

Callicarpa Nudiflora Extract Exerts Anti-Inflammatory Effect on H. Pylori-Associated Gastritis Through Repression of ROS/NLRP3/Caspase-1/IL-1 β Signaling Axis

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***Callicarpa nudiflora* extract exerts anti-inflammatory effect on *H. pylori*-associated gastritis through repression of ROS/NLRP3/caspase-1/IL-1 β signaling axis**

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List of Abbreviations

CHM, Chinese herbal medicine

CagA, cytotoxin-associated gene A

CN, *Callicarpa nudiflora*

DMSO, dimethyl sulfoxide

FBS, fetal bovine serum

HAG, *H. pylori*-associated gastritis

H. Pylori, *Helicobacter pylori*

HpSS1, *Helicobacter pylori* Sydney Strain 1

LDH, lactate dehydrogenase

MIC, minimal inhibitory concentration

MOI, multiplicity of infection

NF- κ B, nuclear factor (NF)- κ B

PBS, phosphate buffer saline

ROS, reactive oxygen species

TNF- α , tumor necrosis factor α

Abstract

Helicobacter pylori (*H. pylori*) is a major pathogenic factor for the development of gastric diseases including chronic gastritis and gastric cancer. *Callicarpa nudiflora* (CN), an air-dried leaves extract of *Callicarpa nudiflora* Hook. & Arn., has been found to exhibit a broad-spectrum antibacterial effect. In our study, we extracted the active ingredient from air-dried leaves of *Callicarpa nudiflora*, detected the effect of CN against *H. pylori*-infected GES-1 cells *in vitro*, and elucidated the underlying mechanism. GES-1 cells were cocultured with HPSS1 at MOI = 100:1 and treated with different concentrations of CN. Results indicated that CN not only significantly decreased cellular lactate dehydrogenase leakage, but also markedly attenuated *H. pylori*-induced cell apoptosis and ROS production in GSE-1 cells, therefore protecting gastric epithelial cells against injuries caused by *H. pylori*. CN also inhibited the secretions of inflammatory factors, such as tumor necrosis factor- α (TNF- α), IL-1 β , IL-6 and IL-8. Furthermore, CN remarkably decreased the expression levels of NLRP3, PYCARD, active Caspase-1. In conclusion, CN exhibited highly efficient protective effect against *H. pylori*-induced gastritis and cell damage; Mechanismly, CN suppressed *H. pylori*-triggered inflammatory response and pyroptosis through depressing ROS production and NLRP3 inflammasome activation via ROS/NLRP3/IL-1 β signaling axis.

Keywords

Callicarpa nudiflora, *Helicobacter pylori*, gastric epithelial cell, cell apoptosis, inflammation

Introduction

Helicobacter pylori, a gram-negative spiral bacterium, infects almost half population of the world at one time or another [1]. In most cases, patients infected with *H. pylori* have no symptoms. Only ~30% of the infected patients will progress to clinical symptoms [2]. *H. pylori* colonizes human gastric epithelial cells, which causes gastric mucosal layer edema and neutrophil infiltration, leads to chronic gastritis, atrophic gastritis, even develop to gastric cancer [3-5]. *H. pylori* is not only cause of more than 70% gastritis [6-8], but also the main carcinogen of gastric cancer by World Health Organization [9]. *H. pylori* was first isolated and described in 1983 by Barry Warren and Robin Marshall, who won the 2005 Nobel Prize in physiology or medicine “for their discovery of the bacterium *H. pylori* and its role in gastritis and peptic ulcer disease” [10]. *H. pylori* can express multiple pathogenic virulent factors, one of which is cytotoxin-associated gene A (CagA). CagA activates the expression of the nuclear factor (NF)- κ B of gastric epithelial cells and then triggers proinflammatory cytokines expression [11]. *H. pylori* can also induce the release of pro-inflammatory factors and the associated oxidative damage. For instance, pro-inflammatory factors IL-8 could activate and aggregate neutrophils, thereby inducing ROS production [12]. Excessive ROS could activate NLRP3 inflammasome, which triggers the cleavage of pro-IL-1 β , then transforming into activated IL-1 β [13, 14].

Currently, the preferred regimen for *H. pylori*-associated gastritis (HAG) is triple therapy: two antibiotics combined with a proton pump inhibitor; however, this treatment has some shortcomings such as drug resistance, gastrointestinal flora imbalance and indigestion [15, 16]. Therefore, it is urgent to develop effective, low toxicity drugs for the therapy of *H. pylori* infection. Chinese herbal medicine (CHM) has been widely employed for eliminating infection for its multi-target effects and fewer side effects [17, 18]. Some herbs showed their promising anti-infection potential in *H. pylori*-associated gastritis [19-23].

Callicarpa nudiflora Hook. & Arn., belonging to the genus of *Callicarpas linn.* of the

family Verbenaceae, the dry leaves of which is used as a traditional Chinese herbal medicine. It is mainly distributed in Guangdong, Hainan, and Guangxi provinces in China [24]. The mainly chemical constituents include phenylpropanoids, flavonoids, triterpenoids, diterpenoids, cycloene ether terpenoids, phenolic acids and their glycosides and sterols [25]. According to “the Supplement of Compendium of Materia Medica” and Chinese Pharmacopoeia 2015, it can not only relieve various symptoms such as poisonous toxins, gangrene, laryngeal paralysis, poisonous swelling, pain and wind-evil, but also treat respiratory tract infections, hepatitis and bleeding [26-30]. Because of its broad-spectrum antibacterial effect, *Callicarpa nudiflora* Hook. & Arn. exerts varying degree of inhibition on *Staphylococcus aureus*, *Salmonella typhi*, *Pneumococcus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Shigella* [31, 32]. In our previous study, we prepared *Callicarpa nudiflora* extract (CN) from the dry leaves of *Callicarpa nudiflora* Hook. & Arn., analyzed its mainly chemical constituents and evaluated corresponding pharmacological activity [25].

In present research, we focused on its pharmacological activity in inflammation. We detected the therapeutic effect of CN on *H. pylori*-associated gastritis (HAG) in GES-1 cells; further, we explored the molecular mechanism of how CN exerted its anti-inflammatory effect. Hopefully, this project will provide a candidate drug for the treatment of clinic *H. pylori*-associated gastritis (HAG).

Results and Discussion

Preparation of *Callicarpa Nudiflora* Extracts (CN)

The voucher specimen (SZG00048161) was deposited in the Plants Herbarium of Fairy Lake Botanical Garden (Shenzhen, China) (Fig. 1). *Callicarpa nudiflora* was prepared according to the method of China Pharmacopoeia 2015. The air-dried leaves of *Callicarpa nudiflora* were smashed into powder (approximately 80 mesh). One kilogram of powder was extracted with two liters water, boiled to approximately 100°C and kept of 1 hour. The extracted fluid

was filtered and the residue was boiled for another 1 hour with 1.5 liters water. The collected decoction was combined, then freeze-dried (Eyela FDU-2110, Eyela Corp, Tokyo, Japan) to gain *Callicarpa nudiflora* powder (268 g). Next, we identification of chemical constituents of CN by UPLC-ESI-Q-TOF-MS (Table 1). Additionally, we analyzed four main compounds of CN by UPLC spectrum, of which acteoside accounts for 33.26%, forsythoside B 0.98%, 5,4'-dihydroxy-3,7,3'-trimethoxyflavone 0.42%, luteolin 0.33% (Fig. 2). In the in vitro study, *Callicarpa nudiflora* was dissolved in DMSO, and DMSO was used for control (the volume of DMSO < 0.5% in all experiments).

CN showed anti-HPSS1 activity in vitro

Because of the broad-spectrum antibacterial effect of CN, we first detected the inhibitory effect of CN on HPSS1, and the minimal inhibitory concentration (MIC) test showed that the MIC value of CN on HPSS1 was 2.5 mg/mL (Fig. 3A) .

Effects of CN on GES-1 cells

In order to figure out toxic and negative effects of CN on gastric mucosa epithelial cells GES-1, we conducted CCK-8 assay with increasing CN concentrations. Results showed that CN presented toxic effects at 45 μ g/mL, moreover higher concentration caused stronger toxicity (Fig. 3B).

CN had therapeutic effect rather than protective effect

In order to further explore the role of CN in GES-1 infected by HPSS1, we first explored the complex number of infections between HPSS1 and GES-1. According to the experimental results, we selected MOI of 100 for the subsequent experimental study (Fig. 3C) . On this basis, we further studied the effect of CN before and after HPSS1 infection, and the results showed that the effect of CN after HPSS1 infection was better (Fig. 3D) . Therefore, we

chose to use CN for the treatment of HPSS1 infected GES-1 cells in the following studies.

CN counteracted the damage of *H. pylori* to GES-1 cells

To research the effects of CN on cell physiology and survival after incubation with *H. pylori*, the morphology, viability and the percentage of LDH leakage of HP-infected GES-1 cells was analyzed. In vitro, GES-1 cells of control group were in good condition, the cell morphology of model group deteriorated and the number decreased, CN mitigated the situation (Fig. 4A). The cell viability of HP infection decreased significantly, and CN treatment increased the cell viability in a concentration dependent manner (Fig. 4B). The damage of HP infection on cells was also manifested in the release of LDH, The release of LDH in HP-infected cells increased significantly, and the release of LDH in CN group decreased gradually with the increase of concentration (Fig. 4C).

