Glycine tabacina Extract Alleviates Inflammatory Bowel Disease via NF-κB, JNK and Nrf2 Signaling Pathways

by

Yongkai LIANG

Master of Philosophy

2024

澳門大學
UNIVERSIDADE DE MACAU
UNIVERSITY OF MACAU

Institute of Chinese Medical Sciences

University of Macau

Glycine tabacina Extract Alleviates Inflammatory Bowel Disease via NF-κB, JNK and Nrf2 Signaling Pathways

by

Yongkai LIANG

SUPERVISOR: Dr. Chengwei HE

Master of Philosophy

in

Chinese Medicinal Science

2024

Institute of Chinese Medical Sciences

University of Macau

煙豆提取物通過 NF-κB,JNK,及 Nrf2 相關通路緩和發炎性炎症疾病

梁永楷

指導老師：何承偉副教授

哲學碩士（中藥學）

2024

中華醫藥研究院

澳門大學

Declaration

I declare that the thesis here submitted is original except for the source materials explicitly acknowledged and that this thesis as a whole or any part of this thesis has not been previously submitted for the same degree or for a different degree.

I also acknowledge that I have read and understood the Rules on Handling Student Academic Dishonesty and the Regulations of the Student Discipline of the University of Macau.

Signature:_____ Date:_____

Acknowledgements

The authors would like to thank all colleagues who contributed to this study.

Thanks Professor Chengwei He for providing advices, comments, and contribution to the whole research project.

Thanks Professor Hua Yu and his student for running HPLC-UV and identifying pure compounds in GTE.

Thank all groupmates (Lihua Tan, Zhimin Miao, Yanbei Tu, Yuxin Zhao, Jinmiao Zhong, Xiaobin Cui, Wendi Yue, Boyang Qiu) in Dr. He's research group and my girlfriend (Li Chen) for contribution and help during the whole research experiment.

Abstract

Inflammatory Bowel Disease (IBD) is a disease caused by complex factors leading to the inflammation, fibrosis, fistula, and ulceration of colonic tissues throughout the intestinal track. Some of the major causes are genes, diets, pathogen infection leading to aberrant immune response, side effects of drugs and lifestyles. Symptoms of IBD includes weight loss, diarrhea, and occult bleeding which may lead to anemia and other diseases such as colorectal cancer. The disease cannot be fully cured as for now because many patients are tolerance to the drugs and there are side effects.

In this study, we used an aqueous extract of a natural herb, *Glycine tabacina* (Labill.) Benth (GTE). It is a plant belongs to the bean family and distributed in China, South-East Asia, Australia, and Oceanic countries. The major active components in GTE are isoflavonoids such as daizein, genistein, and genenistin. Based on previous studies of GTE regulating signaling pathways in rheumatoid arthritis, nephritis, and osteoporosis are similar to IBD. Thus, we want to examine GTE with LPS and TNF-α induced human colorectal adenocarcinoma cells (Caco 2) and DSS induced Balb/C mice.

Through the *in vitro* and *in vivo* model, the anti-colitis effects of GTE were identified. GTE maintains the barrier function of intestinal cells through down regulating MMPs and pro-inflammatory cytokines *in vivo* by decreasing phosphorylation of proteins in NF-κB and MAPK/JNK pathways. Oxidative stress in intestinal cells and tissues are down regulated since GTE was able to reduce the level of ROS through enhancing the protein expression of Nrf2 to increase the expression level of anti-oxidants, HO-1 and NQO1. GTE also have the same effects in DSS induced mice, the diseases activities and pathological sites in the colonic tissues are reduced in the presence of GTE treatment. GTE might become a cost effective Chinese traditional medicine for IBD alternative treatment.

Key Words: colitis; natural herb; anti-inflammation; anti-oxidation

摘要

發炎性腸道疾病是由多種複雜的成因導致的從小腸至大腸（直腸）組織和內膜的發炎，纖維化，穿孔，和潰瘍。主要的成因是基因，體外細菌引起的免疫過度反應，飲食，藥物的副作用，和作息。發炎性腸炎的主要症狀是明顯的體重下降，腹瀉，和便血 從而導致貧血和腸癌甚至死亡。目前，只有藥物和手術能治療發炎性腸炎，但不能被完全治癒。因為在臨床試驗中病人接受藥物和手術治療還是會導致腸炎的復發，甚至某些藥物還會導致比較嚴重的不良反應。

新的藥物特別是天然化合物和提取物或許能成為一個有顯著抗腸炎效果的藥物。煙豆是豆科大豆屬植物，主要分佈在中國，東南亞，澳大利亞，和一些太平洋島國。它的主要活性成分包含一些異黃酮，比如染料木素，染料木苷，和大豆苷元。基於前期使用煙豆提取物治療風濕性關節炎，腎炎，骨質酥鬆實驗，我們希望通過脂多糖或腫瘤壞死因子α誘導的人源腸癌細胞模型和葡聚糖硫酸鈉誘導的 BALB/c 小鼠模型驗證煙豆提取物緩解腸炎的作用。因為前期實驗證明了煙豆提取物通過調節類似於腸炎的炎症通路治療不同的慢性炎症，並且目前沒有任何對於煙豆提取物治療腸炎的研究，所以我們決定使用腸炎模型。

通過體外和體內腸炎模型的實驗，我們篩選了三個有效並且安全的煙豆提取物劑量顯著地降低了腫瘤壞死因子α和白介素-6 的 mRNA 和蛋白水平。从而降低了基質金屬蛋白酶的蛋白水平和它們對緊密連結蛋白的降解，這樣腸壁的滲透性就得到了緩解。減少炎症因子進一步地制止了 NF-κB 和 MAPK/JNK 通路的激活，從而導致 p65 和 c Jun 無法通過細胞核轉移去轉錄下游促炎蛋白的基因。另一方面，我們還發現了煙豆提取物通過提高 Nrf2 的蛋白水平及入核提高了抗氧化蛋白 HO-1 及 NQO1 的基因轉錄去抑制過氧化物的水平從而達到抗氧化應激的效果。在小鼠身上我們也觀察到了煙豆對體重變輕，腹瀉，和便血等一些腸炎病症的改善。經過一系列的實驗，煙豆提取物在治療腸炎研究中有顯著的效果，或許通過進一步機制和臨床的研究，煙豆提取物可以製備成一種成本低有效的治療多種炎症的天然藥物。

關鍵字：腸炎；天然藥物；抗炎；抗氧化

Table of Contents

List of Tables and Figures

List of Abbreviations

ALB	Albumin
ALT	Alanine Transaminase
AKP	Alkaline Phosphatase
ANOVA	Analysis of Variance
B-actin	Beta actin
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
Caco2	Colorectal Adenocarcinoma Cells
CCK-8	Cell Counting Kit 8
CRC	Colorectal Cancer
DCFH-DA	2', 7'-Dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DSS	Dextran Sulfate Sodium
ELISA	Enzyme Linked-Immunosorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescence isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTE	Glycine Tabacina aqueous extract
H&E	Hematoxylin and Eosin
HO-1	heme oxygenase-1
IG	Intragastric Gavage
IBD	Inflammatory Bowel Disease
IkBa	Inhibitor of kB
IL	Interleukin
JNK	c-Jun N-terminus kinase
LPS	Lipopolysaccharide
MAPK	Mitogen activating protein kinase
MMP	Metalloproteinase
MPO	Myeloperoxidase
NF-kB	nuclear factor of kB
NQO1	NADPH quinone dehydrogenase 1
Nrf2	Nuclear factor erythroid 2 related factor 2
OD	Optical Density
PBS	Phosphate buffered saline
PMSF	Phenyl Methane Sulfonyl Fluoride

PVDF	Polyvinylidene fluoride or polyvinylidene difluoride (PVDF)
q-PCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
TAK1(MAP3K7)	Mitogen-activated protein kinase kinase kinase 7
TBST	Tris-buffered saline and Tween-20
TLR	Toll-like Receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor

Chapter 1 Introduction

1.1 Introduction

Inflammatory Bowel Disease (IBD) is an intestinal chronic inflammatory condition referring to Crohn's disease and ulcerative disease with symptoms like abdominal pain, vomiting, diarrhea, hematochezia, etc. The prevalence of IBD had increases drastically in the past few years worldwide, which many development and fully developed countries are in the accelerating to the compounding stages of IBD prevalence [1]. Burdens of IBD includes medication costs and social problems. The population of IBD patients in well developed countries of the west is about 4 million people. The cause of the diseases is still unknown, which could be lots of factors include genes, food, pathogens, and lifestyles [2, 3]. Diet is definitely one of the major factors, unhealthy diet with high fat and sugar undermines the barrier function of the intestine. Previous study revealed individuals with unhealthy diet are more prone to infection leading to colonic injuries and inflammation [4]. IBD is related to multiple diseases because the intestine is one of the organs that metabolism take place, chronic diseases such as obesity, diabetes, and cardio vascular diseases, etc. are related to IBD. Damaged intestinal cells without proper healing or cell death could develop into colorectal cancer. As an example, IBD is found to be correlated to primary sclerosing cholangitis (PSC) in a meta-analysis of multiple epidemiological studies with about seven hundred thousand IBD patients. Average of 2 out of 100 IBD patients have PSC, and it might lead to liver failure, liver cancer, and bile duct cancer [5]. Moreover, epidemiological studies indicate there is close connection between IBD and colorectal cancer (CRC), patients are more likely, at least 2 times easier to develop colorectal cancer than healthy individuals.

In homeostasis, Reactive Oxygen Species (ROS) and cytokines are balanced, serving as anti-bacterial and signaling agents to maintain a healthy microenvironment of intestinal cells from the pathogens [6, 7]. When colon is in the healthy state, intestinal epithelial cells are connected tightly by tight junction proteins such as occludin and claudin families. IBD is a chronic inflammatory disease developed from injuries, pathogens, and other factors activating various cellular inflammatory pathways results from aberrant immune response. Once the aggressive inflammatory response is initiated, the vicious cycle form by ROS, pro-inflammatory cytokines, and many other pro-inflammatory proteins would be activated to further aggravate the inflamed condition [7-9]. Moreover, the abnormal amount of pro-

inflammatory cytokine production from immune cells can damage the epithelial cells and they can also degrade the tight junction proteins in between the epithelial cells. The gaps of intestinal epithelial cells are no longer protected by tight junction proteins, which permits the pathogens and toxins enter the veins and spread throughout the hosts' bodies [10, 11]. Thus, IBD is able to cause many other chronic diseases since risk factors can be uptake into the body leading to serious infection.

1.2 Goals of the Research Study

1. Evaluate the anti-inflammatory function of GTE in Caco 2 cells with safe concentrations.
2. Identify potential signaling pathways GTE regulate.
3. Evaluate the therapeutic effects of GTE with DSS challenged Mice.

1.3 Potential Outcomes and Contribution

1. Verify the effect of GTE in Caco2 cells and DSS model mice.
2. Expanding the usage of GTE in other inflammatory diseases.
3. Discover a natural herb/Chinese medicine treat against colitis with cheaper price, similar effects, and less side effects.

Chapter 2. GTE Ameliorating LPS and TNF-α Induced Inflammation State in Caco 2 Cells through Regulating Pro-Inflammatory Cytokines and Pathways

2.1 Introduction

Colitis is not simply a chronic inflammatory disease with damages on intestinal barriers. Rather, it is an involvement of multiple types of immune cells releasing and receiving cytokines between themselves and the colonic cells. This complex network of cytokines and signaling pathways is complicated to regulate. Immune cells will be recruited by pro-inflammatory cytokines and migrate to the injured area of the colon causing inflammatory storm, increasing the risk of chronic inflammation, bleeding, tumorigenesis and many other symptoms of colitis [12]. TNF-α is one of the most iconic pro-inflammatory cytokines able to activate many classical inflammatory pathways. There is medication for IBD such as TNF-α antagonist blocking the binding site of TNF-α, while studies have shown other monoclonal antibodies and small molecules are more effective than TNF-α antagonist with lower risk of infection [13]. However, these medications are not cost effective, they also need intravenous injection bringing to a concern of other side-effects. As for now, IBD cannot be fully cured by any medication or surgery. Natural Herbs might have similar effects against colitis with cheaper price and less toxicity.

Glycine tabacina (Labill.) Benth (*G. tabacina*) is a floral plant in the bean family originated in Australia. This plant is also found in East Asia, South East Asia, and Oceanic countries. *G. tabacina* is one of the ingredients of 'I-TIAO-GUNG', an ointment medication product in Taiwan region to relieve joint and muscular pain. There have been few studies utilizing the aqueous extract of *Glycine tabacina* (Labill.) Benth (GTE) as medication against rheumatoid arthritis, osteoclastogenesis, and nephrotic syndrome, etc. These studies have proven GTE as a natural herb extract with low toxicity while effective against inflammatory diseases [14-16]. According to data from previous studies [14-16], and the standard compounds, eight major peaks were identified with HPLC-UV at the 254 nm (Fig 2.1 A) and 340 nm (Fig 2.1 B). The HPLC-UV chromatograms of GTE infer this extract contains compounds as follow: daidzin (1), genistin (2), daidzein (3), genistein (4), pratensein (5), 3′-*O*-methylorobol-7-*O*-glycoside (6), coumestrol (7), and glytabastan B (8). These are isoflavones, coumestans, and some other

compounds with anti-inflammatory effects (Fig 2.1). Given the extract has the potential to treat multiple acute and chronic inflammatory diseases, and the research of this medication against IBD has never been carried out yet. Moreover, pro-inflammatory proteins contributing to arthritis are comparable to those of IBD. Therefore, we tried to examine the anti-colitis effect of GTE and identify the proteins regulate by GTE. Due to previous research works already reported the anti-inflammatory abilities of GTE treat against immune cells, thus our studies focused on the effects of GTE on human colorectal adenocarcinoma cell line (Caco 2). Although Caco 2 is a colorectal cell line, it can be differentiated into intestinal epithelial like cells named enterocytes forming clusters of monolayers. The presence of lipopolysaccharide (LPS) or pro-inflammatory cytokines in the medium may trigger the inflammatory signaling pathways allowing us to study the response of Caco 2 cells under inflammatory state and the effectiveness of GTE.

