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Design, synthesis and evaluation of a baicalin and berberine hybrid compound as therapeutic agent for ulcerative colitis

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ABSTRACT

Structural modification of active natural compounds which were originated from Traditional Chinese Medicine (TCM) have showed great advantages in the development of new drugs. In TCM, "Huangqin - Huanglian" is a classic "medicine couple" that has been used to treat intestinal diseases for thousands of years, while baicalin and berberine are the major active compounds of Huangqin and Huanglian respectively. Based on this "medicine couple", we designed and synthesized a new baicalin and berberine hybrid compound (BBH). Its molecular structure was confirmed by spectroscopy. The antibacterial activity of BBH was detected in vitro. Results indicated that the new hybrid compound exhibited the best antibacterial activity for proteobacteria as compared with its original synthetic materials (baicalin and berberine). In vivo, the effect of BBH on ulcerative colitis was also investigated. BBH treatment significantly ameliorated the disease symptoms and prevented the colon damage of ulcerative colitis. Furthermore, BBH showed a significant anti-inflammatory effect through regulating activities of SOD, MPO and expressions of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in colon tissue. Data also suggested that BBH was more superior than baicalin and berberine in ameliorating colonic damage. This indicated that the new hybrid compound BBH showed enhanced efficacy in treating ulcerative colitis.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with high incidence worldwide. Global study reported that the prevalence of UC was range from 2.42 to 298.5/100,000 in North America and Northern Europe, while in developing countries for instance Asia, the prevalence was about 6.3 per 100,000 peoples. In addition, the incidence and prevalence of UC that have increased rapidly, make the situation worse in recent years.[1] As a sub - type of IBD, UC is mainly characterized by repeated flare - ups of inflammation in mucosa and submucosa that involve colon and rectum. UC patients usually suffer abdominal pain, tenesmus, bloody diarrhea, fever, fatigue and weight loss.[2] Moreover, researches found that the risk of developing intestinal cancers were significantly increased in patients with UC.[3–5] Despite still uncertain defined, the pathogenesis of UC is believed to involve dysregulation of immune response in colon and rectum to multiple environmental stimulus such as reactive oxygen mediators,[6,7] arachidonate metabolites, neutrophil infiltration, pro - inflammatory cytokines and intestinal flora imbalance.[8–12] Reactive oxygen species (ROS) are important inflammatory mediators of colonic cells. Under pathological condition of UC, ROS are largely produced by inflammatory cells, neutrophils, and macrophages. Researches indicated

Abbreviations: TCM, Traditional Chinese Medicine; BBH, baicalin and berberine hybrid compound; UC, ulcerative colitis; IBD, inflammatory bowel disease; ROS, reactive oxygen species; SOD, superoxide dismutase; MPO, myeloperoxidase; IL-1β, interleukin - 1β; IL – 6, interleukin - 6; TNF – α, tumor necrosis factor - alpha

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that overproduction of high reactive ROS can induce oxidative stress and cause damage to the integrity of cell membrane.[13] Superoxide dismutase (SOD), an essential natural scavenger for superoxide free radical (O_2^-), is the main defense enzyme that against damages from ROS. Previous studies have confirmed that increased level of SOD in vivo effectively ameliorated symptoms of ulcerative colitis.[14–16] In one hand, Neutrophil - mediated inflammation was proved to play a key role in the development of ulcerative colitis. The correlation between neutrophils and UC has been widely reported.[13,17] The neutrophils among UC patients were abnormally accumulated as compared with health people,[18] and its accumulation in tissue can be expressed by the activity of myeloperoxidase (MPO). Studies confirmed that the activity of MPO in colon mucosa was significantly increased in ulcerative colitis.[13] In the other hand, pro - inflammatory cytokines, such as interleukin - 1β (IL - 1β), interleukin - 6 (IL - 6) and tumor necrosis factor - alpha (TNF - α), play a core role in mediating mucosal and submucosal inflammations in ulcerative colitis[5, 8, 19]. The release of pro - inflammatory cytokines like TNF - α, IL - 1β and IL - 6 were significantly increased in colon of UC patients, and these changes promoted the inflammatory response in mucosa and submucosa of colon.[17,19–21]

It is widely known that the structural optimization of active natural compounds is an effective way to develop new drugs. For example, over 61% of small molecule chemical drugs developed from 1981 to 2002 were derived from natural compounds. In particular, this rate of drugs that used to treat infectious disease rised to 75%.[22,23] Nowadays, structural modification of active natural compounds originated from Traditional Chinese Medicine (TCM) have showed great advantages in the development of potential new drugs. “Medicine couple”, the basic compositional unit of TCM prescription, is a particular combination of two medicinal herbs to enhance efficacy or reduce toxicity.[24] “Huangqin (*Scutellaria baicalensis Georgi*) - Huanglian (*Coptis chinensis Franch*)” is a classic “medicine couple” in TCM prescription that used to treat intestinal disorders.[25] Baicalin and berberine are the major active compounds of Huangqin and Huanglian respectively.[26–28] Previous study found that baicalin - berberine complex precipitated in the water decoction of numerous TCM prescriptions containing “medicine couple” of Huangqin - Huanglian, indicating a combine of these two natural compounds.[25] Recent years, large number of studies aimed to synthesize derivatives of baicalin or berberine, but few studies focus on conjugate synthesis of baicalin and berberine. In present study, we designed and synthesized a baicalin and berberine hybrid compound (BBH). Structure of this new hybrid compound was conformed by spectroscopy (UV, ^{1}H NMR, ^{13}C NMR and HRMS spectra). In vitro, antibacterial activity of BBH was detected. In vivo, we investigated the therapeutic effect of BBH on ulcerative colitis by using dextran sodium sulfate (DSS) - induced UC model mice.

2. Materials and methods

2.1. Chemicals and reagents

In present study, berberine (purity ≧98%) and baicalin (purity ≧95%) were purchased from Sigma - Aldrich (Shanghai; China). Mueller Hinton broth (MHB) and Mueller Hinton agar (MHA) were purchased from Qingdao Haibo Biotechnology Co., Ltd. (Qindao, China). Strains of Staphylococcus aureus (261112-5), Streptococcus hemolyticus (32210), Streptococcus pneumoniae (32215), Candida albicans (98001), Pseudomonas aeruginosa (10211), Escherichia coli (44113-5), Typhoid bacillus (50071-16) and Salmonella paratyphi B (0039) were purchased from Beijing Institute of Biological Products. DSS (molecular weight 36, 000-50, 000) was purchased from MP Biomedicals company. Hemoccult kit, SOD activity assay kit and MPO chemical chromatometry kit were brought from Jiancheng Bioengineering Institute (Nanjing; China). Hematoxylin and eosin (H & E) staining kits and special aldehyde-fuchsin orange G staining kits were purchased from Beyotime Biotechnology (Shanghai; China). Total RNA extract agents (RNAsimple; DP419) and FastKing RT Kits (With gDNase) (KR116) were purchased from Tiangen (TIANGEN BIOTECH CO., Ltd; Beijing; China). RT - qPCR reactions kits used in present study were purchased from Takara (TAKARA Biomedical Technology Co., Ltd., Beijing; China). Antibodies of IL-1β (ab234437), IL-6 (ab229381) and TNF-α (ab92324) were purchased from Abcam (Shanghai, China). The secondary antibody of Tubulin (30301ES60) was purchased from YEASEN (Shanghai, China). All other chemicals and reagents were of analytical grade and purchased from Aldrich Chemical Co. (Beijing, China). Major instruments used in present study included HITACHI high - performance liquid chromatograph (HPLC) including HITACHI 1110 pump, 1410 ultraviolet detector and 1210 automatic sampler; UV - 1601 ultraviolet - visible spectrophotometer (Beijing Ruili Analysis Instrument Company); Bio - Rad Merlin infrared spectrometer; Agilent 1100 LC/MSD system; AVANCE - 300 superconducting nuclear magnetic resonance instrument (Germany BRUKER). ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature with tetramethylsilane (TMS) as an internal standard and chloroform - d ($CDCl_3$) as solvent.

