Original Article

Tiaochang Xiaoyan extract tablets ameliorate chronic inflammation by activating macrophage lysosomes in chronic colitis rats

Shiying Wang[1,2]^, Chun Guo[1], Tao Zhang[3], Cailing Zhong[1], Xiying Zhao[1], Yisheng Su[1], Wei Wei[3], Beiping Zhang[1]^

[1]Department of Gastroenterology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China; [2]Guangdong Province Engineering Technology Research Institute of Traditional Chinese Medicine, Guangzhou, China; [3]Department of Gastroenterology, Beijing Key Laboratory of Functional Gastrointestinal Disorders Diagnosis and Treatment of Traditional Chinese Medicine, Wangjing Hospital, China Academy of Chinese Medical Sciences, Beijing, China

Contributions: (I) Conception and design: B Zhang, S Wang; (II) Administrative support: B Zhang, W Wei; (III) Provision of study materials: S Wang, C Guo, T Zhang, C Zhong, X Zhao, Y Su; (IV) Collection and assembly of data: S Wang, C Guo; (V) Data analysis and interpretation: T Zhang, C Zhong, X Zhao, Y Su; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Wei Wei. Department of Gastroenterology, Beijing Key Laboratory of Functional Gastrointestinal Disorders Diagnosis and Treatment of Traditional Chinese Medicine, Wangjing Hospital, China Academy of Chinese Medical Sciences, No. 6, Wangjing Middle Ring South Road, Chaoyang District, Beijing 100102, China. Email: sxxtyy@sina.com; Beiping Zhang. Department of Gastroenterology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, 111 Dade Road, Yuexiu District, Guangzhou 510120, China. Email: doctorzbp@163.com.

Background: *Tiaochang Xiaoyan tablet (TCXYT)* is a traditional Chinese medicine prescription derived from the *Xianglian pill*, which is a traditional Chinese medicine for treating chronic dysentery recorded in the *Taiping Huimin Heji Bureau* [1078–1085]. For many years, *TCXYT* has been used to treat ulcerative colitis, however, its therapeutic mechanism is still unclear. In the present study, we used colonic lamina propria macrophages (LPM) and mouse-derived macrophage cell line RAW264.7 cells as the research objects, with the aim of exploring the therapeutic effects and mechanisms of *TCXYT* on colitis.

Methods: We used 2,4,6-trinitrobenzenesulfonic acid (TNBS) to induce a rat model of chronic colitis, and normal rats as the control. The disease activity index (DAI) and colonic histopathological changes of rats were used to evaluate the severity of colitis. Rats were divided into the control group; model group; high, middle-, and low-dose TCXYT group; and the hydroxychloroquine sulfate group. *TCXYT* was administered by gavage on the 3rd day after model replication and lasted for 7 days. The doses used for the high-, middle-, and low-dose *TCXYT* groups were 0.8, 0.4 and 0.2 g/kg, respectively. Enzyme-linked immunosorbent assay was used to detect the serum concentration of cytokines. Western blot was used to detect the expressions of Toll-like receptor 9 (TLR9), myeloid differentiation primary response 88 (MyD88), interleukin (IL) receptor-associated kinase (IRAK) 1, and IRAK4 in colonic LPM and RAW264.7 cells. Immunofluorescence was used to detect lysosomal activity. The chemical constituents of *TCXYT* were separated and identified based on Q-Orbitrap high resolution LC/MS data.

Results: *TCXYT* promoted the repair of colonic mucosal injury, attenuated inflammation, increased lysosome activity in macrophages, and decreased the DAI in rats with colitis compared with those in the model group. *TCXYT* decreased the serum concentrations of IL-1β and tumor necrosis factor-α (TNF-α), increased those of IL-4 and IL-10, and decreased the TLR9, MyD88, IRAK1, and IRAK4 protein levels in LPM and RAW264.7 cells compared to the model group.

Conclusions: *TCXYT* could ameliorate colon inflammation and $CD11c^+$ macrophage infiltration in rats with chronic colitis. This effect may be mediated by activating lysosomes in macrophages by inhibiting the TLR9/MyD88/IRAK signaling pathway.

^ ORCID: Shiying Wang, 0000-0002-1005-0381; Beiping Zhang, 0000-0003-2979-9226.

Keywords: *Tiaochang Xiaoyan tablet* (*TCXYT*); ulcerative colitis; macrophages; lysosome; inflammation

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Introduction

Ulcerative colitis (UC) is a chronic and progressive colonic inflammatory disease with recurrent mucosal inflammation and mucosal damage. Recurrent inflammation in colonic mucosa is the most important pathological factor that leads to the occurrence of mucosal ulcers (1,2), and has been linked to increased risk of ulcer-associated colorectal cancer (3). It was found in previous studies that the occurrence of colonic mucosal inflammation in UC patients is closely related to mucosal hypoxia, abnormal activation of inflammatory cells, and autophagy disorder (4,5). Abnormal colonic mucosal inflammation is not only related to the toxin and pathogen translocation to submucosa that is caused by the increased permeability of UC colonic mucosa but is also closely related to the clearance of apoptotic body and necrotic fragments and abnormal immune mediators, and then destroy the immune homeostasis (6,7). Some studies have suggested that the accumulation of apoptotic fragments can lead to tissue damage and the destruction of immune homeostasis, which further leads to abnormal autoimmune and immune homeostasis (8,9). In addition, it was discovered that mucosal damage in colonic mucosa of UC is related to abnormal immune homeostasis (7,10), which is caused by the formation of an abnormal immune complex in colon mucosa. In this process, phagocytosis of macrophages plays an important role in removing pathogenic microorganisms, the clearance of apoptotic body, and other abnormal immune mediators, and maintaining the immune homeostasis of colonic mucosa (9). The lysosome is considered an important organelle in macrophages, which plays an important role in regulating the immune functions of macrophages. Previous studies have found that the maturation of macrophage lysosomes is important for the stabilization of macrophage-mediated autophagy, immune presentation, and other functions (11,12). However, the mechanism of lysosomal function in macrophages is unclear. The Toll-like receptor (TLR) signaling pathway plays an important role in the development of colonic mucosal inflammation in UC (13). Previous studies have found that the activation of the TLR9 signaling pathway in colonic lamina propria macrophages (LPM) can significantly inhibit macrophage autophagy, aggravate the inflammatory response, and increase the apoptosis of mucosal epithelial cells (13-16). Furthermore, the dysfunction of lysosomal activity in LPM has been found to be the main cause of macrophage dysfunction (14). In addition, the TLR9 signaling pathway is involved in the regulation of autophagy of LPM in UC (13), but the effect of the TLR9 signaling pathway on the regulation of lysosomal structure and the function of LPM is unclear. Therefore, the aim of the present study was to explore the role and mechanism of lysosomes in LPM on chronic inflammation.

UC belongs to the categories of diarrhea, dysentery, and intestinal wind of traditional Chinese medicine. Chronic recurrence belongs to the categories of rest dysentery and chronic dysentery of traditional Chinese medicine, which are characterized by long unhealing and easy recurrence. According to the theory of traditional Chinese medicine, it is generally believed that ulcerative colitis is mainly caused by spleen and kidney deficiencies and stagnation of dampness, heat, phlegm, and qi (17). The onset of UC is believed to be related to pathogenic toxins in the body (18). It is believed that the pathogenic toxins are the root of UC, which can cause UC. Therefore, in terms of treatment, detoxification is considered the main method of treatment. Detoxification is used throughout the whole process of UC and is supplemented by heat and damp clearance, qi activation, and blood circulation. *TCXYT* is derived from the Xianglian pill, which is a traditional Chinese medicine for treating chronic dysentery recorded in the Taiping Huimin Heji Bureau [1078–1085]. *TCXYT* consists of *Radix Astragali*, *Lindera aggregata*, *Rhizoma coptidis*, *Oldenlandia diffusa and coix seed*. Our previous research found that *TCXYT* can effectively inhibit the chronic inflammation of colonic mucosa and promote the repair of colonic ulcers in patients with ulcerative colitis; the mechanism may be related to inhibiting the autophagy function of colon cells by regulating the balance of pro-inflammatory and anti-inflammatory factors (19-21). Our findings indicated that *TCXYT* can regulate the lysosomal activity of macrophages

in the colon of rats with chronic colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). We conducted the following research.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/apm-21-250).

Methods

Animals

Sprague-Dawley rats (140–160 g, certificate No. 4400210019232) were obtained from the Experimental Animal Center of Southern Medical University, Guangzhou, China (license No. scxk-Guangdong-2006-0015). The Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Guangzhou University of Chinese Medicine approved all of the procedures involving the rats (animal ethics approval No. 2016021-2). Experiments were performed in compliance with the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine's guidelines for the care and use of animals. We quantitatively analyzed the weight, fecal texture, and other metrics of the rats. The rats were housed in a pathogen-free environment and were allowed to acclimatize for 7 days before use. These rats were divided into the control group (n=6); model group; the high-, middle-, and low-dose *TCXYT* groups; and the hydroxychloroquine sulfate (HS) group. (The dosage of TCXYT in the trial is calculated based on the clinical dosage. For example, the dosage for rats is 60 mg/kg × 70 kg × 0.018/200 g =378 mg/kg. In the experiment, each rat weighed about 400 g, and given 2.5 mL of the drug solution with a concentration of 0.06 g/mL by gavage. Therefore, the dosage of each rat was 375 mg/kg. In order to facilitate the configuration of the drug, 0.4 g/kg of the trial drug was used as the medium dose, 1/2 times was the low dose, and 2 times was the high dose.)

