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Research Article

Garidisan Regulates the Balance of Th17/Treg Cell in Ulcerative Colitis Rats Induced by Immunization and 2,4,6-Trinitrobenzenesulfonic Acid

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Abstract

Introduction: Garidisan is a mongolian traditional medicine containing Wild Poppy and Artemisia Frigida Willd, which is commonly used in Mongolia to treat Ulcerative Colitis (UC). Clinical evidence shows that Garidisan has a good curative effect on UC, however its mechanism are still unclear. The balance of Th17/Treg cell is important in the occurrence and development of inflammatory bowel disease. This study aims to evaluate the regulatory effect of Garidisan on Th17/Treg cell balance in UC model rats induced by immunization and 2,4,6-trinitrobenzenesulfonic acid (TNBS).

Methods: Ninety Wistar rats were randomized to seven groups: Control, UC model, low-dose Garidisan, moderate-dose Garidisan, high-dose Garidisan, sulfasalazine and Bupiyichangwan. UC model was established (except for controls) through immunization and TNBS. Basing on the rats Disease Activity Index (DAI), Colonic Mucosal Damage Index (CMDI), Histopathological Scores (HS), and Hematoxylin and Eosin (H&E) staining to check the therapeutic effect of Garidisan on UC rats. The levels of interleukin-6(IL-6), IL-17, IL-23 and IL-10 were examined by ELISA. The ratio of $CD4^+IL\text{-}17^+$ T cells / $CD4^+CD25^+Foxp3^+$T cells in peripheral blood of UC rats was determined by flowcytometry.

Results: Garidisan could significantly reduce DAI, CMDI, and HS of UC rats. H&E staining showed that Garidisan could repair UC rats damaged mucous membrane and significantly reduce IL-6, IL-17 and IL-23 content, increase IL-10 content in UC Rats peipherial blood, reduce the ratio of $CD4^+IL\text{-}17^+$ T cells /$CD4^+CD25^+Foxp3^+$ T cells in UC rats peipherial blood.

Conclusions: Garidisan could regulate $CD4^+IL\text{-}17^+$/ $CD4^+CD25^+Foxp3^+$ T cell's balance in peipherial blood of UC rats, which further ameliorate UC.

Keywords: Garidisan; Ulcerative colitis; Th17 cell; Th17/Treg cell balance

Abbreviations: UC: Ulcerative Colitis; SASP: Sulfasalazine; TNBS: 2,4,6-Trinitrobenzenesulfonic Acid; H&E: Hematoxylin and Eosin; DAI: Disease Activity Index; CMDI: Colonic Mucosal Damage Index; HS: Histopathological Scores; IL: Interleukin; CD: Cluster of Differentiation; Th: T helper cell; Treg: Regulatory T Cell; Foxp3: Forked Head/Wing-Shaped Spiral Transcription Factor 3

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Introduction

Ulcerative Colitis (UC) is a type of Inflammatory Bowel Disease (IBD), which pathogenesis is not completely clear. Growing evidence shows that the genesis of UC is related to the integrity of mucosal epithelium, the imbalance of immune regulation, genetic susceptibility and the imbalance of intestinal microbiome [1].

Studies indicate that the excess Th17 cells and Th17-related factors play an important role in the pathogenesis of UC [2]. Many reports have indicated that interleukin -6(IL-6) and IL-23 could induce Th17 polaraization [3,4]. Th17 cells produce IL-17 that is a pro-inflammatory cytokine and stimulate a strong chronic immune inflammatory response [4]. Treg cells are related to the pathogenesis of IBD [5]. Treg cells are important in maintaining immune homeostasis and response to commensal bacteria in IBD. Treg cells can prevent intestinal inflammation and reduce the expression of Th17-related cytokines [6]. Treg cells can also produce IL-10 that is anti-inflammatory factor and increases Treg cells differentiation [7,8]. Some studies have shown that Th1/Th2 and Th17/Treg cell balance is associated with UC [9,10].

In clinical practice, immunosuppressive agents, anti-inflammatory drugs (e.g., antibiotics, 5-amino salicylic acid, and corticosteroids), and biological agents (e.g., infliximab and adalimumab) are commonly used to treat UC. Although combination drug therapies offer some efficacy, bring various severe side effects, and the disease recurs easily after drug withdrawal [1,11,12]. Thus, developing specific therapies with fewer side effects and less recurrence is of interest.