2.2. Synthesis of baicalin - berberine hybrid compound (BBH)

2.2.1. Synthesis of berberrubine (BBB)[29]

BBB was synthesized according to previous study.[29] Berberine (1 g, 2.69 mmol) was weighted and dissolved in N, N - dimethylformamide (DMF) with a solid - liquid ratio of 1:25 (g/mL). After microwave irradiated for 15 min at 400 W, the reaction mixture was immediately diluted with distilled water (1.5 times volume to DMF) and refrigerated to crystallization at 4 °C. Crystals were washed with petroleum ether and evaporated in a vacuum to obtain BBB. The filtrate was also collected and purified using PIPO - 02 macroporous resin, then evaporated to isolate BBB. With this method, BBB was obtained with 93% yield (0.862 g). The purity of BBB ≧ 98%.

2.2.2. Synthesis of 9 - o - (4 - bromoethane) berberine hydrochloride (compound X4)

BBB (15 g, 46.55 mmol), dissolved in 600 ml acetonitrile, was added to 1, 4 - dibromobutane (80 ml). The reaction was carried out for 1 h within a temperature range of 85–90 °C and the reaction mixture was immediately evaporated to crystallization. Crystals were filtered, washed with acetonitrile and acetic ether, and evaporated again to obtain compound X4 (17.61 g). With this method, the yield of compound X4 was 92% and the purity ≥ 98%.

2.2.2.1. 9 - o - (4 - bromoethane) berberine hydrochloride. Yellow crystals; UV: λmax 202.0, 227.8, 264.7, 346.8, and 428.7 nm; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.80 (1H, s, H - 1), 7.09 (1H, s, H - 4), 3.68 (2H, t, J = 5.8 Hz, H - 5), 4.95 (2H, t, J = 5.8 Hz, H - 6), 9.77 (1H, s, H - 8), 8.00 (1H, d, J = 9.1 Hz, H - 11), 8.20 (1H, d, J = 9.1 Hz, H - 12), 8.95 (1H, s, H - 13), 6.18 (2H, s, H - 15), 4.06 (3H, s, H - 16), 4.31 (2H, t, J = 7.5 Hz, H - 17), 2.01 (2H, m, H - 18), 2.09 (2H, m, H - 19), and 3.21 (2H, t, J = 7.2 Hz, H - 20); ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 105.33 (C - 1), 150.30 (C - 2), 149.72 (C - 3), 108.32 (C - 4), 130.59 (C - 4a), 28.12 (C - 5), 56.97 (C - 6), 145.20(C - 8), 137.35 (C - 8a), 120.13 (C - 9), 147.58 (C - 10), 120.34 (C - 11),123.34 (C - 12), 132.90 (C - 12a), 126.53 (C - 13), 142.60 (C - 14),121.49 (C - 14a), 101.99 (C - 15), 55.21 (C - 16), 73.29 (C - 17), 26.22 (C - 18), 28.78 (C - 19), and 34.87 (C - 20); ESI - MS: m/z, $[C_{23}H_{23}BrNO_4]^+$, 456.08 $[M - Br]^+$.

2.2.3. Synthesis of BBH

Compound X4 (6.81 g, 43.28 mmol) and baicalin (5.51 g, 35.80 mmol) were dissolved in DMF (340 ml) and triethylamine (5 ml). After mixing, the reaction was carried out at 86 °C for 45 min. Reaction solution was then immediately filtered, cooled to room temperature and adjusted the pH to 2.0–3.0 by using HCL. After adding twice the amount of distilled water, the reaction solution was refrigerated overnight to crystallization. Crystals were then collected and washed with

distilled water to neutral pH. After washed with petroleum ether, neutral crystals were evaporated in vacuum at 80 °C (5.38 g). With this method, BBH was obtained with a 53% yield.

2.2.3.1. HPLC analysis. HPLC was used to monitor the synthesis reaction of BBH. Samples of baicalin, compound X4 and BBH were analyzed using YMC - Pack ODS – AQ (250 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phases consisted of acetonitrile (45%) and 0.2% formic acid aqueous solution (55%). The flow rate was set at 1.0 ml/min. The sample injection volume was 20 μL. Purity of BBH was also detected using HPLC under the same conditions.

2.2.3.2. BBH. BBH, yellow solid; UV: λmax 205.2, 217.7, 273.4, 333.4, and 440.7 nm; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.76 (1H, s, H - 1), 7.09 (1H, s, H - 4), 3.42 (2H, t, J = 5.8, H - 5), 4.85 (2H, t, J = 5.8, H - 6), 9.31 (1H, s, H - 8), 7.96 (1H, d, J = 9.1, H - 11), 8.00 (1H, d, J = 9.1, H - 12), 8.81 (1H, s, H - 13), 6.18 (2H, s, H - 15), 3.76 (3H, s, H - 16), 4.45 (2H, t, J = 6.3 Hz, H - 20), 1.80 (2H, m, H - 18), 1.93 (2H, m, H - 19), 4.22 (2H, t, J = 18.6 Hz, H - 17), 12.39 (1H, s, 5′ - OH), 8.50 (1H, s, 6′ - OH), 8.05 (2H, d, J = 4.5 Hz, 2″, 6″), 7.54–7.57 (2H, m, 3″, 5″), 7.59 (1H, m, 4″), 7.09 (1H, s, 3′), 6.90 (1H, s, 8′), 5.40–5.59 (3H, m, 2‴,3‴,4‴ - OH), 5.26 (1H, d, J = 4.2 Hz, 1‴), 4.44 (1H, d, J = 6.3 Hz, 5‴) and 3.16–3.33 (3H, m, 2‴, 3‴, 4‴); ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 105.50 (C - 1), 149.67 (C - 2), 148.72 (C - 3), 108.25 (C - 4), 130.49 (C - 4a), 26.25 (C - 5), 56.42 (C - 6), 144.28 (C - 8), 137.20 (C - 8a), 120.04 (C - 9), 147.50 (C - 10), 120.37 (C - 11), 122.65 (C - 12), 132.66 (C - 12a), 125.93 (C - 13), 142.05 (C - 14), 120.76 (C - 14a), 101.99 (C - 15), 55.25 (C - 16), 73.16 (C - 17), 24.28 (C - 18), 25.91 (C - 19), 64.59 (C - 20), 162.96 (C - 2′), 104.27 (C - 3′), 182.16 (C - 4′), 149.25 (C - 5′), 131.93 (C - 6′), 150.62 (C - 7′), 93.24 (C - 8′), 146.33 (C - 9′), 105.69 (C - 10′), 130.49 (C - 1″), 126.03 (C - 2″, 6″), 128.96 (C - 3″, 5″), 130.04 (C - 4″), 99.71 (C - 1‴), 72.68 (C - 2‴), 74.94 (C - 3‴), 71.14 (C - 4‴), 75.20 (C - 4‴) and 168.99 (C - 4‴); ESI - MS: m/z, $[C_{44}H_{40}NO_{15}]^{+}$, 822.24.