Reagents

TNBS and lipopolysaccharide (LPS) were purchased from Sigma (St Louis, MO, USA). The Lysosome Staining Kit was purchased from Abnova (Taipei City, Taiwan, China). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-1β, IL-4, IL-10, and tumor necrosis factor-α (TNF-α) were from R&D Systems (Minneapolis, MN, USA). HS (an inhibitor of autophagy and TLR7/9) was obtained from Selleck Chemicals (Houston, TX, USA). Mouse interferon-γ (IFN-γ), IL-1R-associated kinase (IRAK) 1, and IRAK4 were from Cell Signaling Technology (Danvers, MA, USA). CD11c (ab11029), Microtubule Associated Protein 1 Light Chain 3 Beta (LC3B, ab192890), TLR9 (ab134368), and myeloid differentiation primary response 88 (MyD88) antibodies, goat anti-rabbit antibodies, and rabbit anti-mouse antibodies were from Abcam (Cambridge, UK).

Drugs

TCXYT is a herbal preparation that consists of *Radix Astragali seu Hedysari*, *Radix Linderae*, *Rhizoma Coptidis*, *Herba Hedyotis* and *Semen Coicis* (Table S1). In addition, all herbal medicines were purchased from Lingnan Traditional Chinese Medicine Co. Ltd. (Guangzhou, China) and provided by the Department of Pharmacy at The Second Affiliated Hospital of Guangzhou University of Chinese Medicine. Quality of herbal medicines was tested according to the standards of the Pharmacopoeia of the People's Republic of China [2015] before the experiment.

Sample preparation and Q-Orbitrap high resolution LC/MS analysis

The methods and further details used have been described elsewhere (22,23). The extract of TCXYT was powdered and passed through 100-mesh sieves. An aliquot of 50 mg of powder was extracted in 10 mL of 70% methanol (v/v) for 30 min by ultrasonication (40 kHz, 300 W). The sample was maintained at room temperature for 5 min, and the supernatant was filtered through a 0.22-μm membrane before use. An aliquot of 1 μL was injected for analysis.

A UHPLC Ultimate 3000 instrument coupled with a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used. Samples were separated on a Welch Ultimate Polar reversed-phase C18 column (150×2.1 mm 1.8 μm). Mobile phase A is an aqueous solution containing 5% methanol-0.1% formic acid and 2 mmol/L ammonium formate; and mobile phase B was a methanol solution containing 15% isopropanol+0.1% formic acid. A gradient elution program was used as follows: 0–5 min, 20% A; 5–10 min, 20–50% A; 10–15 min, 50–80% A; and 15–25 min, 80–95% A. The flow rate was 0.30 mL/min, and the column temperature was maintained at 35 ℃. The mass spectrometer was operated in the

(+/–) electrospray ionization (ESI) mode. The parameters were as follows: spray voltage: 3.8kV, sheath gas pressure: 40 arb, Aux gas pressure: 10 arb, capillary temperature: 350 ℃, heater temperature: 300 ℃, scan mode: full MS (Mass spectrometry) (resolution 70,000), and scan range: m/z 100–1,500. The data collected by high-resolution liquid quality were collected by CD2.1 (Thermo Fisher), and then the database was retrieved and compared (mzCloud, mzVault, ChemSpider).

Lysosome staining

RAW64.7 cells and LPM (100 μL/well, 1.0×10^5/mL) were cultured in 96-well plates for 24 h. Lysosome staining was detected with the lysosome staining kit (Abnova, KA4111, Taibei City, Taiwan, China). When cells were properly fused, the cells were transferred to the corresponding culture medium and 100 μL of Lyso Green working solution (20 μL of 500× Lyso Green stock solution in 10 mL of live cell staining buffer) was added as described previously (8). The cells were incubated at 37 ℃ in an atmosphere containing 5% CO_2 for 1 h. Finally, the cells were visualized under a fluorescence microscope with a fluorescein isothiocyanate (FITC) filter set (excitation and emission at 490 and 525 nm, respectively).

Lysosome activity

Lysosome activity was assayed as described previously (24). Briefly, RAW264.7 cells were solubilized in 25 μL of 0.1% Triton X-100. Next, the lysates were incubated with 150 μL of 10 mM p-nitrophenyl phosphate (Sigma, USA) for 1 h at 37 ℃. The reaction was stopped by adding 50 μL of 0.2 M borate buffer, and absorbance of the mixture at 405 nm was determined using a spectrophotometer. Relative lysosome activity (%) was calculated as the ratio of the absorbance at 405 nm of *TCXYT*-treated cells to that of control cells multiplied by 100%.

Immunofluorescence

Colonic tissue or treated cells were fixed with 4% (w/v) paraformaldehyde (Sigma, USA), and blocked and incubated with an anti-CD11c antibody (1:100) overnight at 4 ℃. The cells were then washed in phosphate-buffered saline (PBS). After incubation with a secondary FITC-conjugated antibody and 4',6-diamidino-2-phenylindole (DAPI; Sigma, USA), the cells were rewashed in PBS, mounted in anti-fade reagent, and observed under an Olympus microscope, as described previously (25,26).

Statistical analysis

Data were analyzed using IBM SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and were presented as mean ± standard error of mean. One-way analysis of variance or a general linear model with repeated measures was used to analyze the data of ≥3 groups, and the least significant difference post-hoc test was used for multiple comparisons. Student's *t*-test was used to analyze differences between two groups. $P<0.05$ indicated statistical significance.

TNBS-induced chronic colitis, the disease activity index (DAI), ELISA, histological analysis and Western blot are described elsewhere (27,28).

The isolation of colonic LPM, cell culture, cell viability, and proliferation have been described elsewhere (29-31).

Results

UPLC/UV chromatograms of the TCXYT extract

The *TCXYT* extract was separated within 20 min on a C18 column (150×2.1 mm 1.8 μm) eluted with acetonitrile, methanol, and water containing 0.1% formic acid. The compounds were detected in the (–) ESI mode, and their MS/MS spectra were analyzed in an untargeted manner. Although *TCXYT* exhibited only a few major peaks in the HPLC/UV analysis, a number of minor compounds could be observed in the enlarged chromatogram (*Figure 1*). A total of 174 compounds were identified from *TCXYT* (Table S2). The chemical analysis of *TCXYT* extract served as the quality control for the reproducibility of the animal experiment.

TCXYT significantly ameliorates inflammation and colonic mucosal injury

After the colitis model was established, *TCXYT* administration was initiated at day 3 for 7 consecutive days (*Figure 2A*). Compared with the control group, colonic mucosal injury was aggravated in the model group, as evidenced by increased DAI, IL-1β, and TNF-α serum levels, and decreased IL-4 and IL-10 levels (*Figure 2B,C,D,E,F,G*). Treatment with *TCXYT* for 7 days significantly ameliorated injury to the colonic mucosa (*Figure 2G*). *TCXYT* was also found to significantly decrease

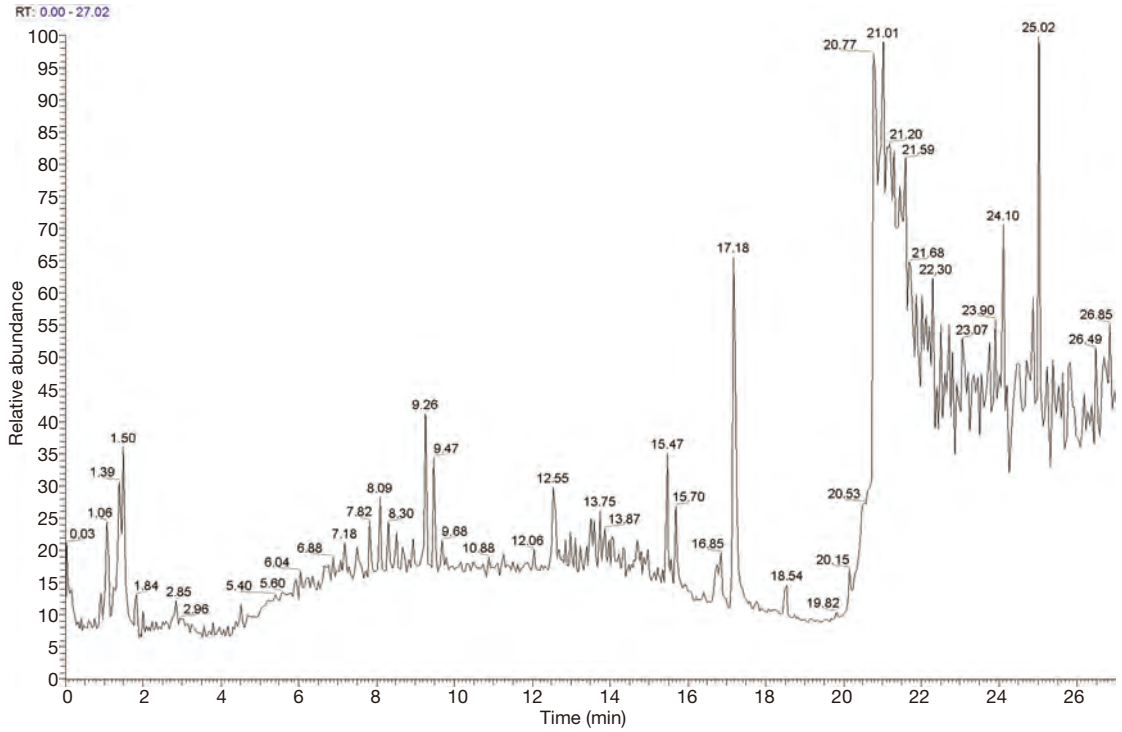

Figure 1 Ultra-high-performance liquid chromatography/UV of *Tiaochang Xiaoyan tablet* (*TCXYT*) *extract* (270 nm).

the DAI and the level of serum pro-inflammatory cytokines in a dose-dependent manner (*Figure 2B,C,D*), and increase the level of anti-inflammatory cytokines (*Figure 2E,F*).