CN reduced the apoptosis of *H. pylori*-infected GES-1 cells

The apoptosis of *H. pylori*-infected GES-1 cell with or without CN was assessed by flow cytometry with Annexin V/PI double staining. Data manifested that HP infection caused 21.73% apoptosis in GSE-1 cells. However, CN treatment prevent GSE-1 cells from apoptosis in a concentration-dependent manner (Fig. 5A & B). To further clarify the anti-apoptotic mechanism , we detected apoptosis-related proteins by western blotting. Results showed that *H. pylori* infection accumulated the cleaved PARP (89 kDa) and activated caspase-3 (17 kDa), which were considered as apoptotic markers. After CN treatment, cleaved PARP (89 kDa) and activated caspase-3 (17 kDa) were decreased in a concentration-dependent manner (Fig. 5C & D).

CN decreased ROS production of *H. pylori*-induced GES-1 cells

DCF-DA fluorescence assay was used to evaluate intracellular ROS levels. HP infected

GES-1 cells significantly increased the levels of intracellular ROS, CN reduced ROS levels in *H. pylori*-stimulated GES-1 cells in a dose-dependent manner (Fig. 6A & B).

CN alleviated inflammatory response of *H. pylori*-infected cells

The effects of CN on the production of cytokines (IL-1 β , IL-6, IL-8 and TNF- α) in *H. pylori*-infected GES-1 cells were shown in Fig. 7A, B, C & D. Cytokines were raised sharply by HP infection, treatment with 100, and 200 μ g/ml CN significantly reduced the production of cytokines. Besides, HP stimulation unregulated the inflammatory protein levels of NLRP3, cleaved Caspase 1 (10 kDa), and PYCARD ($p < 0.001$). Interestingly, CN decrease *H. pylori*-stimulated NLRP3, cleaved Caspase 1 (10 kDa), and PYCARD levels (Fig. 7E & F) ($p < 0.05$).

Callicarpa nudiflora Hook. & Arn. is a genus of *Callicarpa linn.* of the family Verbenaceae, and it is widely distributed in South China [24]. We have analyzed its main chemical constituents and pharmacological activity in our previous work [25]. Here, we demonstrated the anti-inflammatory effect of CN in *H. pylori*-associated gastritis (HAG), further, we found that CN exerted anti-inflammatory response through depressing *H. pylori*-induced ROS/NLRP3/IL-1 β signaling axis activation.

There are some publications about pharmaceutical activity of CN, such as mosquito larvicidal activities [33], hepatoprotective effects [34], wound healing effects [35], dispersing edema and hemostasis [36], but there is no report on whether CN can protect cells from undergoing apoptosis. It's fact that *H. pylori* infection cause immune cells and epithelial cells to undergo apoptosis [37, 38]. In the present study, we employed flow cytometry and western blotting, finding that CN reversed *H. pylori*-caused cell apoptosis in GSE-1 cells in a concentration-dependent manner. This clues us that CN can protect cells from apoptosis during the bacteria infection. About this point, CN could be developed a valuable clinic ancillary drug to counteract side effects of front-line chemical drugs in the future.

Functional experiments showed that CN reduced ROS production triggered by *H. pylori* infection in GSE-1 cells. What is more, we profound explored its anti-inflammatory mechanism by measuring the expression level of inflammatory factors IL-1 β , IL-6, IL-8, TNF- α through ELISA test and detecting pivotal proteins level of ROS/NLRP3/IL-1 β signaling through western blotting. All these evidences suggested that CN had anti-inflammatory effect in *H. pylori*-associated gastritis cell model.

NLRP3 inflammasome, nucleotide-binding domain and Leucine-rich repeat containing receptors and the pyrin and HIN domain containing 3, belongs to the NLR protein family, which contains 22 members in human. The NLRP3 inflammasome was initially found to be activated by ATP and toxins [39]. Subsequently, a wide series of stimuli of damage-associated molecular patterns was identified, such as microbial, RNA viruses, ROS, excess glucose, amyloids, urate and cholesterol crystals [40-47]. PYCARD, also named ASC/TMS1, is a bipartite protein that consists of a pyrin domain (PYD) and a caspase recruitment domain (CARD) domain motif. Once activated, NLRP3 recruits PYCARD to form inflammasome, where PYCARD interacts with the CARD of procaspase-1 and converts it to active casprocase-1, which converts the cytokine precursors pro-IL-1 β and pro-IL-18 into the mature IL-1 β and IL-18, eventually it causes pyroptosis [48]. Thus, nuclear PYCARD polymerization or oligomeritization are considered to be critical mechanisms of NLRP3 inflammasome activation [49, 50].

Conclusions

In our study, CN reversed *H. pylori*-induced ROS and NLRP3 increase in GES-1 cells, as a result, NLRP3 cannot recruit adequate PYCARD into nuclear to form ASC polymerization, speck formation and inflammasome, which inhibited *H. pylori*-induced procaspase-1 activation, in turn, prevent the maturation of IL-1 β . To be summarized, CN suppressed *H. pylori*-triggered inflammatory response and pyroptosis through depressing ROS production

and NLRP3 inflammasome activation via ROS/NLRP3/IL-1 β signaling axis.

Materials and Methods

Materials and Chemicals

Campylobacter Karmali Agar Base (CM0935B, OXOID, United Kingdom) and Brain heart infusion broth (CM1136, OXOID, United Kingdom). Sterile defibrated sheep blood (C035, Chun Du Biotechnology, China). Polymyxin B sulfate (P105490, Aladdin, China), Trimethoprim (TMP) (T129928, Aladdin, China), Amphotericin B (A105482, Aladdin, China), Vancomycin (V105495, Aladdin, China). RPMI-1640 (11875-093, Gibco, United States), FBS (10091-148, Gibco, United States), Penicillin-Streptomycin (15070-063, Gibco, United States) and 0.25% Trypsin-EDTA (25200-056, Gibco, United States). Cell Counting Kit-8 (BS350B, Biosharp, China). Lactate dehydrogenase kit (A020-1, Nanjing Jiancheng Bioengineering Institute, China). Apoptosis Assays Kit (KGA108, KeyGEN BioTECH Corp., Ltd, China). ROS Assay Kit (S0033, Beyotime Institute of Biotechnology, China). ELISA kits for IL-1 β (RK00001, Abclonal, China) and IL-8(RK00011, Abclonal, China) and IL-6 (E-EL-H0102c, Elabscience, China), TNF- α ELISA kits (E-EL-H0109c, Elabscience, China). Sodium orthovanadate (S1873, Beyotime Institute of Biotechnology, China), RIPA Lysis Buffer (P0013B, Beyotime Institute of Biotechnology, China) and Enhanced BCA Protein Assay Kit (P0010, Beyotime Institute of Biotechnology, China). Antibodies against PYCARD (DF6304, Affinity Bioscience, United States), NLRP3 (DF7438, Affinity Bioscience, United States). Antibody against GAPDH (5174S, Cell signaling technology, United States), Antibody against PARP1 (66520-1-AP, Proteintech, China), Antibody against Caspase-1 (SC-56036, Santa Cruz, United States), Antibody against Caspase-3 (9662S, Cell signaling technology, United States).

***H. pylori* Strains and Growth Condition**

HPSS1 was provided in a frozen state by National Centers for Disease Control, and maintained on blood agar plates and brain heart infusion broth supplemented with 5% Sterile defibrated sheep blood at 37°C under 10% CO₂, 5% O₂ and 85% N₂.

Cell Culture and *H. pylori* Infection

GES-1 cells were purchased from iCell Bioscience Inc, Shanghai. Cells were propagated in RPMI-1640 media with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin at 37°C and 5% CO₂. The cells were passaged when they reached 80% confluence. Without Penicillin-Streptomycin, the cells were then divided into four groups: control, model (*H. pylori*) and CN 100, and 200 (100, and 200 µg/mL CN with *H. pylori*) groups. In the CN groups, GES-1 cells were cocultured with *H. pylori* at MOI = 100 for 24 hours in a cell incubator and then treated with different concentrations of CN for other 24 hours.

Determination of MIC

The inhibitory effect of *Callicarpa nudiflora* extract (CN) (0.0025~40 mg/mL) on HPSS1 was evaluated by determination of minimal inhibitory concentration (MIC) according to the Chinese Pharmacopoeia 2010.

Evaluation of GES-1 Cell Viability After Incubation With *H. pylori*

The cell viability of GES-1 cell exposed to *H. pylori* and CN was evaluated by CCK-8 assay and percentage of LDH leakage.

CCK-8 Assay

GES-1 cells which were in logarithmic growth phase with good growth state, and were inoculated into 96-well plate with 5000 cells. Add 100 µL of culture medium to each well, and place them in an incubator of 5% CO₂ at 37°C for overnight cultivation. Then the cells

were treated with ascending concentration of CN according to groups. After the time required for cell culture, PBS was used to rinse 4 times, and 100 μ L fresh medium containing 10 μ L CCK-8 reagent was added to each well, keep culturing at 37°C for 4 hours. The absorbance OD₄₅₀ was determined by multifunctional microplate reader (Flexstation 3, Molecular Devices, United States).