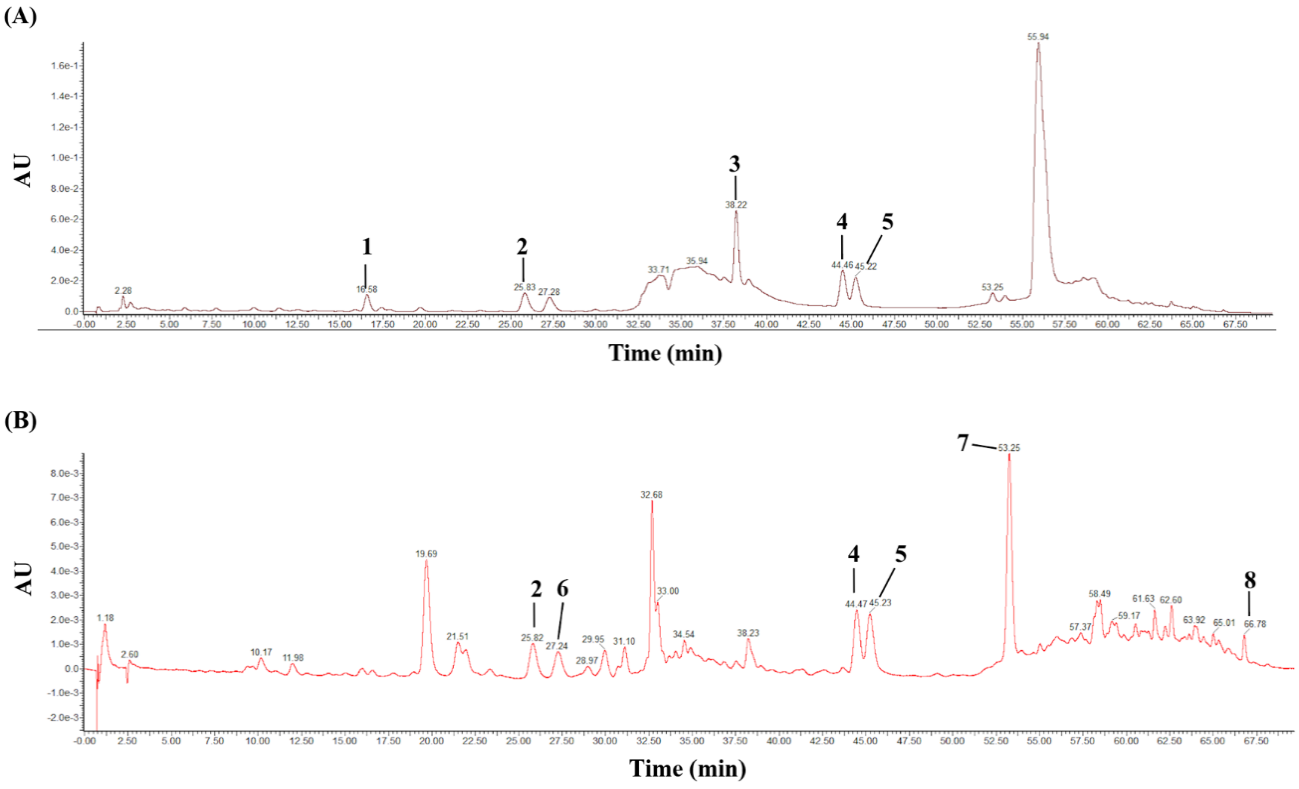


Fig 2.1 HPLC-UV chromatogram of GTE at 254 nm **(A)** and 340 nm **(B)** indicating the pure componds in GTE. daidzin (1), genistin (2), daidzein (3), genistein (4), pratensein (5), 3′-*O*-methylorobol-7-*O*-glycoside (6), coumestrol (7), and glytabastan B (8).

2.2 Methods and Materials

Cell Culture

Human colon carcinoma cell line (Caco 2) is obtained from American Culture Type Collection (ATCC). The cells are cultured in T25 and T75 culture flask with Dulbecco's Modified Eagle's medium (DMEM) added with 10% of Fetal Bovine Serum and 1% of mixed penicillin and streptomycin. Caco2 cells are cultured for one to two weeks for them to differentiate into enterocytes monolayers. Some of the experimental design are based on previous study done by Wu et al [17].

Treatment and Model

After differentiated into monolayers, lipopolysaccharide (1 μg/mL) purchased from Sigma-Aldric (St. Louis, MO, USA) is used as the stimulator for *in vitro* model of inflammation. The ethanol extraction of *G. tabacina* used in this study is the same batch from previous study [15]. Then GTE is dissolved in double distilled water and sonicated. Three concentrations (25 μg/mL, 50 μg/mL, 100 μg/mL) of GTE is used as the therapeutic drug.

Cell Viability

Caco 2 monolayers are cultured in 96 well plates, each well contains 1×10^5 cells with 100 μL of DMEM overnight. 8 concentrations of GTE are added to the wells for 24 hours. Remove the medium on the next day, add 100 μL of DMEM with 10% cell counting kit 8 (CCK8) solution to each well. Read the plate under microplate reader at wavelength of 450 nm after 1.5-2 hours. The OD values are recorded, cell viability is calculated by following equation:

$$\frac{OD\ of\ treatment - OD\ of\ blank}{OD\ of\ control - OD\ of\ blank}\ X\ 100 = \textbf{Relative Cell Viability (\%)}$$

Cytokine Release Determination

Caco2 monolayers are subculture into 48 well plates purchase from Eppendorf, 2×10^5 cells each well with 1 mL of DMEM overnight. Exposed cells with GTE and LPS for 24 hours, then collect the medium. Human TNF-α and IL-6 Enzyme Linked Immunosorbent Assay (ELISA) kits are purchased from Biolegend (CA, USA). The procedure of the experiment is performed according to the manufacture's protocols.

HPLC-UV analysis of GTE

Separation of GTE was performed by a Waters Arc HPLC (Waters, Milford, MA, USA) coupled with an Atlantis T3 3 μm column (3.0 × 150 mm) at a flow rate of 0.35 mL/min. The mobile phases were composed of methanol (A) and water with 0.1% formic acid (B) using the following gradient program: 0 - 26 min, 34% A; 26 -27 min, 34% - 48% A; 27 - 45min, 48%-52% A; 45-52min, 52% A; 52-55min, 52%-64% A; 55 - 57 min, 64% - 92% A; 57-70 min, 92%-95% A. UV spectra were recorded from 190 nm to 400 nm, and the UV detector was set at 254 nm and 340 nm.

Immunoblotting

Cells are subculture into small dishes (60 mm) or 6 well plates with 1.2×10^6 cells each dish or wells with 5 mL of DMEM overnight. Cells are collected into tubes after treatment of 4 hours and 24 hours with GTE and LPS. Add 20 μL of RIPA buffer with 1% of cocktail and 1% of PMSF (Beyotime, China) on ice and vortex. After 15 minutes, the tubes are centrifuge with 14000 rpm at 4℃ for 30 minutes. Supernatants are collected, and concentration of protein extract are determined by BCA kit (Thermofischer, MA, USA). After concentration adjustment, 5x loading buffer (Beyotime, China) is diluted with the protein extracts. Tubes are shake for 650 rpm in a hot bath of 100°c for 8 minutes. The samples (25 μg-30 μg) are separated through Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis at 70 v for 20 minutes, then 120 v for 1 hour and 15 minutes. Then the separated proteins are transfer on PVDF membrane after submerging into methanol at 100 v for 150 minutes and 120 v for 90 minutes. Then blot with 2.5% of non-fat milk powder (Biorad, USA) for 1 hour or QuickBlock Buffer (Beyotime, China) for 15 minutes. Incubate the membrane with primary antibodies (1:1000) purchased from Cell Signaling Technology overnight at 4℃. Then wash with tris-buffered saline with 0.1% of TWEEN 20 (TBST) and shake for 5 minutes. Repeat the step 3 times. Then incubate in secondary antibody (1:2000) purchased from Beyotime for 1 hour. Then wash with tris-buffered saline with 0.1% of TWEEN 20 (TBST) and shake for 5 minutes. Take pictures with Bio-Rad ChemiDoc imaging systems (Biorad, USA) with chemiluminescence mode and colorimetric mode. Results are analyzed with ImageJ and generate graphs with Graphpad Prism 9.

Immunofluorescence

Caco 2 monolayers are cultured into 96 well plate 1×10^4 cells per well with 100 μL of DMEM. After treatment with GTE, the cells are wash with phosphate buffered saline (PBS) 3

times. 100 μL Paraformaldehyde is added to each well incubate for 15 minutes. Then wash 3 times with PBS. Add 100 μL of 0.1% of TritonX-100 and PBS incubate for 15 minutes. Wash with PBS for 3 times. Incubate with 0.3% of TritonX-100, 1% of BSA mixed into 100 μL of PBS for 1 hour. Then incubate with primary antibodies (CST, USA) diluted according to manufacturer's instructed concentration overnight at 4℃. Wash with PBS 3 times. Incubate with secondary antibodies, Alexa 488 or Alexa 568 (1:500) for 1 hour in room temperature. Wash 3 times with PBS. Add 50 μL antifade mounting medium with DAPI (Beyotime, China) and 50 μL of PBS. Images were captured with Opera Phenix High Content Screening System.

Isolation of Nucleus and Cytoplasm

Caco 2 monolayers are cultured in 60 mm culture dishes, about 1.5×10^6 cells per dish. After treatment and stimulation, cells are collected to tubes. Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) are used to lyse the cells, isolate nuclear and cytoplasmic proteins. The experiments are done according to the manufacture's protocol.

Permeability Assay

Caco 2 cells were cultured at the top chambers of transwells. After treatment and stimulation, medium is removed. FITC-Dextran (4 kDA) (MedChemExpress, New Jersey, USA) was added to the upper chamber, the apical sides of Caco 2 monolayers. Then after 15 minutes, collect the medium from the bottom wells. Read the plate with microplate reader with fluorescence mode excitation wavelength: 485 nm and emission wavelength: 538 nm. Then calculate relative fluorescence intensity.

ROS Detection

After GTE treatment and LPS stimulation, mediums were removed and washed with PBS. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldric, USA) is diluted according to the manufacture's instruction and added to the cells for 20 minutes and incubate in dark. Then ROS fluorescence images were captured by fluorescence microscope using the green fluorescent channel and fluorescence area is calculated by ImageJ. With the same condition, a separate experiment was carried out. The plate was read in a microplate reader with fluorescence mode, excitation wavelength: 488 nm and emission wavelength: 525 nm.

RNA isolation, cDNA synthesis, qPCR

After stimulation and treatment, 1 mL of RNAi TRIZO (TAKARA, Japan) is added to each well of cells. Collect the samples, add 200 μL of chloroform. Vortex 5 second, rest for 10 minutes. Centrifuge 12000 rpm at 4°C for 15 minutes. Then collect the clear part of the supernatant to a new tube. Add the same volume of isopropyl alcohol as the clear solution. Vortex and Rest. Centrifuge 12000 rpm at 4°c for 10 minutes. Remove the supernatants, wash with ethanol twice, centrifuge 7500 rpm at 4°C for 5 minutes twice. Then remove the ethanol. Add 10 μL of DEPC water. The concentration and purity of RNA are detected using NanoDrop one c (ThermoFischer Scientific, USA).

The RNA is diluted according to 1000 ng/ul, and reverse transcribe into cDNA using reverse transcription kit (TAKARA, Japan) and PCR c1000 machine (BioRad, USA). Then cDNA samples are mixed with Primers (Guangzhou IGE Biotechnology, Guangzhou, China), and SYBR Green PCR MasterMix (Applied Biosystems, ThermoFischer, USA). All the temperatures and times for reverse transcription and RT-qPCR are set according to manufacturer's protocols. Results are generated in Quant Studio 7 Flex. (Applied Biosystems, ThermoFischer, USA).

Primers:

TNF-α Forward	5′- CCTCTCTCTAATCAGCCCTCTG -3′
TNF-α Reverse	5′- GAGGACCTGGGAGTAGATGAG -3′
IL-6 Forward	5′- TGAGTGCACCCAAGATCCAT -3′
IL-6 Reverse	5′- TAACCAAGATCCCTGCCCTG -3′
Occludin Forward	5′- TTTGTTTCATTGCCGCGTTG -3′
Occludin Reverse	5′-CGTAGAGTCCAGTAGCTGCA -3′
Claudin 5 Forward	5′- CTCTGCTGGTTCGCCAACAT -3′
Claudin 5 Reverse	5′- CAGCTCGTACTTCTGCGACA -3′

Statistical Analysis

All of the quantification data are calculated, analyzed and graph are generated with Image J and GraphPad Prism 9. All of the experiments are repeated trice and statistical significance is calculated by using one-way ANOVA test.

2.3 Results

2.3.1 Safe and Effective Concentration of GTE Decrease Pro-Inflammatory Cytokines

Although the toxicity of GTE was examined, and safe concentrations are confirmed in previous studies [14-16], usage of GTE on Caco2 cells has not been reported yet. Thus, CCK-8 kit was used to evaluate the cell viability, then find three safe concentrations for further experiments. Only the highest concentration 200ug/mL of GTE is toxic to Caco2 (Fig 2.2 A). Given that we choose the highest three safe concentrations, 25 μg/mL, 50 μg/mL, and 100 μg/mL of GTE for further *in vitro* study. Next, we performed qPCR and ELISA kit to measure the mRNA and protein levels of TNF-α and IL-6. Both cytokines are stimulators of inflammation and their levels rise up within 24 hours after LPS exposure. GTE is discovered to have the ability lower both the mRNA (Fig 2.2 B) and protein levels (Fig 2.2 C) of the two pro-inflammatory cytokines in Caco2 cells and in the medium. These two are key pro-inflammatory cytokines contributing to pathogenesis of IBD since they are able to recruit and activate immune cells; they can also interrupt the barrier function of intestinal cells through degrading tight junction proteins. Serving as ligands, they could activate NF-κB and MAPK pathways through tumor necrosis factor receptors (TNFR) and interleukin-6 receptor (IL-6R).