TCXYT reduces the infiltration of CD11c⁺ macrophages in colonic mucosa

Compared with the control group, the degree of CD11c⁺ macrophage infiltration in the model group was more severe (*Figure 3A*), and the number of CD11c⁺ cells in the lamina propria was greater (*Figure 3B*). Compared with the model group, the infiltration degree and number of CD11c⁺ macrophages in the high-, middle-, and low-dose *TCXYT* groups and the HS group decreased. The *TCXYT* group was dose dependent, and there was no significant difference between the middle-dose group and the high-dose group (*Figure 3A,B*). In short, *TCXYT* can reduce the infiltration of CD11c⁺ macrophages and the number of CD11c⁺ cells in the lamina propria in rats with chronic colitis.

TCXYT promoted the activation of lysosome in LPM

The number and activity of lysosomes in LPM in the experimental colitis groups decreased (*Figure 4A,B,C*). After 7 days of treatment with *TCXYT*, the number and activity of lysosomes in LPM significantly increased compared with those in the model group (*Figure 4A,B,C*). In addition, *TCXYT* regulated the lysosomal activity in LPM in a dose-dependent manner (*Figure 4C*). The expression of LC3B in LPM significantly decreased in the colitis groups compared with the control group (*Figure 4D*). However, after 7 days of treatment with *TCXYT*, the expression of LC3B in LPM significantly increased compared with the model group. These effects were more significant in the medium- and high-dose groups than in the low-dose group (*Figure 4A,B,C,D*).

TCXYT increases the viability, but not the proliferation, of RAW264.7 cells

The viability of RAW264.7 cells was reduced, and their proliferation was reduced significantly, by LPS+IFN-γ (*Figure 5A,B*). However, the viability of RAW264.7 cells increased significantly with *TCXYT* in a dose-dependent manner (*Figure 5C*), and their proliferation was unaffected by treatment with *TCXYT* for 24 and 48 h (*Figure 5D*). *TCXYT* also significantly increased the viability of RAW264.7 cells stimulated with LPS (10 μg/mL) plus

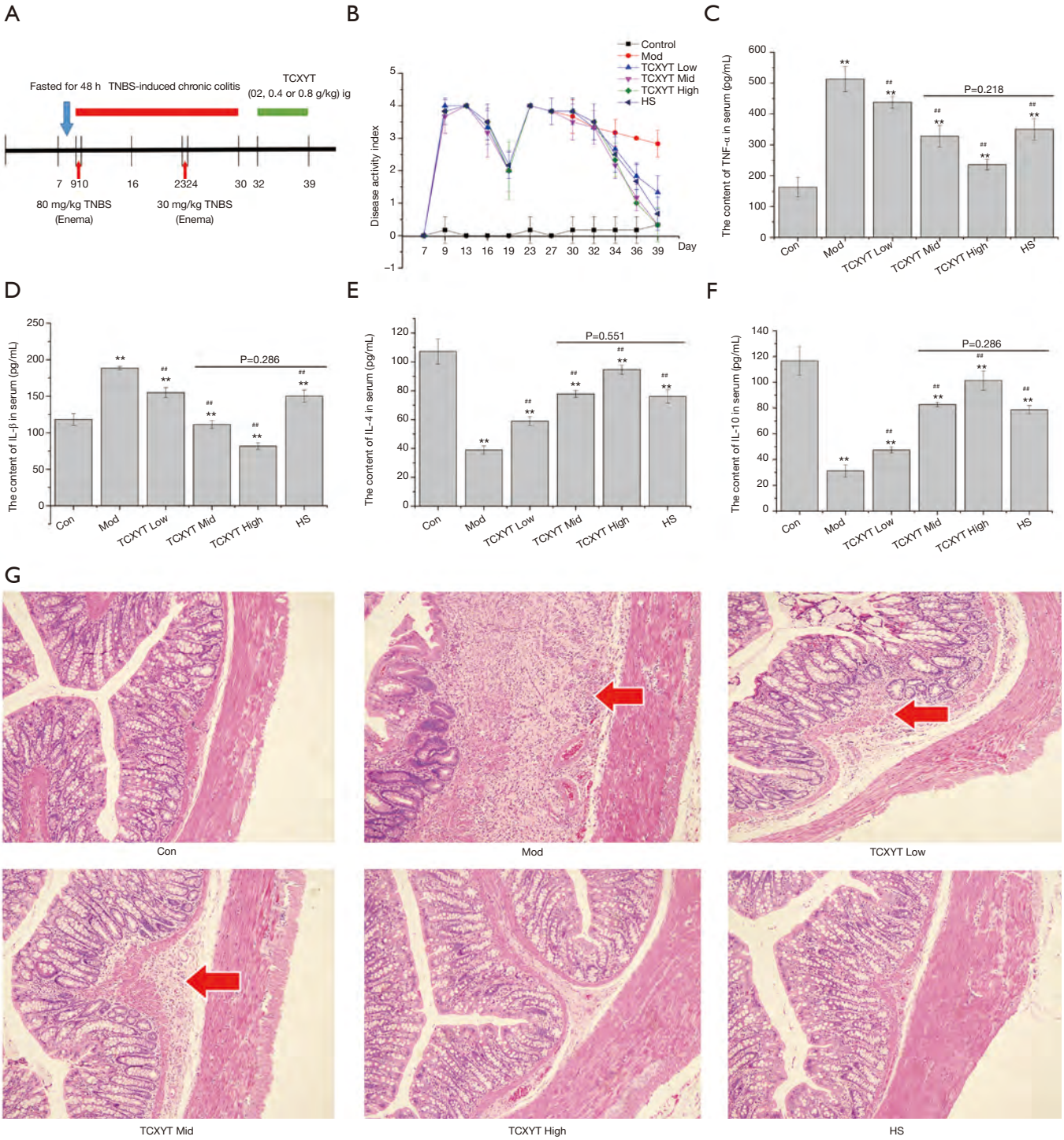

Figure 2 *Tiaochang Xiaoyan tablet* (TCXYT) ameliorates inflammation and colonic mucosal injury. (A) *TCXYT* administration was initiated at day 3 for 7 consecutive days. (B,C,D,E,F) Colonic mucosal injury; daily activity index (DAI) scores; and interleukin (IL)-1β, tumor necrosis factor-α, IL-4, and IL-10 levels. (G) Histopathological analysis of colon tissue (magnification ×400). The red arrows indicate colonic mucosal inflammatory injuries. Data are presented as mean ± standard error of mean of 3 independent experiments performed in triplicate. **, P<0.01 *vs.* control; ##, P<0.01 *vs.* model. Con: Control group; Mod: Model group; *TCXYT* Low, Mid and High: low-, middle-, and high-dose *TCXYT*; HS: HS group (treated with HS).