In China, people always use plant and plant-based drugs treating various diseases since recorded history. Many Chinese medicines have certain clinical effects in treating UC, but their pharmacological effects are not clear and need further research. Garidisan, containing Wild Woppy (Papaver nudicaule L.) and Artemisia Wrigida Willd, is usually used to treat UC in Mongolia, China. Clinical evidence allegedly suggests that Garidisan is useful to treat UC with less side-effect and fewer recurrences. Our studies have shown that Garidisan can improve the ulcer healing quality, regulate the Th1/Th2 balance of UC rats induced by immunization with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [13]. Garidisan also can regulate intestinal microbioma [14]. However, whether Garidisan regulates Th17/Treg balance in UC rats remains unclear.

Materials and Methods

Animal Preparation

Ten Specific-Pathogen-Free (SPF) grade New Zealand rabbits (male: female = 1:1) and 87 SPF grade Wister rats with body weight of 200±10g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. [Beijing, China, SCXK (Beijing) 2012-0001].

Mongolian Garidisan Composition and Preparation

Mongolian Garidisan, containing 15g P. nudicaule L. and 24g A. frigid Willd, were purchased from an herbal medicine procurement and supply company in Inner Mongolia and identified by associate professor Almaz, Institute of Chinese Minority Traditional Medicine, Minzu University of China, Beijing, China. P. nudicaule L. and A. frigid Wild was weighed according to appropriate proportions, followed by adding 22 volumes of 70% ethanol for 5-h reflux extraction to concentrate the extract to three concentrations of 0.70, 0.35, and 0.18g/ml crude drugs and store in the dark at 4°C. SASP (Sine [Tianping] Pharmaceutical Co., Ltd., Shanghai, China) and Bupiyichangwan (Guangzhou Chen Li Ji Pharmaceutical Factory Co., Ltd., Guangdong Province, China) were independently ground into powders and filtered through 100-mesh sieves to prepare 0.036g/ml SASP and 0.16g/ml Bupiyichangwan suspension in distilled water for later use.

UC Modeling

Total protein extraction and concentration determination: The total protein was extracted from rabbit colon by Radio Immunoprecipitation Assay (RIPA) lysis buffer (PCode: P0013C, Beyotime Institute of Biotechnology, Shanghai, China) and protease inhibitor Phenylmethanesulfonyl Fluoride (PMSF). The protein concentration determination was used Bicinchoninic Acid (BCA) protein concentration determination kit (Pro: P0010S, Beyotime Institute of Biotechnology, Shanghai, China).

Induction of UC modeling: Equal amounts of Freund's adjuvant (PCode: F5881, Sigma-Aldrich, St. Louis, MO) completely mixed with the extracted protein. 8 mg antigen solution was injected into the toes and groins of rats except 13 for control group on days 1 and 15. Meanwhile 1.5 ml saline was injected into the toes and groins of control rats. All rats were fasted for 1d until day 28. Then, after anesthetizing with isoflurane inhalation, rats except for control group were administrated 100mg/kg mixture containing equal volumes of TNBS (PCode: 1001910376, Sigma-Aldrich. co. USA) and 50% ethanol vialocal enema for the modeling of typical UC. Control rats were given 1 ml 25% ethanol via local enema. After enema rats were gotten TNBS began to diarrhea and hematochezia, and got weight loss and other symptoms of UC. The rat disease activity index was subsequently recorded every day according to table 1 [15].

Table 1: Scoring of disease activity index.

Weight loss(%)	Stool consistency	Occult/gross bleeding	Score
Normal	Normal	Normal	0
1~5			1
5~10	Loose stools	Hemoccult+	2
10~20			3
>20	Diarrhea	Gross bleeding	4

Note: Normal stools = well-formed pellets, Loose stools = pasty and semi-formed stools which do not stick to the snus, and diarrhea = liquid stools that stick to the anus.

Table 2: Colonic mucosal damage indices scoring criteria.