As a consequence of inflammation, ulceration, and injuries caused by abnormal immune response, fibrosis and fistula occurs in the colon. The modification of colonic structure leads to production of cellular debris and other components can be refer as extracellular matrix (ECM), which leads to the secretion of matrix metalloproteinase (MMP) to degrade them. MMPs are shown to interrupt barrier function in various diseases and some are able to activate pro-inflammatory cytokines [18-20]. Thus, the level of collagenase (MMP 1) and gelatinase (MMP 9) should have a positive correlation to inflammation. Treating Caco 2 cells with GTE decrease the expression level of MMPs, who were enhanced by TNF-α. (Fig 2.2 B) Given GTE has negative effect on pro-inflammatory cytokines and MMPs in the safety range of concentrations, the therapeutic potential of GTE against colitis is confirmed.

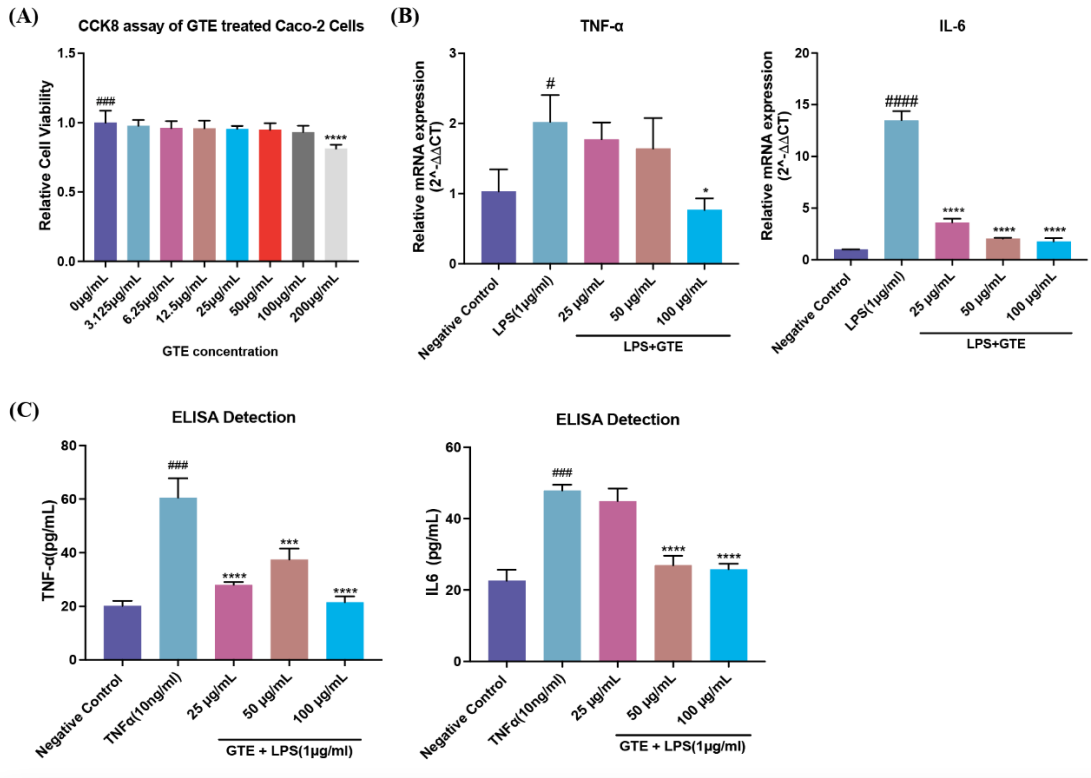
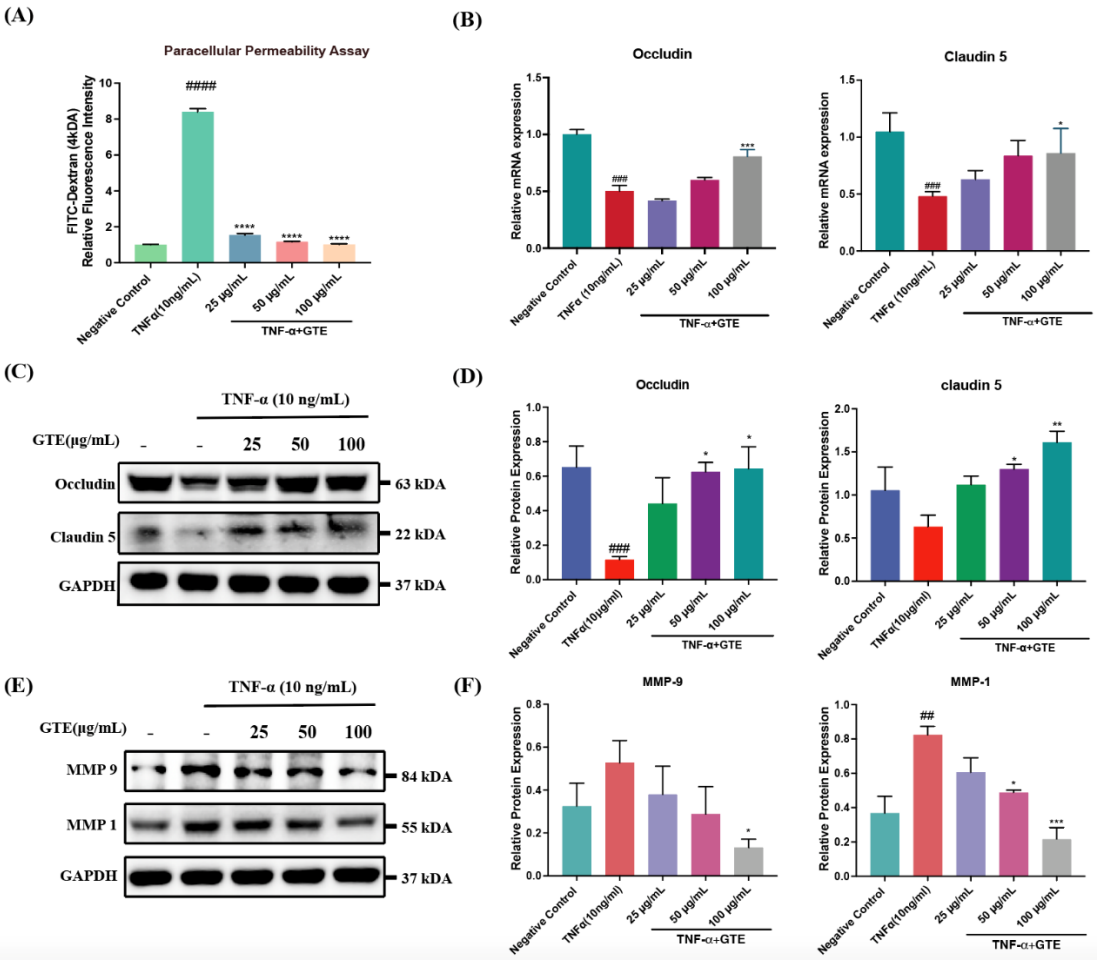


Fig 2.2 Safety and Effects of GTE. Cell Viability under 8 different concentration of GTE, 0 μg/mL is the negative control for comparison **(A).** mRNA level of TNF-α and IL-6 in Caco 2 cells **(B).** Secreted TNF-α and IL-6 from Caco2 cells in the medium **(C).** Statistical significance is compared between GTE treatment groups to 0μg/mL or LPS groups: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to model group. # compared to negative control group. Data are shown as mean ±SD (*n*=3).

2.3.2 GTE Decrease the Protein Expression of MMPs, Enhance the mRNA and Protein Levels of Tight Junction Proteins

As a consequence of inflammation, ulceration, and injuries caused by abnormal immune response, fibrosis and fistula occurs in the colon. The modification of colonic structure leads to production of cellular debris and other components can be refer as extracellular matrix (ECM), which leads to the secretion of matrix metalloproteinase (MMP) to degrade them. MMPs are shown to interrupt barrier function in various diseases and some are able to activate pro-inflammatory cytokines [18-20]. Thus, the level of collagenase (MMP 1) and gelatinase (MMP 9) should have a positive correlation to inflammation. Treating Caco 2 cells with GTE decrease the expression level of MMPs, who were enhanced by TNF-α (Fig 2.2A).

After differentiation, the morphology of Caco2 are characterized as monolayer clusters. Aforementioned, these monolayers of enterocytes are connected by various tight junction proteins, and they are degraded when exposed to aberrant level of pro-inflammatory cytokines and MMPs. Lower expression of tight junction protein is leading to severe infection and related diseases because the pathogens could take the advantage of paracellular transportation [10, 21]. Firstly, the ability of GTE ameliorating the intestinal barrier function is examined by using transwell assay with FITC labelled dextran (4 kDA). The monolayers of Caco 2 forms clusters which are attached tightly, and it is difficult for the FITC-dextran to pass through the gap defends by the tight junction proteins. Under the effect of TNF-α, great amount of dextran migrates from the apical side to the basal side of monolayers. Fluorescence intensity of FITC decrease around 8-fold after GTE treatment comparing to the model group (Fig 2.3 A). Herein, TNF-α (10ng/mL) model group is established. Both qPCR and immunoblotting evince TNF-α decrease mRNA (Fig 2.3 B) and protein level (Fig 2.3 C&D) of Claudin-5 and Occludin, which are two symbolic tight junction proteins. In about 24 hours of treatment with GTE, the mRNA and protein levels of the two increase dependently on concentration increment. As a consequence of inflammation, ulceration, and injuries caused by abnormal immune response, fibrosis and fistula occurs in the colon. The modification of colonic structure leads to production of cellular debris and other components can be refer as extracellular matrix (ECM), which leads to the secretion of matrix metalloproteinase (MMP) to degrade them. MMPs are shown to interrupt barrier function by degrading tight junction proteins in various diseases and some are able to activate pro-inflammatory cytokines [18-20]. Thus the level of collagenase (MMP 1) and gelatinase (MMP 9) should have a positive correlation to inflammation. Treating Caco 2 cells with GTE decrease the expression level of MMPs, who were enhanced by TNF-α (Fig 2.3 E,F). Through fluorescence imaging of Occludin in Caco2 cells, less Occludin immunofluorescence signals were observed in TNF-α group than GTE treatment groups (Fig 2.3 G). Through regulation the protein expression of pro-inflammatory cytokines and MMPs, the protein expression of tight junction proteins was enhanced by GTE, thereby reducing the intestinal permeability to protect the host from pathogen and other risk factors via the colon.


(A)
Paracellular Permeability Assay
FITC-Dextran (4kDA) Relative Fluorescence Intensity
Negative Control
TNFα(10ng/mL)
25 μg/mL
50 μg/mL
100 μg/mL
TNF-α+GTE
(B)
Occludin
Claudin 5
Relative mRNA expression
(C)
TNF-α (10 ng/mL)
GTE(μg/mL)
Occludin
Claudin 5
GAPDH
63 kDA
22 kDA
37 kDA
(D)
Occludin
claudin 5
Relative Protein Expression
(E)
MMP 9
MMP 1
84 kDA
55 kDA
(F)
MMP-9
MMP-1

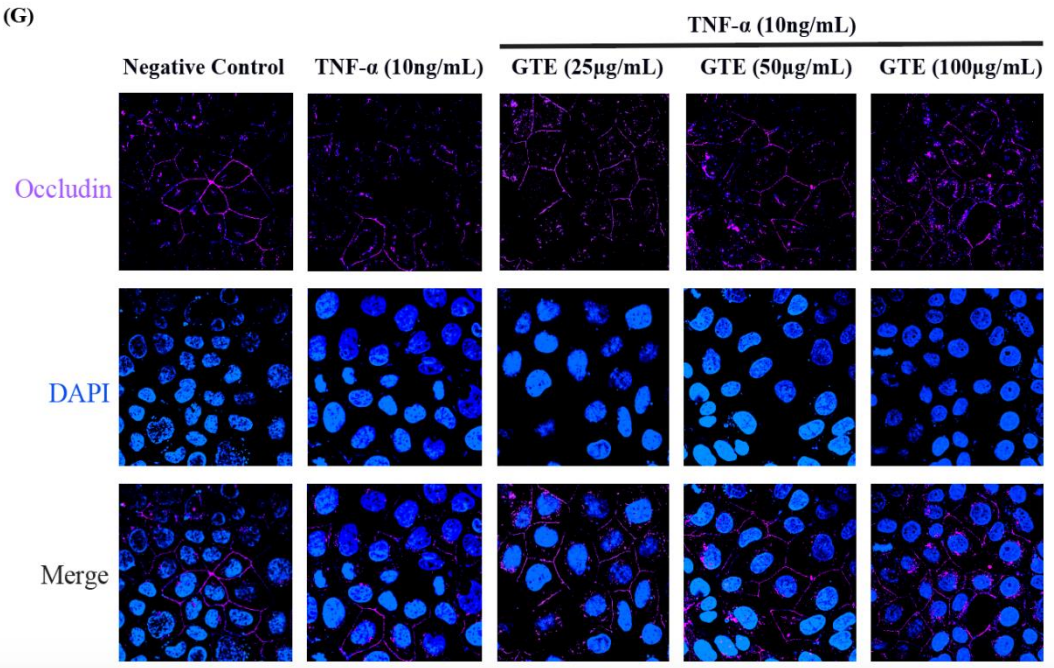

(G)
TNF-α (10ng/mL)
Negative Control
TNF-α (10ng/mL)
GTE (25μg/mL)
GTE (50μg/mL)
GTE (100μg/mL)
Occludin
DAPI
Merge

Fig 2.3 GTE decrease paracellular permeability through inhibiting MMPs to degrade tight junction proteins. Paracellular permeability assay perform with FITC-Dextran (4kDA) added to the monolayer of Caco 2 cells in transwells, the fluorescence intensity is measured (A). mRNA level of Occludin and Claudin 5 (B). Immunoblotting showing protein expression of tight junction proteins (C). Quantification of tight junction protein immunoblotting results (D). Immunoblotting of MMPs (E). Quantification of MMPs immunoblotting (F). Immunofluorescence against occludin of Caco 2 cells (G). Statistical significance is compared between GTE treatment groups to 0μg/mL or LPS groups: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to model group. # compared to negative control group. Data are shown as mean ±SD (*n*=3).