2.3. In vitro antibacterial test

Baicalin, berberine and BBH were prepared and diluted with culture medium accordant with ratios of 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640. These drug mediums were then sterilized using circulating steam. With pure water as the control, the minimal inhibitory concentrations (MIC) of treatments against eight kinds of common pathogenic microorganisms were tested through micro - dilution method. Four to five colonies were picked from purified strains, inoculated to the nutrient broth medium, and cultured in an incubator at 37 °C for 8 h. Afterwards, it was diluted to the liquid containing 1.5 × 105 CFU/mL bacteria. In sterilized 96 - well polystyrene micro - well plate, 0.1 ml of each strain liquid were added to holes containing different concentrations of treatment agents or pure water. After incubated at 37 °C for 24 h, the results were observed.

2.4. In vivo experiment

2.4.1. Mice

Specific pathogen - free (SPF) male BALB/c mice (18–22 g) were purchased from Experimental Animal Center of Guangdong [SCXK (Guangdong) 2013-0034]. Mice were housed in SPF condition under a 12 h light - dark cycle with ad libitum access to water and food. All experiments and procedures were carried out according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China. Every effort was made to minimize the number of animals used and their suffering.

2.4.2. DSS - induced ulcerative colitis and treatments

In present study, mice were randomly divided into 5 groups (n = 8): control group; UC model group; baicalin - treated group (50 mg/kg); berberine - treated group (50 mg/kg) and the BBH - treated group (50 mg/kg). DSS was dissolved in pure water to obtain a 5% DSS solution. To develop mouse model of UC, mice in model and treatment groups were allowed free access to 5% DSS solution as drinking water for 7 days, the solution was freshly prepared. UC mice started to receive treatments from day 1 and lasted for 14 days. Dose of berberine was set according to previous study,[30] while the concentrations of baicalin and BBH were in line with berberine. Mice in control and UC model groups were administered with pure water.

Table 1
Disesase activity index scoring system.

Score	Weight loss (%)	Fecal bleeding	Stool consistency
0	0–2	None	Normal
1	> 2–5	–	
2	> 5–10	Hemoccult +	Soft
3	> 10–15	–	
4	> 15–20	Visible bleeding	liquid

2.4.3. Disease activity index (DAI)

Both weight loss and appearance of fecal bleeding were recorded every day. Severity of ulcerative colitis was assessed using DAI. The score distribution was adapted from previously study with slight modification as shown in Table 1.[31] Occult blood in the feces was measured using Hemoccult kit.

2.4.4. Tissue collection and processing

After 14 days treatments, mice were sacrificed. Entire colon were collected, the length and weight were measured. Organs of spleen and cecum were removed and weighed.

2.4.5. Colonic damage

Fat and mesentery of colon were cleaned on ice - cold plate. Colon was longitudinally opened and scored for macroscopically visible damage according to the criteria described in Table 2.[32] After macroscopic observation, one cm of the distal colon was cut and fixed in PBS buffered 10% formalin for 24 h. Distal colon were then embedded in paraffin, cut into 5 μm thick sections and stained with H & E. Histological scoring (HS) was given to each microscopic field according to the histological disease scoring system (Table 2), the number of fields per section were large than 12.

2.4.6. Activities assay of SOD and MPO

Activities of SOD and MPO in colon were measured using related biochemistry assay kits. SOD activity was assayed by xanthine oxidase method while MPO was measured by colorimetric method. The absorbance was determined at 550 nm by using microplate reader.

2.4.7. Real - time quantitative PCR (RT - qPCR)

Total RNA was extracted from colonic tissue using TRIzoI reagent, followed by first strand cDNA synthesis. RNA quantification were performed using the SYBR Premix Ex Taq$^{@}$ with BIORAD CFX96 (BIO - RAD, USA). All RT - qPCR reactions were carried out in triplicate. GAPDH house keeping gene was used as a reference standard. Relative expression levels of the target genes were calculated by $2^{-\Delta\Delta Ct}$ method. For mRNA expression, data were first normalized relative to the expression of GAPDH, and then relative to the model group. Primers used in real - time quantitative PCR were shown in Table 3.

2.4.8. Western blotting (WB)

Total protein of colon was extracted and fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Then, the membranes were blocked with 5% nonfat milk in Tris - buffered saline with Tween - 20 (TBST) for 1 h at room temperature. Membranes were incubated with

Table 2
Colonic damage scoring system.

Observation method	Score	Description
Macroscopic	0	No damage
	1	Hyperaemia, no ulcers
	2	Linear ulcer with no significant inflammation
	3	Linear ulcer with inflammation at one site
	4	Two or more sites of ulceration/inflammation
	5	Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation extending > 1 cm along the length of the colon
	6	If damage covered > 2 cm along the length of the colon
Microscopic	0	Normal colonic tissue
	1	Inflammation or focal ulceration limited to the mucosa
	2	Focal or extensive ulceration and inflammation limited to the mucosa and the submucosa
	3	Focal or extensive ulceration and inflammation with involvement of the muscularis propria
	4	Focal ulceration and transmural inflammation with involvement of the serosa
	5	Extensive ulceration and transmural inflammation with involvement of the serosa
	6	Focal or extensive ulceration and transmural inflammation, and perforation

antibodies (IL - 1β, IL - 6 and TNF - α) with a ratio of 1:1000 at 4 ˚C overnight. Then, membranes were rinsed thrice with TBST and incubated with secondary antibody for 1 h at room temperature. Protein bands were visualized with Thermo Fisher Scientific SuperSignal West Femto Maximum Sensitivity Substrate (Rockford, USA) and captured using an Image Quant LAS 4000 imaging system (Shanghai, China).

2.4.9. Mast cell detection

Colon was fixed in formalin and paraffin - embedded, sectioned into 5 μm thick, and stained with special aldehyde - fuchsin orange G to detect mast cell.

