

Xanthones from *Gentianopsis paludosa*(Hook. f.) Ma ameliorates colonic epithelial-mesenchymal transition through regulating autophagy

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Abstract

Ethnopharmacological relevance: Ulcerative colitis (UC) is an immune-mediated, idiopathic, chronic, recurrent inflammatory bowel disease inflammatory bowel diseases (IBD). *Gentianopsis paludosa* (Hook. f.) Ma, a traditional Tibetan medicine, is commonly used to treat hepatitis, cholecystitis, diarrhea, gastroenteritis, conjunctivitis, scabies, swollen poison and other diseases. The research group replicated the ulcerative colitis (UC) rat model through (2,4-dinitrobenzenesulfonic acid, DNBS in the early stage, and it was clear that xanthone is the main active components, but the mechanism is still unclear and needs to be further elucidated.

Aim of the study: Inflammatory bowel disease (IBD) is considered as one of the most intractable diseases worldwide. Intestinal fibrosis, as the most serious complication of IBD, can easily lead to intestinal scarring, intestinal stricture and even intestinal obstruction. Therefore, there is an urgent need to accelerate the development of anti-fibrotic drugs for IBD. The purpose of this study was to investigate the protective effect of xanthone from *Gentianopsis paludosa* (GPX) on the colonic epithelial-mesenchymal transition and its specific mechanism.

Methods: The damage to colonic epithelium was caused by intraperitoneally injecting DNBS, and the therapeutic potential of GPX on colonic fibrosis was detected, which was verified by macroscopic evaluation of rats (disease activity index, DAI; colonic mucosal damage index, CMDI), histopathological staining and Western blotting. In addition, the effect of GPX was tested on NCM460 cells *in vitro*. The expression levels of EMT-related proteins, mRNAs and autophagy-related proteins, mRNAs in colon epithelial cells were detected by immunofluorescence assay, western blotting, quantitative real-time PCR and other techniques, and the possible mechanisms were explored.

Result: GPX significantly inhibited colonic fibrosis, as preliminarily demonstrated by DNBS-induced body weight gain, DAI, CMDI score reduction and colonic fibrosis area reduction in rats. Moreover, GPX could down-regulate the expression of mesenchymal transition-related genes and proteins, and up-regulate the level of autophagy in colonocytes. Mechanistic studies showed that GPX could affect EMT by regulating autophagy, as evidenced by autophagy inhibitors (3-MA) or agonists (Rapa) that inhibit or enhance this effect of GPX.

Conclusion: Herein, the evidence was provided to support that GPX could effectively inhibit colonic fibrosis, and its mechanism of action may be related to the inhibition of epithelial-mesenchymal transition and the regulation of autophagy in intestinal epithelial cells.

1. Introduction

Ulcerative colitis (UC) is a chronic relapsing inflammatory bowel disease clinically characterized by diarrhea and rectal bleeding, which is often accompanied by mucosal pus, abdominal cramps, fever, and rectal emergency [1]. It has been listed as one of the intractable diseases by the World Health Organization (WHO) [2]. Fibrosis would promote tissue repair as an adaptive response to inflammatory episodes.

However, it would lead to excessive scarring of the intestinal wall and intestinal stenosis or even intestinal obstruction as an excessive response to chronic inflammation characterized by massive production and deposition of extracellular matrix (ECM)^[3–4]. Although great progress has been made in drug research for the treatment of colon diseases, there is no effective anti-fibrotic drug on the market so far. Compared with the latest progress in anti-inflammatory therapy, the anti-fibrotic therapy still needs to be further developed. Currently, endoscopic intervention or surgery, such as strictureplasty or segmentectomy, is the main treatment method for clinically symptomatic patients, but frequent hospitalization and surgery not only affect the prognosis of patients, but also bring a high socioeconomic burden^[5]. A growing number of studies have shown that the isolation of anti-fibrotic drugs from natural medicines is an appreciable approach. Xanthone is a class of phenolic compounds with diverse biological activities, such as anti-viral, anti-inflammatory, anti-tumor and anti-hepatitis, myocardial, kidney and other organs organ cell fibrosis, etc^[6–9]. Our previous study found that the Tibetan medicine *Gentianopsis paludosa* (Hook. f.) Ma has a good therapeutic effect on ulcerative colitis and its complications^[10], and the main active ingredient is xanthone^[11]. Based on the previous research, in this study, xanthone was obtained by extraction and isolation from *G. paludosa*, and used to explore whether the xanthone compounds from *G. paludosa* (GPX) can regulate the autophagy of colonic epithelial cells, and thereby inhibit the occurrence and development of cell Epithelial-mesenchymal transition (EMT) by a combination of in vitro and in vivo experiments. The obtained result was expected to provide scientific basis for its clinical application and further research and development.

2. Materials And Methods

2.1 GPX preparation

The herb was collected from Dacao Beach, Zhangxian County, Dingxi District, Gansu Province and identified as *G. paludosa* by Shangzhi Zhang, professor of Gansu University of Traditional Chinese Medicine. The herb was soaked overnight with 8 times the amount of 95% ethanol, and extracted under reflux for 2 h. The extract solution was filtered and the residue was extracted under reflux once again according to the above procedure. The two extract solutions were combined and concentrated into the crude extract with a concentration of $1.5\text{g}\cdot\text{mL}^{-1}$ under the reduced pressure. The extract was suspended in deionized water, and extracted successively with petroleum ether, ethyl acetate and n-butanol. The ethyl acetate extract was purified by column chromatography on DM130 macroporous resin producing xanthone powder with a purity $\geq 51\%$, The product was sealed and stored at low temperature for future use^[12].