Gross morphologies	Symptoms	Score
Correlation with the surrounding tissues during sampling	No adhesion	0
	Mild adhesion (Colon and other tissues were easily peeled)	1
	Serious adhesion	2
Inflammation and ulceration	No ulceration and inflammation	0
	Local congestion, no ulceration	1
	1 ulcer without congestion or thickening of intestinal wall	2
	1 ulcer with inflammation	3
	≥ 2 ulcers with inflammation	4
	> 2 ulcers and > 1 cm inflamed area	5
	Ulcers with > 2 cm inflamed area; increase 1 score for each additional 1 cm lesion	6–8

Table 3: Histological scoring criteria.

	Score 0	Score 1	Score 2
Acute inflammatory cell infiltration	No	Mild increase	Severe increase
Chronic inflammatory cell infiltration	No	Mild increase	Severe increase
Submucosal edema	No	Patchy form	Fusion form
Ulcer formation	No		Yes

Table 4: Effect of Garidisan on Th17, Treg related factors in UC Rats peripheral blood (M±SEM, n=6).

Group	IL-6(ng/L)	IL-17(pg/L)	IL-23(ng/L)	IL-10
UC model	132.84±6.62***	51.83±1.57***	43.24±1.23***	14.36±1.08***
Normal control	67.64±4.38###	11.04±1.53###	11.40±3.18###	35.45±0.71###
Low-dose Garidisan	103.45±3.86***##	28.19±1.61***###	26.07±0.99**###	25.22±0.81***###
Moderate-dose Garidisan	97.09±3.63**###	29.21±1.04***###	30.47±3.32***#	23.20±0.42***###
High-dose Garidisan	107.91±2.81***##	28.43±1.80***###	29.91±2.49***##	25.21±1.45***###
Sulfasalazine	83.81±4.51###	18.83±1.85*###	22.33±2.82*###	20.30±1.17***##
Bupiyichangwan	114.44±5.79***	21.99±1.53***###	33.30±1.86***	24.92±0.39***###

*p<0.05, **p<0.01, and ***p<0.001 compared with normal controls. #p<0.05, ##p<0.01, and ###p<0.001 compared with the UC model group

Table 5: The ratio of IL-17/ IL-10 and the ratio of Th17/Treg cells.

Group	IL-17/IL-10	Th17/Treg cells
UC model	3.72±0.30***	1.73±0.06***
rmal control	0.31±0.05###	0.23±0.03###
Low-dose Garidisan	1.13±0.10**###	0.57±0.05***###
Moderate-dose Garidisan	1.26±0.04***###	0.74±0.07***###
High-dose Garidisan	1.13±0.06**###	0.80±0.02***###
Sulfasalazine	0.94±0.10*###	0.76±0.06***###
Bupiyichangwan	0.89±0.07###	0.38±0.05*###

*p<0.05, **p<0.01, and ***p<0.001 compared with normal controls. #p<0.05, ##p<0.01, and ###p<0.001 compared with the UC model group

Drug Administration and Samples Collections

Four days after UC modeling, three rats from UC group were randomly selected to evaluate the UC model. Survived rats with colitis induced by immunization and TNBS were randomly divided into 6 groups (UC, low-dose, moderate-dose and high-dose Garidisan, sulfasalazine (SASP), and Bupiyichangwan). Then rats were dealt with 7g/kg Garidisan(high-dose Garidisan), 3.5g/kg Garidisan(moderate -dose Garidisan) and 1.8g/kg Garidisan(low-dose Garidisan), 0.36g/kg SASP, 1.6g/kg Bupiyichangwan or 10ml/kg distilled water(control, UC) by gavage for 28 days, 1 time per day. The dosages of Garidisan for high-, moderate- and low-dose Garidisan are 2, 1, 0.5 times of adult dose in clinic. The dosages of SASP and Bupiyichangwan for SASP and Bupiyichangwan group respectively are 1 times of adult dose in clinic. After last administration, all rats were fasted for 1d then anesthetized with isoflurane inhalation (2.0%), followed by blood collection from the heart to collect serum for measuring cytokines (from clotted blood) and for flow cytometry (blood sample stored in anticoagulants). Subsequently, taking colon tissues from different treatment groups rats for morphological detection. Colon tissues dissected from six rats in different treatment groups were raised in cold normal saline, followed by assessment and recording of colon mucosa damage index (CMDI) [16] in a double-blind manner (see calculation standard in Table 2).