2.4.10. Statistical analysis

Statistical analysis was performed using SPSS soft ware version 20.0 (IBMCorp., Armonk, NY, USA). Statistical tests of one - way or two - way analysis of variance (ANOVA) followed by Scheffe's test or unpaired Student's *t*-test were used. All data were expressed as mean ± standard error of the mean (S. E. M.). Graphical representations were generated using Graph Pad Prism 6 soft ware (Graph Pad Soft ware Inc., San Diego, CA, USA). P-values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Design and synthesis of BBH

The synthesis of BBH was performed according to the strategy shown in Scheme 1. In this study, the microwave method was used to synthesize berberrubine (BBB) from berberine with DMB as the reaction medium. BBB was then dissolved in acetonitrile and reacted with 1, 4 - dibromobutane at 85–90 ˚C for 1 h to obtain 9 - o - (4 - bromoethane) berberine hydrochloride (compound 4X) with a yield of 92% (purity ≥ 98%). For the synthesis of BBH, compound X4 and baicalin were dissolved in DMF and triethylamine to react for 45 min at 86 ˚C, the pH of reaction solution was adjusted to 2.0–3.0 and refrigerated overnight to crystallization. The crystals were washed with distilled water to neutral pH and evaporated to dry up. With this method, BBH was obtained with a 53% yield. In present study, HPLC was used to monitor the synthesis reaction, and the results confirmed that baicalin and berberine were synthesized into a new hybrid compound by chemical bonding (Supplementary data). HPLC assessment also showed that with this synthesis strategy, BBH was obtained with a purity ≧ 95% (Supplementary data). The structure of BBH was confirmed by UV, ^{1}H NMR, ^{13}C NMR and HRMS spectroscopy (Supplementary data).

3.2. Vitro antibacterial activity of BBH

In recent years, the relationship between intestinal bacteria and ulcerative colitis has gained much attention. Studies found that patients with ulterative colitis manifested increased enrichment of proteobacteria.[33,34] In present study, we conducted in vitro experiment to investigate the antibacterial activity of BBH. As shown in Fig. 1, berberine and BBH showed good inhibitory effects on all of 8 kinds bacteria, but baicalin had no noteworthy inhibition effects. In addition, compared with berberine, BBH manifested better inhibition effects on candida albicans and proteobacteria in present study (Pseudomonas aeruginosa, Escherichia coli, Typhoid bacillus and Salmonella paratyphi B). These results suggested that the newly synthesized hybrid compound BBH exhibited the best inhibition effect on proteobacteria as compared with its original synthetic materials of baicalin and berberine.

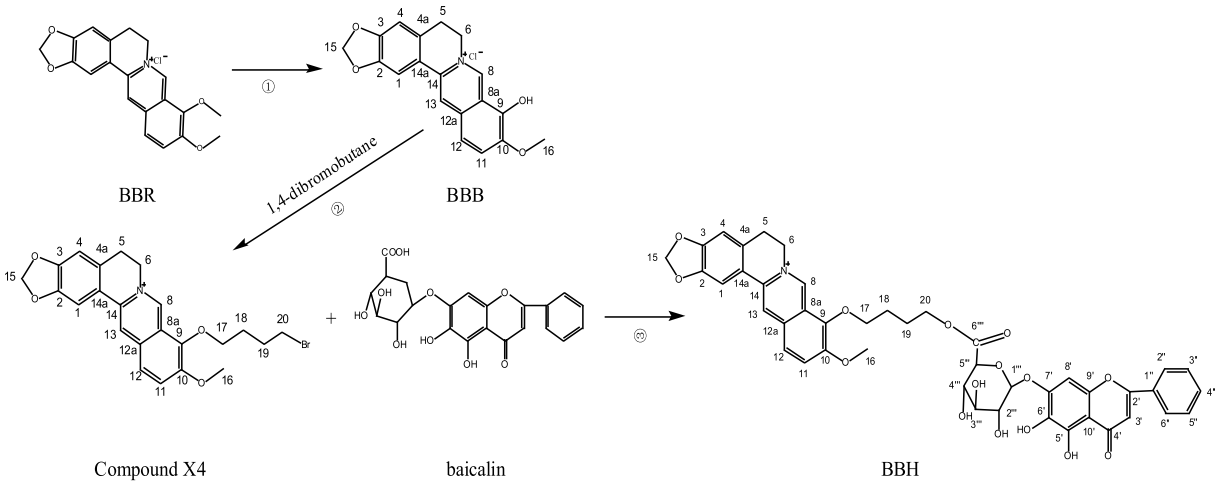
3.3. Effects of BBH on ulcerative colitis

3.3.1. Effects of BBH on disease activity score of UC mice

In present study, ulcerative colitis was induced by giving 5% DSS in drinking water over a 7 - day period. To test and compare effects on disease symptoms of ulcerative colitis, baicalin, berberine and BBH were administrated to DSS - induced UC mice. According to previous studies,[35,12] the mainly disease parameters of DSS - induced ulcerative colitis were marked by body weight loss, pasty - to - liquid grossly bloody stool and rectal bleeding. The DAI scoring system, observe symptoms include weight loss, fecal bleeding and stool consistency, is used to evaluate severity of ulcerative colitis. In present study, body weight, stool consistency and fecal bleeding were recorded every day and DAI was scored according to scoring system presented in Table 1. As shown in Fig. 2, BBH treatment significantly ameliorated the DAI of UC mice from day 5, berberine also manifested the effects from day 8. However, baicalin treatment failed to show noteworthy effects on symptoms of UC mice. These results demonstrated that BBH exhibited

Table 3
Primers used in real - time quantitative PCR.

Gene	Forward primer	Reverse primer	Organism
IL - 1β	TTGGTGATGTCGGTGCTCTT	GGACTCACGGCAGAAGTTCA	Mouse
IL - 6	CTGCCGTGGTACAGAACTGG	CCAGGTGCTGTGGAGTATGC	Mouse
TNF - α	GGATGGCCACTGTGAATAACTG	TCGAGGACATCGCTCTCTCA	Mouse



Scheme 1. Synthesis of BBH. Reagents and conditions: ① solution: DMF; condition: microwave power (400 W), 15 min; yield: 93%. ② solution: acetonitrile; condition: reflux, 85 – 90 °C, 1 h; yield: 92%. ③ solution: DMF and triethylamine; condition: reflux, 45 min at 86 °C; yield: 53%.

the best potential therapeutic effects on symptoms of ulcerative colitis as compared to its original molecules of baicalin and berberine.

3.3.2. The effect of BBH on colon index of UC mice

Alterations in architectural organization of colon, such as increased barrier permeability and decreased thickness of the colonic mucus layer, are major contributors to disease pathology of UC.[36,37] Decreased colon index (colon length/colon weight) suggested alterations in architectural organization of colon in UC mice, moreover, reflected the degree of inflammation.[32] As shown in Fig. 3 and Table 4, DSS - induced UC model mice showed a significant reduction in colon length and increment in colon weight as compared with normal mice. Berberine and BBH treatments showed remarkable inhibition effects on alteration of colon weight. While mice in BBH group apparently regained their colon length and relieved their colon edema. However, baicalin treatment with a dose of 50 mg/kg exhibited no noteworthy effect on colon index of UC mice. In treatment groups, compared with baicalin and berberine, BBH indicated the best effects on both the length and weight of colon in UC mice, suggesting that, under the same concentration, BBH exhibited the highest therapeutic effects in preventing the alterations in colonic architectural organization of UC mice.