LDH Measurement

LDH assay was used to quantity LDH release from cells. According to the directions, we collected the cell culture supernatant, centrifuged at 3000 rpm for 10 minutes, and measured the OD of the supernatant directly. The LDH concentration in the medium was measured at 440 nm. LDH activity (U/L) = (determination of OD — control OD)/(standard OD — blank OD) * concentration of standard (2 mmol/L) *1000.

Apoptosis Detection

GES-1 cells in the logarithmic growth phase with good growth state were inoculated into 6-well plates with 10^5 /well. 2 mL of culture medium was added to each well and cultured overnight in a 5% CO₂ incubator at 37°C. Then the cells were processed in according to groups. The cell pellets were collected, washed in PBS, resuspended cells with 500 μ L binding buffer, 5 μ L AnnexinV-FITC and 5 μ L PI was added and mixed. At room temperature, the reaction was kept out of light for 5~15 minutes. To be detected by Flow cytometry (Beckman coulter, cytoFLEX, United States).

ROS Measurement

GES-1 cells in the logarithmic growth phase with good growth state were inoculated into 6-well plates with 10^5 /well. 2 mL of culture medium was added to each well and cultured overnight in a 5% CO₂ incubator at 37°C. Then the cells were processed according to groups.

The cell pellets were collected, washed in PBS, added 1 mL DCFH-DA which was diluted with serum-free medium to 10 M, Incubated at 37°C for 20 minutes, and mixed once every 3 minutes. Then the cells were washed twice with serum-free medium, resuspended with PBS, to be tested by Flow cytometry (Beckman coulter, cytoFLEX, United States).

ELISA assay for IL-6, IL-8, IL-1 β and TNF- α

Supernatants from GES-1 cell cultures were collected and centrifuged to remove cell debris. The concentration of IL-6, TNF- α , IL-1 β and IL-8 in the culture supernatants was determined by using a cytokine specific ELISA kit per the manufacturer's instructions. All assays were performed in triplicate in three independent experiments (FlexStation® 3, Molecular Devices, United States).

Western Blot

Total proteins were extracted with RIPA lysis buffer (Beyotime) with phosphatase and protease inhibitors, and the protein concentrations were detected with BCA Protein Assay Kit (Beyotime). SDS-PAGE was used to separate lysates, and then the proteins were transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 2 hours at room temperature, and incubated with primary antibody overnight at 4°C. After being incubated with secondary antibody, the signals were detected with ECL reagents. Antibodies used were: GAPDH, PYCARD, PARP1, Caspase-1, NLRP3, Caspase-3.

Statistical Analysis

The data was analyzed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Parametric tests were used to compare different treatments with Unpaired t-test. The data are expressed as the mean $\pm$ standard error, and the differences were considered significant as indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns: not significant.

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Compliance with ethical standards

Conflict of Interest The authors declare that there are no conflict of interest.

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Figure 1 The plant specimen picture of *Callicarpa nudiflora*.

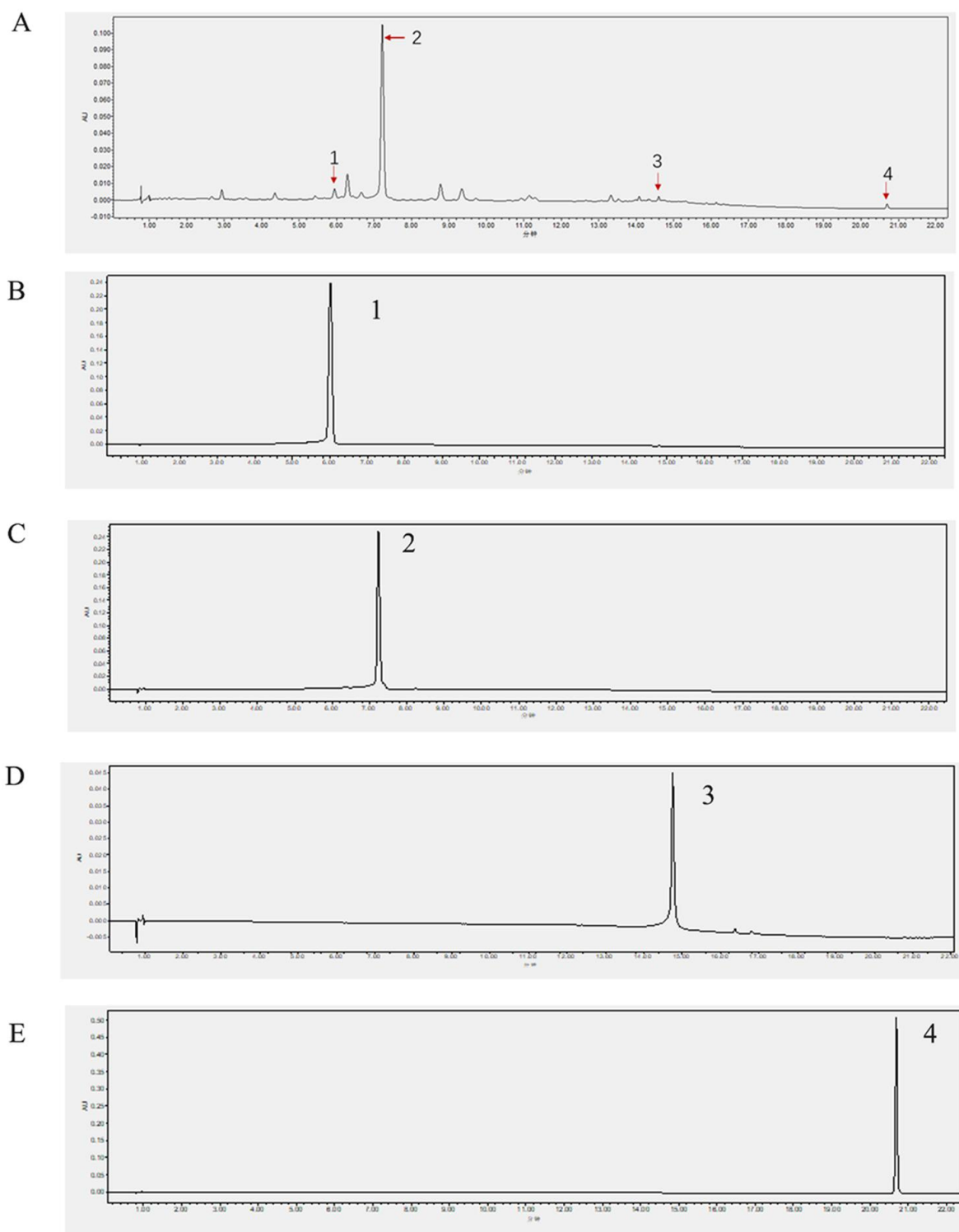


Figure 2 The UPLC spectrum of four main compounds. (A) 1: Forsythoside B (0.98%), 2: Acteoside (33.26%), 3: Luteolin (0.33%), 4: 5,4'-dihydroxy-3,7,3'-trimethoxyflavone (0.42%). (B-E) The UPLC spectrum of Forsythoside B. (B) Acteoside, (C) Luteolin, (D) 4: 5,4'-dihydroxy-3,7,3'-trimethoxyflavone, (E) standard sample.