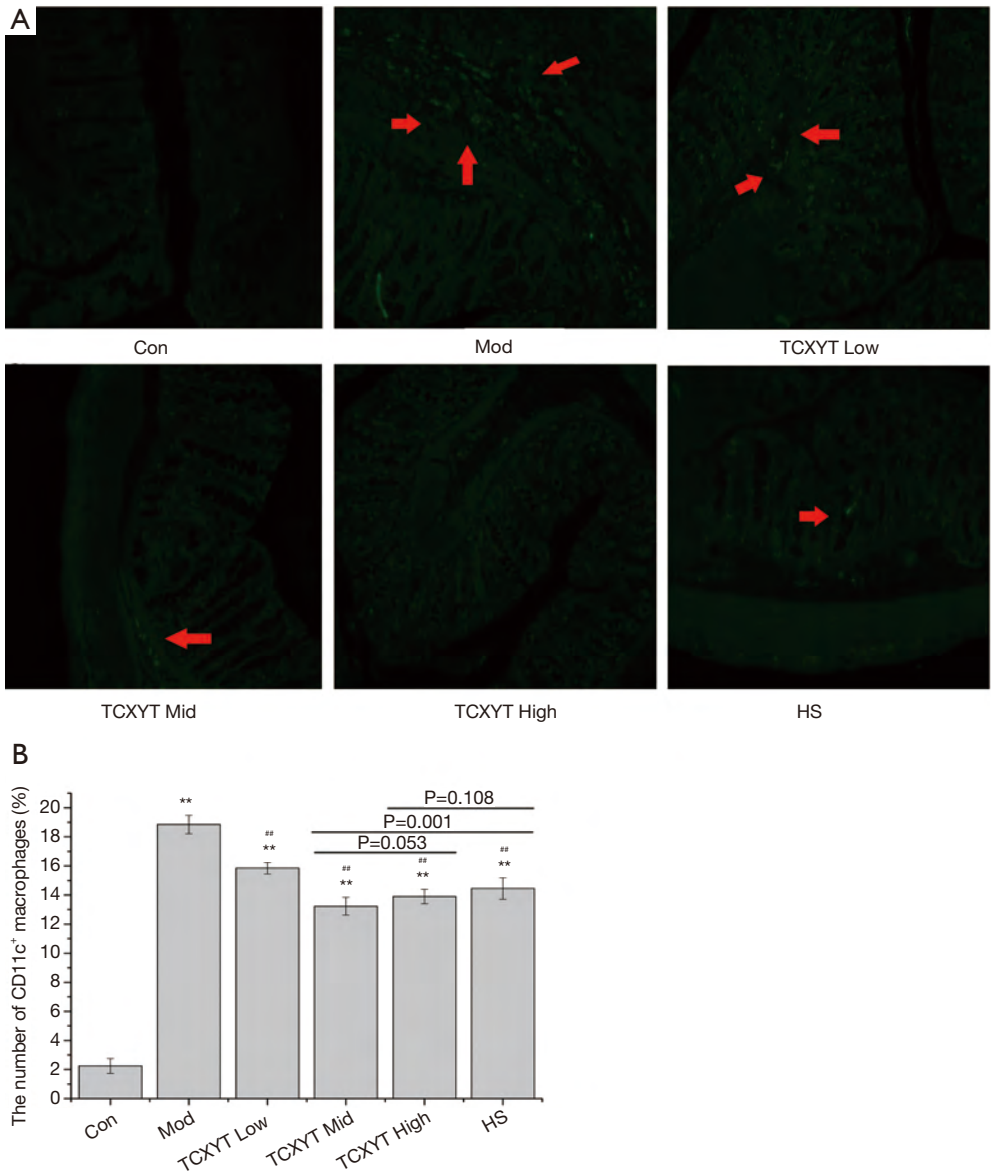

Figure 3 *Tiaochang Xiaoyan tablet* (*TCXYT*) reduces CD11c$^+$ macrophage infiltration in the colonic mucosa. (A) Infiltration of CD11c$^+$ macrophages (CD11c$^+$ macrophages was showed by the arrows, magnification ×400); (B) number of CD11c$^+$ lamina propria macrophages in colon tissue. Data are presented as mean ± standard error of mean of 3 independent experiments performed in triplicate. One-way analysis of variance and Student's *t*-test. **, P<0.01 *vs.* control; $^{\#\#}$, P<0.01 *vs.* model. The red arrows indicate CD11c$^+$ lamina propria macrophages. Con: Control group; Mod: Model group; *TCXYT* Low, Mid and High: low-, middle-, and high-dose *TCXYT*; HS: HS group (treated with HS).

IFN-γ (10 ng/mL) in a dose-dependent manner, but did not influence their proliferation. On the basis of this, we used *TCXYT* at 0.12, 0.06, and 0.03 g/mL in subsequent experiments.

TCXYT activates lysosomes in RAW264.7 cells

The number and activity of lysosomes significantly increased with *TCXYT* compared with the control group in a dose-dependent manner (*Figure 6A,B,C*).

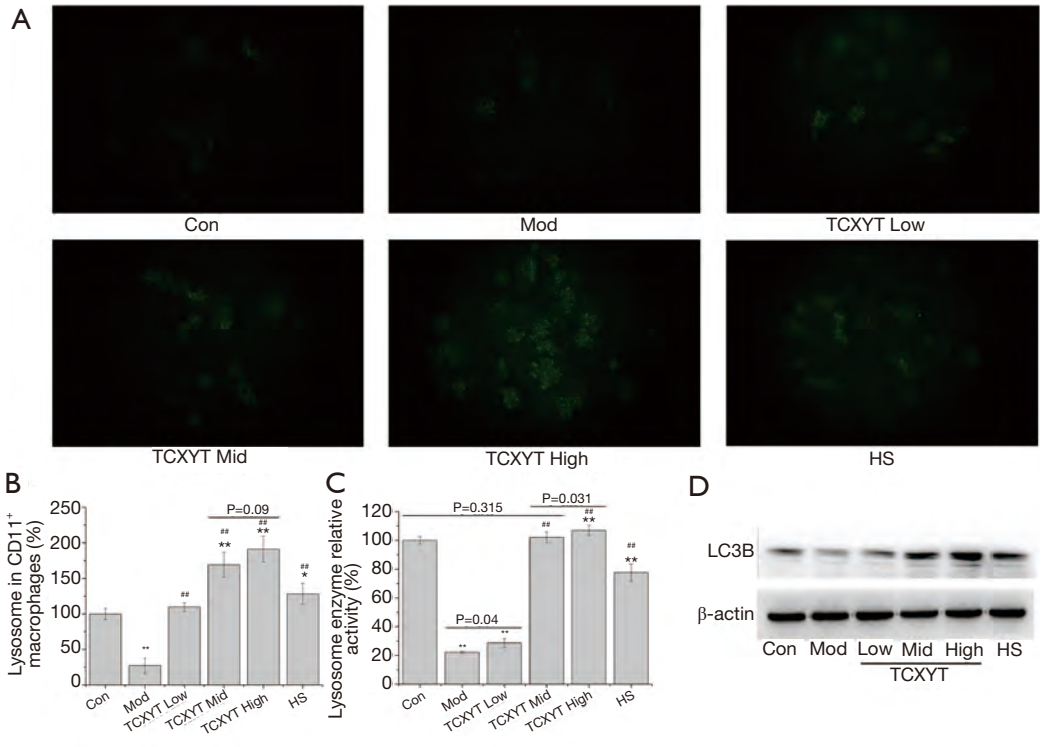

Figure 4 *Tiaochang Xiaoyan tablet* (*TCXYT*) promotes the activation of lysosomes in (*in vitro*). (A) Fluorescence images of lysosomes in lamina propria macrophages (LPM) (magnification ×200); (B) ratio of the number of lysosome in LPM; (C) lysosomal activity in LPM; (D) expression of LC3B in LPs. Data are presented as mean ± standard error of mean of 3 independent experiments performed in triplicate. **, P<0.01 *vs.* control; ##, P<0.01 *vs.* model. Con: Control group; Mod: Model group; Low, Mid and High: low-, middle-, and high-dose *TCXYT*; HS: HS group (treated with HS).

The effects in the middle- and high-dose groups were similar and were superior to those of the low-dose group (*Figure 6A,B*). However, the expression of LC3B in RAW264.7 cells did not differ markedly among the treatment groups (*Figure 6D*).

TCXYT also significantly increased the number and activity of lysosomes in RAW264.7 cells stimulated with LPS+IFN-γ in a dose-dependent manner compared with cells treated with only LPS+IFN-γ (*Figure 6C,E,F*). The expression of LC3B in RAW264.7 cells treated with LPS+IFN-γ was lower than that in the control group, but increased with *TCXYT* treatment (*Figure 6G*). However, there was no significant difference between the medium- and high-dose groups.

Therefore, *TCXYT* ameliorated inflammation in rats with chronic colitis, possibly by increasing the number and activity of lysosomes in macrophages.

TCXYT regulates the TLR9/MyD88/IRAK signaling pathway

The TLR9 signaling pathway plays a central role in the regulation of mucosal innate immunity, particularly of macrophage autophagy, which is implicated in the pathogenesis of UC (13). In the present study, the TLR9, MyD88, IRAK1, and IRAK4 protein levels in LPM were significantly increased in the groups with colitis compared with the control group. *TCXYT* significantly decreased the TLR9, MyD88, IRAK1, and IRAK4 protein levels in LPM in rats with colitis in a dose-dependent manner. In addition, the effects in the medium- and high-dose groups were superior to those in the low-dose group (*Figure 7A*).