2.2 Reagents and antibodies

Rapamycin (Rapa, 18098372), MedChemExpress, USA; 3-methyladenine (3-MA, N1012A), Dalian Meilun Biotechnology Co., Ltd.; 2,4-Dinitrobenzenesulfonic acid (DNBS, 556971), Aladdin Reagent Co., Ltd., Shanghai; Pifenidone (PNDF, S290702), Selleck, USA; Rpmi-1640 Medium (AF32246309) and PBS (AF29526788), HyClone, USA; HE staining kit (G1120), Masson's trichrome staining kit (G1340),

Lipopolysaccharide (LPS, 408Z033), Autophagy Staining Detection Kit (20201127), Solebo Technology Co., Ltd., Beijing; Anti-e-cadherin Antibody (40860), SouthernBiotech, USA; Anti-N-Cadherin (GB11135), Xavier Biotechnology Co., Ltd. Company, Wuhan; Anti-col I (BS-0578R) Boaosen Biotechnology Co., Ltd., Beijing; Anti-col III (GTX111643), Anti-E-Cadherin (GTX42718), ZO-1 (GTX43488), α -SMA (GTX43040), LC3B (GTX42592), GeneTex, USA. Anti-Beclin-1 (AB207612), anti-p62 (55274-1-AP), Proteintech, USA; anti-Ic3b (BM4827), Broad Bioengineering Co., Ltd., Wuhan. TRIeasy™ Total RNA Extraction reagent (10606ES60), Hifair® II 1st Strand cDNA Synthesis SuperMix for qPCR (11123ES60), Hieff® qPCR SYBR Green Master Mix (11203ES08), Cell Counting Kit-8 (CCK8, C1905401) and Fetal Bovine Serum (FBS, 504050519), Yisheng Biotechnology Co., Ltd. Shanghai.

2.3. Induction of chronic colitis-associated intestinal fibrosis and GPX treatment

The principles and protocols of animal research were reviewed and approved by the Animal Ethics Committee of Gansu University of Traditional Chinese Medicine. Wistar male rats with a body weight of 220 ± 20 g were purchased from Changzhou Kevins Laboratory Animal Co., Ltd. (Changzhou, China), license number: SCXK (Su) 2021-0013. The rats were reared in the Specific Pathogen Free (SPF) laboratory of Gansu University of Traditional Chinese Medicine, with the site license number SYXK (Gan) 2021-0004, and housed in environmentally controlled breeding room (temperature: $20 \pm 2^\circ\text{C}$, humidity: $60 \pm 5\%$, 12-h dark/light cycle). Water and food were given ad libitum throughout the study period. Rat colonic fibrosis was induced as previously reported with a slight modification (Di Marco et al. 2003). The mice were fasted for 24 h with free access to water before operation.

Rats were injected intracolonicly with $300 \mu\text{L} \cdot (100 \text{ g})^{-1}$ of 50% ethanol containing 25% DNBS solution or 50% ethanol as illustrated in Fig. 1A. Intracolonic administration was achieved by inserting a polyethylene catheter (2 mm in outer diameter) 8–10 cm into rectum under anesthesia. After administration of DNBS, rats were held in headfirst position for 3 min to prevent the solution from leaking out. Rats were then returned to their cages with free access to food and water. GPX treatment begins with the first DNBS induction. DNBS-treated mice were randomly divided into five groups and gavaged daily (5 mL/kg/d) with either vehicle (0.9% saline) or different concentrations of GPX ($138.6, 69.3, 34.7 \text{ mg} \cdot \text{kg}^{-1}$) and perfenidone (PNFD) $107.7 \text{ mg} \cdot \text{kg}^{-1}$ for another 20 days while control mice were gavaged daily with 0.9% saline by the same procedure. Colonic tissues were collected after euthanasia.

2.4 Disease activity index (DAI) and histological scoring

Rat's bodyweight, stool features, and fecal occult blood were monitored throughout the experiment on alternate days. The disease activity index (DAI) was evaluated according to the previously established criteria^[13]. The length and weight of colons were determined. Colon gross morphological damage index (CMDI) score was calculated according to the previously established criteria^[14]. Scores of two gross morphology values were averaged for statistical analysis.

2.5 Histopathological examination

The colonic samples were fixed in 4% paraformaldehyde fixative solution, and embedded in paraffin. Then the serial sections with 5- μ m thickness were subjected to hematoxylin/eosin (H&E) staining. Finally, the slides were examined under a light microscope (with $\times 100$ magnification) to observe the morphological changes. Ten random microscopic fields from each tissue section were digitally captured under fixed microscope illumination settings. At least six colonic tissues were selected and analyzed for each animal.

2.6 Masson Staining

The paraffin blocks of colonic tissue were cut in 5 μ m thickness, and sections were subjected to Masson trichrome staining following the manual. Photographs were taken by light microscope. Under the microscope, the collagen fibers were stained blue, and the cytoplasm and muscle fibers were stained red. Then the percentage of collagen fiber in the overall area was calculated.

2.7 Cell Cultures and treatment

NCM460 cell line was purchased from Beijing BNA Chuanglian Biotechnology Research Institute (Beijing, China). It was grown in DMEM high glucose medium supplemented with 10% FBS and maintained at 37°C in a humidified incubator with 5% CO₂. Experimental cells were processed as follows: (1) the control group was treated with DMEM medium (2) the model group was treated with 2.5 μ g/ml LPS for 14 day. (3) the GPX group PFND group GPX + 3-MA group and GPX + Rapa group were treated with different concentrations of GPX PFND 0.5mM 3-MA + GPX and 10 nM Rapa dissolved in DMEM medium for 48h after the treatment with 2.5 μ g/ml LPS for 14 day .

2.8 Cell viability assays

Cell viability assays were performed using the CCK-8 kit, according to the manufacturer's instructions. Briefly, cells were seeded at a density of 5×10^4 cells per well in 96-well plates and incubated overnight to allow adherence. Cells were then treated with GPX (25, 50, 100, 200, 300, 400 μ g/mL) for 48 h, respectively, and incubated with CCK-8 solution at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate reader. Each experiment was repeated 3 times, and each sample was measured 6 times in parallel.

2.9 Immunofluorescence staining

To observe the expression E-cadherin, Vimentin and α -SMA in cells, NCM460 were grown on cover glass and treated as described in the figure legends. Cells were then fixed in 1% formaldehyde and permeabilized with 0.1% triton x100. The cells were probed with E-cadherin, Vimentin and α -SMA antibodies followed by Alexa Fluor 488 secondary antibodies, respectively. To visualize the nuclei, cells were then treated with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Cells were washed with PBS and coverslips were mounted with mounting solution onto glass slides; the slides were then examined under a fluorescent microscope .