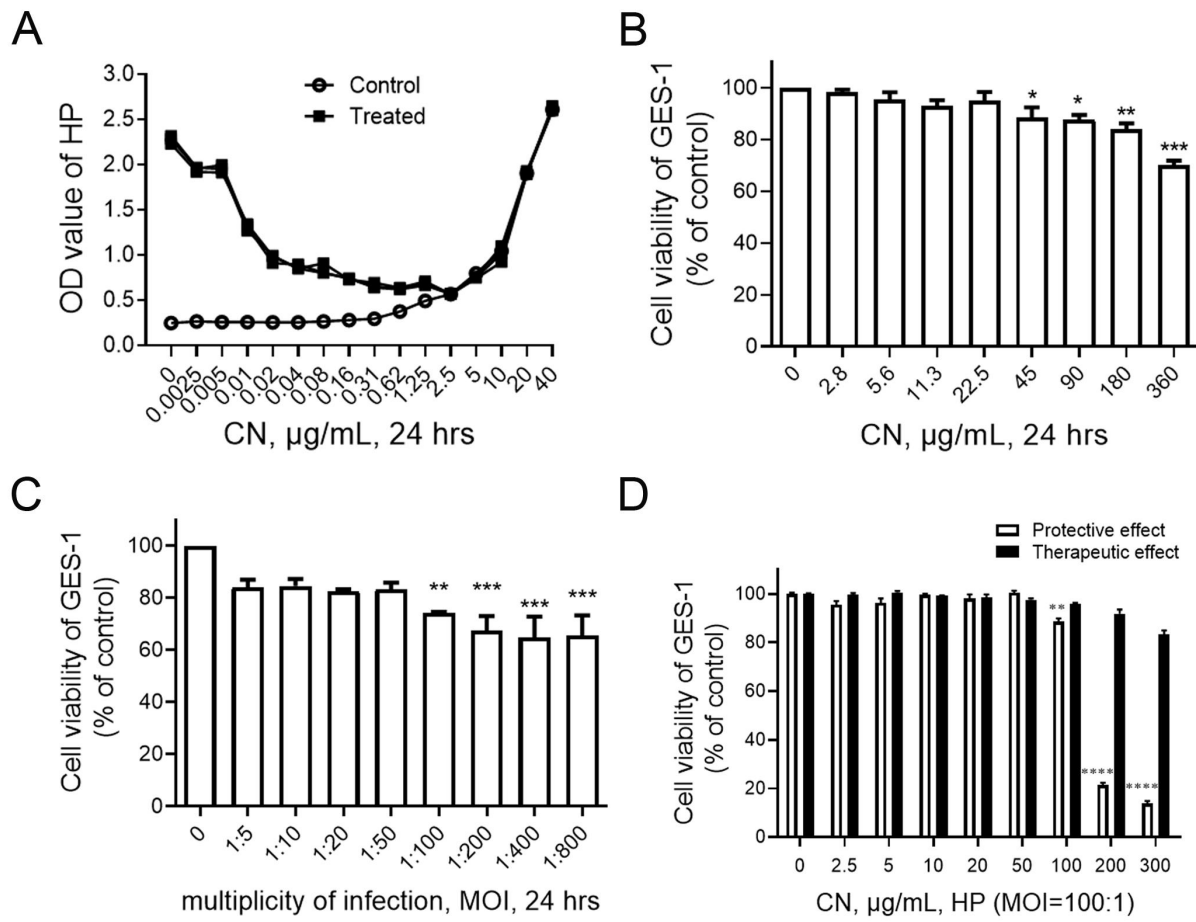


Figure 3 CN showed anti-HPSS1 activity in vitro. (A) The MIC value of CN on HPSS1. (B) Toxicity determination of CN to GES-1. (C) Determination of the proportion of GES-1 infected with HPSS1. (D) Preliminary comparison of CN prevention and treatment effects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$ compared with the control group.

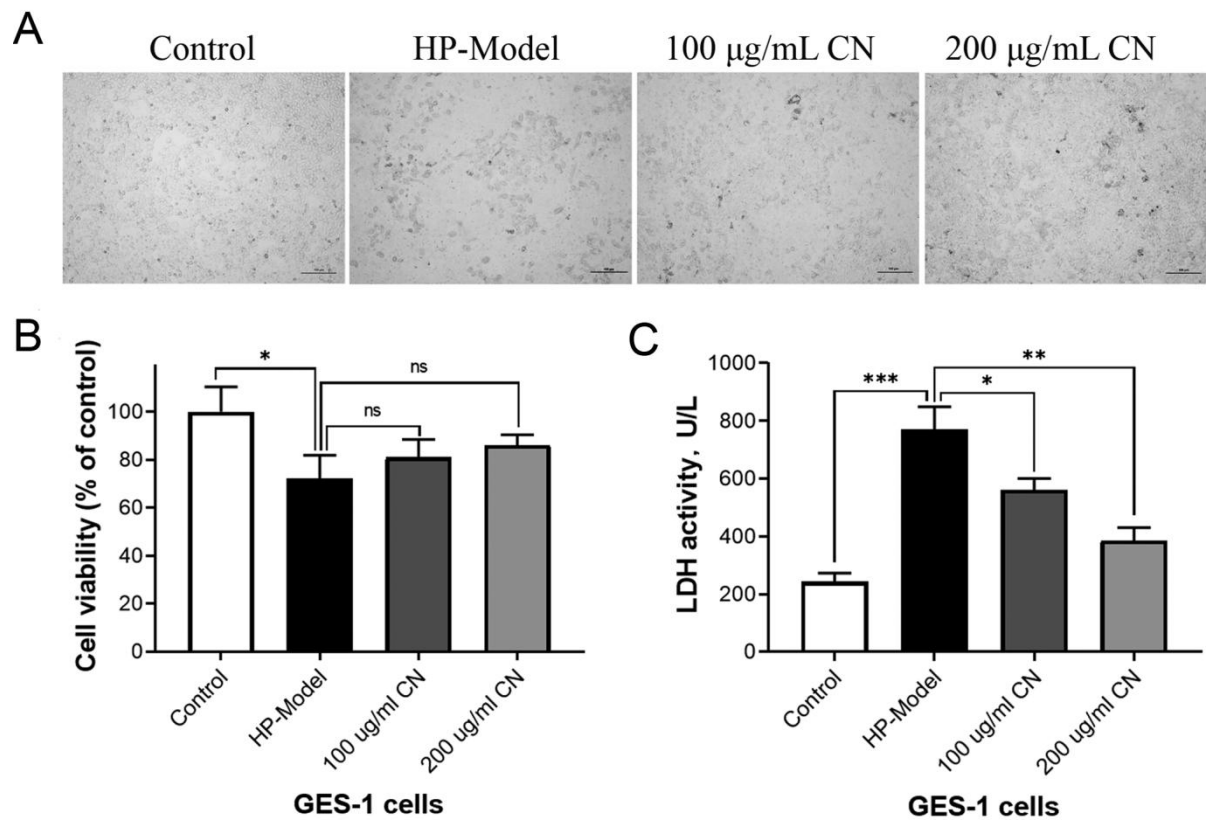


Figure 4 CN reduced the damage of HP to GES-1. (A) Morphological characterization of GES-1. Scale bar, 100 μm . (B) The effects of CN on cell viability of HP-infected GES-1. (C) The effects of CN on the percentage of LDH leakage of HP-infected GES-1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the specified group.

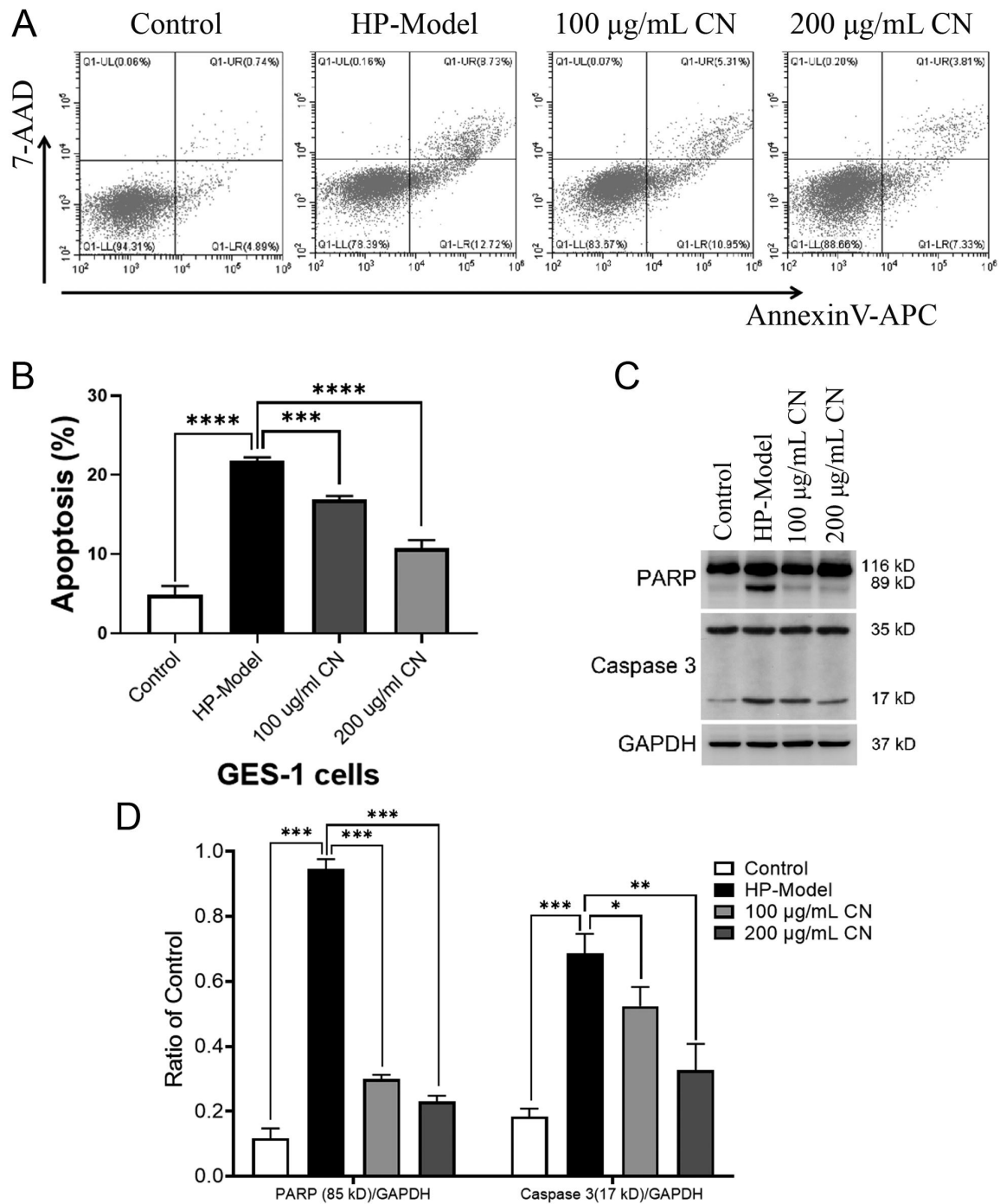


Figure 5 CN reduced the apoptosis of HP-infected GES-1. (A, B) Apoptosis detected by Flow cytometry. (C, D) Western blotting results of PARP and Caspase 3 proteins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the specified group.