The TLR9, MyD88, IRAK1, and IRAK4 protein levels in RAW264.7 cells treated with LPS+IFN-γ were significantly increased compared with those in the control group. *TCXYT* significantly decreased the TLR9, MyD88,

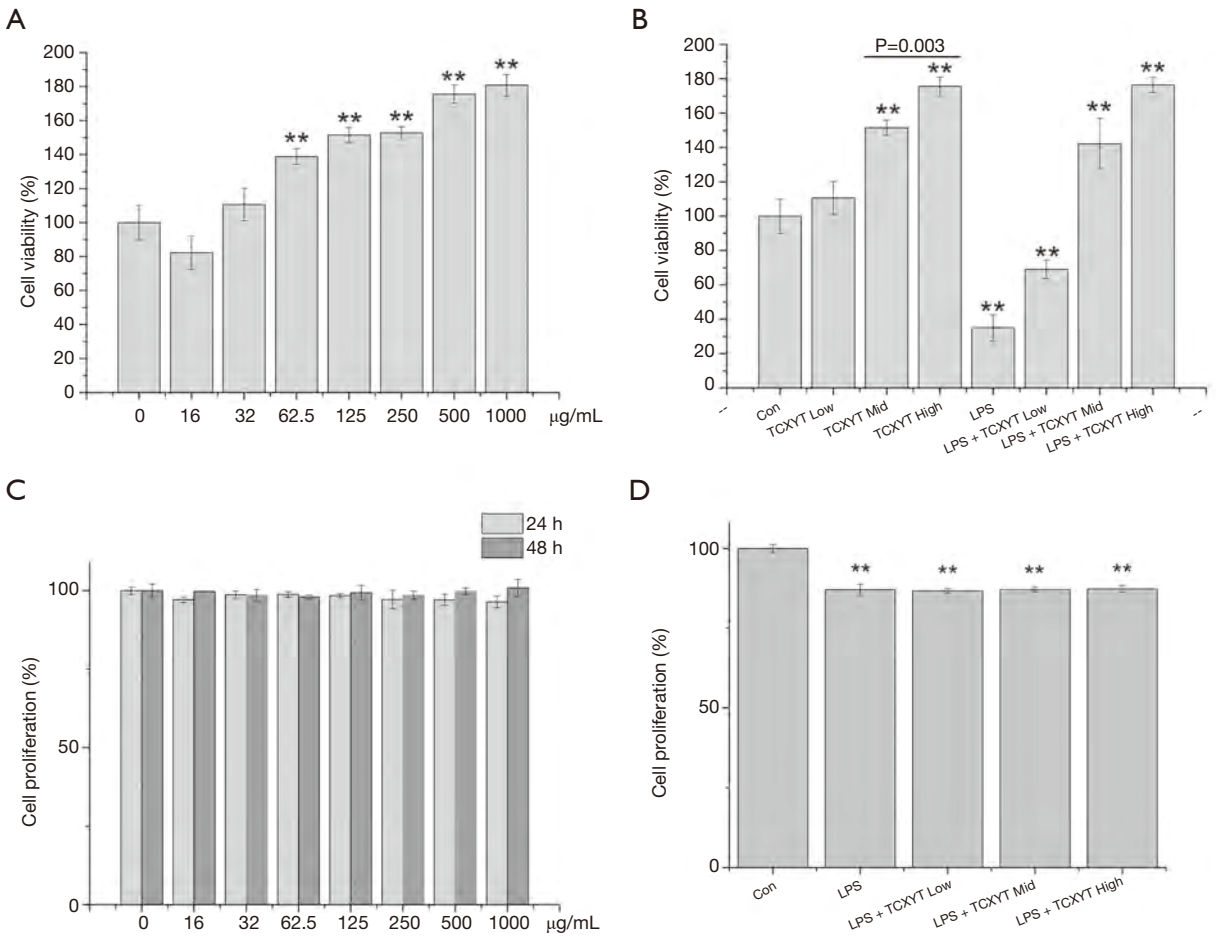

Figure 5 *Tiaochang Xiaoyan tablet* (*TCXYT*) reduces the viability, but not the proliferation, of RAW264.7 cells. (A) Viability of RAW264.7 cells at different concentrations; (B) RAW264.7 cell relative viability in different stimulation groups; (C) proliferation of RAW264.7 cells at different concentrations; (D) RAW264.7 cell relative proliferation in different stimulation groups. Data are presented as mean ± standard error of mean of 3 independent experiments performed in triplicate. **, P<0.01 *vs.* control. Con: Control group; LPS: LPS group (stimulated with LPS); *TCXYT* Low, Mid and High: low-, middle-, and high-dose *TCXYT*.

IRAK1, and IRAK4 protein levels in a dose-dependent manner (*Figure 7B*).

Inhibition of the TLR9 signaling pathway ameliorates inflammation and activates lysosomes

HS, an inhibitor of autophagy and TLR7/9, is an anti-malarial agent that is also used to treat inflammatory conditions (32). When RAW264.7 cells stimulated with LPS+IFN-γ were treated with HS for 24 h, the TLR9, MyD88, IRAK1, and IRAK4 protein levels significantly decreased, and the LC3B protein level increased, compared with cells stimulated with LPS+IFN-γ (*Figure 8A*). Similarly, after treatment with HS for 7 days, the TLR9, MyD88, IRAK1, and IRAK4 protein levels in LPM significantly reduced compared with those in the model group (*Figure 8B*). HS treatment also increased the expression of LC3B and the lysosomal number and activity in LPM (*Figure 4A,B,C,D*). Inflammation and colonic mucosal injury were also significantly ameliorated compared with the model group (*Figure 2C,E,F,G*).

Discussion

TCXYT is a herbal preparation that suppresses inflammation in the colonic mucosa of patients with UC (19). The

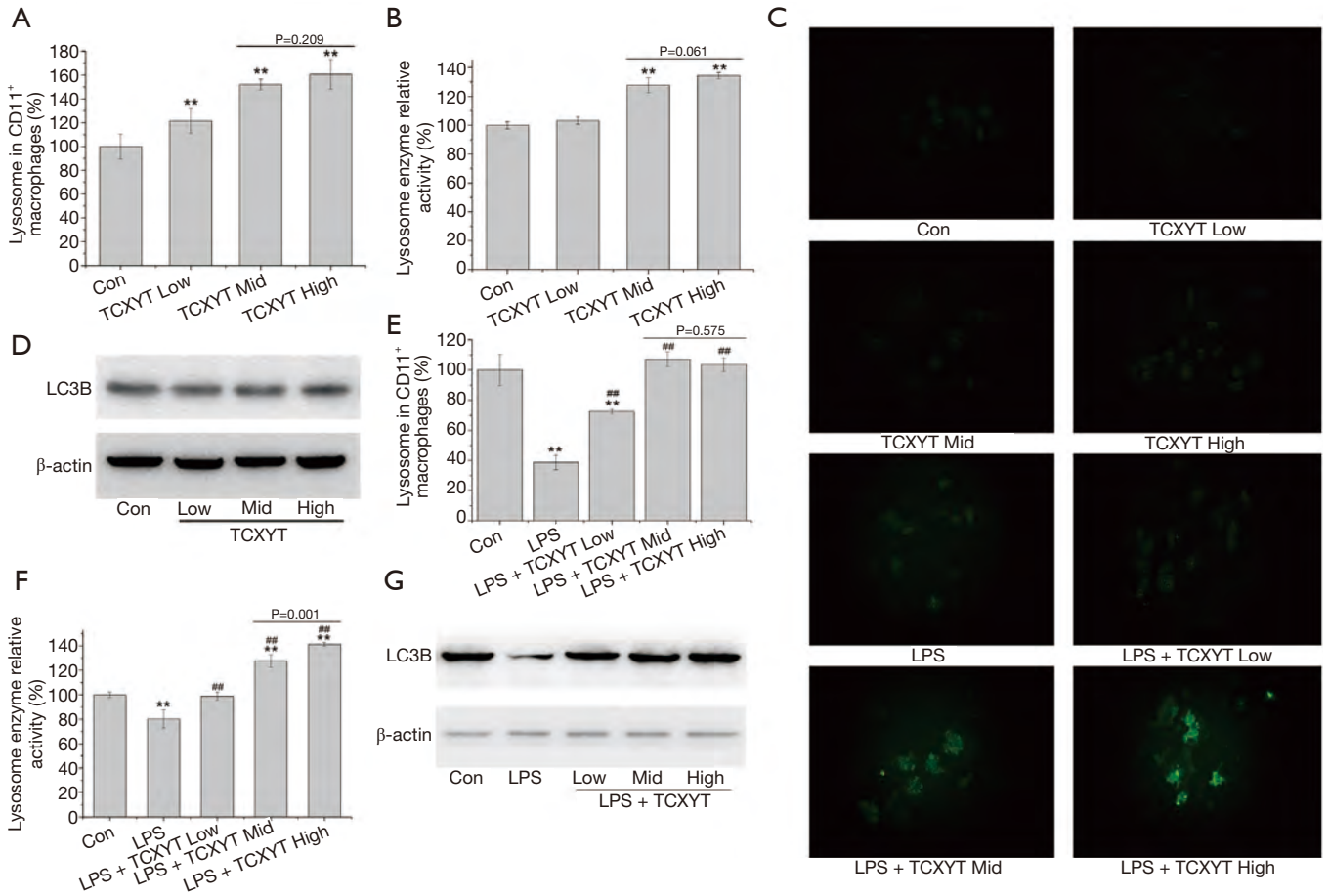

Figure 6 Effects of *Tiaochang Xiaoyan tablet* (*TCXYT*) on lysosomes in RAW264.7 cells. (A) Effects of low-, middle-, and high-dose *TCXYT* on the number of lysosomes in RAW264.7 cells; (B) effects of low-, middle-, and high-dose *TCXYT* on the lysosomal activity in RAW264.7 cells; (C) effects of low-, middle-, and high-dose *TCXYT* on the number of lysosomes in RAW264.7 cells stimulated by lipopolysaccharide (LPS)+interferon-γ (IFN-γ) (magnification ×200); (D) fluorescence micrographs of lysosome activity in RAW264.7 cells stimulated by LPS+INF-γ; (E) effects of low-, middle-, and high-dose *TCXYT* on the expression of LC3B in RAW264.7 cells; (F) effects of low-, middle-, and high-dose *TCXYT* on the lysosomal activity in RAW264.7 cells stimulated by LPS+INF-γ; (G) effects of low-, middle-, and high-dose *TCXYT* on the expression of LC3B in RAW264.7 cells stimulated by LPS+INF-γ Data are presented as mean ± standard error of mean of 3 independent experiments performed in triplicate. **, P<0.01 *vs.* control; ##, P<0.01 *vs.* model. Con: Control group; LPS: LPS group (stimulated with LPS); *TCXYT* Low, Mid and High: low-, middle-, and high-dose *TCXYT*.

bioactive components of *TCXYT* and their contents vary in composition and extraction methods. To clarify the chemical composition and to evaluate the quality of *TCXYT*, we used HPLC/UV to analyze the composition of *TCXYT* used in the present study. The components of *TCXYT* were detected, indicating that *TCXYT* is a mixture of compounds. The components and their contents are in line with the provisions of Pharmacopoeia of the People's Republic of China and meet the requirements of good clinical practice in China.

