.

Unlike their synthetic counterparts, most Chinese herbal medicines have not been pharmaceutically evaluated to define their pharmacodynamic, pharmacokinetic properties as well as key parameters such as optimal, effective, and toxic dosing. Consequently, unduly increased doses or prolong administrations of TCM herbal formula can cause adverse reactions (Cheng and Leung, 2012). In this study, the safety parameters and dose-efficacy relationship between CQCQD and severity scale in a mouse CER-AP model were also assessed for the first time. Using the human-mice dose equivalent coefficient, we determined that the clinically-proven optimal human dose in mice is 5.75 g/kg. Using this as a reference point, a four-dose regimen ranging from low to high was derived (5, 10, 15, and 20 g/kg). The results suggest CQCQD was most effective in reducing systematic inflammatory parameters in pancreas and lung at 10 g/kg (approximately twice of the reference dosage). The other CQCQD lower dosages were not as effective as 10 g/kg, and the 20 g/kg regimen was associated with increased lung injury and elevated serum IL-6 levels, indicating potential toxic effects.

There are several limitations to this study. As we focused on TLR4/NLRP3 related pathways, we may have underestimated the impact of other pro-inflammatory or signaling pathways such as the anti-oxidant property also possess by CQCQD. Indeed, the CQCQD has a wide range of active ingredients including flavonoids, anthraquinones, iridoids, phenols, lignans, and corresponding glycosides, most of which have been reported to be capable of reducing AP severity via antagonizing reactive oxygen species by activating nuclear factor erythroid-2-related factor 2/antioxidant response element, an intrinsic antioxidative stress pathway (Shapiro et al., 2007). Therefore, much more work is needed to understand the complex mechanisms and full range of presumed therapeutic targets between AP and CQCQD.

In conclusion, the present study demonstrated that CQCQD could alleviate the severity of experimental AP at least in part via inhibition of TLR4 and the NLRP3 inflammasome. Pharmacological network analysis and experimental validation identified that four CQCQD bioactive compounds (emodin, rhein, baicalin and chrysin) are responsible for these effects. Emodin, rhein and chrysin were also found to work synergistically to mitigate cell injury and suppress TLR4/NLRP3 related pathways in cultured macrophages.

Author contributions

QX, WH and DD obtained funding, conceptualized this study and supervised students; YW, CH, TL, RW, WC, JY, GL, LY and NS performed experiments, acquired data and analyzed data; YW, CH and WH drafted the manuscript; XF and LD had important intelligence input; RS, JAW, JH and ARP critically revised the manuscript; all authors read and approved the final version of the manuscript before submission.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2020.153328.

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