2.3.3 GTE Down Regulates Phosphorylated Proteins in NF-κB and MAPK/JNK Pathways

NF-κB pathway is an essential molecular pathway with various proteins regulating cell survival, apoptosis, inflammation, and other biological functions. This pathway had been studied deeply and frequently in various diseases, especially diseases related to inflammation [22]. In our study, our major focus is the canonical NF-κB pathway down stream of Toll Like Receptor 4 (TLR4), which is the receptor bind with lipopolysaccharide (LPS). After LPS stimulation, the TLR4 on the Caco-2 cells trigger downstream protein activation, one of the proteins is TAK1. TAK1 can be phosphorylated to phosphorylate other proteins such as IKKα/β/γ in the NF-κB pathway, JNK and P38 in the MAPK pathways [23, 24]. Phosphorylation of IKKα/β phosphorylates IkBα, which causes the ubiquitination of IkBα follow by proteasomal degradation in the cytoplasm. IkBα does not degrade and sequester the NF-κB family proteins (P65, P50, etc.) in the cytoplasm when the cell is not in an inflammatory state. On the other hand, the dimers of P65 and P50 will be phosphorylated and enhance the transcription of various pro-inflammatory genes when cells are in the inflammatory state [22]. The protein level of phosphorylated TAK1 and total level of TAK1 is determined by immunoblot. After LPS stimulation, the phosphorylation of TAK1 is increased, while phosphorylated TAK1 is decreased in the co-treatment groups of GTE and LPS (Fig 2.4 A,B). Similar trends are observed in the immunoblotting of phosphorylated IKKα/β (Fig 2.3 A,B), NF-κB p65, and IkBα (Fig 2.4 C,D). These results revealed the chain

reaction of phosphorylation in the NF-κB pathway, once GTE decreased the level of phosphorylated TAK1, all of its downstream phosphorylate form of proteins decreased.

The Mitogen-activated protein kinase (MAPK) pathway is another classic, major, frequently studied pathway in research areas of inflammation and cancer. MAPK contains three major sub-pathways (JNK, ERK, and p38), and phosphorylation is chain reaction, which is similar to NF-κB pathway mentioned previously. Under the stimulation of LPS, TNF-α, and other factors, each kinase will phosphorylate the downstream kinase to further activate other proteins or eventually transcription factors [25]. TAK1 phosphorylation can also phosphorylate JNK, and the downstream target of JNK is c-Jun. The phosphorylation of c Jun cause itself to dimerize with c-fos in combination called AP-1 serving as a pro-inflammatory transcription factor. As expected, the decrease of phosphorylated TAK1 in GTE treatment group is negatively affecting the protein level of phosphorylated JNK (Fig 2.4 E,F). Similarly, the downstream target of JNK, phosphorylated c-Jun also had lower expression levels in the GTE treatment groups (Fig 2.4 E,F). The phosphorylation of proteins in both pro-inflammatory signaling pathways are down regulated by GTE in Caco 2 cells, this might explain the increase of pro-inflammatory cytokines secretion from Caco 2 cells. NF-κB and MAPK signaling pathways are constantly activated to produce more cytokines forming a vicious cycle as a consequence of LPS stimulation, while GTE was observed to inhibit the generation of cytokines to further activates the signaling pathways.

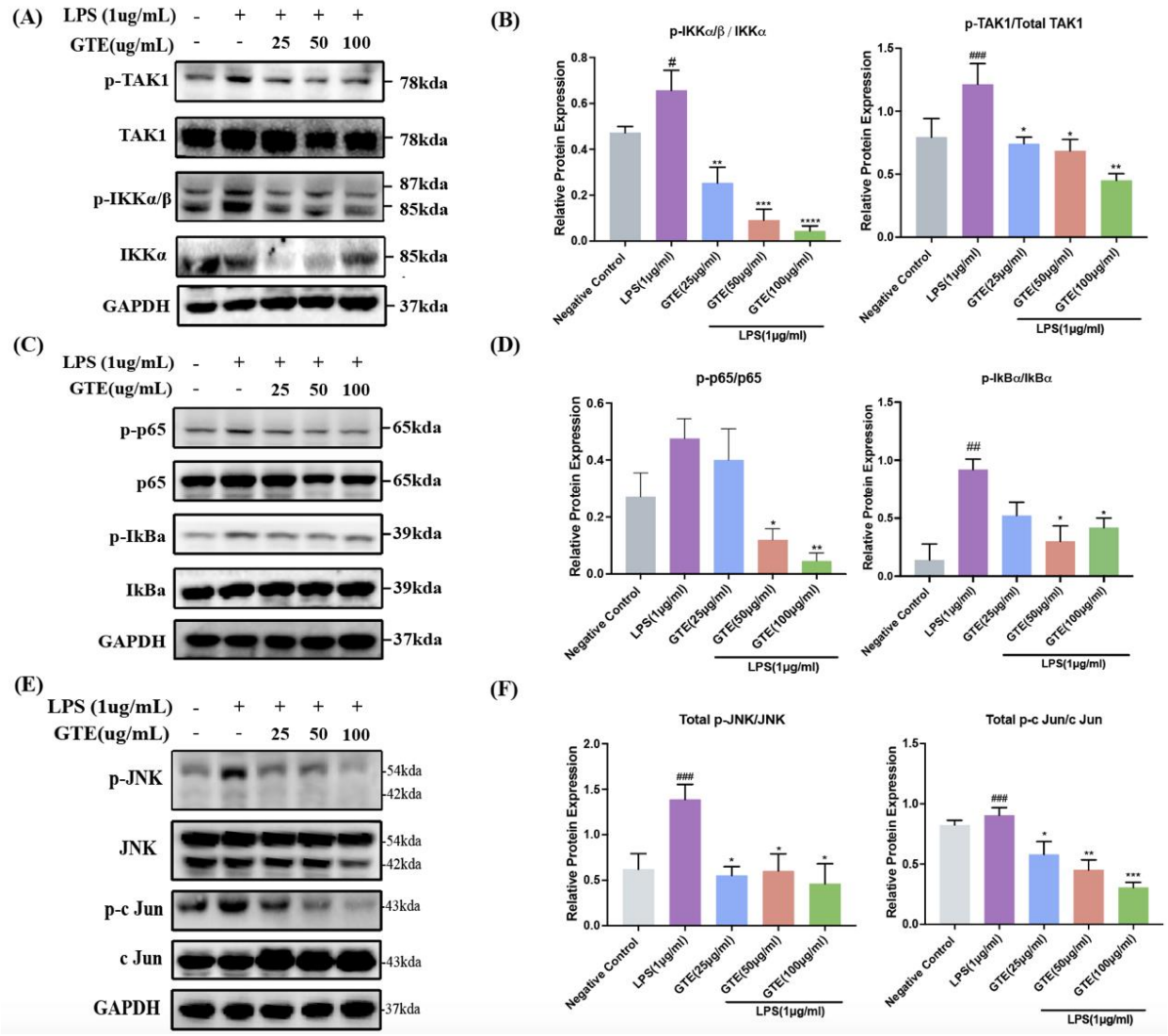


Fig 2.4 GTE down regulates NF-κB and MAPK/JNK pathway proteins. Immunoblotting and quantification of phosphorylated and total NF-κB proteins, TAK1 and IKKα/β (A&B), p65 and IkBα (C&D), JNK and c Jun (E&F). Statistical significance is compared between GTE treatment groups to 0μg/mL or LPS groups: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to model group. # compared to negative control group. Data are shown as mean ±SD (*n*=3).

2.3.4 GTE Inhibited the Nuclear Translocation of p65/ JNK/c Jun

In order to further confirm the anti-inflammatory ability of GTE through the NF-κB pathway in Caco-2 cells, the isolation of nuclear and cytoplasmic protein is performed. Nuclear

translocation of p65 is the typical symbol for cell inflammation, immunoblotting of nuclear P65 indicates GTE lowers the number of P65 in the nucleus[22]. Oppositely, the cytoplasmic protein level of p65 is increased in the GTE and LPS co-treatment group along with cytoplasmic IkBα (Fig 2.5 A). In accordance to the immunoblotting of nucleus/cytoplasm isolation, we can visualize the preventative effects of GTE on NF-κB nuclear translocation through immunofluorescence. The LPS challenged group manifests cyan fluorescence results from the merging of P65 green fluorescence and the DAPI staining of nucleus, an indication of P65 nuclear translocation. While, the p65 green fluorescence are outside of the nucleus in other groups, negative control group and GTE/LPS co-treatment groups (Fig 2.5 B). Through these examination and observation, GTE is capable of inhibiting the P65 translocation and activating transcription of inflammatory cytokines by decreasing whole series of phosphorylated protein expression levels. Some studies have shown the phosphorylation of JNK follow by nuclear translocation leads to inflammatory related diseases, such as cancer and obesity *in vitro* and *in vivo*. Revealing the importance of JNK pathway, and it is critical to regulate this pathway to impede diseases [26, 27]. The anti-inflammatory activity of GTE was discovered through downregulating the JNK/c Jun MAPK pathway. Furthermore, JNK and its target c Jun undergoes nuclear translocation to promote pro-inflammatory cytokine transcription. Nuclear and Cytoplasm isolation and immunoblot indicates GTE prevents large amount of phosphorylated JNK and phosphorylated c Jun entering the nucleus, and both of the two proteins are left in the cytoplasm (Fig 2.5 C,D). Visual wise, immunofluorescence confirms the phosphorylated c Jun entering the nucleus after LPS stimulation without GTE, while there is greater amount of phosphorylated c-jun in the cytoplasm in all concentration GTE treatment groups (Fig 2.5 E). Since p65, JNK, and c Jun are proteins exert their pro-inflammatory function once they are phosphorylated, thus GTE was examined if it has the ability to inhibit this process. Clearly with the data presented, GTE decrease the phosphorylation level of these protein to prevent their translocation.

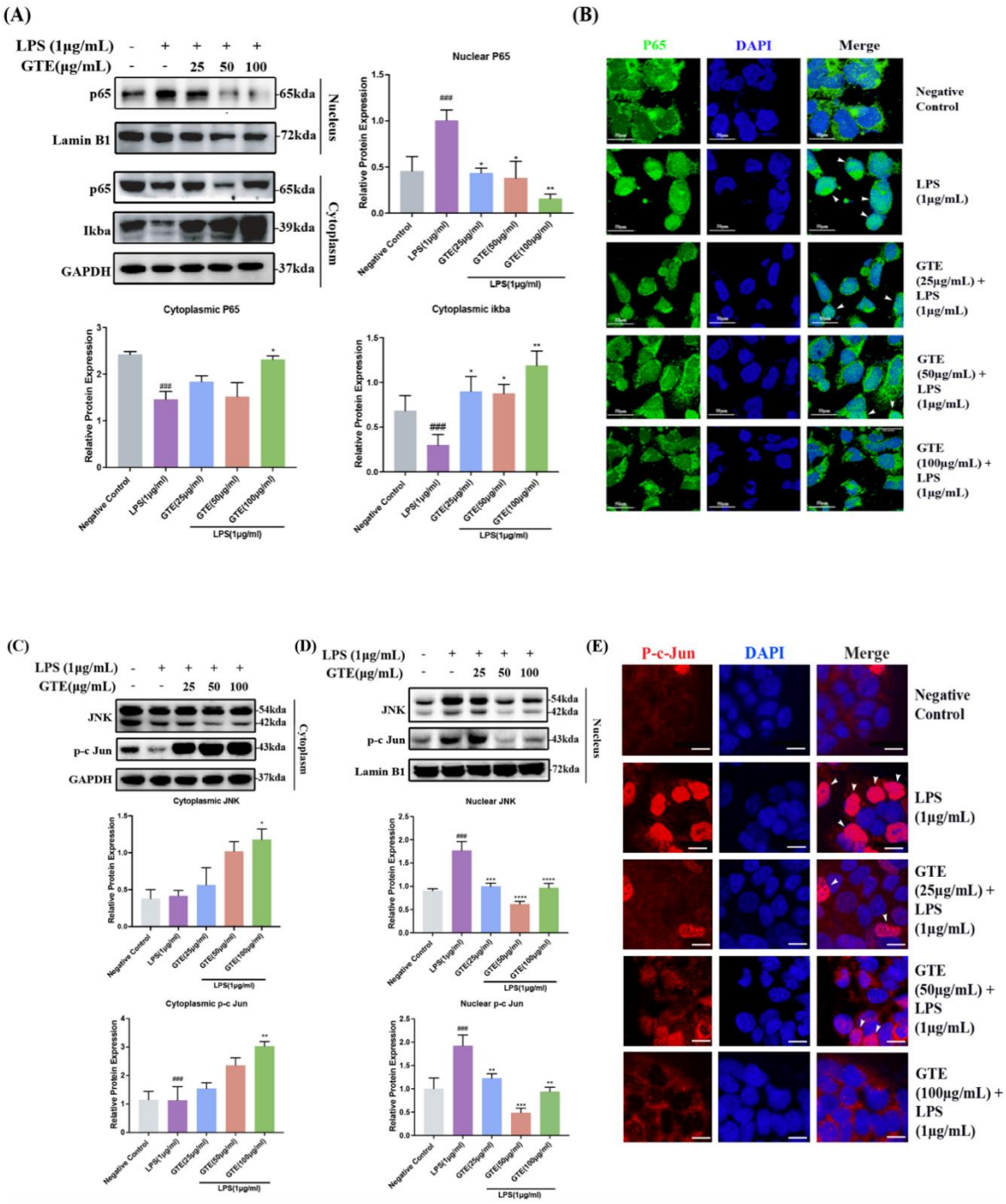


Fig 2.5 Prevention of p65, JNK, and c Jun undergoing nuclear translocation.

Immunoblotting and quantification of nuclear p65 and cytoplasmic p65 and IkBα (A). Immunofluorescence of p65 in Caco 2 cells (B). Immunoblotting and quatification of cytoplasmic JNK and p-c Jun (C). Immunoblotting and quatification of cytoplasmic JNK and p-c Jun (D). Immunofluorescence imaging against phosphorylated c-Jun (E). Statistical significance is compared between GTE treatment groups to 0μg/mL or LPS groups: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to model group. # compared to negative control group. Data are shown as mean ±SD (*n*=3).