Macrophages, a type of myeloid cell, play a pivotal role in the innate immune response against pathogens. In patients with UC, a heterogeneous population of inflammatory cells is present in colon tissues, particularly $CD11c^+$ macrophages (33,34). Although the role of macrophages in the development of UC has been explored, the effect and its mechanism are unclear. However, it is well known that increased macrophage infiltration in colon tissue and abnormal polarization are related to the development of inflammation in UC (33). In the present

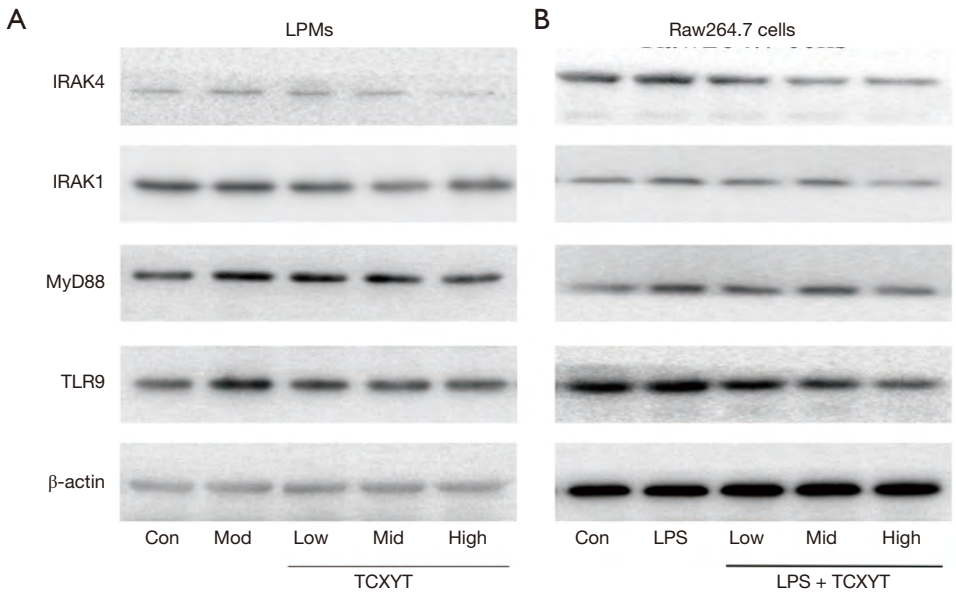

Figure 7 *Tiaochang Xiaoyan tablet* (*TCXYT*) regulates the Toll-like receptor 9 (TLR9)/myeloid differentiation primary response 88 (MyD88)/interleukin receptor-associated kinase (IRAK) signaling pathway. (A) TLR9, MyD88, IRAK1, and IRAK4 protein levels in lamina propria macrophages were significantly upregulated in the experimental colitis groups and significantly decreased by *TCXYT* in a dose-dependent manner. (B) TLR9, MyD88, IRAK1, and IRAK4 protein levels in RAW264.7 cells treated with lipopolysaccharide + interferon-γ were significantly increased compared with the control group, and were significantly decreased with *TCXYT* in a dose-dependent manner. Con: Control group; Mod: Model group; LPS: LPS group (stimulated with LPS); *TCXYT* Low, Mid and High: low-, middle-, and high-dose *TCXYT*.

study, compared with the control group, the infiltration of $CD11c^+$ macrophages in the colon of rats with chronic colitis was significantly increased, and the mucosal damage was aggravated. After 7 days of *TCXYT* treatment, the degree of $CD11^+$ macrophage infiltration and tissue injury in the colon tissue of the high-, middle-, and low-dose *TCXYT* groups improved at different degrees. Therefore, *TCXYT* alleviates inflammation and promotes the repair of colon mucosal injury by reducing the infiltration of $CD11c^+$ macrophages in the colon.

Lysosomes in macrophages are not only degradative organelles but also play a central role in nutrient sensing, metabolism, and cell-growth regulation (35). Lysosomal number and activity, two important elements of lysosome function, are related to the activity and polarization of macrophages (36,37). The lysosomal activity of $CD11c^+$ macrophages in the colon of patients with UC was significantly inhibited (38), but the mechanism is unknown. The number and activity of lysosomes in LPM isolated from the colon tissue of rats with chronic colitis significantly decreased, the level of pro-inflammatory cytokines increased, and anti-inflammatory cytokines decreased. In addition, *TCXYT* significantly increased the number and activity of lysosomes in $CD11c^+$ macrophages. However, *TCXYT* increased the viability, but not the proliferation, of $CD11c^+$ macrophages *in vitro*, suggesting that its anti-inflammatory activity was mediated by effects on lysosomes in macrophages, rather than on macrophage proliferation directly; however, the mechanism is unclear.

TLRs play central roles in the regulation of macrophage-mediated mucosal innate immunity and in the pathogenesis of UC (31). TLR9 is a conserved transmembrane receptor that recognizes pathogen-associated molecular patterns, and initiates an immune response by modulating lysosomal activity in macrophages (39). TLR9 is an important component of the TLR9/MyD88/IRAK signaling pathway, and its activation triggers the production of cytokines and chemokines, which are important in the development of inflammation in UC (31,40). In addition, the lysosomal activity in macrophages is regulated by the TLR9/MyD88 signaling pathway in macrophages (41). IRAK1 and IRAK4 are important factors in the TLR9/MyD88 signaling pathway and play a key role in the activation of macrophages (42,43). However, the role of IRAKs (i.e.,

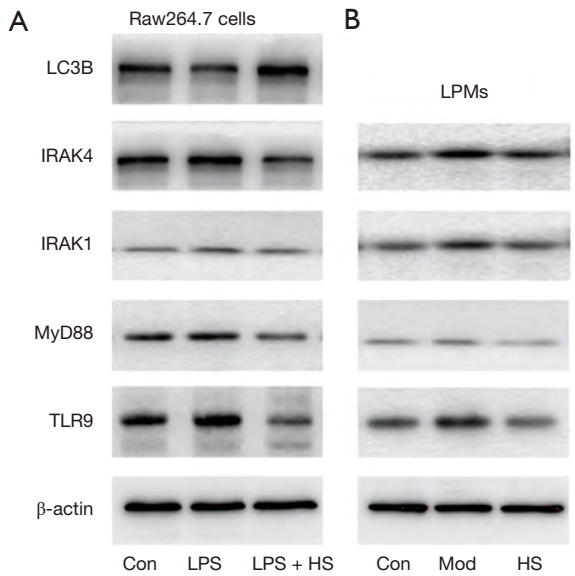

Figure 8 Inhibition of the Toll-like receptor 9 (TLR9) signaling pathway ameliorates inflammation and activates lysosomes in macrophages. (A) In RAW264.7 cells stimulated with lipopolysaccharide (LPS) + interferon-γ (IFN-γ) and treated with hydroxychloroquine sulfate (HS) for 24 h, the TLR9, myeloid differentiation primary response 88 (MyD88), interleukin receptor-associated kinase (IRAK) 1, and IRAK4 protein levels significantly decreased compared with those in cells only stimulated with LPS+IFN-γ. (B) TLR9, MyD88, IRAK1, and IRAK4 protein levels in lamina propria macrophages treated with HS significantly decreased compared with those in the model group. Con: Control group; Mod: Model group; LPS: LPS group (stimulated with LPS).

IRAK1 and IRAK4) in macrophages in the development of UC is unknown. We found that lysosomal activity in macrophages was inhibited in RAW264.7 cells stimulated with LPS+IFN-γ, and in LPM from the colons of rats with chronic colitis, the IRAK1 and IRAK4 protein levels increased significantly and the TLR9/MyD88 signaling pathway was activated. However, the IRAK1 and IRAK4 protein levels significantly decreased and lysosomal activity in macrophages significantly increased with HS-mediated suppression of the TLR9/MyD88 signaling pathway. Therefore, the lysosomal activity in macrophages may be inhibited by activating the TLR9/MyD88/IRAK signaling pathway. In addition, *TCXYT* reduced the infiltration of $CD11c^+$ macrophages in colon tissue and increased lysosomal activity in macrophages by inhibiting the TLR9/MyD88/IRAK signaling pathway.