3.3.3. Effects of BBH on organs weight of UC mice

In present study, DSS - induced UC mice demonstrated big decreased cecum weight and elevated spleen weight as compared with normal mice. In treatment groups, berberine ameliorated the abnormal weight change of cecum but showed no significant effect on spleen weight of UC mice (Fig. 4A & B). But, similar spleen and cecum weights to the normal mice were observed in BBH - treated UC mice. In addition, a noteworthy difference was found between berberine and BBH - treated groups. In results of organs weight, 50 mg/kg dose of baicalin also failed to reveal obvious effects. Previous studies reported that UC mice indicated tissue damage related weight loss of cecum and hyperactivity of spleen caused by immune overactivation.[38] Our results presented that BBH exerted significant therapeutic effects on pathological weight changes of cecum and spleen which were caused by DSS, and suggested a potential regulatory effect of BBH on inflammation of UC mice.

3.3.4. Effects of BBH on colon damage of UC mice

Defects of colonic epithelial cells as well as mucous barrier are strongly implicated in the pathogenesis of ulcerative colitis. DSS is a common chemical which used to mimic UC - like phenotype of colon damage. DSS which can induce chemical injury in colonic epithelial cells as well as macrophages makes the colonic lamina propria and submucosal compartment exposed to luminal antigens as well as enteric bacteria and triggers inflammation.[2] Colon damage was evaluated according to the scoring system shown in Table 2. The macroscopic damage scoring of colon indicated that, compared with normal mice, UC model mice manifested visible colon damage features, such as obvious edema, ulceration, sever inflammation, thinning and easy ruptured colon wall (Fig. 5A). While such symptoms were significantly alleviated

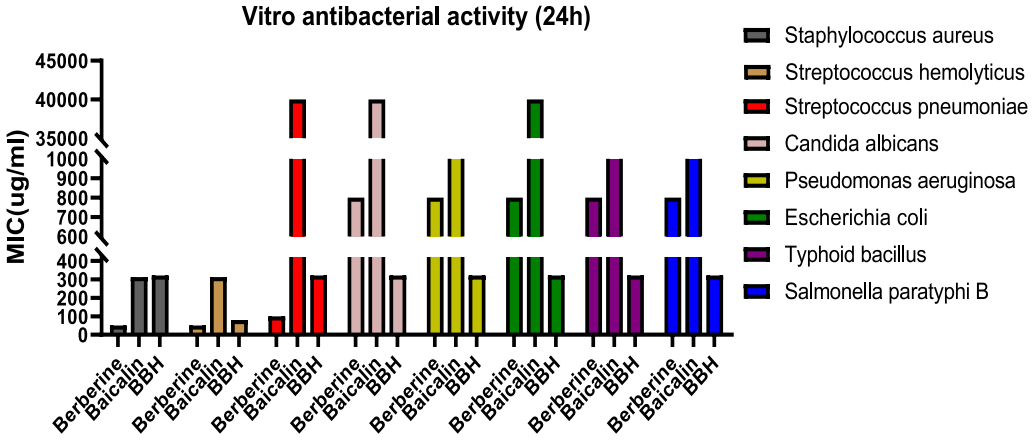


Fig. 1. Vitro antibacterial activity. 8 kinds of bacteria were used in present study to evaluate the antibacterial activity of BBH. Baicalin and berberine were used for comparison.

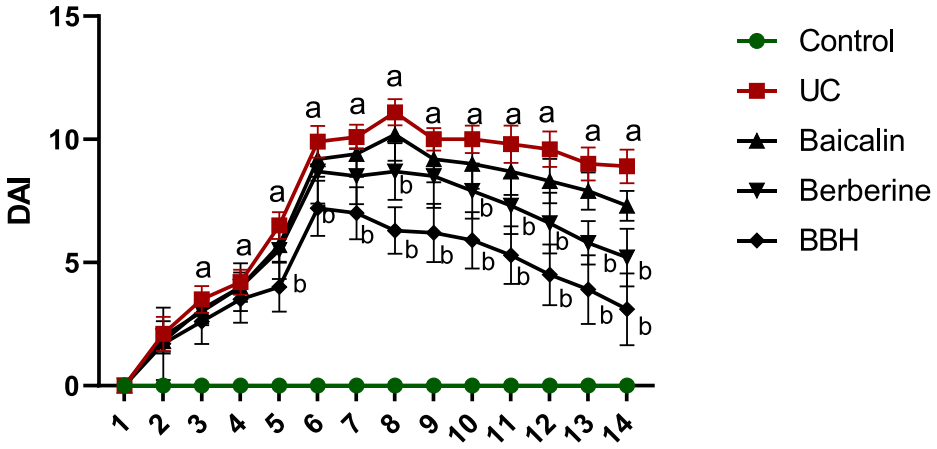


Fig. 2. Effects of BBH on disease activity score of UC mice. Body weight, stool consistency and fecal bleeding were recorded every day and DAI was scored. Data were represented as means ± S. E. M. (n = 8). a means P < 0.05 when UC model group vs control group; b means P < 0.05, when treatment groups vs UC model group.

in BBH - treated UC mice. Histological examination of colon was conducted after H & E staining. Compared with normal mice, UC mice showed severe mucosal damage, crypt shortening, ulceration, gland destruction, loss of goblet cells, disappearance of glandular epithelium and infiltration of inflammatory cells in mucosa as well as lamina propria. In treatment groups, the histological scores of berberine and BBH - treated mice were both decreased when compared to UC mice (Fig. 5B). As shown in Fig. 5C, berberine and BBH significantly reduced the colon damage, promoted regeneration of intestinal mucosa and inhibited inflammation in colon. Although baicalin, to some extent, inhibited mucosal damage and pathological inflammation of UC mice, its therapeutic efficiency was still not strong enough to remarkably ameliorate ulcerative colitis. As compared to berberine, BBH - treated UC mice also presented outstanding low histological score. All these results indicated that BBH had the best therapeutic effects of reducing DSS - induced colon damage.

3.3.5. Anti - inflammatory effects of BBH on colon tissue of UC mice

Previous studies have confirmed that oxidative stress played an important role in the pathogenesis of UC through affecting inflammatory process. Oxidants can directly increase the number of neutrophils and macrophages that will induce inflammatory process subsequently.[13] Under the normal condition, body keeps a balance between oxidants and antioxidants which is important for the function of intestinal mucosal barrier. SOD is an antioxidase that protects cells against ROS - induced damage by combating and blocking free radicals.[39] In the pathological state of ulcerative colitis, the balance of oxidants and antioxidants is broken, and one of its major indications is the decreased SOD level. In present study, to analyse the effect of BBH on antioxidant defense, activity of SOD in colon was measured. Results reported that the activity of SOD in UC model mice was significantly decreased in colon as compared with normal mice. While, UC mice which were treated with BBH manifested remarkably increased SOD

Table 4
The effect of BBH on colon index.