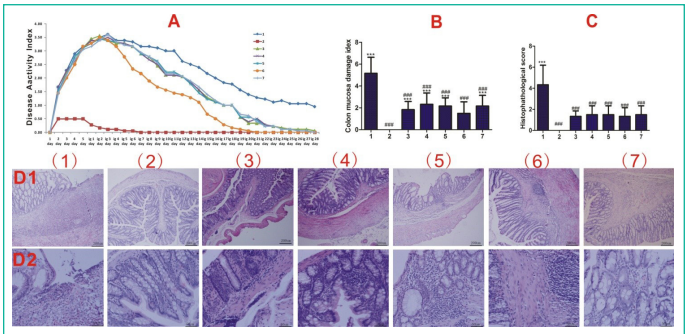

Figure 1: Treatment efficacy of Garidisan in UC rats (A) DAI for rats after UC modeling; (B) colonic mucosal damage indices for rats in different treatment groups; (C) HS for rats in different treatment groups, (D) Histological examination of colon tissue of experimental animals (H&E), 1 representing 100× magnification and 2 representing 400× magnification. Notes: (1) represents the UC model group; (2) represents normal controls; (3) represents the low-dose Garidisan group; (4) represents the moderate-dose Garidisan group; (5) represents the high-dose Garidisan group; (6) represents the SASP group; and (7) represents the Bupiyichangwan group; *P<0.05, **P<0.01, and ***P<0.001 compared with normal controls; # P <0.05, ## P<0.01, and ###P<0.001 compared with the UC model group.

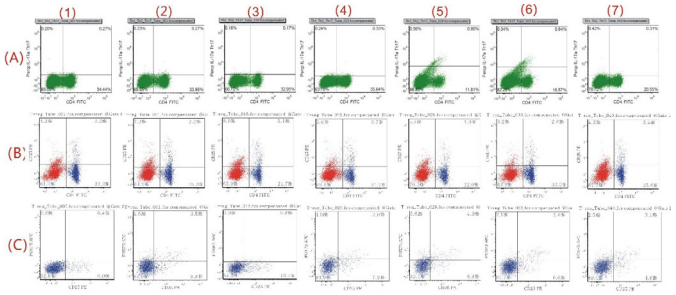

Figure 2: Effects of Garidisan on Th17 cells and Treg cells in peripheral blood. (A) Flow cytometry for $CD4^+IL\text{-}17^+T$ cells in peripheral blood of experimental animals; (B) Flow cytometry for $CD4^+CD25^+T$ cells in peripheral blood of experimental animals; (C) Flow cytometry for $CD4^+CD25^+$ $Foxp3^+T$ cells in peripheral blood of experimental animals. Notes: (1) represents the UC model group; (2) represents normal controls; (3) represents the low-dose Garidisan group; (4) represents the moderate-dose Garidisan group; (5) represents the high-dose Garidisan group; (6) represents the SASP group; and (7) represents the Bupiyichangwan group.

Morphological Assessment

Colon tissues morphological detection were embedded by paraffin, made into paraffin slices, stained with conventional H&E and photographed structures under light microscopy (olympus, BX517-PHD-J11). Two experts double blind evaluated H&E stained tissue sections according to the evaluation criteria listed in Table 3 to obtain histopathological scores (HS, Table 3) [17].

ELISA Assay

Blood samples without anticoagulants were stored at room temperature for 2h and centrifuged at 3,000rpm for 15 min to

collect the serum. Serum interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17 (IL-17) and interleukin-23 (IL-23) were measured according to the manufacturer's instructions of ELISA kit (Beijing Winter Song Boye Biotechnology Co., Ltd., Beijing, China).

Flow Cytometry Assay

CD4+ CD25+ Foxp3+ T cells assay: Anticoagulated blood (500µl) was labeled using FITC anti-Rat CD4, PE anti-Rat CD25 and antibodies again intracellular protein Foxp3 according to the manufacturer's instructions. Briefly, anticoagulated blood and antibodies again surface protein CD4, CD25 were mixed and incubated for 20 min at room temperature in the dark. 2ml red cell lysate were added into the samples to remove red cells. After adding 500µl Fixation buffer and 2µl Permeabilization Wash Buffer and centrifuging 5 min at 300×g to collect sediment, appropriate amount of cell factor AlexaFlour647 anti-RatFoxp3 was added and incubated for 20 min at room temperature in the dark. The Sample was washed with 2µl Permeabilization Wash Buffer and resuspended at 500ul cell washing buffer before detecting by flow cytometry (BD LSRII Flow cytometry instrument, BD Corporation, USA).