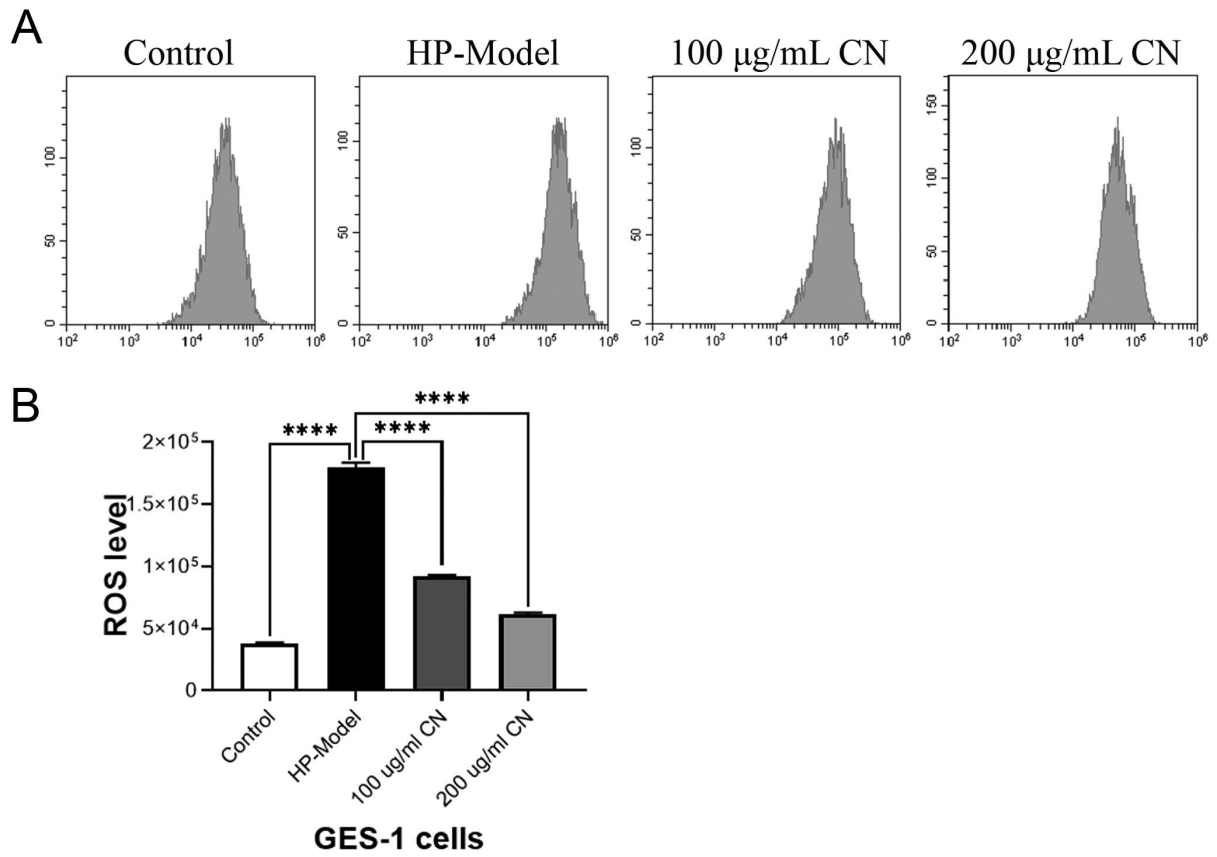


Figure 6 CN decreased the ROS production of HP-induced GES-1. (A) ROS production detected by Flow cytometry. (B) Quantitative analysis of DCFH-DA accumulation by FACS.

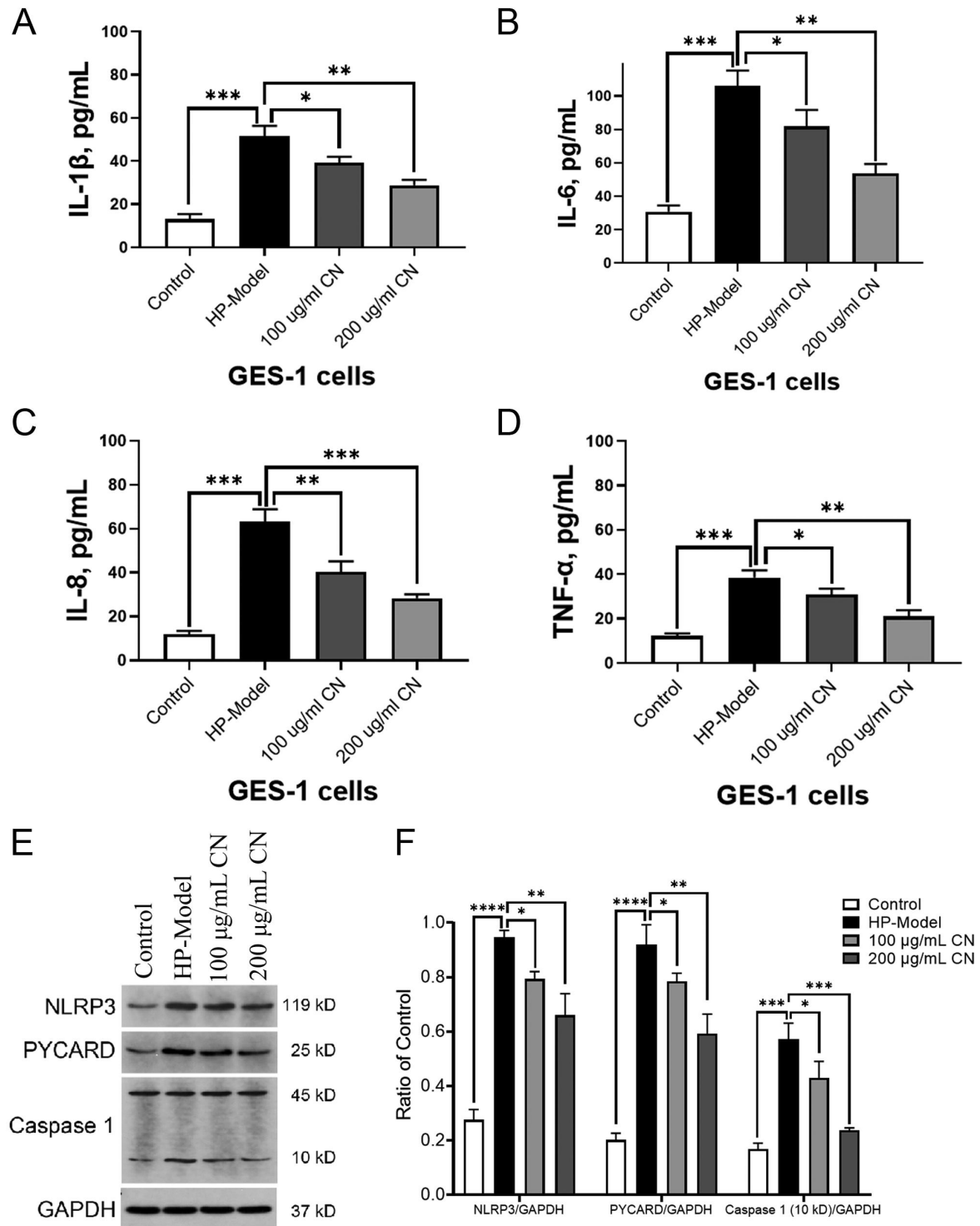


Figure 7 CN alleviated inflammatory response of HP-infected GES-1. (A-D) Cellular cytokine productions detected by ELISA. (E-F) Western blotting results of NLRP3, PYCARD and Caspase 1 proteins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the specified group.

Table 1 Identification of chemical constituents of *Callicarpa nudiflora* by UPLC-ESI-Q-TOF-MS

No.	T _R (min)	Identification	Negative ion (m/z)	ion	Positive ion (m/z)	ion	Molecular formula	Molecular weight (Da)	MS/MS (m/z)	Chemical type
			Indicate d	pp m	Indicate d	pp m				
1	2.05*	catalpol	397.090 4	-0.7	-	-	C ₁₅ H ₂₂ O ₁₀	362.121 3	-	I
2	5.16	caffeic acid	179.034 7	-1.7	-	-	C ₉ H ₈ O ₄	180.042 3	-	PA
3	5.35	8-acetyl-harpagide	405.139 9	-0.9	429.135 6	-1.2	C ₁₇ H ₂₆ O ₁₁	406.147 5	(-)165.0552 (+)149.0578	I
4	5.82	6'-O-trans-caffeoylcatalpol	523.145 6	-0.2	547.142 3	0.1	C ₂₄ H ₂₈ O ₁₃	524.153 0	(-)323.0765,161.0241 (+)325.0899,163.0369,135.0416	I
5	6.06*	verminoside	523.145 6	-0.3	547.139 9	-2.3	C ₂₄ H ₂₈ O ₁₃	524.153 0	(-)323.0766,179.0345,161.0242 (+)163.0380	I
6	6.56	β-hydroxyacteoside	639.193 0	-0.1	663.189 0	-0.5	C ₂₄ H ₂₈ O ₁₃	524.153 0	(-)475.1400,179.0347,161.0244,151.0400 (+)163.0383, 135.0421	P