Groups	Colon length (cm)	Colon weight (g)	Colon index (cm/g)
Control	11.156 ± 0.592	0.265 ± 0.027	42.858 ± 4.304
UC model	8.350 ± 1.111**	0.369 ± 0.023**	22.608 ± 2.655**
Baicalin	8.740 ± 0.488	0.369 ± 0.065#	24.172 ± 3.907#
Berberine	8.950 ± 0.575	0.315 ± 0.033^	28.175 ± 4.414^
BBH	9.288 ± 0.662^	0.311 ± 0.038^^	29.769 ± 5.581^^

Data were represented as means ± S. E. M. (n = 8). **P < 0.01 when UC model group vs control group; ^P < 0.05, ^^P < 0.01 when treatment groups vs UC model group; #P < 0.05 when baicalin or berberine - treated groups vs BBH - treated group.

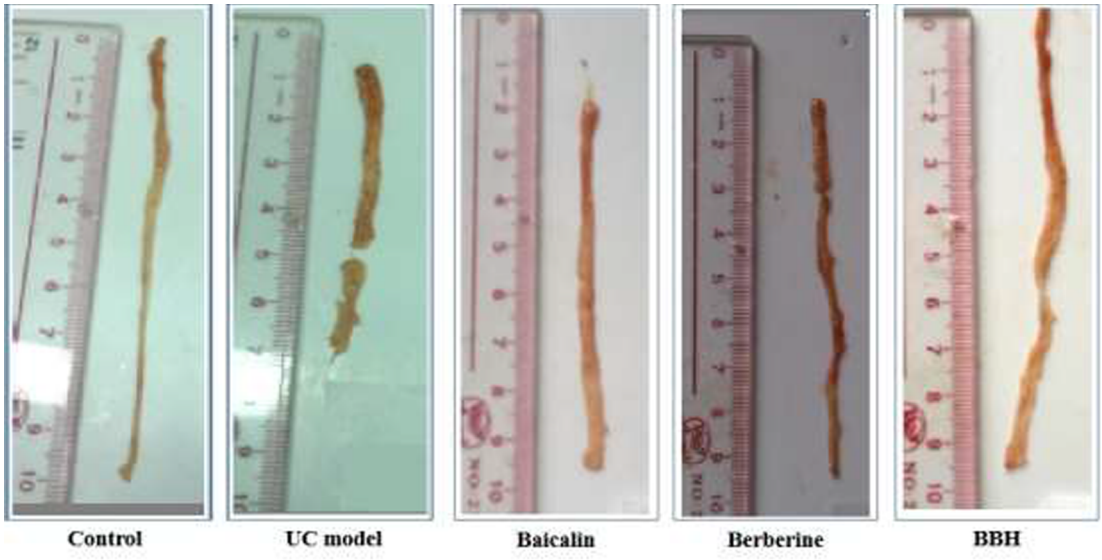


Fig. 3. The effects of BBH on colon length of UC mice.

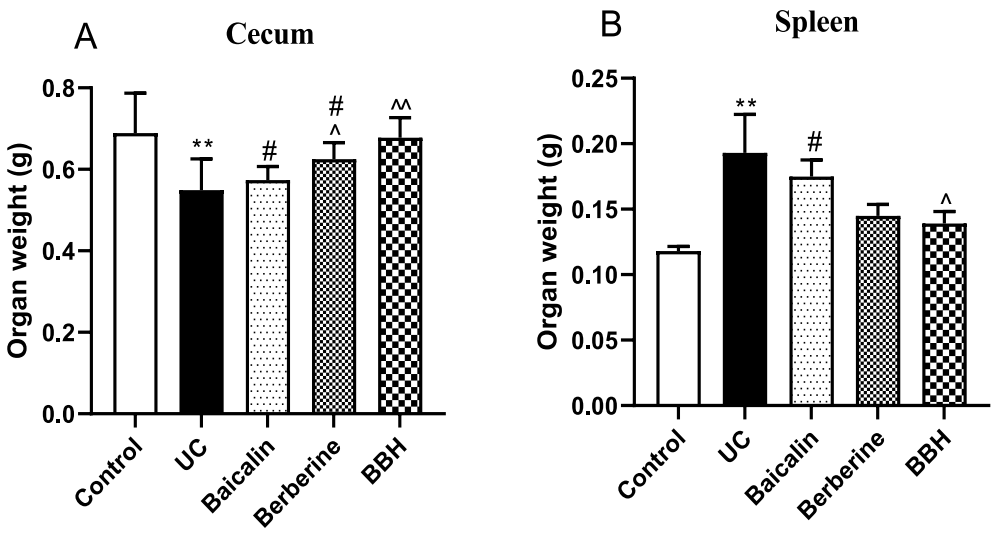


Fig. 4. Effects of BBH on organs weight of UC mice. Organs weight of cecum (A) and spleen (B) of mice in all groups were detected. Data were represented as means ± S. E. M. (n = 8). **P < 0.01 when UC model group vs control group; ^P < 0.05, ^^P < 0.01 when treatment groups vs UC model group; #P < 0.05 when baicalin or berberine - treated groups vs BBH - treated group.