2.3.5 GTE Decrease Reactive Oxygen Species Level to inhibit Oxidative Stress through Up Regulating Nrf2 Signaling Pathway

The metabolites of oxygen, reactive oxygen species (ROS) are agents damaging tissues and cells after injury when there is an exceed amount of ROS. The response of cells after myriad amount of ROS generation is oxidative stress, which lead to inflammation [28]. ROS production is the key to evaluate the anti-oxidative ability of GTE. ROS also triggers the activation inflammatory pathways (NF-κB and JNK pathway) and production of pro-inflammatory cytokines (TNF-α) [29]. Given that, DCFH-DA was used for observing the ROS production under fluorescent microscope. The green fluorescence is indication of cellular ROS, LPS group has much more green fluorescence compare to negative control and the highest concentration of GTE treatment group. Add on to the fluorescence imaging, intracellular ROS is also quantified with microplate reader using the same staining. GTE could significantly reduce the production of ROS from LPS induction (Fig 2.6 A). Some research had divulged Nrf2 can regulate pro-inflammatory cytokines and inflammatory state of macrophages [30]. Thus, we want to examine if GTE is able to amplify the expression of Nrf2 to inhibit oxidative stress and inflammation in Caco2 cells. Immunoblotting shows the Nrf2 protein level is lower after LPS stimulation, while GTE can enhance Nrf2 expression (Fig 2.6 B). Nrf2 is sequestered in the cytoplasm by Keap1, while it is the transcription factor undergoes nuclear translocation to promote the transcription of antioxidants HO-1 and NQO1 [31]. Therefore, we test if GTE further affects the protein expression of the two antioxidants in order to reason GTE decrease the amount of ROS in Caco2 cells. Immunoblotting confirms the protein expression of HO-1and NQO1 had similar trend as Nrf2 (Fig 2.6 B). As mentioned above, Nrf2 exerts its anti-oxidative function in the nucleus as a transcription factor, thus investigating the effects of GTE regarding the nuclear translocation of Nrf2 can further explain and evince its function as an anti-oxidative herb. Compartmentalization of Caco2 cells into nucleus and cytoplasm is performed along with immunofluorescence against Nrf2. After Caco2 cells incubation with LPS, nuclear Nrf2 expression level diminish, more Nrf2 accumulates in the cytoplasm. However, these phenomena are exact opposite in the GTE treatment groups observed by immunoblots (Fig 2.6 C) and immunofluorescence (Fig 2.6 D). In conclusion, GTE decrease the level of ROS during inflammation and oxidative stress of Caco2 cells through enhancing Nrf2, then enhancing the antioxidants HO-1 and NQO1.

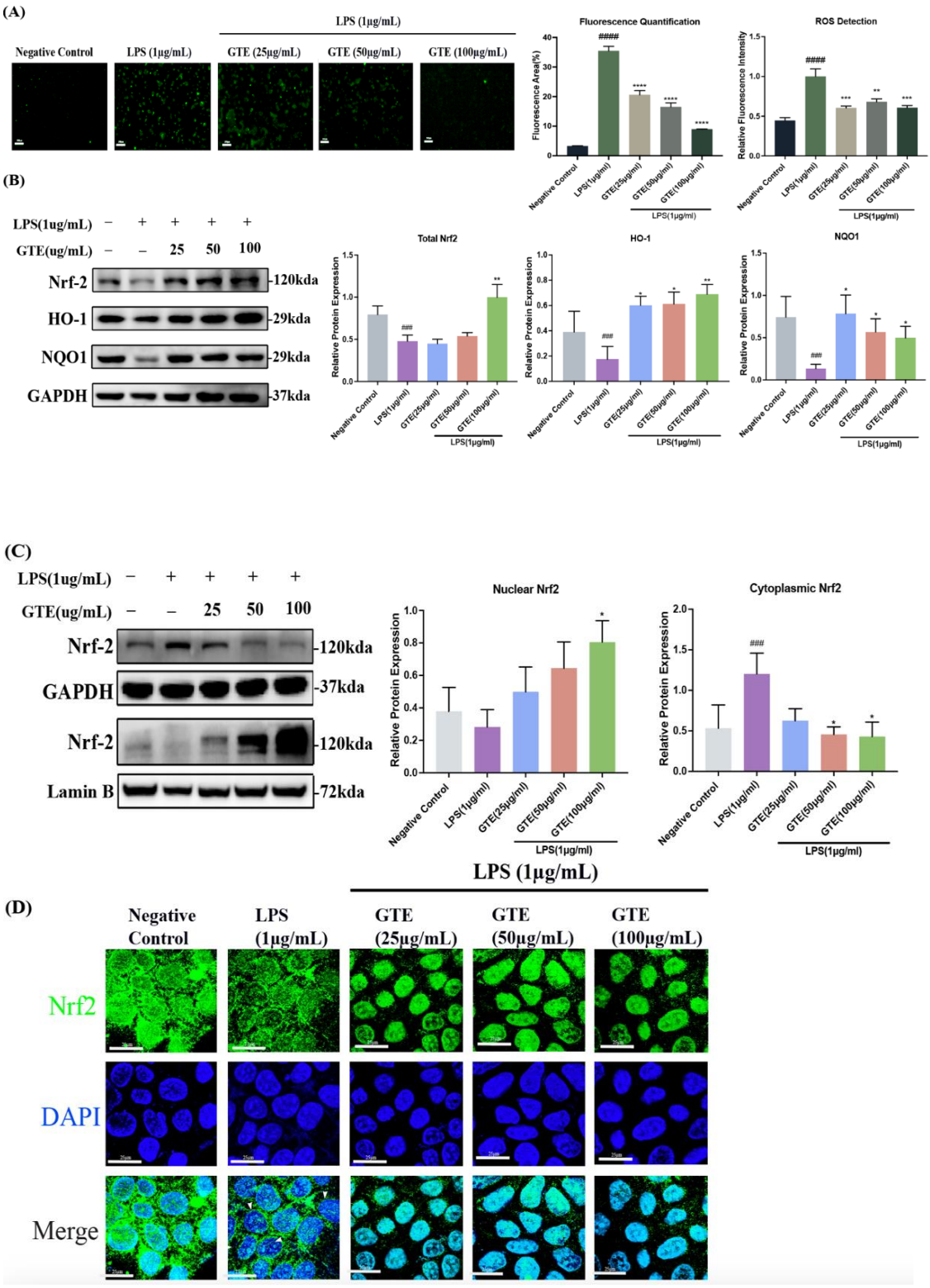


Fig 2.6 GTE function of anti-oxidation. Fluorescence Images of DCFH-DA labeled ROS in Caco 2 cells, quantification of fluorescence area percentage, and quantification of relative fluorescence Intensity of cellular ROS in treatment groups compare to LPS group (A). Immunoblotting and quantification of total proteins of oxidative stress pathway, Nrf2, HO-1, and NQO1 (B). Immunoblotting and quantification of nuclear and cytoplasmic Nrf2 (C).

Immunofluorescence imaging of Nrf2 cellular localization (D). Statistical significance is compared between GTE treatment groups to 0μg/mL or LPS groups: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to model group. # compared to negative control group. Data are shown as mean ± SD (n=3).

Chapter 3 Therapeutic Function of GTE against DSS induced Inflammatory Bowel Diseases Mice

3.1 Introduction

Many previous researches reported the use of dextran sodium sulfate (DSS) to establish *in vivo* models for studying IBD. The specific mechanism of DSS activation of colitis in mice is still unknown, while the effects of it can be evaluated through macroscopic analysis and detection of proteins. Macroscopic analysis is through daily observation of the weight changes, texture of feces, and hematochezia of mice. These are classic symptoms of colitis observed in human patients [6, 32, 33]. Watery and soft feces are indicating intestine fail to absorb the water in food, diarrhea is considered infection with pathogens. Another characteristic of IBD in mice is shortening of colon length due to inflammation and damage of intestinal tissues. Histopathology analysis also reveals the destruction of DSS to colonic structure and condition. Since DSS induced tissue damages in mice colons, oxidative stress and inflammation occur as a consequence of colon injuries. Tight junction proteins connecting the intestinal epithelial cells are also damaged, the lack of barrier function cause more severe infection in the colon of mice; inflammation and oxidative stress is once more activated. Although, the *in vitro* study of GTE on Caco 2 cells is carried out, *in vivo* study is more convincing to clarify the ability of GTE treating against colitis in living animal. The goal of *in vivo* study is to investigate the therapeutic effects of GTE on DSS challenged mice and to support the data of *in vitro* study.

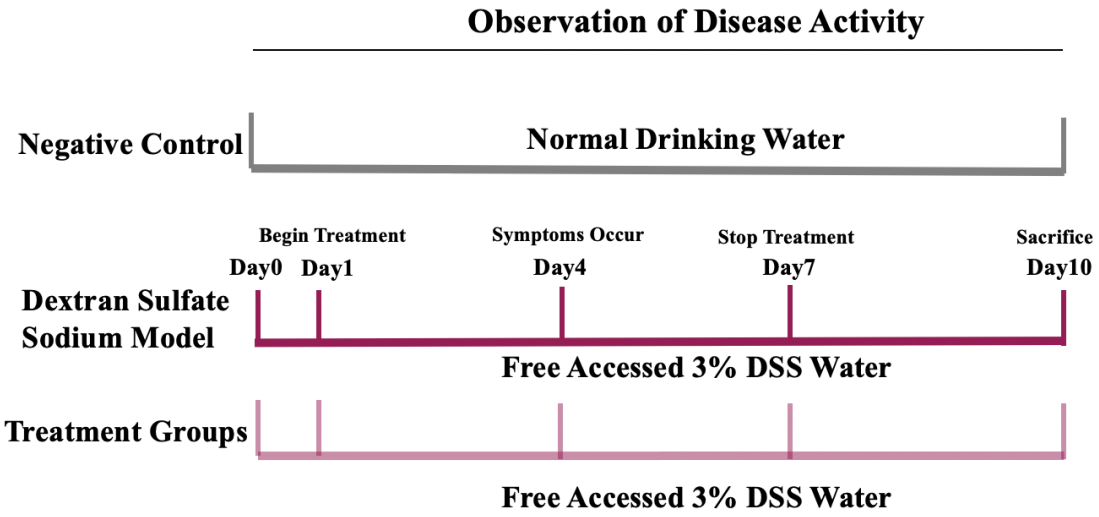
3.2 Method and Materials

Drug

3% DSS was purchased from MD Biomedical, USA. It was diluted in water for inducing IBD model. GTE was extracted based on previous study, it is diluted with water for 125 mg/kg and 250 mg/kg. 200 mg/kg of Sulfasalazine is diluted with water, it is the positive drug used in the study to confirm the accuracy of methods and experiments.

Design of Animal Study

In order to develop the colitis condition in Balb/c mice, DSS is administered through daily drinking water. The negative control group are mice with normal drinking water without any medication. The positive control group is Sulfasalazine (200 mg/kg), a classic clinical anti-inflammatory drug for ulcerative colitis and Crohn's diseases treatment [34, 35]. Two treatment group of GTE (125 mg/kg and 250 mg/kg). All drugs are administered by intragastric gavage (IG) to better simulate the actual intake of the medicine through the whole gastric intestinal tract in reality. From day 1, mice are accessed to DSS drinking water freely, treatment groups received their IG once per day. On Day 7, DSS drinking water will be replaced by normal drinking water. The mice will be sacrifice on day 10 (Fig 3.1). Daily measurement of the body weights, observation of stool texture, and occult bleeding is recorded. At the end of the experimental session, disease activity score will be calculated based on Table 1. The groups with DSS water all have a dramatic weight change on day 6. Throughout the whole experimental period, DSS challenged group had the most weight lost up to about -20% from day 0. The high dosage GTE treatment and Sulfasalazine group have significant difference in percentage of weight lost comparing to the DSS challenged group, which is around -10% from day 0. The condition and texture of mice feces recordings indicate the development of DSS challenged mice model is successful with mice having soft feces starting on day 4, diarrhea on day 6, and various degrees of bleeding from the anus on day 7.



1. **Sulfasalazine (200mg/kg)**
2. **GTE (125mg/kg)**
3. **GTE(250mg/kg)**

Fig 3.1 *In vivo* model establishment and drug administration design.

Table 3.1 Disease Activity Score Criteria

	Body Weight Loss (%)	Stool Texture	Hematochezia
0	0%	Solid	No Bleeding
1	1%-5%	-	-
2	5%-10%	Soft, Sticky,	-
3	10%-20%	-	Stool with Blood
4	20% or above	Diarrhea, Fluidic	Bleeding

Histopathological Analysis

One cm segment of colon is cut and put into 4% paraformaldehyde. Put in between parafilm and cross sectioned are cut and stained with hematoxylin and eosin (H&E). Pictures of cross section is taken under a microscope with bright field. The histopathological score is calculated based on Table 3.2.

Table 3.2 Histopathological Score Criteria

Category	0	1	2	3
Crypt Damage	No Damage, A Little Space between Mucosa	Small Branching of Crypt, Little bit out of Shape	-	Total Loss of Mucosa and Crypt Structure
Inflammatory Cell Migration	No Inflammatory cells	Accumulation in Lamina Propria	Observed in the bottom of Mucosa	Large Number of Inflammatory Cells in the Mucosa
Decrease of Goblet Cells	No Lost	-	-	Large Number of Goblet Cell Lost
Thickening of Colonic Wall	Regular Thickness	-	-	Significantly Enlarge

Colon Length

After sacrificing the mice and they are dissected, whole colon is separated from the bodies. Pictures are taken individually on a piece of white paper.

Cytokine Release Determination

About 1cm of colon from each mouse are wash with PBS and cut into smaller pieces on ice. Then homogenized with PBS. The supernatants are used as samples. Human TNF-α and IL-6 Enzyme Linked Immunosorbent Assay (ELISA) kits are purchased from Biolegend (CA, USA). The procedure of the experiment is performed under the manufacture's protocols.