CD4+ IL17+ T cells assay: The same amount of anticoagulated blood and 1640 medium were mixed in 24-well culture plate. The sample were added Cell Stimulation Cocktail, including 50ng/ml PMA, 1ug/ml Ionomysin, 5ug/ml BFA. And then the samples were incubated for 4 hours at 37°C temperature. Subsequently 200µl sample culture and appropriate amount of FITC anti-Rat CD4 antibody was taken to the up-flow sample tube, mixed thoroughly and incubated for 20min at room temperature in the dark. Then according to the manufacturer's instructions, adding PerCP-Cy5.5 anti-Rat IL-17A. Last, the Sample was washed with 2µl Permeabilization Wash Buffer and resuspended at 500ul cell washing buffer before detected by flowcytometry (BD LSRII Flowcytometry instrument, BD Corporation, USA).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL). ANOVA was used for analysis. Statistical significance was determined for $P<0.05$.

Results

Therapeutic Effect of Garidisan on UC Rats

Animal weight, activity, and fecal character in the control group were normal throughout the course of the experiment. UC animals had loose, bloody, or occult-blood stools. DAI for UC rats in each group was elevated and maximized on day 4 after the corresponding treatment. DAI of different-treatment and UC model groups began to decline on day 5 following the corresponding treatment. Wherein, the DAI in the SASP group was the fastest decline and first to reach normal. DAI in other drug treatment groups were similar with SASP group (Figure 1A). Garidisan could reduce CMDI and HS of UC rats. Significant differences in CMDI was found between treatment groups and the UC model group ($P<0.001$, Figure 1B). Significant differences in HS was found between different treatment groups and the UC model group ($P<0.001$, Figure 1C). The rats of UC model group had obvious ulcerations in mucosal tissues, whereas mucosal epithelia of UC rats after treatment were intact, but there are still some inflammatory cells in the mucous lamina propria (Figure 1D). These indicated that Garidisan and positive drug had certain therapeutic effect on ulcerative colitis.

Garidisan Regulated Th17/Treg Cell Balance in UC Rats

ELISA indicated that the concentration of IL-17, IL-6 and IL-23 in peripheral blood of experimental rats in different treatment groups were higher than that in the control group and lower than that in the UC group. Compared with the UC model group, the decrease of IL-6 content in the Garidisan and sulfasalazine group was significantly different ($P<0.001$), however the decrease of IL-6 in the Bupiyichangwan group was not significantly different. Compared with the UC group, the decrease of IL-17 content in each administration group was significantly different ($P<0.001$). Compared with the UC group, the decrease of IL-23 in the low-dose Gridisan and sulfasalazine group was significantly different ($P<0.001$), the decrease of IL-23 in the high-dose Gridisan group was significantly different ($P<0.01$), the decrease of IL-23 in the moderate-dose Gridisan group was also significantly different ($P<0.05$). IL-10 contents in peripheral blood of different treatment groups was significantly lower than that in the control group and significantly higher than the UC group ($P<0.001$) (Table 5). These result indicated that Garidisan could inhibit th17 cells differentiation and maintenance and icrease the contens of anti-inflammatory facer IL-10 in UC rats.

The ratio of IL-17/IL-10 in different treatment groups was significantly lower than that in the UC group ($P<0.001$) (Table 5). $CD4^+IL\text{-}17^+Th17$ cells, $CD4^+CD25^+Foxp3^+Treg$ cells in peripheral blood of all experimental animals was measured with flow cytometry, the results indicated that the $CD4^+IL\text{-}17^+$ Th17/ $CD4^+CD25^+Foxp3^+$ Treg cell ratio in treatment groups were significantly lower than in UC group($P<0.001$) (Table 5, Figure 2). These results indicated that Garidisan and positive drugs could regulate the balance of Th17/Treg cell in UC rats.