2.10 Monodansylcadaverin(MDC)

After overnight culture in a 6-well plate (2.5×10^5 cells/well, 1 mL medium/well), the cells were treated with LPS for 21 day and GPX,PFND,GPA + 3-MA and GPX + RAPA for an additional 48 hours, the cells were incubated with MDC (50 μ mol/L) at 37°C for 30 minutes. After incubation, the cells were washed two times with precooling PBS, and immediately observed and pictured under a fluorescence microscope (excitation wavelength of 355 nm and emission wavelength of 512 nm).

2.11 Western blot analysis

Tissues and cells lysates were homogenised in radio immunoprecipitation assay lysis buffer, and total protein concentrations were determined using a BCA Protein Assay Kit. Equal amounts of protein extracts were separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were then transferred to methanol-preactivated polyvinylidene difluoride membranes. Membranes were then blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C. After washing with TBST, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, proteins were visualised using an enhanced chemical luminescent system, and bands were imaged using a chemiluminescence imaging system. The relative expression level of target protein was indicated by the ratio of target protein to internal reference β -actin or GAPDH gray value.

2.12 RNA isolation and quantitative real-time PCR

Total RNA in NCM460 cells were extracted with TRIzol Reagent according to the manufacturer's instructions. cDNA was synthesized by ReverTra Ace qPCR RT Kit. The mRNA levels of ZO-1, E-cadherin, N-cadherin and α -SMA were measured via the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as endogenous control) using quantitative reverse-transcription qRT-PCR. Real-time PCR was performed with SYBR Green Master Mix using FTC-3000P system. Relative levels were calculated using the comparative-Ct method ($\Delta\Delta$ Ct method) (Yang et al.2021). The primer sequences can be found in Table 1.

Table 1
Primers for qRT-PCR.

gene	Primer sequence (5'-3')
E-cadherin	ACGTATACCCTGGTGGTTCA
	AAATCCAAGCCCGTGGTGG
Vimentin	TCCGCACATTGAGCAAAGA
	AACTTACAGCTGGGCCATCG
ZO-1	AGGCGTAGTAGGTCGGGTTT
	CCAGAGATCACACTTCAGGAAC
α -SMA	TACTGTTCCGCTCCCAGAAG
	TCATCGCCATAGCAAACCCG
beclin 1	AGCTAAGGGGGTCGGTATGT
	TGTGGCGACAACTCCAAACT
P62	GGAGATGAGGCTCCGCAAT
	GCTGGTAAACGTCCACAACG
bcl-2	AAAAATACAACATCACAGAGGAAGT
	TCCCGGTTATCGTACCCTGT
LC3B	TTCCGAGTTGCTGACTGACC
	CCCTTGTAGCGCTCGATGAT
GAPDH	GTCAACGGATTTGGTCGTATTG
	CATGGGTGGAATCATATTGGAA

2.13 Statistical analysis

The experimental data were expressed as means $\pm$ SD. The software SPSS Statistics 21.0 was used for statistical analysis. One-way ANOVA was adopted and $P < 0.05$ was considered statistically significant. And ruskal-Wallis tests were performed to analyze the histopathology scores. Graphpad prim 9.0 was used for plotting.

3. Results

3.1 Protective effect of GPX against DNBS-induced colitis

In this study, DNBS-induced chronic colitis rat model was used to evaluate the effect of GPX. As expected, weight loss and increased DAI scores were observed in DNBS-induced mice compared with the control group, whereas the treatment with GPX or PNFD after modeling caused significant recovery of body weight loss (Fig. 1B) and DAI scores (Fig. 1C). Furthermore, it was found that the DNBS-induced colitis group had severe colonic ulceration and greater colonic wet mass index compared with the control group, whereas GPX treatment significantly rescued DNBS-induced colonic injury (Fig. 1D) and reduced the wet mass index and CMDI scores (Fig. 1E and F). Histopathological examination showed that in the DNBS-induced colitis group, the structure of colon was severely disordered with reduced goblet cells and a large number of infiltrated inflammatory cells. These symptoms were significantly improved after GPX treatment (Fig. 1G). The results of Masson staining showed that in the blank control group, the colonic muscle fibers were arranged as a whole, and the blue staining of collagen fibers mainly appeared in the submucosa, and some narrow blue-stained bands are seen between endogenous muscle smooth muscle fibers. In the DNBS-induced model group, blue-stained collagen increased, and the arrangement of colon muscle fibers was disordered. In the GPX intervention group, with the increase of the dose, the area ratio of collagen fibers decreased significantly, and the arrangement of colon muscle fibers tended to be neat to different degrees (Fig. 1H and I). The above results confirmed that GPX had a certain protective effect on against the development of colonic fibrosis in chronic colitis.

3.2 Inhibition effect of GPX on epithelial-mesenchymal transition in colon tissue

To further confirm the effect of GPX on colonic epithelial-mesenchymal transition, the levels of E-cadherin, N-cadherin, α -smooth muscle actin (α -SMA), collagen type I (Col I) and collagen type III (Col III) in colon tissue was measured by western blot analysis. As shown in Fig. 2A, TNBS can significantly increase the expression of α -SMA, N-cadherin, Col I, and Col III proteinse, while reduce the expression of E-cadherin protein. However, GPX and PFND could reverse this effect, indicating that GPX has the effect of inhibiting colonic epithelial-mesenchymal transition.