Conclusions

The composition and stability of *TCXYT* are elevated by chromatography. In the study, the results showed that *TCXYT* can be used as a qualified clinical drug which had stable drug composition and physical-chemical properties. In addition, *TCXYT* is promising for the treatment of UC, as it ameliorates inflammation and $CD11c^+$ macrophage infiltration in the colon of rats with chronic colitis. *TCXYT* may promote the activation of lysosomes in macrophages by inhibiting the TLR9/MyD88/IRAK signaling pathway. However, several issues warrant further study, including how lysosomes regulate the differentiation of macrophages and determination of the exogenous regulator of macrophages in the development of UC. These issues must be resolved if *TCXYT* is to be used to ameliorate inflammation in the colon mucosa of patients with UC.

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Footnote

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approved all of the procedures involving the rats (animal ethics approval No. 2016021-2). Experiments were performed in compliance with the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine's guidelines for the care and use of animals.

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Supplementary

Table S1 Herbal composition of Tiaochang Xiaoyan tablet

Latin name	English name	Chinese name	Dosage	Place of origin	Lot number
Radix Astragali seu Hedysari.	*Milkvetch root*	*Huang-Qi*	15 g	Nei Mongol, China	1804001
Radix Linderae	*Combined spicebush root*	*Wu-Yao*	15 g	Hunan, China	1804001
Rhizoma Coptidis	*Coptis Root*	*Huang-Lian*	10 g	Sichuan, China	1804001
Herba Hedyotis	*Hedyotis*	*Bai hua she she cao*	20 g	Hunan, China	1712001
Semen Coicis	*Coix seed*	*Yi yi ren*	30 g	Guizhou, China	1803001

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Table S2 Main chemical components of *Yiaozhong Xiaoyan tablet (TCSXT)* extract