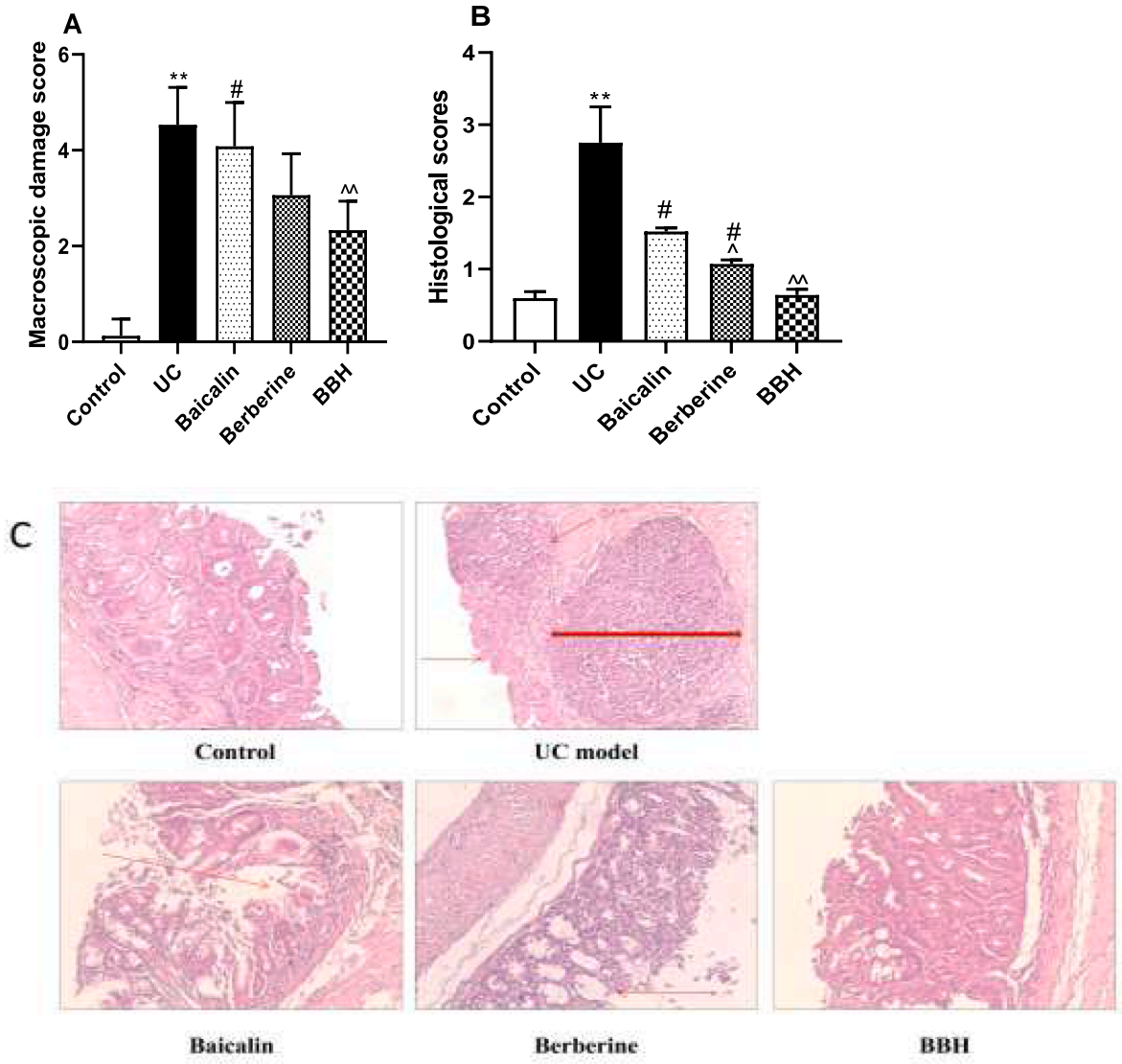


Fig. 5. Effect of BBH on colon damage of UC mice. Colon damage was evaluated according to colonic damage scoring system. (A) Macroscopic damage scores. (B) Histological scores. (C) H & E staining of colon (10 * 10). Data were represented as means ± S. E. M. (n = 8). **P < 0.01 when UC model group vs control group; ^P < 0.05, ^^P < 0.01 when treatment groups vs UC model group. #P < 0.05 when baicalin or berberine - treated groups vs BBH - treated group.

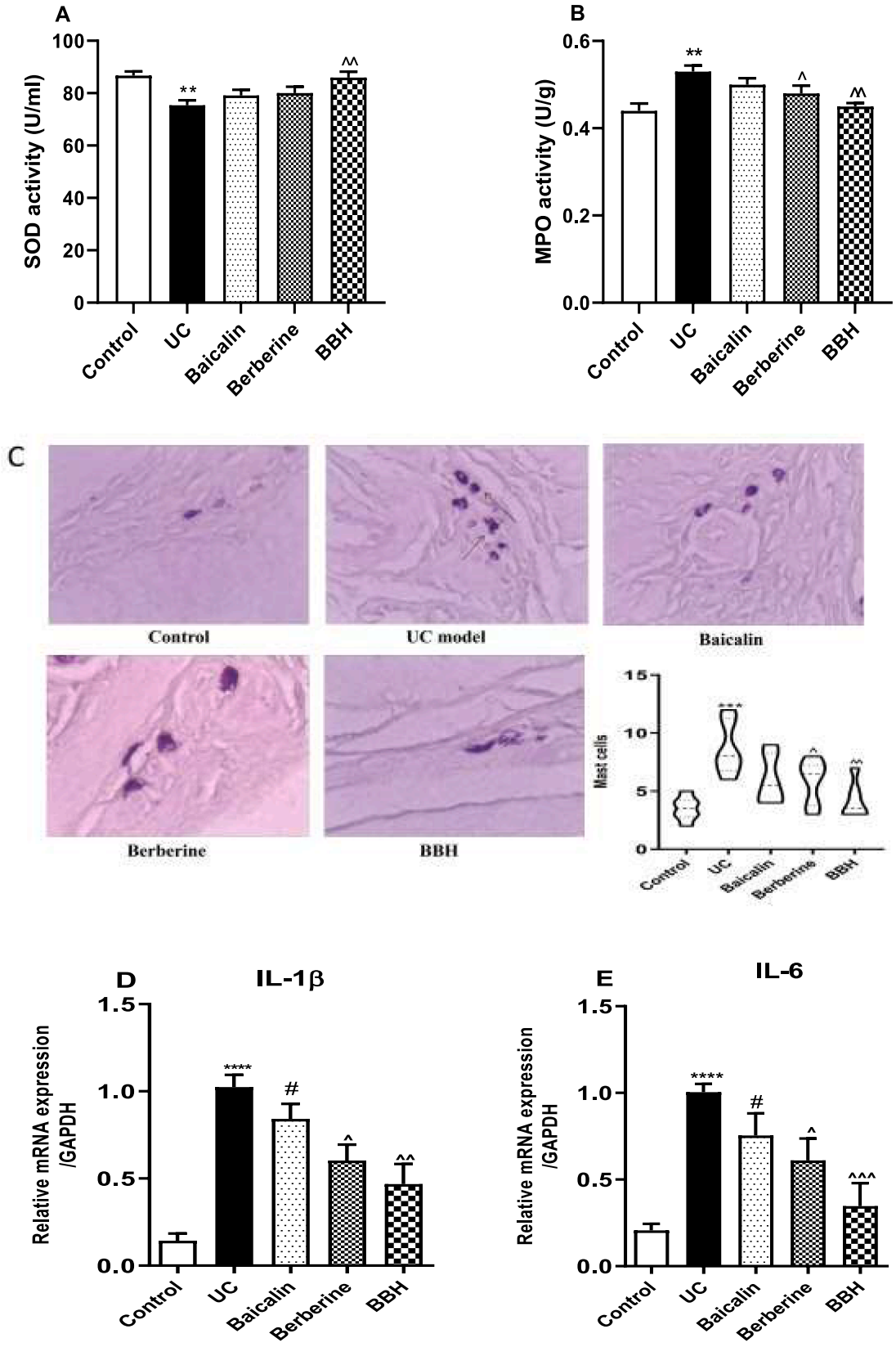


Fig. 6. Anti - inflammatory effect of BBH in colon tissue of UC mice. After 14 days of treatments, colon of mice were collected, activity of SOD (A) and MPO (B) were detected using relative biochemistry assay kits. Colon special aldehyde - fuchsin orange G staining and statistical analysis of colon MCs were also conducted (C) (n = 8). mRNA expressions of IL - 1β (D), IL - 6 (E) and TNF - α (F) were analyzed by using RT - qPCR method. Protein levels of IL - 1β, IL - 6 and TNF - α in colon were analyzed by using WB (G). Data were represented as means ± S. E. M. (n = 8). **P < 0.01, ***P < 0.001 and ****P < 0.0001when UC model group vs control group; ^P < 0.05, ^^P < 0.01 and ^^^P < 0.001 when treatment groups vs UC model group; #P < 0.05 and ##P < 0.01 when baicalin or berberine - treated groups vs BBH - treated group.