7	6.67	β -hydroxyacteoside	639.192 9	-0.2 3	663.189 3	-0.2 16	C ₂₉ H ₃₆ O 3	640.200 3	(-)529.1556,475.1447,179.0347,161.0243, 133.0291 (+)163.0385,135.0438	P
8	7.18*	6-Hydroxyluteolin-7-O- β -D-glucoside	463.087 7	-1.0 3	465.101 3	-1.5 12	C ₂₁ H ₂₀ O 5	464.095 5	(-)301.0346 (+)303.0492, 301.0330	F
9	7.88	6-O- <i>trans</i> -feruloylcatapol	537.160 4	-1.7 9	561.155 9	-2.0 13	C ₂₅ H ₃₀ O 6	538.168 6	(-)285.0391, 175.0394 (+)449.1033,287.0542,177.0543,163.0382,133.0855	I
10	8.04*	forsythoside B	755.240 2	-0.3 8	779.233 8	-3.1 19	C ₃₄ H ₄₄ O 7	756.247 7	(-)593.2084,179.0344,161.0242,135.0446 (+)163.0383,135.0449	P
11	8.37*	nudifloside	523.145 0	-1.3 5	547.141 5	-0.8 13	C ₂₄ H ₂₈ O 0	524.153 0	(-)361.0918,161.0240,133.0290 (+)363.1072,163.0383,135.0427	I
12	8.60*	luteoloside	447.092 8	-1.1 0	449.107 0	-0.9 11	C ₂₁ H ₂₀ O 6	448.100 6	(-)285.0397 (+)287.0548,153.0166	F
13	8.73*	lutedin-7-O-neohesperidoside	593.151 6	0.3 2	595.165 2	-0.6 15	C ₂₇ H ₃₀ O 5	594.158 5	(-)285.0395 (+)287.0541	F
14	8.77*	acteoside	623.197 9	-0.3 5	647.194 5	-0.1 15	C ₂₉ H ₃₆ O 4	624.205 4	(-)461.1660,315.1079,179.0346,161.0243,113.0242 (+)163.0388,135.0436	P
15	9.52	isoacteoside	623.197 6	-0.9 9	647.193 9	-0.7 15	C ₂₉ H ₃₆ O 4	624.205 4	(-)461.1656,315.1070,179.0345,161.0241,135.0446 (+)163.0384,135.0430	P
16	9.70	parvifloroside B	623.197 8	-0.5 5	647.193 5	-1.2 15	C ₂₉ H ₃₆ O 4	624.205 4	(-)461.1651,179.0345,161.0241,135.0444,133.0289 (+)163.0375,135.0419	P
17	9.96*	lutedin-4'-O- β -D-glucoside	447.092 8	-1.1 3	449.106 3	-1.6 11	C ₂₁ H ₂₀ O 6	448.100 6	(-)285.0400, 133.0288 (+)287.0541	F

18	10.42	chrysoeriol-7-O- β -D-glucoside	461.108	-1.6	463.121	-2.0	C ₂₂ H ₂₂ O	462.116	(-)255.0293	F
			2		5		11	2	(+)301.0687	
19	10.51	10-O- <i>trans</i> -p-coumaroylgeniposidic acid	519.150	-0.4	-	-	C ₂₅ H ₂₈ O	520.158	(-)339.0867,313.1072,163.0395,161.0238	I
			6				12	1		
20	10.62	luteolin-3'-O- β -D-glucopyranoside	447.093	-0.2	449.106	-1.2	C ₂₁ H ₂₀ O	448.100	(-)285.0401, 133.0291	F
			2		6		11	6	(+)287.0544,153.0166	
21	11.22	6'-O- <i>trans</i> -p-coumaroyl-8-epiloganic acid	521.165	-1.5	545.161	-1.2	C ₂₅ H ₃₀ O	522.173	(-)297.1125, 195.0654,163.0396	I
			6		8		12	7	(+)179.0689,165.0536	
22	11.68	martynoside	651.229	-0.5	675.224	-1.0	C ₃₁ H ₄₀ O	652.236	(-)175.0396,161.0238,160.0161	P
			1		9		15	7	(+)195.0632,177.0543,163.0736	
23	12.06	luteolin	285.040	-1.3	287.054	-0.4	C ₁₅ H ₁₀ O	286.047	(-)179.0344,133.0291	F
	*		1		9		6	7	(+)339.1055	
24	14.02	5,4'-dihydroxy-3,7,3'-trimethoxyflavone	343.081	-1.9	345.096	-0.7	C ₁₈ H ₁₆ O	344.089	(-)328.0577,313.0347	F
	*		7		2		7	6	(+)330.0726,315.0487	

*: standard compounds, I: iridoid glycosides, F: flavanones, P: phenylethanoid glycoside, PA: phenylpropionic acid

Figure 1

中国科学院植物所

植物标本馆 [SZG]

Shenzhen Farley Herbarium

采集人: 李博, 李博, 李博

采集号: 2000

采集日期: 2000/02

采集地点: 海南五指山

海拔: 1000

经纬度: 109° 10'

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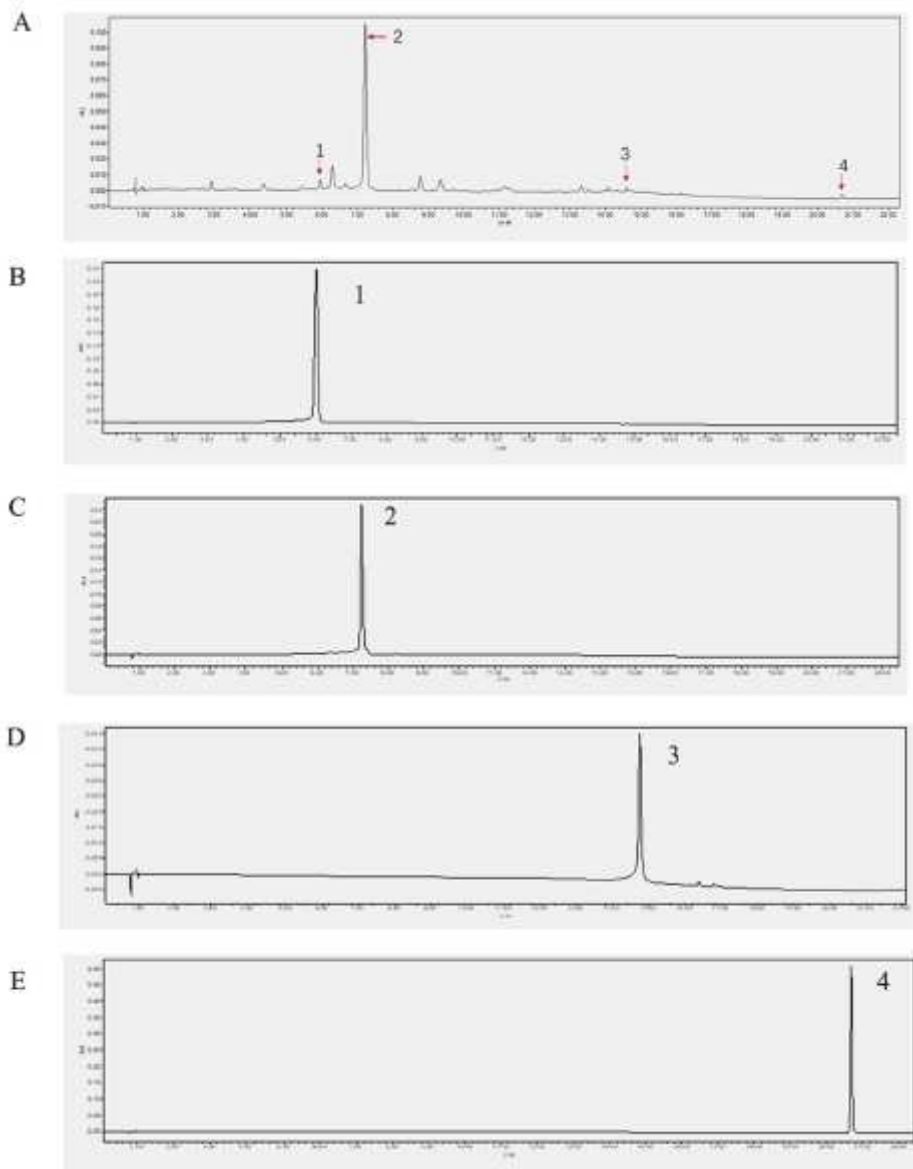


Figure 2

The UPLC spectrum of four main compounds. (A) 1: Forsythoside B (0.98%), 2: Acteoside (33.26%), 3: Luteolin (0.33%), 4: 5,4'-dihydroxy-3,7,3'-trimethoxyflavone (0.42%). (B-E) The UPLC spectrum of Forsythoside B. (B) Acteoside, (C) Luteolin, (D) 4: 5,4'-dihydroxy-3,7,3'-trimethoxyflavone, (E) standard sample.