Discussion

Studies have shown that TNBS-induced UC model is one of the commonly used models for studying IBD, and TNBS-induced chronic UC model ulcers can persist for 49 days [18]. The animal model used in this experiment is firstly to make the whole body of the animal in the state of immune response, and then TNBS stimulation, so that the body of systemic immune abnormalities and local inflammatory lesions coexist. Tan Yan et al. confirmed that the duration of this animal model could reach 12 weeks [19]. So in this experiment, the UC model group continuously had ulcerations throughout the experimental processes (from TNBS induction to the end of the experiments in a total of 32 days). And H&E staining results also showed that ulcers existed throughout the whole experiment (Figure 1D (1). This experiment demonstrated that Garidisan can effectively relieve the immunization with TNBS induced UC rats by reducing the DAI, CMDI, HS and repairing the damaged mucosa of UC rats (Figure 1A-D).

Studies have shown that the incidence of UC is closely related to immune disorders. Several studies have shown Th17-related factors is higher in peripheral blood of UC patients than in peripheral blood of nomal people. Recent studies suggest that Th17 cells and related cytokines are important factors in the pathogenesis of UC and Crohn disease [19]. During the development of UC, the number of pro-inflammatory Th17 cells generally increases [20]. IL-6 can promote the differentiation of Th17 cells, which produce the pro-inflammatory cytokines IL-17 and IL-23. IL-17 promotes further expansion of the inflammatory response, while IL-23 in turn promotes Th17 cells

differentiation and maintains the phenotype of Th17 cells [3]. The results of this experiment showed that the contents of pro-inflammatory factors IL-6, IL-17 and IL-23 in peripheral blood of UC model group were significantly higher than that in other groups, and the contents of IL-6, IL-17 and IL-23 in peripheral blood of UC rats could be decreased at Galidisan groups. This suggests that Garidisan can inhibit spread of inflammatory response by reducing the content of factors related to Th17 cells differentiation and phenotypic maintenance and reducing Th17 cells production (Table 4).

Treg cell is one of T cell subsets with both immunoregulatory and immunosuppressive functions, Treg cells can inhibit inflammation by regulating the activity of innate and acquired immune cells during the immune response. CD25 is one of the characteristic markers of Treg cells, and forked head/wing-shaped spiral transcription factor 3(Foxp3) is currently recognized as a Treg cell specific marker [21,22]. $CD4^+CD25^+Foxp3^+T$ cells not only regulate autoimmune responses but also suppress a variety of pathological, inflammatory responses to a wide spectrum of non-self antigens [23]. Treg cells secrete IL-10, IL-35 and so on to enhance immune tolerance along with cell-contact suppression. During the development of UC, the number of $CD4^+CD25^+Foxp3^+T$ cells generally decreases [20]. This study showed that the expression of IL-10 in the UC model groups decreased compared with the control group, Garidisan and positive drugs could increase the content of IL-10 in peripheral blood of UC rats (Table 4).

Studies have shown that Treg cells and Th17 cells have opposite functions in regulating immune responses. Growing evidences have shown that destroying Th17/Treg cell balance is a crucial contributor for the IBD pathogenesis. In the UC, increased pro-inflammatory Th17 and decreased anti-inflammatory Treg led to the imbalance of Th17/Treg cell thus triggering the inflammation [24,25]. In this study showed that Garidisan could reduce the ratio of IL-17 / IL-10. Flow cytometry results showed that Garidisan could reduce the ratio of CD4+IL-17+T cells / CD4+CD25+Foxp3+T cells in peripheral blood of UC rats. This results are similar to the results of Salidroside and Taurohyodeoxycholic acid alleviates UC [25,26]. This suggests that Garidisan possibly protected rats against ulcerative colitis by regulating the balance of Th17/Treg cell (Figure 2, Table 5).

Th17/Treg cell balance plays important role in the pathogenesis of IBD and autoimmune disease. In the meantime it is a potential targets for UC therapy. Many herbal medicines such as YiyiFuziBaijiang formula [27], Qing-Chang-Hua-Shi granule [28], Baitouweng decoction [29] and plant extracts such as Salidroside [25], Huangshan Floral Mushroom Polysaccharide [30], Taurohyodeoxycholic acid [26] can alleviate UC by regulating Th17/Treg cell balance.

In summary, our findings provide evidence that Garidisan ameliorates immunization with TNBS induced colitis. The underlying mechanism may be related to the reduction of IL-6, IL-17 and IL-23 which are Th17 cells differentiation and property maintenance factors, increase IL-10 which is Treg cells related factor and the regulation of Th17/Treg cell balance.

Author Statements

Conflict of Interest Statement

The authors declare that they have no competing interests.

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