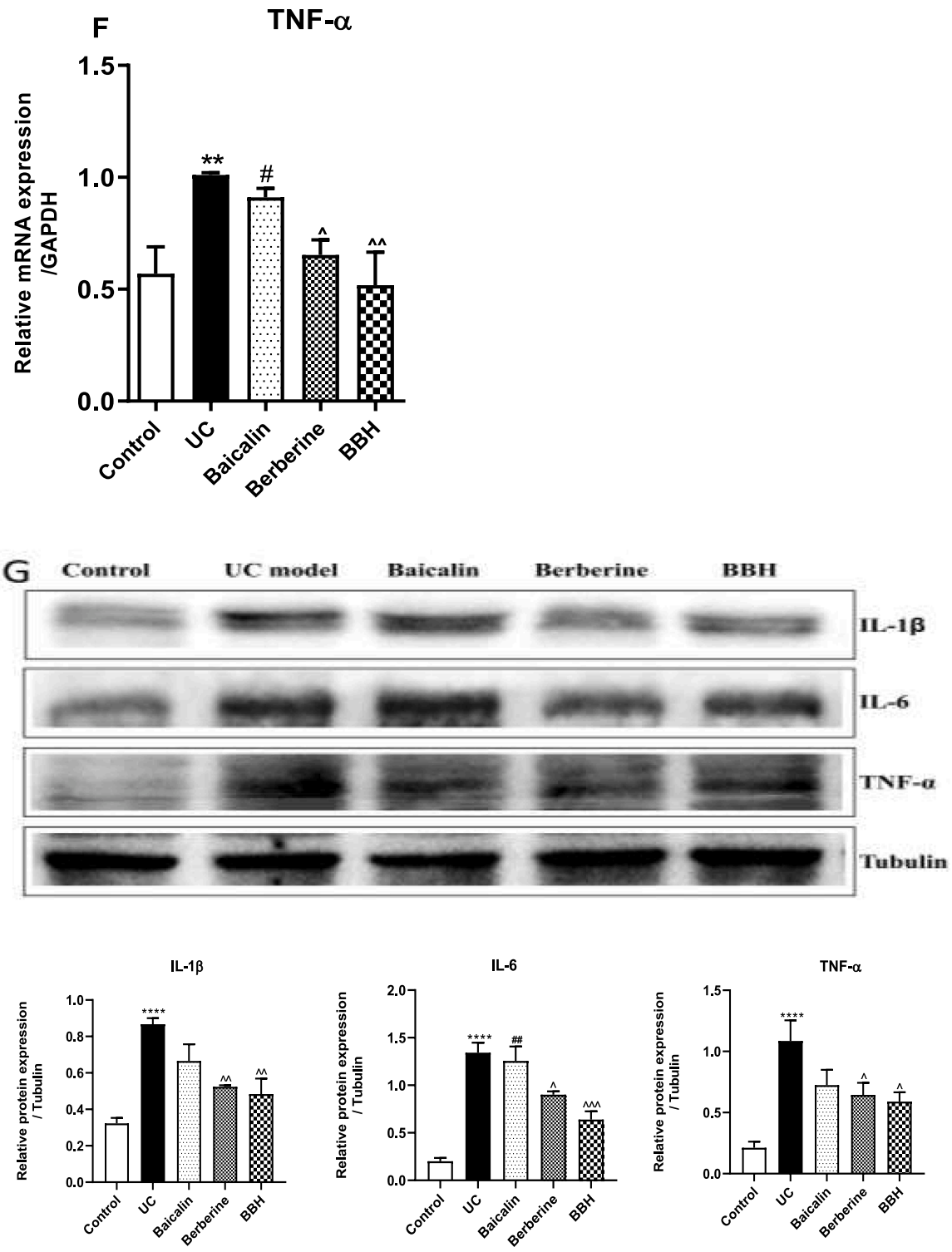


Fig. 6. (*continued*)

level in colon. The date indicated a inhibition effect of BBH on oxidative stress in colon of UC mice (Fig. 6A). As a marker for accumulation of tissue - related neutrophils, MPO is closely related to neutrophil infiltration. In ulcerative colitis, the activity of MPO is a direct indicator to follow inflammatory processes in colonic tissue.[39,40] As shown in Fig. 6B, that UC mice revealed obviously increased MPO activity in colon, suggested higher colonic neutrophil infiltration. While, UC mice treated with BBH and berberine significantly reduced the MPO activity in colon, and the BBH had better effect than berberine. This indicated a noteworthy anti - inflammatory effect of BBH in treating ulcerative colitis. Inflammation has been known to play a core role in the induction and persistence of ulcerative colitis. Over - expression of pro - inflammatory cytokines such as IL - 1β, IL - 6 and TNF - α are closely linked to intestinal inflammation and seem to act an important role in the disease process.[41] Acute intestinal injury can affect the gut immune function through inducing over-secretion of TNF - α, IL - 6, IL - 1β and other cytokines.[42] Mast cells (MCs) are important components of immune system, and responsible for secretion of multiple inflammatory mediators. Their degranulation occurs under pathological condition of UC, and results in inflammatory infiltration in intestines or impacts

intestinal function.[43–45] Controlling inflammation is essential in preventing ulcerative colitis, and treatments of ulcerative colitis that focus on symptom management are mainly aim to relieve inflammation.[12,41,46] In present study, berberine and BBH reduced the number of infiltrated mast cells in colon of mice with UC (Fig. 6C). We next detected the effects of BBH on pro - inflammatory cytokines in colon. Our results showed that DSS - induced UC mice expressed significant higher mRNA and protein expressions of IL - 1β, IL - 6 and TNF - α in colonic tissue. Berberine and BBH treatments remarkably decreased the mRNA and protein levels of IL - 1β, IL - 6 and TNF - α in colon of UC mice. While BBH exhibited better efficiency. Besides, baicalin showed no noteworthy effects on mRNA and protein expressions of above cytokines in colon (Fig. 6D - G). Our results indicated that BBH exhibited significant anti - inflammatory effect through down-regulating pro-inflammatory cytokines in colon of UC mice.

4. Conclusion

In present study, we designed and synthesized a new hybrid compound of baicalin and berberine as well as investigated its potential therapeutic effects on ulcerative colitis. In in vitro antibacterial test, BBH showed a broad - spectrum antibacterial activity, and as compared with its original synthetic compounds (baicalin and berberine), BBH exhibited the best antibacterial activity against proteobacteria. Based on this experiment, we further conducted in vivo study to evaluate the therapeutic effects of BBH on murine model of ulcerative colitis. The treatment of UC mice with BBH demonstrated significant and beneficial influences. BBH prevented DSS - inducing colon damages and improved the symptoms of colitis. Furthermore, BBH exhibited noteworthy anti - inflammatory effects on UC mice through inhibiting activity of MPO and protein expressions of pro - inflammatory cytokines (TNF - α, IL - 1β and IL - 6) in colon tissue. In addition, BBH markedly improved the SOD activity in UC mice, suggesting an inhibitory effect of BBH on oxidative damage. Data also suggested that BBH was more superior than baicalin and berberine in treating UC mice. In conclusion, our study provided insights into the therapeutic effects of a new baicalin and berberine hybrid compound which designed according to a “medicine couple” of TCM. We found that BBH manifested the best therapeutic effects on UC mice as compared with its original synthetic compounds. But its underlying mechanism require further investigations.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115697.

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