3.3 Inhibition effect of GPX on epithelial-mesenchymal transition in NCM460 cells

To verify the cytotoxicity of GPX on NCM460 cells, the cell viability was assessed using the CCK-8 assay. The cell viability decreased significantly at GPX concentration of 300 μ g/ml, whereas was not affected at GPX concentrations less than 200 μ g/ml (Fig. 3A). Hence, 200 μ g/mL of GPX was used as the highest concentration for *in vitro* efficacy. Similarly, the effect of GPX at different concentrations on EMT markers in NCM460 cells was investigated by the immunofluorescence assay. when the concentration of GPX was >50 μ g/mL, it could significantly reduce the fluorescence intensity of Vimentin and α -SMA in NCM460 cells, and enhance the fluorescence intensity of E-cadherin (Fig. 3B). Therefore, 50, 100, and 200 μ g/ml of GPX were used in our subsequent in vitro experiments. These results indicated that GPX markedly

inhibited the interstitial transition of NCM460 cells. To verify the regulatory effect of GPX on EMT at the cellular level, the mRNA expression and protein levels of E-cadherin, Vimentin, ZO-1 and α -SMA in NCM460 cells were measured. Compared with that in the control group, ZO-1 and E-cadherin mRNA expression in the model group decreased, while Vimentin and α -SMA mRNA were significantly up-regulated (Fig. 3C). However, such an effect was significantly reversed followed by GPX treatment. In line with the qRT-PCR results, GPX notably rescued the protein levels of related markers relative to those of the model group, with the best efficacy at 200 μ g/ml (Fig. 3D-E).

3.5 Regulation of GPX to autophagy levels

3.5.1 Regulation of GPX to autophagy levels in colonic tissue

To explore whether GPX inhibits EMT by regulating autophagy, the expression levels of autophagy regulation-related proteins LC3B (LC3-II/LC3-I), Beclin-1, and p62 in the colon tissue were determined. Compared with the normal control group, the expressions of LC3B and Beclin-1 proteins in the model control group were significantly down-regulated, while the p62 protein was significantly up-regulated. GPX and PNFD could reverse this effect (Fig.5A). The results showed that GPX could improve the autophagy level in colon cells of rats with UC intestinal fibrosis.

3.5.2 Regulation of GPX to autophagy levels in NCM460 cells

To further clarify whether GPX inhibited colonic epithelial-mesenchymal transition by regulating the level of autophagy, an autophagy inhibitor (3-MA) and an autophagy agonist (Rapa) were used for verification. The expression of autophagosome was observed by cadaverine (MDA) staining, and the mRNA and protein levels of Bcl-2, Beclin-1, p62 and LC3B in NCM460 cells were measured. MDC staining analysis showed that compared with the control group, the intracellular green fluorescence in the model group was weakened. Compared with the model group, the intracellular green fluorescence intensity was enhanced after GPX and PNFD intervention. The green fluorescence in the 200 mg/mL GPX and 200 mg/mL PNFD group were obvious (Fig.6A). Compared with the control group, the mRNA expressions of Bcl-2, LC3B and Beclin-1 decreased and the expression of p62 was up-regulated in the model group, however, GPX and PNFD intervention reversed this effect (Fig. 6B). Consistent with the qRT-PCR results, GPX significantly rescued the protein levels of related markers relative to the model group, with the most significant expression at 200mg/mL.

Rapa and 3-MA could enhance or inhibit the effect of GPX. As expected, in the autophagy inhibitor group (3-MA), the effect of GPX in regulating autophagy was inhibited, while in the autophagy agonist group

(Rapa), GPX increased the level of autophagy more significantly. The results of the combination of *in vivo* and *in vitro* experiments further indicated that GPX could inhibit colon fibrosis by regulating the level of autophagy.

4. Discussion

Ulcerative colitis (UC) is a chronic relapsing and protracted disease that can cause the body to repair the colon wall continuously and repeatedly, resulting in abnormal proliferation of colonic fibroblasts^[15], increased ECM synthesis and secretion, and decreased degradation. Excessive deposition leads to changes in the original normal structure and tissue morphology, intestinal stenosis, intestinal shortening, and the formation of intestinal fibrosis^[16-17]. Although the development of intestinal fibrosis is associated with inflammation in the affected site, current clinical anti-inflammatory therapies are far from being able to treat intestinal strictures, which may be related to our insufficient understanding of the mechanisms of intestinal fibrosis formation^[18]. It is worth mentioning that currently commonly used drugs for the treatment of UC intestinal fibrosis, such as aminosalicylic acids, steroids and immunosuppressants, have limited therapeutic effects and large adverse reactions^[19-20]. Therefore, it is imperative to understand the mechanism of chronic inflammation-induced epithelial-mesenchymal transition leading to intestinal stricture formation and to identify alternative or adjunctive treatment options for colonic fibrosis. Our study suggests that GPX is a potentially viable treatment option for colonic fibrosis.

Ethnic medicine is rich in resources and has unique efficacy in the treatment of diseases, especially intractable diseases, with small side effects. It is an effective way to develop new anti-UC intestinal fibrosis drugs based on ethnic medicine. Tibetan medicine *G. paludosais* has been widely used for the treatment of hepatitis, cholecystitis, febrile plaques, diarrhea, gastroenteritis, conjunctivitis, scabies and other diseases^[21,22]. It is reported that xanthone, flavonoid, and triterpenoid in *G. paludosa* have anti-tumor, antidiarrheal, anti-inflammatory, analgesic, bacteriostatic, antioxidant, and hepatoprotective effects. The previous research of our research group found that the ethyl acetate extract of *G. paludosa* could reduce the expression of TGF- β and reduce the degree of colon fibrosis. The specific mechanism of action, however, is still unclear. In this study, xanthone was enriched using macroporous resin, the were replicated, and its effect on intestinal inflammation and epithelial-mesenchymal transition was investigated on DNBS-induced rat colitis model and the LPS-induced NCM460 cell EMT model. The result of *in vivo* experiment showed that GPX significantly improved the course and macroscopic manifestations (DAI, colon weight, length, adhesions, strictures, and ulcers) of TNBS-induced chronic colitis, as well as the histological severity of colonic wall fibrosis. The *in vitro* experiments showed that GPX could improve the morphology of LPS-induced EMT model of NCM460 cells and down-regulate the fluorescent expression of mesenchymal transition markers. It is suggested that GPX can be beneficial to the recovery of intestinal wall function by inhibiting colonic epithelial-mesenchymal transition.