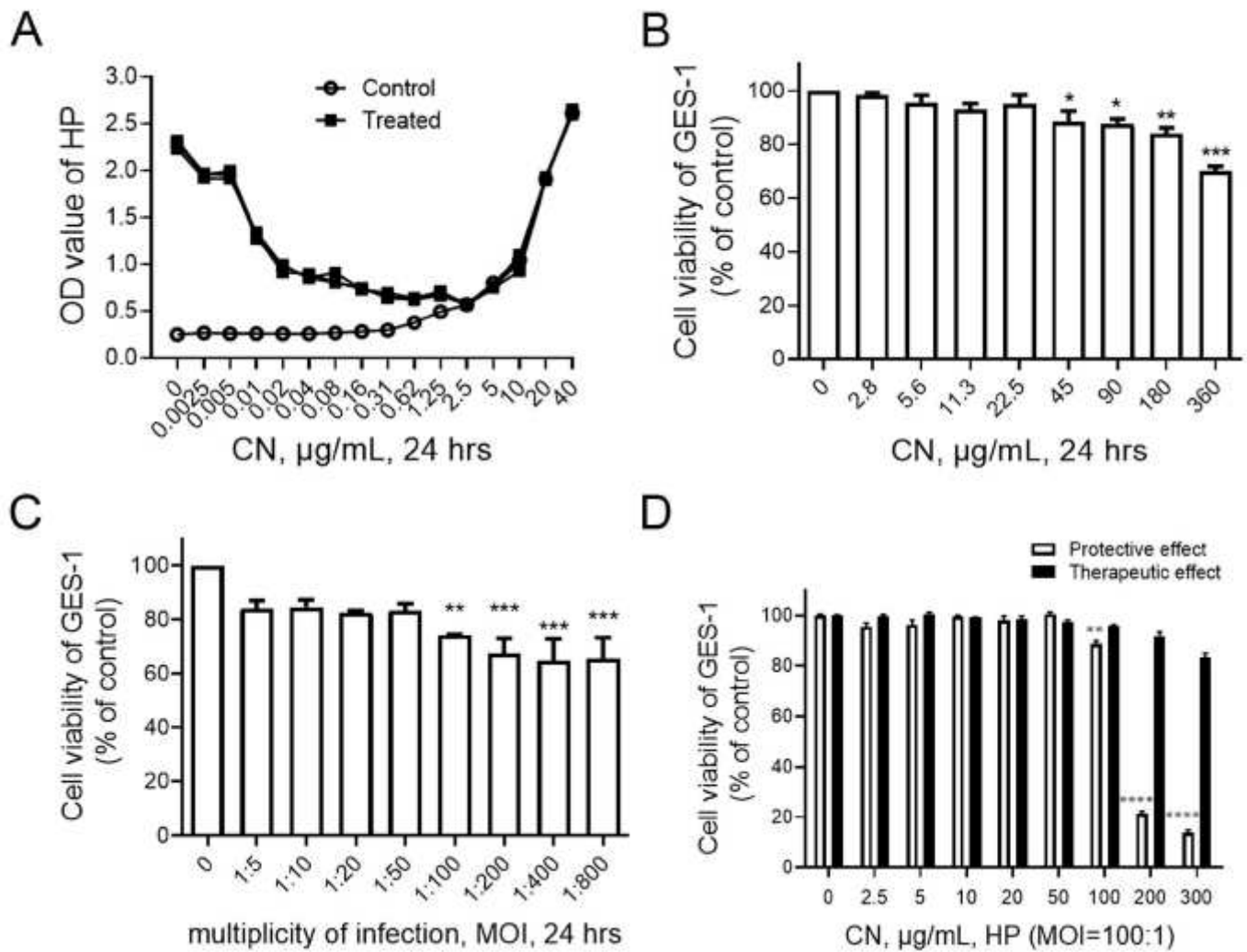


Figure 3

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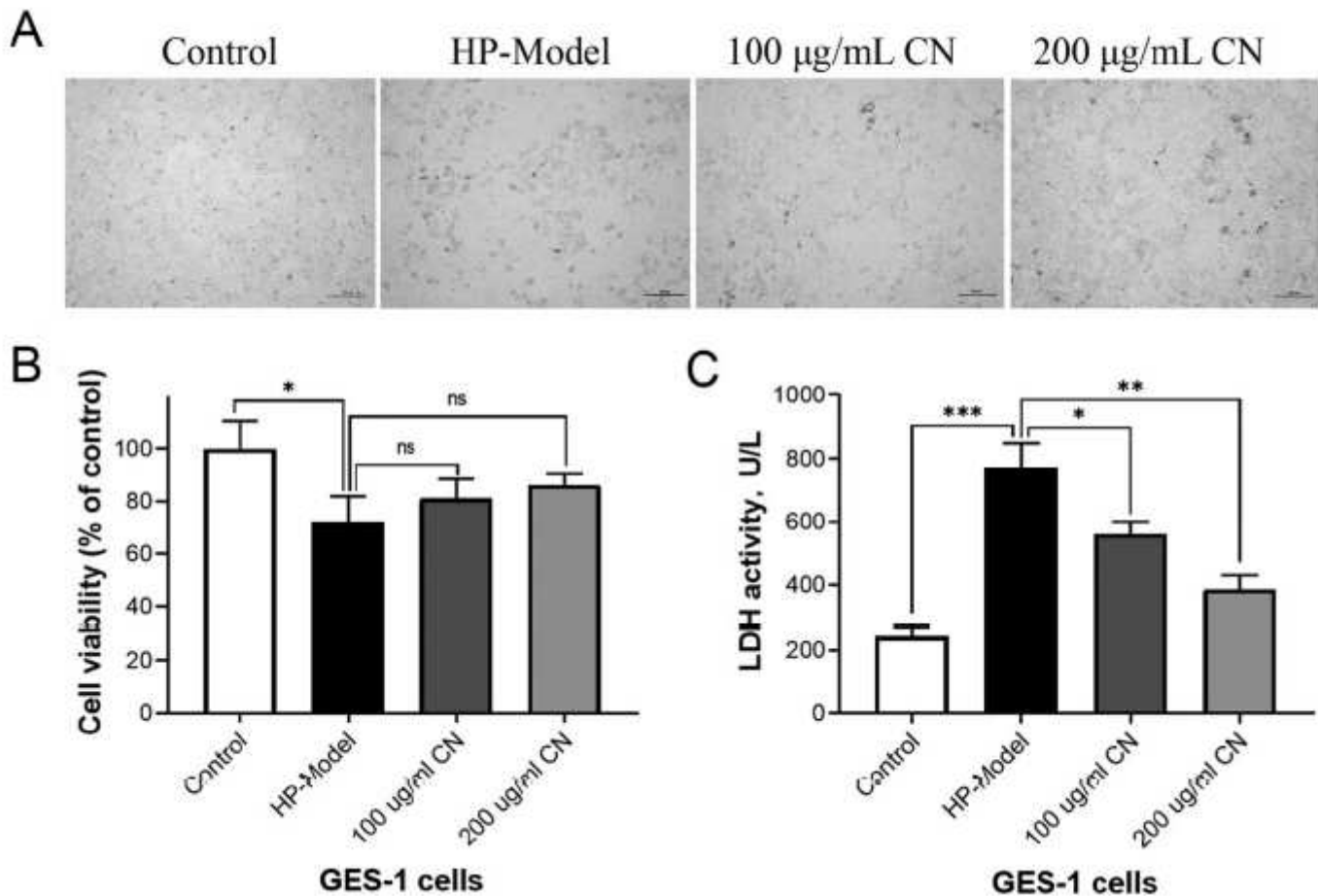


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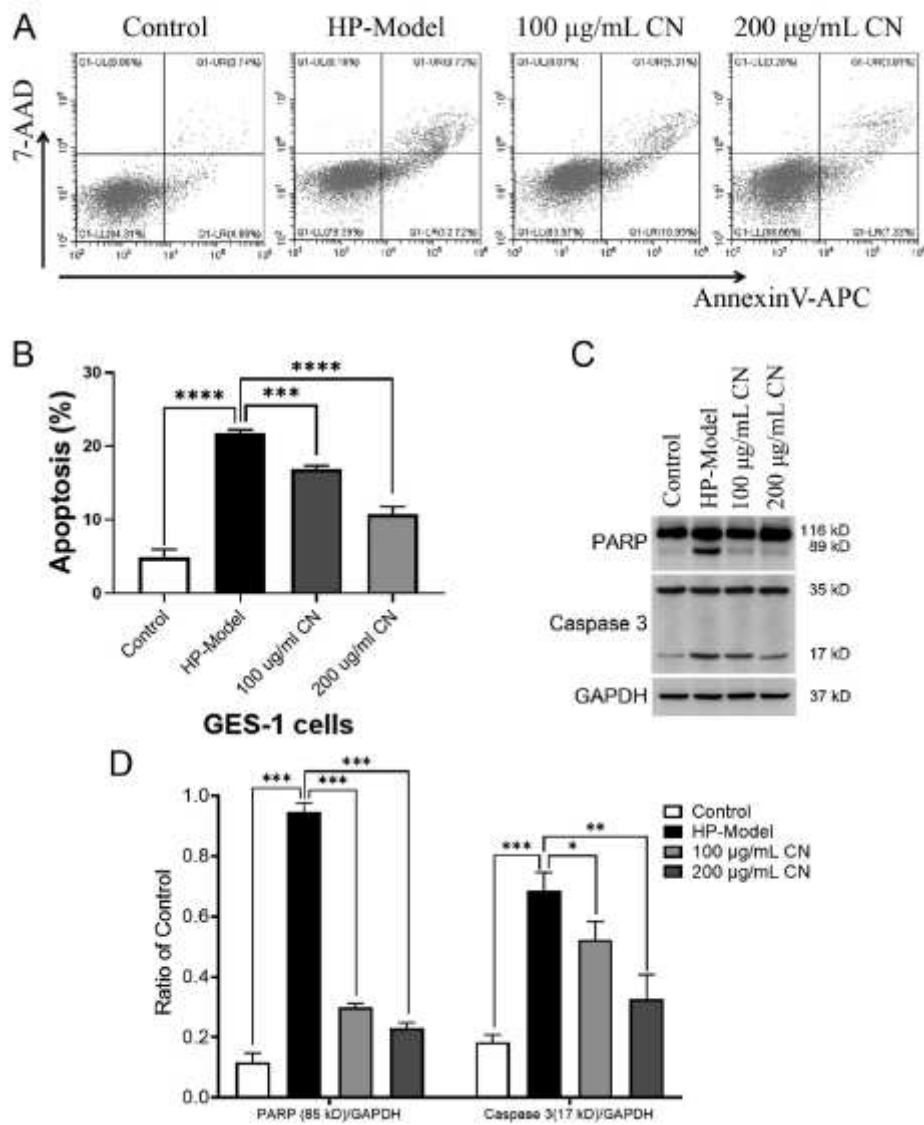