No.	Retention time (minimum)	Area (maximum)	Formula	Molecular weight	Name
1	1.014	851905.051	C6 H14 N2 O2	146.1057	DL-Lysine
2	1.085	2084841.67	C6 H9 N3 O2	155.06967	L-Histidine
3	1.088	77920697.6	C5 H12 N4 O3	176.09096	L-Canavanine
4	1.091	576947.596	C9 H20 N2 O2	188.15266	N6,N6,N6-Trimethyl-L-lysine
5	1.109	947714.453	C7 H15 N O3	161.10523	DL-Carnitine
6	1.113	31174111.7	C6 H14 N4 O2	174.11185	DL-Arginine
7	1.119	4827890.75	C6 H13 N O5	179.07954	D-Glucosamine
8	1.178	248671.276	C7 H14 N2 O3	174.10049	N-Acetylornithine
9	1.193	189415.329	C7 H15 N O2	145.11043	Acetylcholine
10	1.202	525769.026	C10 H19 N O4	217.13169	Propionylcarnitine
11	1.254	1534833.42	C3 H7 N O3	105.04307	D-Serine
12	1.268	14620239.8	C4 H8 N2 O3	132.05358	Asparagine
13	1.278	1545457.27	C4 H9 N O3	119.05858	Threonine
14	1.301	299118.409	C6 H11 N O3	145.07403	4-Acetamidobutanoic acid
15	1.305	9184726.94	C5 H9 N O4	147.05314	L-Glutamic acid
16	1.321	6788148.17	C4 H7 N O4	133.03759	L-Aspartic acid
17	1.321	2254608.67	C9 H17 N O7	251.10045	Muramic acid
18	1.323	1275889.55	C9 H13 N3 O5	243.08552	Cytidine
19	1.335	737309.153	C6 H13 N O5	179.07954	Kanosamine
20	1.353	5947307.66	C11 H15 N5 O4	281.11107	2′-O-Methyladenosine
21	1.362	1886193.76	C6 H14 O6	182.07905	L-Iditol
22	1.364	14316910.8	C5 H11 N O2	117.07919	Betaine
23	1.372	706507.028	C10 H13 N O3	195.08966	N-Acetyldopamine
24	1.373	6462121.21	C10 H18 N4 O6	290.12247	Argininosuccinic acid
25	1.376	1019714.24	C8 H15 N O6	221.0899	N-Acetyl-D-galactosamine
26	1.395	48715428.9	C5 H9 N O2	115.06359	Proline
27	1.407	743063.223	C6 H10 O7	194.04177	Galacturonic acid
28	1.407	4731840.36	C11 H19 N O9	309.10585	N-Acetylneuraminic acid
29	1.418	1036821.52	C8 H16 N4 O3	216.12222	N-Acetylarginine
30	1.432	7601658.62	C6 H12 O7	196.05743	Gluconic acid
31	1.445	3140574.36	C18 H32 O16	504.16894	D-Raffinose
32	1.456	3977688.15	C6 H6 O3	126.03186	5-Hydroxymethyl-2-furaldehyde
33	1.474	356987.817	C8 H9 N O4	183.05323	4-Pyridoxic acid
34	1.48	13146742.9	C7 H13 N O2	143.09463	DL-Stachydrine
35	1.482	16893287.3	C12 H22 O11	342.11584	α,α-Trehalose
36	1.491	630266.766	C6 H10 O8	210.03684	D-Saccharic acid
37	1.495	957480.128	C11 H20 N2 O3	228.14746	Prolylleucine
38	1.536	533566.051	C9 H11 N O3	181.07411	L-three-3-Phenylserine
39	1.537	1170679.82	C5 H5 N5 O	151.04951	Guanine
40	1.543	7712636.75	C6 H11 N O2	129.07913	Pipecolic acid
41	1.569	946918.31	C7 H12 O6	192.06367	D-(-)-Quinic acid
42	1.616	113479.558	C6 H15 N O3	149.10539	Triethanolamine
43	1.633	84164.3581	C6 H13 N O4	163.08466	Bicine
44	1.817	1127450.23	C6 H13 O9 P	260.02972	Glucose 1-phosphate
45	1.995	1258417.31	C6 H5 N O2	123.03229	Nicotinic acid
46	2.306	2521312.268	C5 H4 N4 O3	168.0285	Uric acid
47	2.37	1087566.73	C5 H4 N4 O	136.03866	Hypoxanthine
48	2.404	5700782.36	C6 H13 N O2	131.09477	Leucine
49	2.504	2601739.995	C8 H9 N O	135.06855	Acetanilide
50	2.568	369847.267	C6 H6 O6	174.01655	Trans-aconitic acid
51	2.577	57784033.2	C5 H7 N O3	129.04268	L-Pyroglutamic acid
52	2.831	552223.159	C4 H4 N2 O2	112.02773	Uracil
53	2.88	276983.762	C10 H11 N O3	193.07423	Phenylacetylglycine
54	2.92	323418.136	C6 H11 N3 O	141.09035	L-Histidinol
55	2.981	11721038.4	C10 H13 N5 O4	267.09645	Adenosine
56	3.095	271199.136	C17 H12 Cl F N2 O	314.06342	Nuarimol
57	3.61	4413431.33	C8 H8 O	120.05788	Acetophenone
58	3.776	307462.154	C6 H11 N O2	129.07921	Nipecotic acid
59	4.49	113151.314	C8 H15 N O4	189.10023	2-Aminooctanedioic acid
60	4.526	419888.382	C10 H12 N4 O5	268.08086	Inosine
61	4.654	5301291.55	C9 H11 N O2	165.07905	L-Phenylalanine
62	4.74	300443.532	C10 H14 N5 O7 P	347.06313	3′-Adenosine monophosphate
63	5.578	359566.303	C10 H14 N2 O4	226.0954	Carbidopa
64	5.931	1155035.7	C11 H9 N O2	187.06337	Indole-3-acrylic acid
65	6.035	175369.867	C11 H13 N O4	223.08456	N-Acetyl-L-tyrosine
66	6.042	154793.429	C11 H12 O5	224.06857	Sinapinic acid
67	6.172	3150661.656	C10 H14 N2 O2	194.10582	Nikethamide 1-oxide
68	6.591	49713.4144	C17 H19 N O3	285.13655	Norcodeine
69	6.735	95652.93	C10 H8 O4	192.04254	7,8-Dihydroxy-4-methylcoumarin
70	6.788	270509.405	C19 H21 N O4	327.14697	Hernandine
71	6.887	2373980669	C20 H23 N O4	341.16183	N-Methylhernagine
72	7	402806.553	C10 H9 N	143.07362	6-Methylquinoline
73	7.048	247147.547	C11 H12 O3	192.07886	BMK methyl glycidate
74	7.067	197438.512	C8 H7 N O	133.05276	5-Hydroxyindole
75	7.07	383587.03	C19 H23 N O4	329.16261	Sinomenine
76	7.31	4411193.91	C15 H14 O6	290.07905	Catechin
77	7.693	4850576.02	C20 H21 N O4	339.14696	Papaverine
78	7.697	12481235.2	C21 H25 N O4	355.17794	Glaucine
79	7.821	902085.282	C10 H11 N O3	193.07422	4-Methylhippuric acid
80	7.833	245680.584	C9 H11 N O2	165.07903	Benzocaine
81	7.879	1303765.05	C15 H22 O4	266.15144	Verrucarol
82	8.084	74115119.2	C17 H16 F3 N O2	323.11473	Flutolanil
83	8.1	9542048.78	C20 H42 O11	458.2727	PEG n10
84	8.14	1784550.5	C11 H12 O6	240.06299	(3R,4S)-4,6,8-Trihydroxy-7-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one
85	8.311	4332569.07	C22 H46 O12	502.29891	PEG n11
86	8.318	300156.932	C9 H10 O2	150.06824	4′-Methoxyacetophenone
87	8.347	473669.468	C10 H10 O3	178.06311	4-Methoxycinnamic acid
88	8.405	200137.306	C21 H20 O11	448.1004	Kaempferol-7-O-glucoside
89	8.409	332459.73	C16 H18 O9	354.09458	Neochlorogenic acid
90	8.414	1224657.12	C17 H20 O9	368.11058	(3R,5R)-1,3,5-Trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid
91	8.423	52306256.7	C10 H8 O3	176.04725	4-Methylumbelliferone
92	8.434	84760.9625	C15 H10 O7	302.04222	Robinetin
93	8.703	28668320.5	C27 H30 O16	610.15249	Rutin
94	8.733	2041735.89	C13 H18 O2	206.09073	Ibuprofen
95	8.748	47364845.1	C22 H22 O10	446.12066	Glycitin
96	8.803	534398.729	C10 H13 N O2	179.09476	Phenacetin
97	8.84	516591.244	C28 H58 O15	634.37756	PEG n14
98	8.856	810691.416	C11 H13 N O3	207.08884	N-Acetyl-L-phenylalanine
99	8.877	128638451	C14 H12 O3	228.07837	Resveratrol
100	8.888	144802.43	C9 H8 O3	164.04755	4-Coumaric acid
101	8.907	2411037.27	C15 H12 O4	256.07344	Isoliquiritigenin
102	8.913	253302.339	C9 H6 O2	146.0369	Coumarin
103	8.973	132768.442	C21 H20 O12	464.09559	Quercetin-3β-D-glucoside
104	9.13	45988.0495	C15 H10 O7	302.04277	Quercetin
105	9.134	64687.0043	C10 H9 N O3	191.05813	5-Hydroxyindole-3-acetic acid
106	9.209	1927855.54	C10 H10 O4	194.05796	Meconin
107	9.415	1765942.49	C10 H8 O4	192.04228	6,7-Dihydroxy-4-methylcoumarin
108	9.614	613299.581	C16 H14 O5	286.08411	Sakuranetin
109	9.676	12649322.9	C25 H24 O12	516.12641	4,5-Dicaffeoylquinic acid
110	9.843	6422948.65	C9 H16 O4	188.10393	Azelaic acid
111	9.903	105308.54	C15 H22 O4	266.15192	3-Hydroxy-4-(2-hydroxy-6-methyl-2-heptanyl)benzoic acid
112	9.96	391564.181	C11 H12 O4	208.07371	6-Hydroxy-8-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one
113	10.096	768208.665	C16 H12 O4	268.07344	Formononetin
114	10.141	22539616.5	C22 H22 O9	430.12533	Ononin
115	10.417	196633.46	C7 H5 N O S	151.00925	1,2-Benzisothiazolin-3-one
116	10.766	22507245.3	C14 H14 O4	246.08894	(5S,6S)-5-Hydroxy-4-methoxy-6-[(E)-2-phenylvinyl]-5,6-dihydro-2H-pyran-2-one
117	10.793	419058.666	C10 H18 O4	202.11983	3-tert-Butyladipic acid
118	10.944	30909.2896	C15 H22 O5	282.14681	4-(2,7-Dihydroxy-6-methyl-2-heptanyl)-3-hydroxybenzoic acid
119	10.982	27464672.4	C16 H12 O5	284.06799	Glycitein
120	11.036	2536982.49	C15 H20 O4	264.13609	Ambrosic acid
121	11.559	411763.661	C22 H23 Cl N2 O2	382.14398	Loratadine
122	11.731	34964.0387	C15 H12 O5	272.06871	Naringenin
123	11.829	1158473.03	C10 H10 O4	194.05797	Ferulic acid
124	11.99	23621655.3	C18 H34 O5	330.24034	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid
125	12.377	5801211.66	C15 H20 O4	264.13608	3-Methylene-4-[(2E)-3-methyl-4-(4-methyl-5-oxotetrahydro-2-furanyl)-2-buten-1-yl]dihydro-2(3H)-furanone
126	12.481	92053.4197	C12 H22 O4	230.15142	Dodecanedioic acid
127	12.537	11630427.8	C9 H10 O3	166.06301	Apocynin
128	12.562	71458.3629	C15 H22 O3	250.15672	2-[(2S,4aR,8aS)-2-Hydroxy-4a-methyl-8-methylenedecahydro-2-naphthalenyl]acrylic acid
129	12.728	302718.891	C15 H22 O4	266.15179	1,9b-Dihydroxy-6,6,9a-trimethyl-5,5a,6,7,8,9,9a,9b-octahydronaphtho[1,2-c]furan-3(1H)-one
130	12.97	24890.2411	C15 H22 O	218.16716	Nootkatone
131	12.989	63719.4854	C18 H15 O P	278.0861	Triphenylphosphine oxide
132	13.035	305373.31	C9 H19 N O	157.14674	2,2,6,6-Tetramethyl-4-piperidinol
133	13.156	318209.558	C15 H18 O3	246.12564	3,5a,9-Trimethyl-2,3,3a,4,5,5a,8,9b-octahydronaphtho[1,2-b]furan-2,8-dione
134	13.255	119906.479	C9 H10 O	134.07327	2,4-Dimethylbenzaldehyde
135	13.265	1902819.59	C13 H24 N2 O	224.18877	N,N′-Dicyclohexylurea
136	13.436	96136.7631	C15 H20 O3	248.1411	(3aR,8R,8aR,9aR)-8-Hydroxy-8a-methyl-3,5-bis(methylene)decahydronaphtho[2,3-b]furan-2(3H)-one
137	13.794	109643.92	C15 H24 O	220.1828	(-)-Caryophyllene oxide
138	13.821	17534.1666	C14 H12 O3	228.07863	Trioxsalen
139	13.873	2106417.57	C18 H28 O3	292.2037	9S,13R-12-Oxophytodienoic acid
140	13.945	1063883.67	C11 H14 O	162.10451	Valerophenone
141	14.057	11174726.7	C18 H32 O4	312.22984	(9Z,12Z)-6,8-Dihydroxy-9,12-octadecadienoic acid
142	14.152	84633.5308	C18 H39 N	269.30832	Octadecanamine
143	14.379	338867.245	C18 H34 O4	314.24567	(+/-)12(13)-DiHOME
144	14.933	153711.354	C9 H6 O3	162.03174	7-Hydroxycoumarine
145	14.944	425342.435	C24 H30 O6	414.20719	Bis(4-methylbenzylidene)sorbitol
146	14.976	39657905.7	C15 H10 O5	270.05239	Genistein
147	15.213	669833.621	C12 H11 N	169.08922	Diphenylamine
148	15.518	105128.32	C16 H30 O4	286.21446	Hexadecanedioic acid
149	15.923	42065.0361	C20 H26 O3	314.18826	Kahweol
150	16.341	172959.956	C13 H17 N O	203.13116	Crotamiton
151	16.353	513069.811	C18 H30 O2	278.22466	α-Eleostearic acid
152	16.413	1993012.28	C15 H22 O2	234.16187	3,5-di-tert-Butyl-4-hydroxybenzaldehyde
153	16.78	5787980.42	C10 H10 O2	162.06807	4-Methoxycinnamaldehyde
154	16.782	186332.042	C8 H7 N	117.0582	Indole
155	16.894	133196.861	C15 H19 N O3	261.1366	MDPBP
156	16.895	212250.263	C18 H30 O3	294.21955	9-Oxo-10(E),12(E)-octadecadienoic acid
157	16.896	270327.051	C17 H24 O3	276.17269	Shogaol
158	16.968	732570.078	C15 H22 O2	234.16712	3,5-di-tert-Butylbenzaldehyde
159	17.176	485466.093	C12 H14 O4	222.08916	Monobutyl phthalate
160	17.188	78807.1899	C8 H6 O4	166.0267	Phthalic acid
161	17.19	46388946.9	C16 H22 O4	278.15154	Dibutyl phthalate
162	17.198	214247.191	C8 H7 N O	133.05285	6-Methylbenzoxazole
163	17.541	31923.0691	C14 H26 O4	258.18328	Disobutyl adipate
164	17.71	431333.43	C18 H34 O2	282.25602	Ethyl palmitoleate
165	18.271	74449.2981	C12 H22 O2	198.16214	4-tert-Butylcyclohexyl acetate
166	19.369	649534.665	C16 H33 N O	255.25621	Hexadecanamide
167	19.558	2056387.78	C23 H32 O2	340.23987	2,2′-Methylenebis(4-methyl-6-tert-butylphenol)
168	19.862	55974.8979	C16 H30 O	238.22974	Muscone
169	19.937	100935.801	C18 H34 O4	314.24583	Dibutyl sebacate
170	19.997	211966.37	C20 H32 O2	304.2403	Mesterolone
171	20.242	523726.765	C20 H32 O2	304.2403	Arachidonic acid
172	21.705	848468.574	C18 H37 N O	283.28762	Stearamide
173	23.043	2072873.03	C24 H38 O4	390.27677	Bis(2-ethylhexyl) phthalate
174	26.097	364077.628	C22 H45 N O	339.3502	Docosanamide

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