To evaluate the therapeutic effect of GPX, the expression of these key indicators in colon tissue and NCM460 cells was evaluated. It was found that GPX could down-regulate the expression levels of

interstitial cell marker proteins α -SMA and N-cadherin in colon tissue, and up-regulate epithelial cell marker proteins N-cadherin and collagen Col I and Col III expression levels. GPX could also reduce the protein and mRNA expression levels of Vimentin and α -SMA in NCM46 interstitial cell, and increase the protein and mRNA expression levels of epithelial cell markers E-cadherin and ZO-1. Animal and cell model experiments show that GPX exerted anti-UC intestinal fibrosis effect by regulating the expression of EMT-related genes. In addition, both PFND and GPX at high dose had the best therapeutic effect.

Accumulating evidence suggests that autophagy plays a key role in the pathogenesis of IBD, such as regulation of pathogen clearance and regulation of immune responses. This study preliminarily explored whether GPX inhibited the occurrence and development of cellular EMT by regulating autophagy in colonic epithelial cells. It has been reported that the occurrence of inflammatory response is closely related to autophagy. On the one hand, impaired autophagy can interfere with intestinal barrier function, and affect immune response, endoplasmic reticulum stress and production of ROS, thus causing abnormal inflammatory response, and further promoting the occurrence and development of IBD. Up-regulation of autophagy levels is beneficial to protect cells from tumor necrosis factor-induced apoptosis and inhibit intestinal inflammation^[23-24]. On the other hand, accelerated or excessive autophagy can trigger or aggravate UC, such as colonic neutrophils^[25]. Autophagy of cells has been shown to promote mucositis. In order to further objectively elucidate the role of autophagy in UC, more in-depth research and exploration of the effects of different types of cells on autophagy are still needed. Therefore, clarifying the effect of autophagy level of NCM460 cells on colonic epithelial-mesenchymal transition is a promising strategy for the treatment of intestinal fibrosis in UC. First, the expression levels of autophagy-related proteins (LC3-II/LC3-I, Beclin-1, P62) in rat colon tissue were measured. Surprisingly, compared with the normal control group, the relative expression of LC3-II/LC3-I and Beclin-1 protein in colon tissue decreased significantly while the relative expression of p62 protein increased significantly in the model group. The intervention of GPX reversed this effect. It was preliminarily shown that GPX could up-regulate the level of autophagy in colon cells of UC intestinal fibrosis rats. In order to further clarify whether autophagy is involved in the inhibition of EMT of colon epithelial cells by GPX, the expression of autophagy-related proteins and mRNAs (LC3-II/LC3-I, Beclin-1, Bcl-2, P62) in the NCM460 EMT model was measured. The results showed that compared with the control group, the expression of LC3-II/LC3-I, Bcl-2, and Beclin-1 genes in the model group decreased significantly, while the expression of p62 gene significantly increased. Again, GPX intervention reversed this effect. Based on the above results, autophagy inhibitors and autophagy agonists were involved in the study. The results showed that autophagy agonists and inhibitors could significantly up- or down-regulate the expression levels of autophagy-related genes. This finding further indicated that GPX inhibited the EMT process of epithelial cells by up-regulating the level of autophagy in NCM460 cells, and provided a basis for the mechanism in which GPX exerted a significant curative effect and protective effect in UC intestinal fibrosis.

5. Conclusion

Taken all together, the effect of GPX in improving intestinal fibrosis in UC was confirmed by combining *in vitro* and *in vivo* experiments, and it was further elucidated that its inhibition of colonic epithelial-mesenchymal transition may be related to the up-regulation of autophagy levels.

Declarations

Declaration of competing interest

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

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CRediT authorship contribution statement

LN,KL and YML performed the experiments, selected the literature,and drafted the manuscript. MJ guided them to prepare the manuscript. SQB extensively revised the manuscript. All authors listed have made a substantial, direct, and intellectual contributions to thework, and approved it for publication