Immunoblotting

About 1 cm of colon from each mouse are wash with PBS and cut into smaller pieces on ice. Then homogenized with 500 μL of RIPA buffer with 1% of cocktail and 1% of PMSF. After 15 minutes, the tubes are centrifuge with 14000 rpm at 4°c for 30 minutes. Supernatants are collected, and concentration of protein extract are determined by BCA kit (ThermoFischer, MA, USA). After concentration adjustment, 5x loading buffer (Beyotime, China) is diluted with the protein extracts. Tubes are shake for 650 rpm in a hot bath of 100℃ for 8 minutes. The samples (25 μg-30 μg) are separated through Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis at 70 v for 20 minutes, then 120 v for 1 hour and 15 minutes. Then the separated proteins are transfer on PVDF membrane after submerging into methanol at 100 v for 150 minutes and 120 v for 90 minutes. Then blot with 2.5% of non-fat milk powder (Biorad, USA) for 1 hour or QuickBlock buffer (Beyotime, China) for 15 minutes. Incubate the membrane with primary antibodies (1:1000) purchased from Cell Signaling Technology overnight at 4℃. Then wash with tris-buffered saline with 0.1% of TWEEN20 (TBST) and shake for 5 minutes. Repeat the step 3 times. Then incubate in secondary antibody (1:2000) purchased from Beyotime for 1hour. Then wash with tris-buffered saline with 0.1% of TWEEN20 (TBST) and shake for 5 minutes. Take pictures with Bio-Rad ChemiDoc imaging systems with chemiluminescence mode and colorimetric mode. Results are analyzed with ImageJ.

Metabolic Panels Test

Blood are collected from the mice and centrifuge at 4℃ with 1000 rpm for 20 minutes. All samples are from the serum of mice and detected according to the manufacture's protocols with GPT/ALT kit and AKP/ALP kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); BUN kit (Solarbio Life Science, Beijing, China); ALB kit (Fuxing Changzheng, Shanghai, China).

Myeloperoxidase Detection

Serum and colonic tissue homogenate are used as samples. The MPO levels are detected according to manufacturer's protocol with MPO commercial kit (Nanjing Jiancheng, Nanjing, China).

Organ Index

After sacrificing the mice, hearts, kidneys, spleens, lungs, and livers are dissected from mice. The very organ is recorded. The index is calculated by organ weight divide by total body weight of animal on day 10.

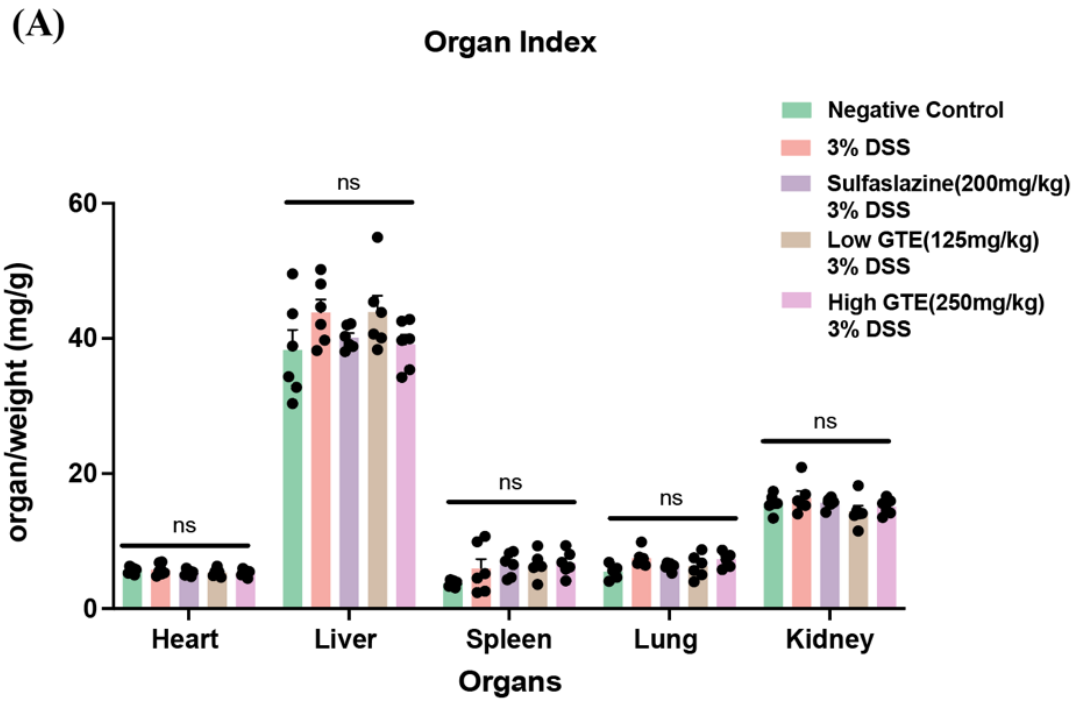
Statistical Analysis

All of the quantification data are calculated, analyzed and graph are generated with Image J and GraphPad Prism 9. All of the *in vivo* experiments are repeated six times] and statistical significance is calculated by using one-way ANOVA test.

3.3 Results

3.3.1 GTE Safe Concentration *In vivo*

In order to ensure the safe and effective concentrations of GTE, organ index of heart, liver, spleen, lung, and kidney is calculated to evaluate the organ toxicity of the drugs. The organ index for all five organs does not show any significance difference comparing within experimental groups (Fig 3.2A). Besides the organ index, alanine transaminase (ALT), alkaline phosphatase (AKP), albumin (ALB), and blood urea nitrogen (BUN) levels in the mice serum are determined with specific kits. These biomarkers are classic indicators for clinical metabolic panel test during the diagnosis of liver, kidney, and other organ injuries or failures. 3% of DSS, both concentration of GTE, and 200 mg/kg of sulfasalazine does not damage the organs significantly as the levels of these biomarkers in the serum are similar within experimental groups (Fig 3.2B).


(A)
Organ Index
Negative Control
3% DSS
Sulfaslazine(200mg/kg) 3% DSS
Low GTE(125mg/kg) 3% DSS
High GTE(250mg/kg) 3% DSS
organ/weight (mg/g)
60
40
20
0
ns
ns
ns
ns
ns
Heart
Liver
Spleen
Lung
Kidney
Organs

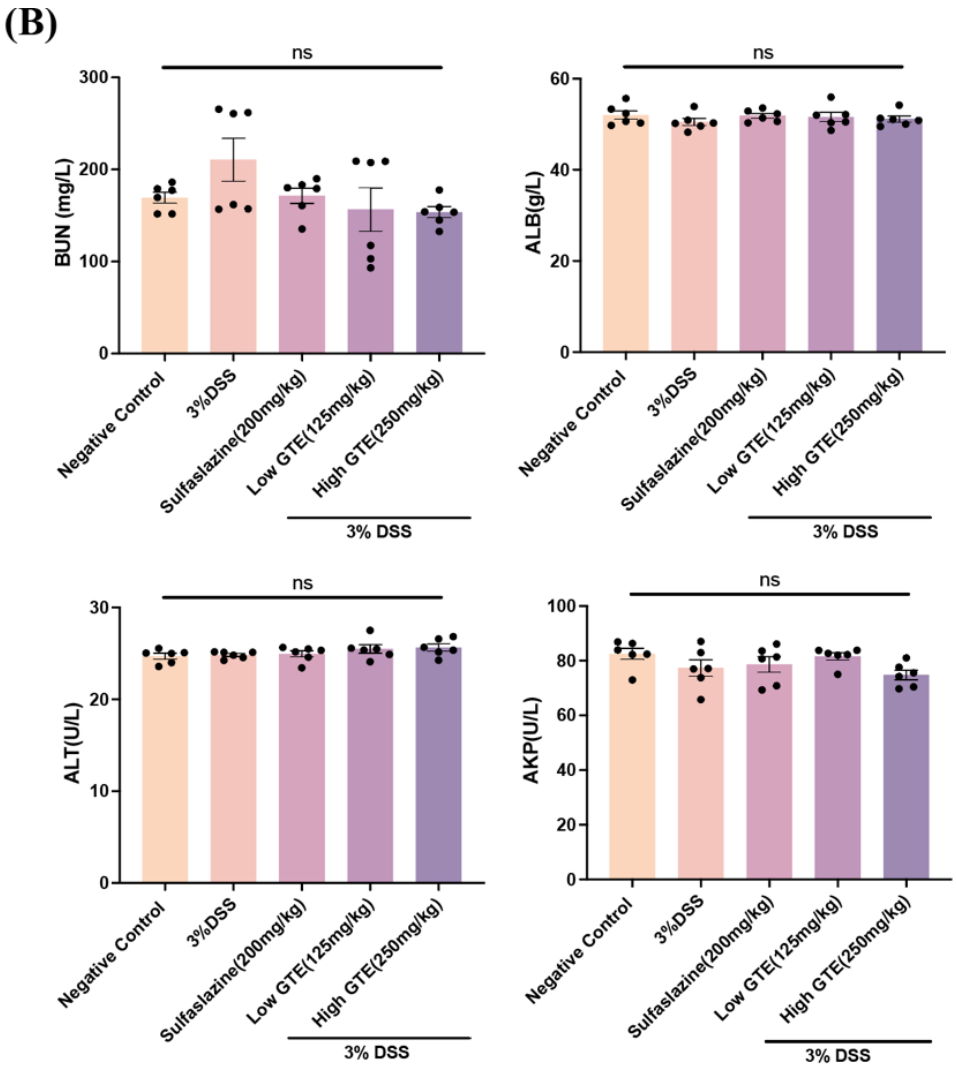

(B)
BUN (mg/L)
300
200
100
0
ALB(g/L)
60
40
20
0
ALT(U/L)
30
20
10
0
AKP(U/L)
100
80
60
40
20
0
ns
Negative Control
3%DSS
Sulfaslazine(200mg/kg)
Low GTE(125mg/kg)
High GTE(250mg/kg)
3% DSS

Fig 3.2 Organ toxicity of DSS and drugs. Organ Index (mg/g) of heart, liver, spleen, lung, and kidney. (A) Levels of serum ALT, AKP, ALB, and BUN. (B) Statistical Significance compare to 0μg/mL or LPS groups. ns refers to no significant difference. Data are shown as mean ± SEM (*n*=6).

3.3.2 GTE Decrease Disease Activity of IBD and Improves the Condition of Colonic Tissue in Inflammatory State

The three symptoms listed in **TABLE 3.1** are observed and recorded daily. The groups with DSS water all have a dramatic weight change on Day 6. Throughout the whole experimental period, DSS challenged group had the most weight lost up to about -20% from Day 0. The high dosage GTE treatment and Sulfasalazine group have significant difference in percentage of weight lost comparing to the DSS challenged group, which is around -10% from Day 0 (Fig 3.3 A). The condition and texture of mice feces recordings indicate the development of DSS challenged mice model is successful with mice having soft feces starting on Day 4, diarrhea on Day 6, and various degrees of bleeding from the anus on Day 7. Intense degrees have a higher score, and vice versa. The graph shows DSS challenged group has the highest score throughout the whole experimental session, and treatment groups have significant lower scores manifesting the therapeutic effects of GTE (Fig 3.3 B). Shrinkage of the colon is a sign of inflammation and injury as shown in DSS group. The average colon length of negative control and other treatment groups are longer than the average colon length of DSS group by at least 2 cm disregarding the variation on the size and weight of mice (Fig 3.3 C). As one of the first responders to infection, neutrophils are recruited to the infectious sites as they increase in numbers, which causes inflammatory symptoms. One major enzyme produce by neutrophils with anti-bacterial function is myeloperoxidase (MPO). Through assessing the level of MPO in colonic tissue, the severity of colitis induced by DSS could be determined in the aspect of neutrophil's number and neutrophil's infiltration to the colonic tissue. The level of MPO remains low in healthy mice, while DSS damaging the lumen of colon cause serious infection leading to great amount of MPO secretion. Sulfasalazine, the classic anti-colitis drug lowers the MPO level as expected. GTE shows a concentration dependent effect on lowering the level of MPO, which both concentration of GTE could effectively alleviate neutrophil secretion of MPO (Fig 3.3 D). The disease activity of mice was induced by DSS through destroying the structure of colon leading to severe infection and inflammatory response. Thus, the destruction of colon is assessed through hematoxylin and eosin (H&E)

staining of the intestinal cross sections. **TABLE 3.2** is a scoring chart for evaluating the degree of pathology of the colon base on crypt loss, goblet cell loss, thickening of colon wall, and infiltration of lamina propria based on previous clinical studies and *in vivo* studies of IBD [32, 36]. The pathological score is quantification of H&E staining indicating DSS induction of undermining colonic tissue is ameliorate by GTE (Fig 3.3 F). Regarding the condition and structure of the inflamed colon induced by DSS, the goblet cells are not observed in mucosa (yellow), crypt is no longer organized (red), and the cell wall width is increased (blue) indicated with arrows (Fig 3.3 E). Great number of immune cells are observed migrating from the lamina propria or the basal side of the mucosa to the inner part indicate by black arrows (Fig 3.3 E). This is a significant sign of aberrant immune response causing fibrosis and other symptoms of inflammation. While other treatment groups, both GTE and Sulfasalazine groups are having similar characteristics of the colon structure compare to negative control group (Fig 3.3 E). GTE have significant protective effects of the colon through observing disease activity and histopathological analysis of DSS induced mice. GTE was able to maintain a healthy state similar to the negative control mice, while remarkable damages were seen in the colon of DSS model group mice.