Figure 5

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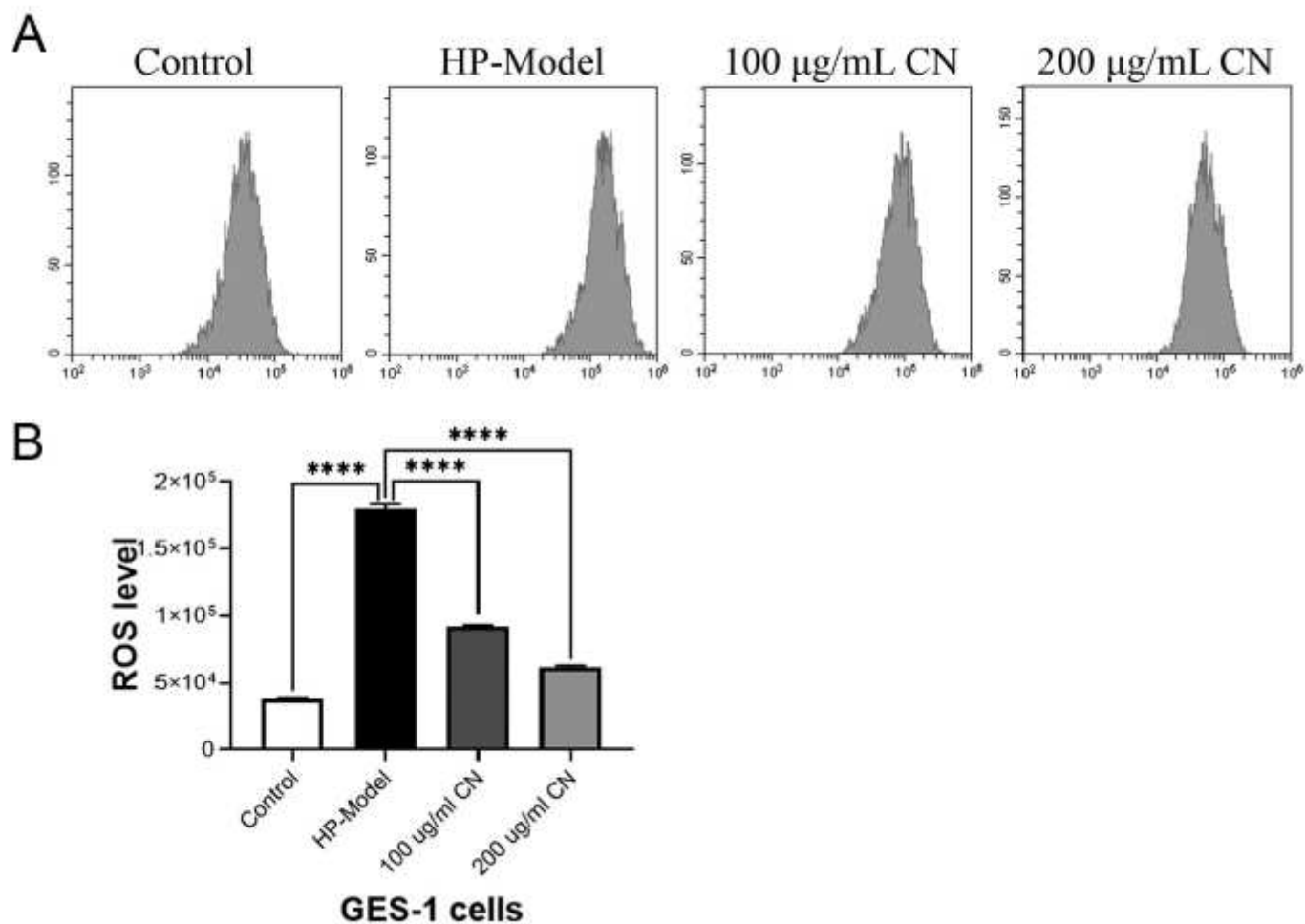


Figure 6

CN decreased the ROS production of HP-induced GES-1. (A) ROS production detected by Flow cytometry. (B) Quantitative analysis of DCFH-DA accumulation by FACS.

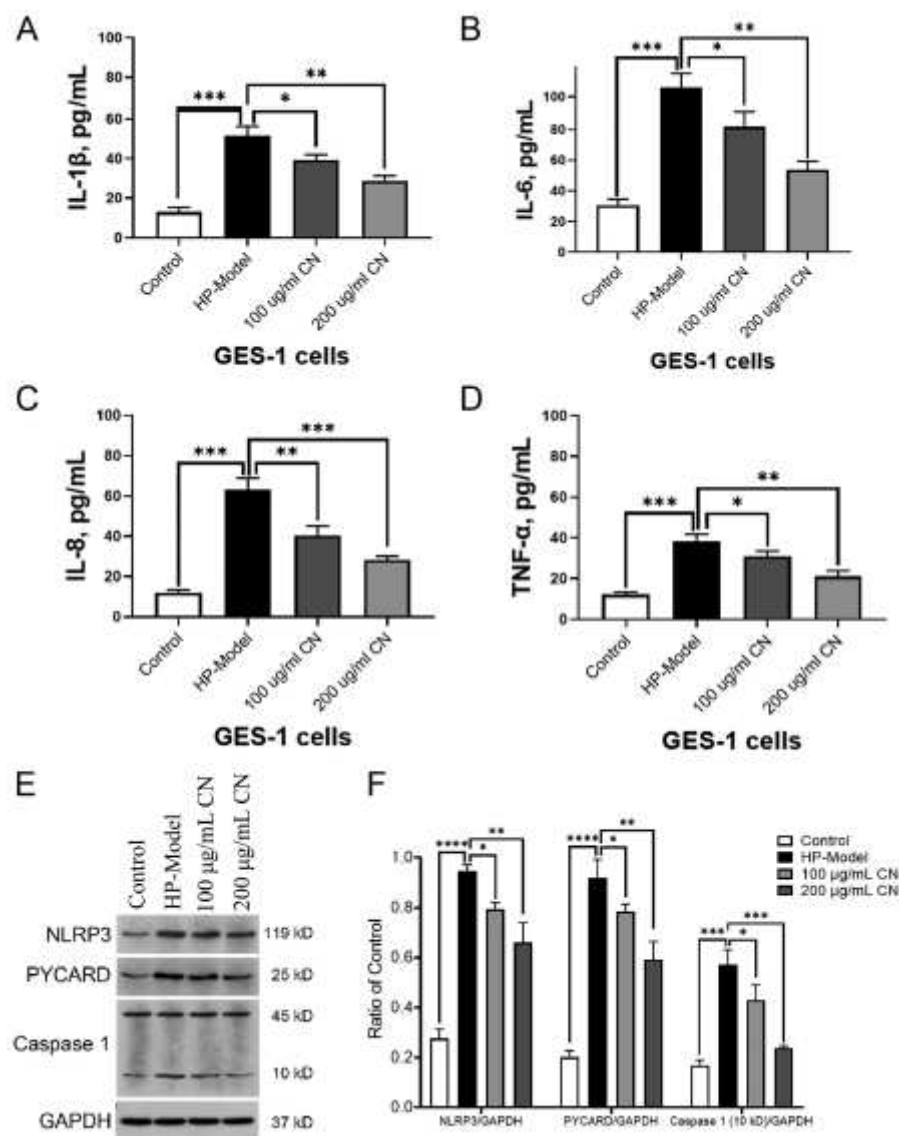


Figure 7

CN alleviated inflammatory response of HP-infected GES-1. (A-D) Cellular cytokine productions detected by ELISA. (E-F) Western blotting results of NLRP3, PYCARD and Caspase 1 proteins. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, compared with the specified group.