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Figures

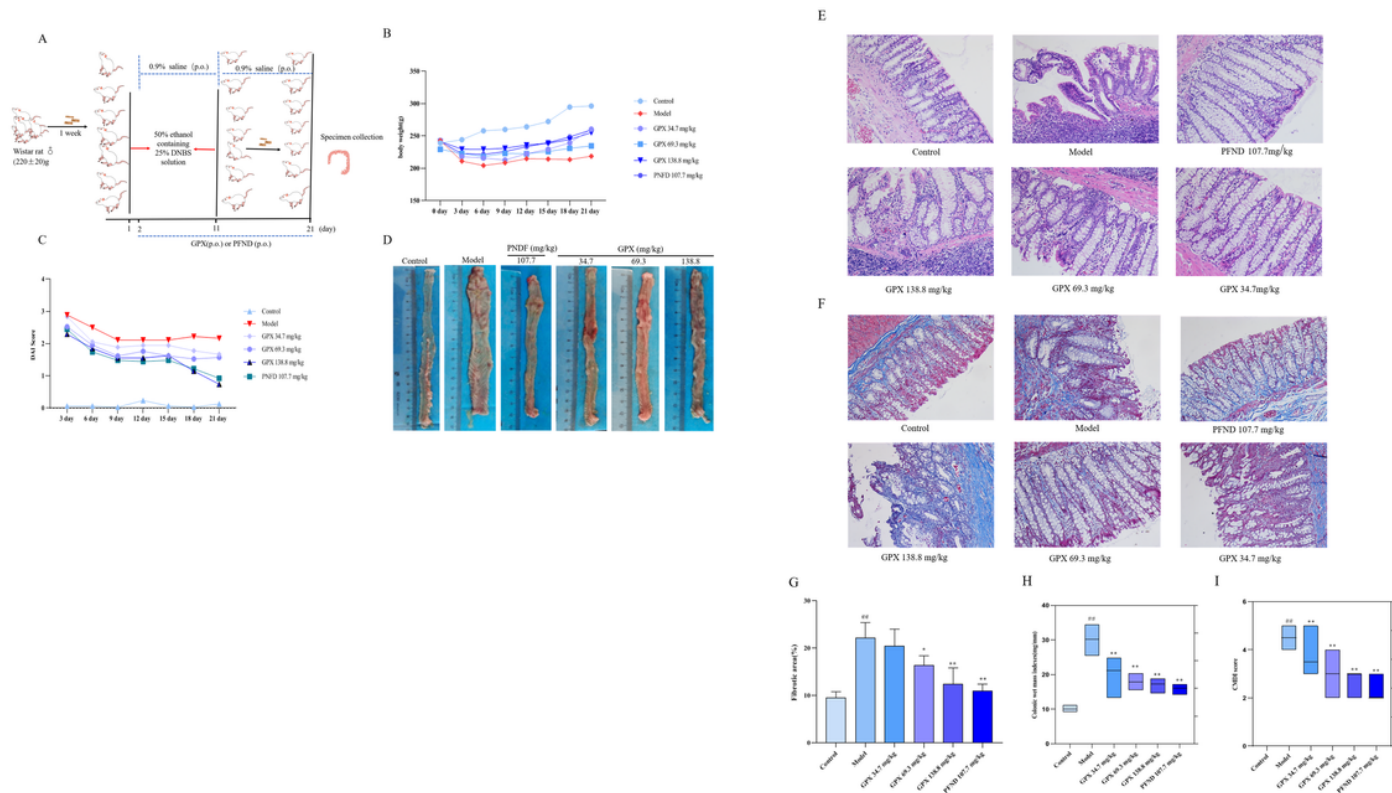


Figure 1

GPX attenuates colitis in the 2,4-Dinitrobenzenesulfonic acid (DNBS)-induced colitis in rat.

(A) Experimental design. (B) Body weight change. (C) Disease activity index. (D) After sacrificing, colon damage was evaluated by colon length. (E) Histological appearance of Rat colonic mucosa (H and E, magnification $\times 100$). (F) Masson's trichrome staining. (G) Quantification of lesion area in Colon with Masson staining (n = 6). (H) Colon wet mass index (colon length/colon mass). (I) Colon gross morphological damage index (CMDI) score of each group. ##p < 0.01 compared with Control group, *p < 0.05, **p < 0.01 compared with the model group.

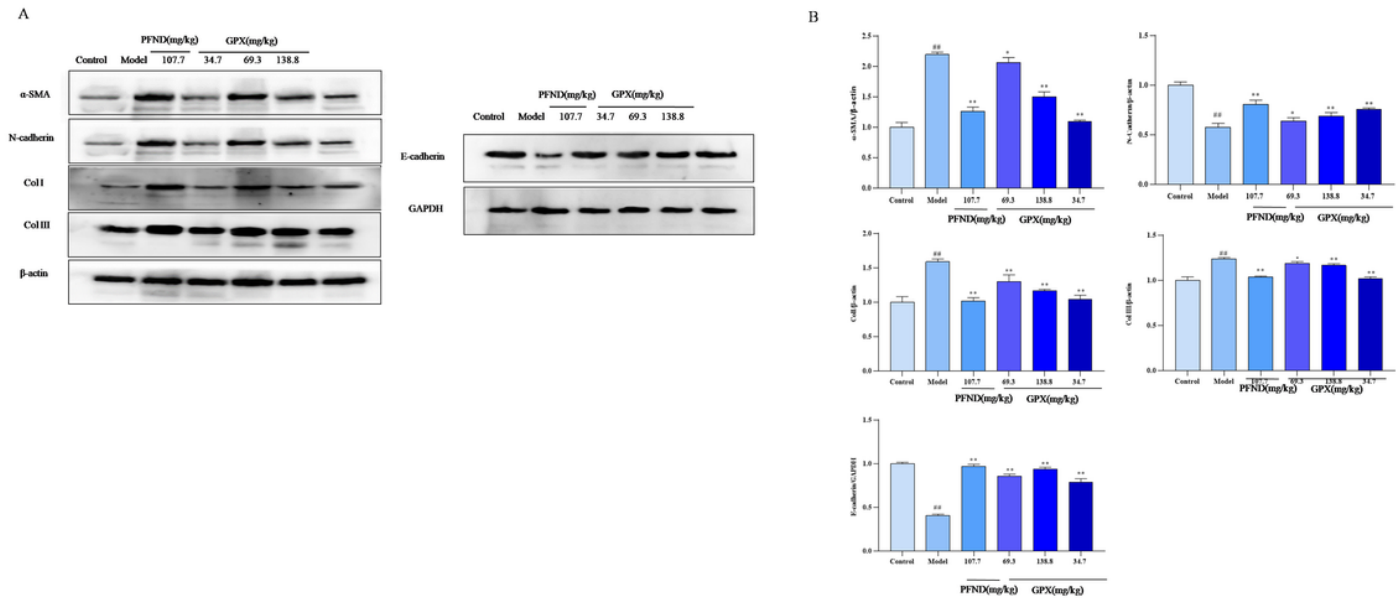


Figure 2

GPX inhibits colonic epithelial-mesenchymal transition. (A) Levels of EMT-related proteins E-cadherin, α-SMA , N-cadherin, ,Col I and Col III in the colon of rats.(B) Densitometric values were normalised to those in the control group and are presented as relative intensity (n = 3)

. ##p < 0.01 compared with Control group, *p < 0.05, **p < 0.01 compared with the model group.