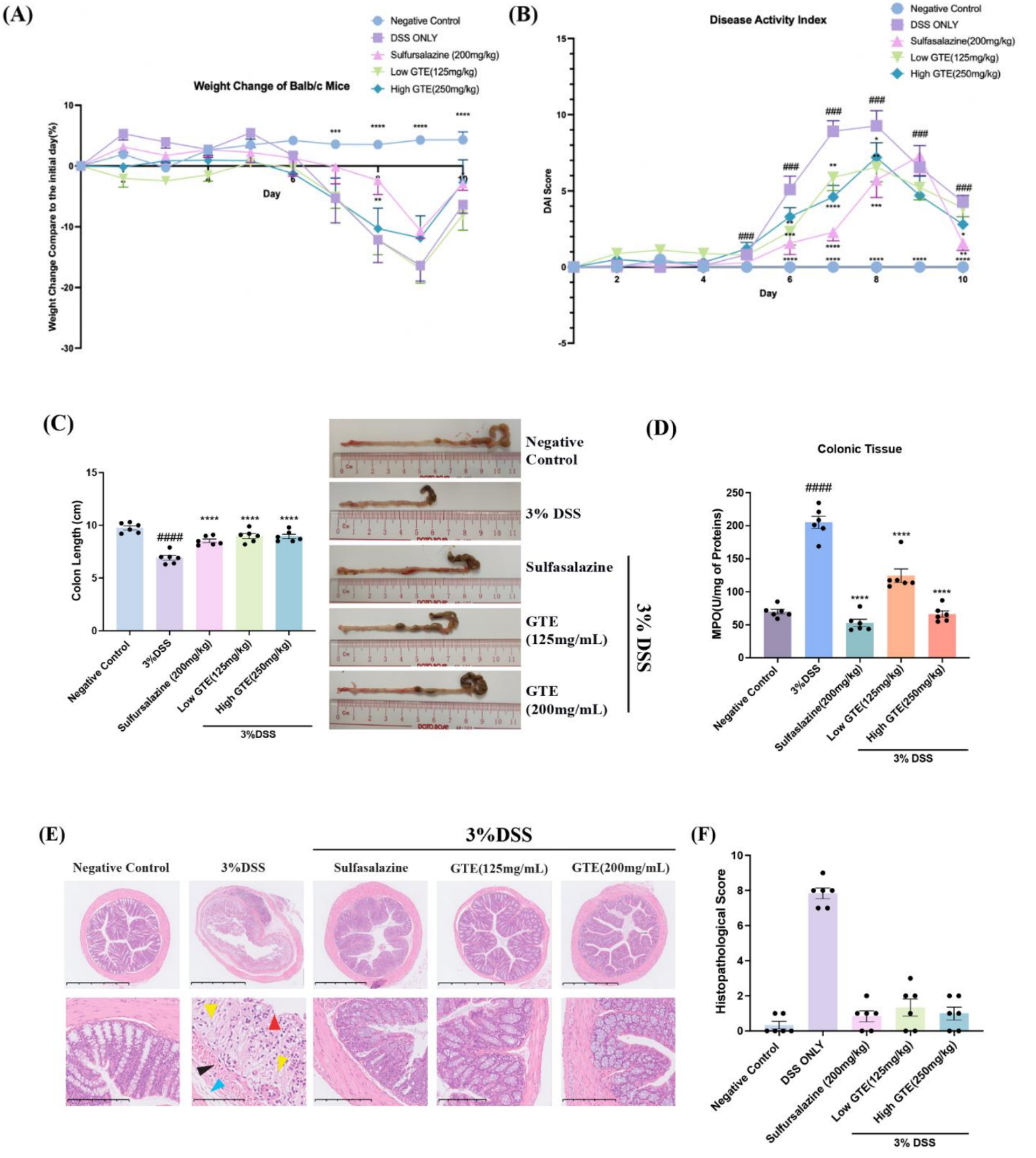

(A)
Weight Change of Balb/c Mice
Negative Control
DSS ONLY
Sulfursalazine (200mg/kg)
Low GTE(125mg/kg)
High GTE(250mg/kg)
Weight Change Compare to the initial day(%)
Day
(B)
Disease Activity Index
DAI Score
Day
(C)
Colon Length (cm)
Negative Control
3%DSS
Sulfursalazine (200mg/kg)
Low GTE(125mg/kg)
High GTE(250mg/kg)
3%DSS
Negative Control
3% DSS
Sulfasalazine
GTE (125mg/mL)
GTE (200mg/mL)
3% DSS
(D)
Colonic Tissue
MPO(U/mg of Proteins)
Negative Control
3%DSS
Sulfasalazine(200mg/kg)
Low GTE(125mg/kg)
High GTE(250mg/kg)
3% DSS
(E)
3%DSS
Negative Control
3%DSS
Sulfasalazine
GTE(125mg/mL)
GTE(200mg/mL)
(F)
Histopathological Score
Negative Control
DSS ONLY
Sulfursalazine (200mg/kg)
Low GTE(125mg/kg)
High GTE(250mg/kg)
3% DSS

Fig 3.3 GTE decrease the disease activities of IBD. Weight change of mice from day 0 to day 10 (A). Disease Activity Score of mice from day 0 to day 10 (B). Colon length of mice after sacrifice and dissection. Colon length pictures (C). MPO level in colonic tissue of mice (D). H&E staining images. Arrows are indicating the disease sites in colonic tissue. Yellow arrow: goblet cell lost; Blue arrow: colonic wall thickening; Red arrow: crypt lost; Black arrow: Immune cell infiltration at the lamina propria (E). Histopathological analysis score (F). Statistical Significance compare to 0μg/mL or LPS groups $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ compared to model group. # compared to negative control group. Data are shown as mean ±SEM (n=6).

3.3.3 GTE Enhance the Protein Expression of Tight Junction Proteins through Decreasing Pro-Inflammatory Cytokines

The main function of epithelial cells is defending against extracellular stimulation such as bacteria, virus, fungi, metabolites, and other pathogens. The receptors of the epithelial cells will trigger downstream pathways activation, NF-κB, MAPK, and oxidative stress pathways. The immune cells surveilling the whole body will also respond to potential infection, causing lamina propria infiltration in the colon [37]. The pro-inflammatory cytokines secreted by these cells degrades the tight junction proteins, infection occur along with the cell permeability increase [10, 11]. First pro-inflammatory cytokine is detected, the anti-inflammatory effect of GTE of *in vivo* study. Similar to the *in vitro* study results, TNF-α and IL-6 production in the colonic tissue are lower in GTE treatment group (Fig 3.4 A). As a consequence, the GTE treatment also increase the protein expression of the two tight junction proteins (Fig 3.4 B). These data explain the histopathological score is low in drug treatment groups as GTE can prevent further infection, putting the vicious cycle of inflammation and oxidative stress to an end.

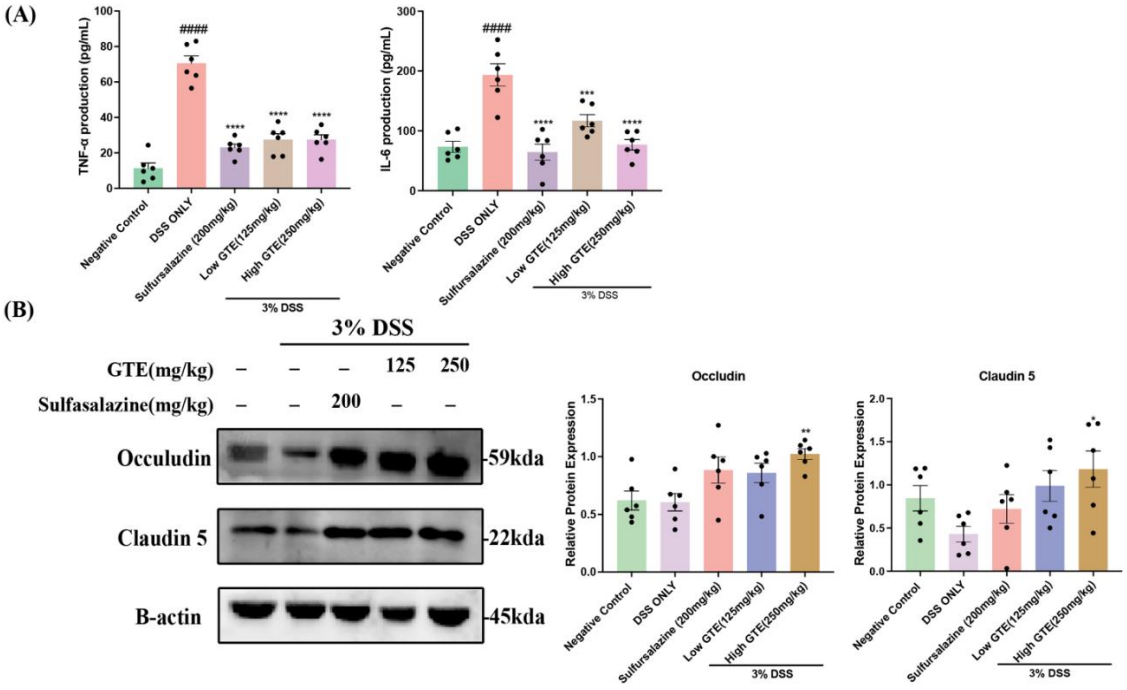


Fig 3.4 TNF-α and IL-6 secretion in the colonic tissue (A). Immunoblotting and quantification of tight junction proteins occluding and claudin 5 (B). Statistical Significance compare to 0μg/mL or LPS groups **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to model group. # compared to negative control group. ns refers to no significant difference. Data are shown as mean ±SEM (*n*=6).

3.3.4 GTE Decrease the Protein Expression of p65/IkBα, JNK/c Jun, and Nrf2/HO-1/NQO1

The involvement of pro-inflammatory cytokines during colitis is complex, various kinds of cytokines are released from different types of immune cells and epithelial cells back and forth. The cytokines are some of the main drivers causing pathologic symptoms and responsible for activating NF-κB and Oxidative pathways worsen the conditioned [12]. Since the enterocytes on lumen side of colon produce pro-inflammatory cytokines leading to further inflammatory response, colonic cytokines protein expression is determining with ELISA kits. TNF-α and IL-6 in colonic tissues are significantly diminish by GTE. The reduction of pro-inflammatory cytokines in colonic tissue might be an implication for inhibition of NF-κB proteins by GTE. The decrease of phosphorylated Ikba and p65 in colonic tissue happen simultaneously. In accordance with the *in vitro* study, change in the expression of pro-

inflammatory cytokines has a great effect on NF-κB pathway (Fig 3.5 A). The JNK and c Jun belonging to the MAPK pathway in the homogenate of colon are affected by GTE the same way as in Caco 2 cells. The phosphorylation of these two MAPK pro-inflammatory proteins are enhanced by DSS induced injuries and inflammation in the colon, while GTE lowers the level of phosphorylation (Fig 3.5 B). As stated by Grisham, the number of anti-oxidant (i.e Nrf2, HO-1, and NQO1) are in deficit when large amount of ROS free radicals is produced in the colon during inflammation [6]. The role of Nrf2 is significant against IBD, the DSS is able to heavily damage the colon of Nrf2 deficient mice, as a consequence of imbalance ROS [38]. Immunoblotting shows its expression of protein increase after GTE treatment even with the exposure to DSS, but less Nrf2 expression in the DSS challenged group. Since Nrf2 is the transcription factor of HO-1 and NQO1, we want to investigate if GTE have anti-oxidation function. The immunoblotting result on colonic tissue is commensurate with *in vitro* study, similar trend with Nrf2, GTE could elevate anti-oxidants against ROS to reduce its damages done to the colon (Fig 3.5 C).

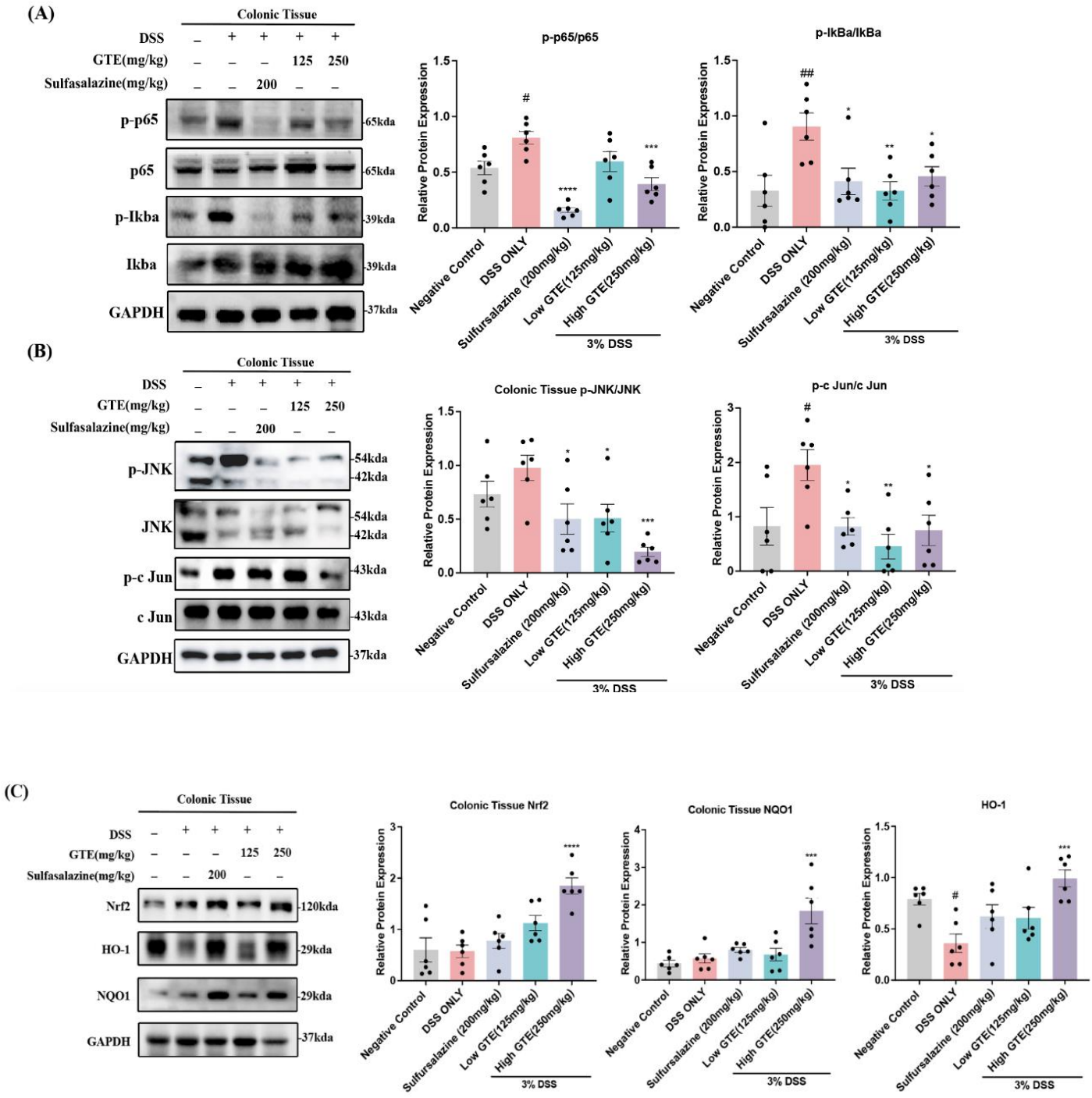


Fig 3.5 GTE decrease phosphorylation of NF-κB and MAPK proteins, while enhance the expression of NRF2/HO-1/NQO-1. Immunoblotting and quantification of phosphorylated p65 and IkBα, total p65 and IkBα of colonic tissue (A). Immunoblotting and quantification of JNK and c Jun of colonic tissues (B). Immunoblotting and quantification of anti-oxidants, Nrf2, HO-1, and NQO1 of colonic tissue (C). Significance compare to 0μg/mL or LPS groups *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to model group. # compared to negative control group. Data are shown as mean ± SEM (n=6).