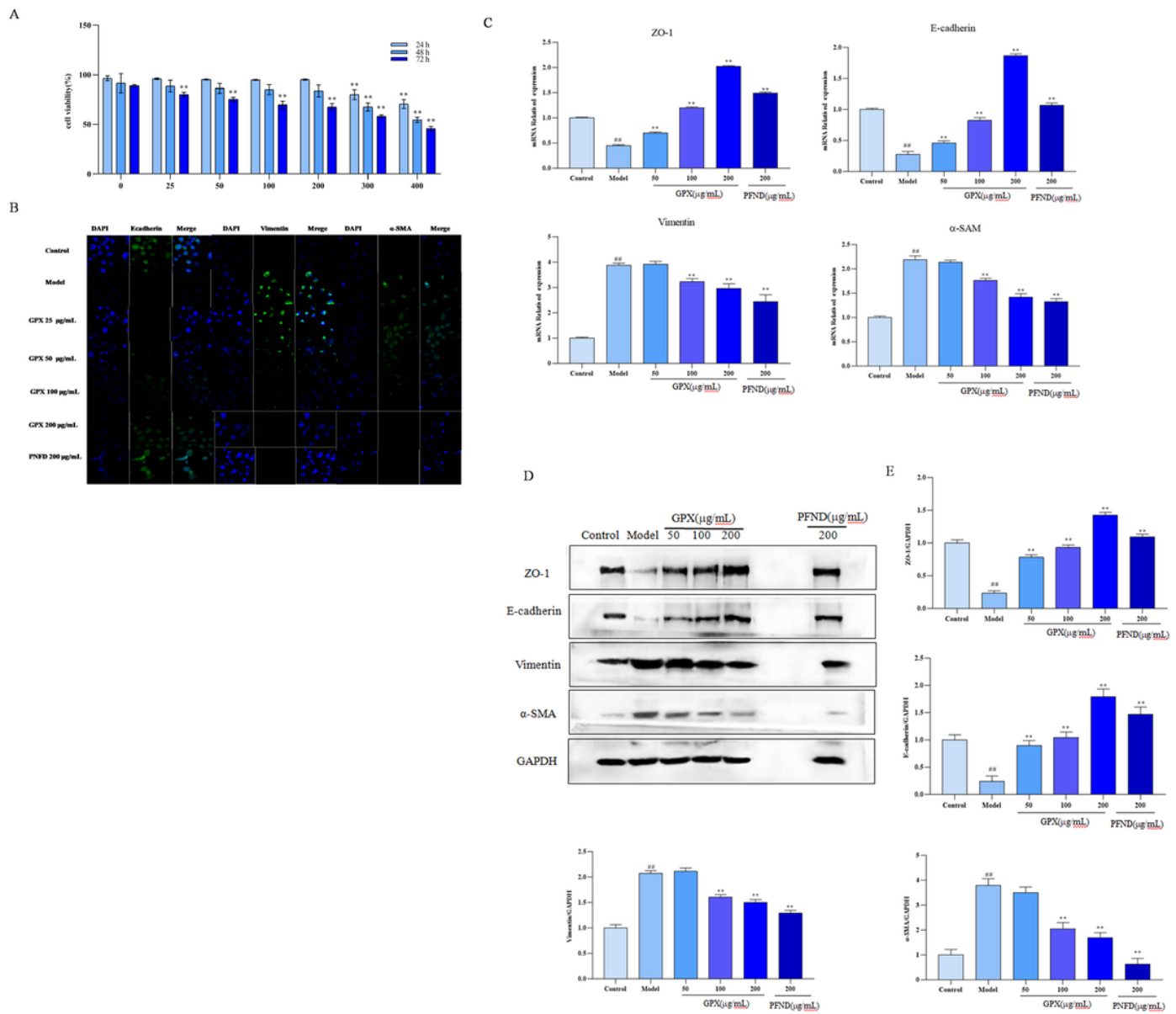


Figure 3

GPX inhibits the interstitial transition of NCM460 cells. (A) Effects of GPX on NCM460 viability. Cells were treated with 0, 25, 50, 100 and 200 µg/mL of GPX for 24,48,72h. Cell viability was then measured using the CCK-8 assay. (B) Effects of GPX on the Fluorescence Expression of Mesenchymal Transition Markers in NCM460. Cells were treated with 0, 25, 50, 100 and 200 µg/mL for 48 h. Fluorescent expression of markers was detected by immunofluorescence techniques. (C) Expression of EMT-related genes (E-cadherin, Vimentin, ZO-1 and α-SMA) in NCM460, as determined using qRT-PCR. (D) Levels of EMT-related proteins (E-cadherin, Vimentin, ZO-1 and α-SMA) in NCM460, as determined using western blotting. (E) Densitometric values were normalised to those in the control group and are presented as relative intensity (n = 3). In figure 3A *p < 0.01, **p < 0.01 compared with blank group. In figure 3C-D ##p < 0.01 compared with Control group, *p < 0.05, **p < 0.01 compared with the model group.

A

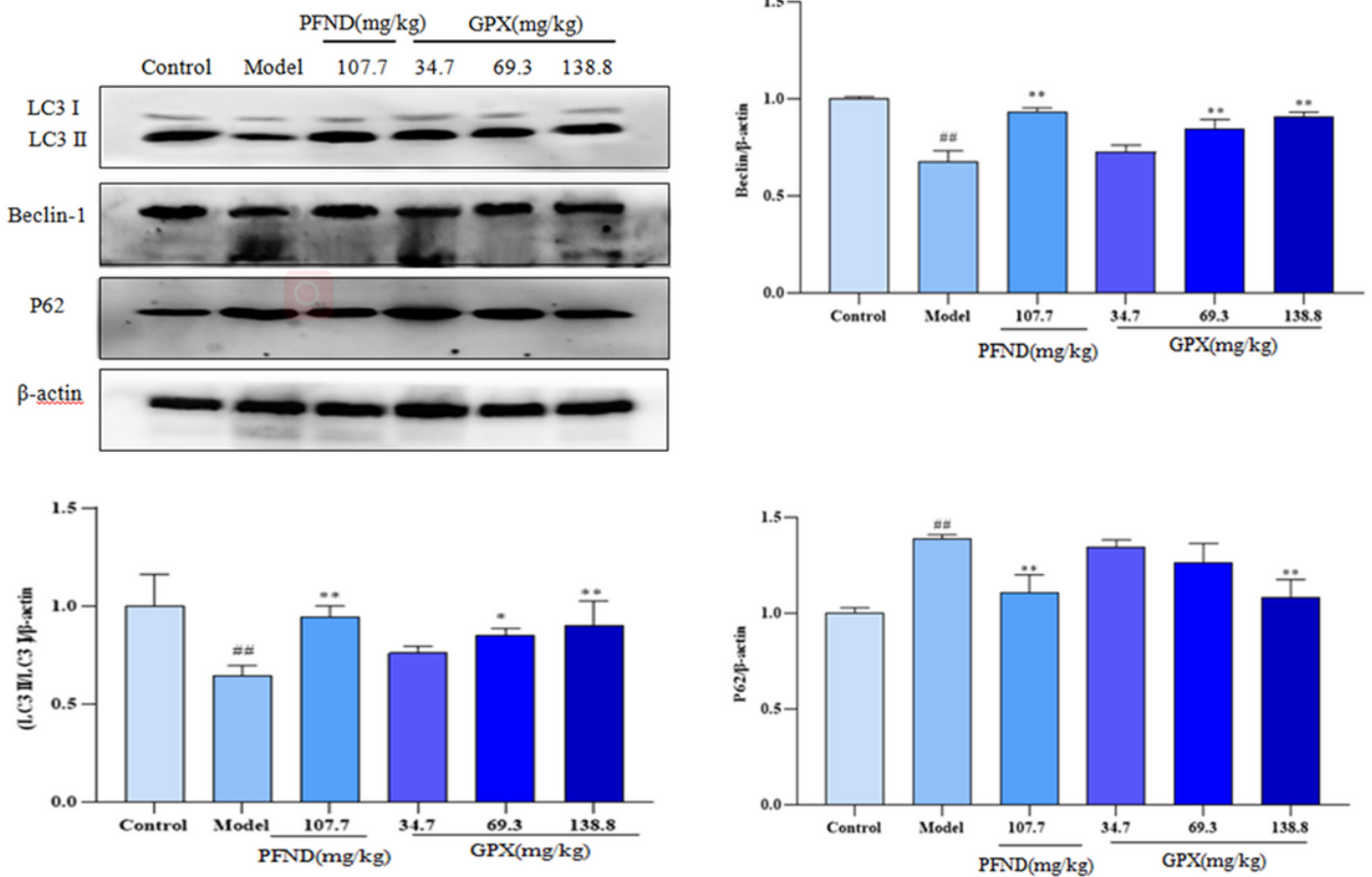


Figure 4

FIGURE 5 | GPX regulates autophagy levels in colonic tissue. (A) Expression of autophagy -related genes (L3B, Beclin-1, and p62) in colonic tissue, as determined using western blotting.. (B) Densitometric values were normalised to those in the control group and are presented as relative intensity (n=3). Data are shown as the mean $\pm$ SEM. ##p < 0.01 compared with Control group, *p < 0.05, **p < 0.01 compared with the model group.

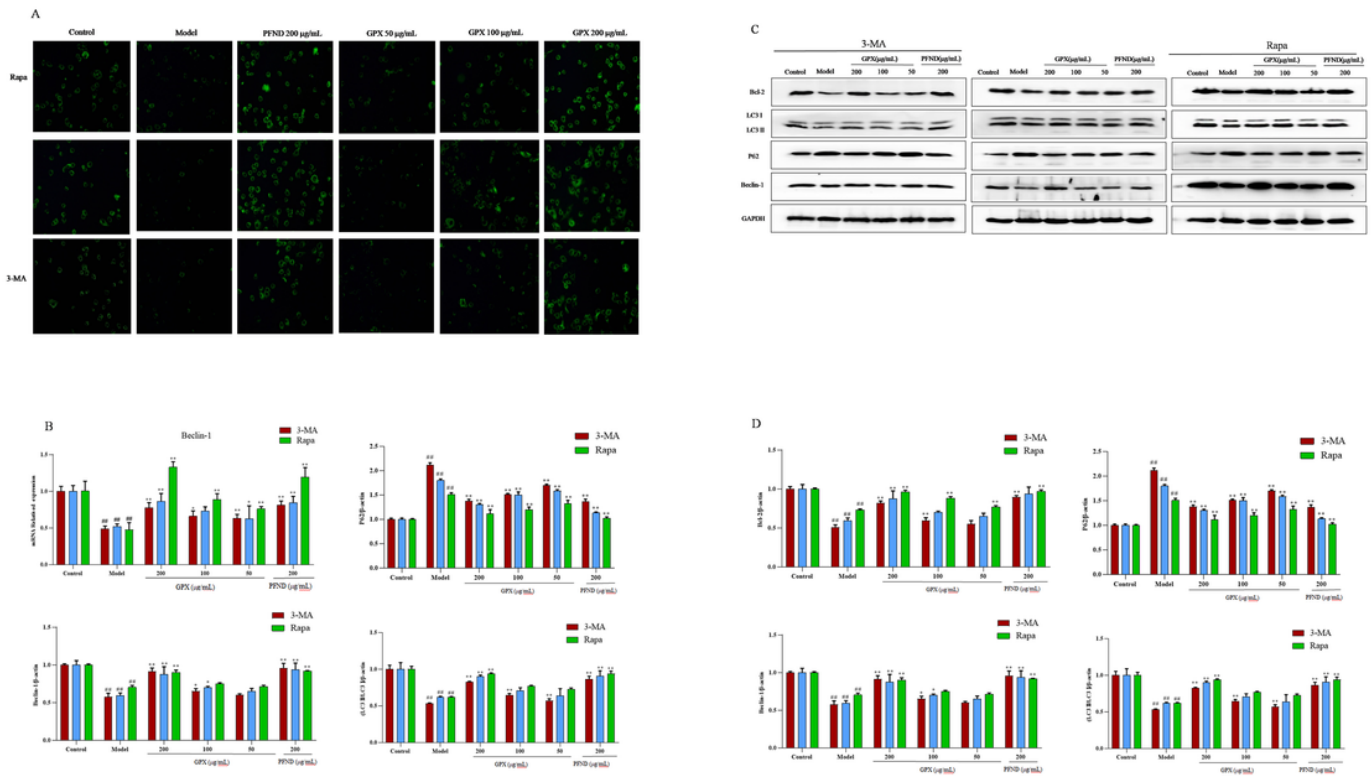


Figure 5

FIGURE 5 | GPX regulated the level of autophagy in NCM460 cells .(A)Autophagic vacuoles in NCM460 cells were visualized by MDC staining(original magnification 200×).(B) Expression of autophagy -related genes (Bcl-2, Beclin-1, p62 and LC3B) in NCM460, as determined using qRT-PCR. (C) Levels of autophagy -related proteins(Bcl-2, Beclin-1, p62 and LC3B) in NCM460, as determined using western blotting.(D) Densitometric values were normalised to those in the Control group and are presented as relative intensity (n = 3). Data are shown as the mean ± SEM. ##p < 0.01 compared with Control group, *p < 0.05, **p < 0.01 compared with the model group.

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