Chapter 4 Conclusion

The current dilemma of IBD is there are treatment for the disease, while it remains to be uncured. There are classic drugs such as sulfasalazine with low price, while there are potential severe risk and side effects such as nausea, heartburn, headache, abdominal pain, which are symptoms and signs similar as IBD. Other novel developed drugs such as TNF-α inhibitors and monoclonal antibodies are more expansive. In addition, the side effects could be serious, clinical studies have shown using these monoclonal antibodies could lead to autoimmune diseases, decrease in the number of platelet and other conditions. Therefore, utilizing the large number of herbs in nature and seek for the effective ones is an alternative solution for IBD. Previous studies revealed the therapeutic effects of GTE against arthritis, nephrotic syndrome, and synovial inflammation by down regulating TNF-α, IL-6, IL-1β, Nitric Oxide, NF-κB pathway, and MAPK pathways. Thus, GTE might have similar effects against IBD through identical signaling pathways. The natural herb glycine tabacina and its aqueous extract are examined in TNF-α or LPS induced Caco 2 cells and DSS induced BALB/c mice. GTE was proven to ameliorate symptoms and conditioned of IBD within the safety concentration.

TNF-α and IL-6 are key pro-inflammatory cytokines contributing to IBD pathogenesis. First, they are signaling proteins released to recruit immune cells and activates immune response. After migration and aggregation of immune cells such as macrophages and neutrophils in the colon, fibrosis and fistula might occur as a result, which are common symptoms of IBD. Next, ECM and cytokines increase protein expression of MMPs to degrade tight junction proteins allowing extracellular factors to invade the host as the intestinal permeability increases. In our current study, three safe and effective concentrations of GTE were identified with LPS or TNF-α induced Caco 2 cells and two concentration with DSS induced BALB/c mice. Pro-inflammatory cytokines secretion and transcription are inhibited by GTE because the phosphorylation in NF-κB and MAPK/JNK are lessen. More specifically, the nuclear translocation of both transcription factors, p65 and c Jun is disrupted by GTE, so the transcription of targeted genes is prevented.

Inflammation and oxidative stress are positively correlated, neutrophils infiltration and injuries of cells are due to the imbalance amount of ROS. We observed the level of ROS in Caco 2 cells and MPO in colonic tissue decreased after GTE was used. In healthy state and LPS induction, we observed low protein expression level of the transcription factor and lack

of nuclear translocation of Nrf2. It was discovered to be enhanced by GTE, and Nrf2 undergoes nuclear translocation to promote the transcription of anti-oxidants HO-1 and NQO1 (Fig 4.1).

The anti-inflammatory effects of GTE at molecular level improves the weight loss, diarrhea, and occult bleeding activities. In addition, the structure of colonic tissues, the percentage of various types of cells are balanced comparing to DSS model group. Overall, GTE is proven to have therapeutic function against IBD and reduce the degree of IBD symptoms and ameliorates the condition of the environment in mice colons under the ruination of DSS on the macroscopic level. Molecularly, GTE prevents nuclear translocation of p65 and c Jun causing the lower level of TNF-α and IL-6. In the aspect of oxidative stress, GTE elevates the expression of Nrf2, HO-1, and NQO1 to disrupt the cycle of inflammation and oxidative stress.

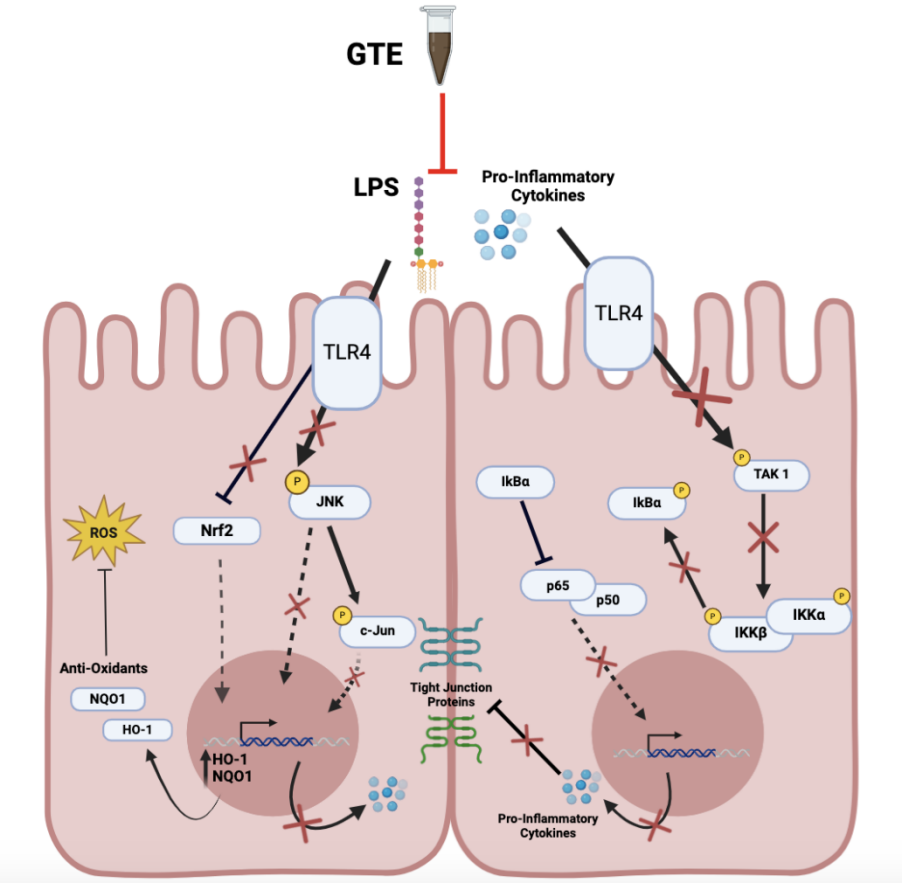


Fig 4.1 GTE inhibiting extracellular risk factors to prevent activation of inflammatory pathways and oxidative stress in intestinal cells.

Some of the limitation of our study:

We did not use single compounds of GTE in the study. Without this compound, we do not understand the specific mechanism and targets of the drug in treatment of IBD. We did not perform molecular assays such as knockdown and overexpression of specific proteins, so we cannot confirm the major pathway regulates by GTE. Lack of co-culture with immune cells to mimic the environment in the colon after lamina propria infiltration.

Future Perspective

Use the isolated compounds for further mechanism and target studies by utilizing immunoprecipitation, docking, and many other assays. Using microbiota to investigate the microbiome and metabolites to explain their role in treatment of IBD. This extract can be developed into products for oral administration to treat IBD.

References

1. Kaplan, G.G. and J.W. Windsor, *The four epidemiological stages in the global evolution of inflammatory bowel disease.* Nat Rev Gastroenterol Hepatol, 2021. **18**(1): p. 56-66.
2. Kaplan, G.G., *The global burden of IBD: from 2015 to 2025.* Nat Rev Gastroenterol Hepatol, 2015. **12**(12): p. 720-7.
3. Jairath, V. and B.G. Feagan, *Global burden of inflammatory bowel disease.* Lancet Gastroenterol Hepatol, 2020. **5**(1): p. 2-3.
4. Adolph, T.E., et al., *The metabolic nature of inflammatory bowel diseases.* Nat Rev Gastroenterol Hepatol, 2022. **19**(12): p. 753-767.
5. Barberio, B., et al., *Prevalence of Primary Sclerosing Cholangitis in Patients With Inflammatory Bowel Disease: A Systematic Review and Meta-analysis.* Gastroenterology, 2021. **161**(6): p. 1865-1877.
6. Grisham, M.B., *Oxidants and free radicals in inflammatory bowel disease.* Lancet, 1994. **344**(8926): p. 859-61.
7. Aviello, G. and U.G. Knaus, *ROS in gastrointestinal inflammation: Rescue Or Sabotage?* Br J Pharmacol, 2017. **174**(12): p. 1704-1718.
8. Dahan, S., et al., *Epithelial: lamina propria lymphocyte interactions promote epithelial cell differentiation.* Gastroenterology, 2008. **134**(1): p. 192-203.
9. Rashidian, A., et al., *Atorvastatin attenuates TNBS-induced rat colitis: the involvement of the TLR4/NF-kB signaling pathway.* Inflammopharmacology, 2016. **24**(2-3): p. 109-18.
10. Ye, D., et al., *MicroRNA regulation of intestinal epithelial tight junction permeability.* Gastroenterology, 2011. **141**(4): p. 1323-33.
11. Rawat, M., et al., *IL1B Increases Intestinal Tight Junction Permeability by Up-regulation of MIR200C-3p, Which Degrades Occludin mRNA.* Gastroenterology, 2020. **159**(4): p. 1375-1389.
12. Friedrich, M., M. Pohin, and F. Powrie, *Cytokine Networks in the Pathophysiology of Inflammatory Bowel Disease.* Immunity, 2019. **50**(4): p. 992-1006.
13. Solitano, V., et al., *Comparative Risk of Serious Infections With Biologic Agents and Oral Small Molecules in Inflammatory Bowel Diseases: A Systematic Review and Meta-Analysis.* Clin Gastroenterol Hepatol, 2023. **21**(4): p. 907-921 e2.
14. Tan, L., et al., *Exploring protective effect of Glycine tabacina aqueous extract against nephrotic syndrome by network pharmacology and experimental verification.* Chin Med, 2020. **15**: p. 79.
15. Tu, Y., et al., *Isolation and Identification of Antiarthritic Constituents from Glycine tabacina and Network Pharmacology-Based Prediction of Their Protective Mechanisms against Rheumatoid Arthritis.* J Agric Food Chem, 2020. **68**(39): p. 10664-10677.
16. Tu, Y., et al., *Glycine tabacina ethanol extract ameliorates collagen-induced arthritis in rats via inhibiting pro-inflammatory cytokines and oxidation.* J Ethnopharmacol, 2019. **237**: p. 20-27.
17. Wu, X.X., et al., *Paeoniflorin Prevents Intestinal Barrier Disruption and Inhibits Lipopolysaccharide (LPS)-Induced Inflammation in Caco-2 Cell Monolayers.* Inflammation, 2019. **42**(6): p. 2215-2225.
18. Biel, C., et al., *Matrix metalloproteinases in intestinal fibrosis.* J Crohns Colitis, 2024. **18**(3): p. 462-478.
19. Shimshoni, E., et al., *ECM remodelling in IBD: innocent bystander or partner in crime? The emerging role of extracellular molecular events in sustaining intestinal inflammation.* Gut, 2015. **64**(3): p. 367-72.
20. Manicone, A.M. and J.K. McGuire, *Matrix metalloproteinases as modulators of inflammation.* Semin Cell Dev Biol, 2008. **19**(1): p. 34-41.
21. Capaldo, C.T. and A. Nusrat, *Cytokine regulation of tight junctions.* Biochim Biophys Acta, 2009. **1788**(4): p. 864-71.
22. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling.* Cell, 2008. **132**(3): p. 344-62.
23. Takaesu, G., et al., *TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway.* J Mol Biol, 2003. **326**(1): p. 105-15.
24. Landstrom, M., *The TAK1-TRAF6 signalling pathway.* Int J Biochem Cell Biol, 2010. **42**(5): p. 585-9.
25. Morrison, D.K., *MAP kinase pathways.* Cold Spring Harb Perspect Biol, 2012. **4**(11).
26. Han, M.S., et al., *JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation.* Science, 2013. **339**(6116): p. 218-22.
27. Maik-Rachline, G., et al., *The nuclear translocation of the kinases p38 and JNK promotes inflammation-induced cancer.* Sci Signal, 2018. **11**(525).
28. Ma, Q., *Role of nrf2 in oxidative stress and toxicity.* Annu Rev Pharmacol Toxicol, 2013. **53**: p. 401-26.

29. Blaser, H., et al., *TNF and ROS Crosstalk in Inflammation.* Trends Cell Biol, 2016. **26**(4): p. 249-261.
30. Kobayashi, E.H., et al., *Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription.* Nat Commun, 2016. **7**: p. 11624.
31. Niture, S.K., R. Khatri, and A.K. Jaiswal, *Regulation of Nrf2-an update.* Free Radic Biol Med, 2014. **66**: p. 36-44.
32. Ghosh, S. and R. Mitchell, *Impact of inflammatory bowel disease on quality of life: Results of the European Federation of Crohn's and Ulcerative Colitis Associations (EFCCA) patient survey.* J Crohns Colitis, 2007. **1**(1): p. 10-20.
33. Singh, S., et al., *Common symptoms and stressors among individuals with inflammatory bowel diseases.* Clin Gastroenterol Hepatol, 2011. **9**(9): p. 769-75.
34. Klotz, U., et al., *Therapeutic efficacy of sulfasalazine and its metabolites in patients with ulcerative colitis and Crohn's disease.* N Engl J Med, 1980. **303**(26): p. 1499-502.
35. Sutherland, L.R., G.R. May, and E.A. Shaffer, *Sulfasalazine revisited: a meta-analysis of 5-aminosalicylic acid in the treatment of ulcerative colitis.* Ann Intern Med, 1993. **118**(7): p. 540-9.
36. Hendrickson, B.A., R. Gokhale, and J.H. Cho, *Clinical aspects and pathophysiology of inflammatory bowel disease.* Clin Microbiol Rev, 2002. **15**(1): p. 79-94.
37. Sartor, R.B., *Microbial influences in inflammatory bowel diseases.* Gastroenterology, 2008. **134**(2): p. 577-94.
38. Khor, T.O., et al., *Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis.* Cancer Res, 2006. **66**(24): p. 11580-4.