JoP Journal of Pharmacopuncture

Journal of Pharmacopuncture 2024;27(2):59-69
pISSN: 2093-6966 • eISSN: 2234-6856
https://doi.org/10.3831/KPI.2024.27.2.59

Review Article

Check for updates

Indigo Naturalis in Inflammatory Bowel Disease: mechanisms of action and insights from clinical trials

Hyeonjin Kim†, Soohyun Jeong†, Sung Wook Kim, Hyung-Jin Kim, Dae Yong Kim, Tae Han Yook, Gabsik Yang*

Department of Korean Medicine, College of Korea Medicine, Woosuk University, Jeonju, Republic of Korea

Received January 25, 2024
Reviewed February 2, 2024
Accepted March 20, 2024

***Corresponding Author**
Gabsik Yang
Department of Korean Medicine, College of Korea Medicine, Woosuk University, 61 Seonneomeo 3-gil, Wansan-gu, Jeonju 54986, Republic of Korea
Tel: +82-63-290-9030
E-mail: yanggs@woosuk.ac.kr

†These authors contributed equally to this work.

This study investigates the therapeutic potential of *Indigo Naturalis* (IN) in treating a Inflammatory Bowel Disease (IBD). The objective is to comprehensively examine the effects and pharmacological mechanisms of IN on IBD, assessing its potential as an novel treatment for IBD. Analysis of 11 selected papers is conducted to understand the effects of IN, focusing on compounds like indirubin, isatin, indigo, and tryptanthrin. This study evaluates their impact on Disease Activity Index (DAI) score, colon length, mucosal damage, and macrophage infiltration in Dextran Sulfate Sodium (DSS)-induced colitis mice. Additionally, It investigate into the anti-inflammatory mechanisms, including Aryl hydrocarbon Receptor (AhR) pathway activation, Nuclear Factor kappa B (NF-κB)/nod-like receptor family pyrin domain containing 3 (NLRP3)/Interleukin 1 beta (IL-1β) inhibition, and modulation of Toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MYD88)/NF-κB and Mitogen Activated Protein Kinase (MAPK) pathways. Immunomodulatory effects on T helper 17 (Th17)/regulatory T cell (Treg cell) balance and Glycogen synthase kinase-3 beta (GSK3-β) expression are also explored. Furthermore, the study addresses the role of IN in restoring intestinal microbiota diversity, reducing pathogenic bacteria, and increasing beneficial bacteria. The findings reveal that IN, particularly indirubin and indigo, demonstrates significant improvements in DAI score, colon length, mucosal damage, and macrophage infiltration in DSS-induced colitis mice. The anti-inflammatory effects are attributed to the activation of the AhR pathway, inhibition of inflammatory pathways, and modulation of immune responses. These results exhibit the potential of IN in IBD treatment. Notably, the restoration of intestinal microbiota diversity and balance further supports its efficacy. IN emerges as a promising and effective treatment for IBD, demonstrating anti-inflammatory effects and positive outcomes in preclinical studies. However, potential side effects necessitate further investigation for safe therapeutic development. The study underscores the need for future research to explore a broader range of active ingredients in IN to enhance therapeutic efficacy and safety.

Keywords: anti-inflammatory, *Indigo Naturalis*, inflammatory bowel disease, intestinal microbiota, immunomodulation, random clinical trial

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract with complex etiology and limited therapeutic options. It is reported that IBD can be caused by defects in genes related to the innate immune function of the epithelium, which plays a crucial role in maintaining intestinal immune homeostasis [1, 2]. Apart from congenital factors, if the intestinal epithelium, acting as a selective permeability barrier, is physically damaged or experiences issues with selective permeability due to environmental stimulation, it can trigger an excessive mucosal immune response, leading to IBD [2]. Furthermore, changes in the composition and function of the intestinal microbiota have been observed in IBD patients [3].

There is accumulating evidence suggesting that this imbalance is a potential cause of IBD, and the control of IBD is associated with the proliferation of beneficial bacteria [4].

Ulcerative colitis (UC), like Crohn's disease, is one of the IBDs and is a chronic idiopathic condition with a low remission rate [5, 6]. UC has a higher incidence than Crohn's disease and is characterized by inflammation limited to the mucosal surface, which begins in the rectum and extends continuously proximally [5]. The main clinical symptoms include bloody diarrhea, stool urgency, fecal incontinence, and abdominal pain [1, 7]. While there is still an epidemiological gradient in UC between Western and Asian countries, the incidence and prevalence of IBD have significantly increased in Asia in the 21st century. The substantial rise in patients over just a few decades is primarily attributed to UC and environmental factors, suggesting a close relationship [8, 9]. Therefore, UC can be understood as a multifactorial disease resulting from the interaction of genetic defects regulating intestinal epithelial immunity, damage to the barrier and mucosa caused by external stimuli (environmental factors, etc.), and an imbalance in the intestinal microflora as the primary causes.

In recent years, traditional herbal medicine has gained traction as a potential source of innovative and effective treatments for inflammatory conditions. Among these, IN has emerged as a compelling candidate for investigation in IBD therapeutics. IN, known as "청대" in Korea and "Qingdai" in Chinese, is a blue powder made from the leaves or stems of *Strobilanthes cusia* (Nees) Kuntze, *Persicaria tinctoria* (Aiton) Spach, and *Isatis tinctoria* L [10]. As of now, 63 compounds have been isolated from IN. Active components include *indirubin* [11-15], *indigo* [11, 15, 16], *tryptanthrin* [17], *isatin* [18], etc. Previous studies have revealed that *indigo*, *indirubin*, and *tryptanthrin* are the main components of IN through HPLC or NMR analysis [19, 20]. These main components have been reported to possess pharmacokinetic and pharmacological effects, including antitumor, anti-angiogenic, anti-inflammatory, and anti-microbial effects [10]. According to the growing interest in IN, related research continuously accumulates, reporting various effects and pharmacological mechanisms. This study aims to explore IN's effects and pharmacological mechanisms on UC comprehensively.

PRECLINICAL EVIDENCE AND MECHANISMS OF ACTION

In this study, 11 papers were selected, encompassing research on both IBD and UC. Compounds of IN used in IBD research include *indirubin*, *isatin*, *indigo*, and *tryptanthrin*. The most frequently employed extract was a mixed extract of IN, and among the individual ingredients, *indirubin* was predominantly utilized. For the experimental model of IBD, mice were used in which the epithelial barrier was chemically damaged with DSS, leading to the spread of pro-inflammatory substances to the periphery and inducing colitis [21]. In DSS-induced colitis, IN improved the DAI score, increased colon length, alleviated mucosal damage, and reduced macrophage infiltration, ultimately ameliorating overall colitis lesions (Table 1).

1. Anti-inflammatory mechanisms

Studies have demonstrated that compounds of IN may exert anti-inflammatory effects by modulating key pathways involved in the inflammatory cascade. This includes the inhibition of pro-inflammatory cytokines and signaling molecules associated with the pathogenesis of IBD.

Indirubin and *indigo* play a crucial role in regulating multiple inflammatory pathways by activating the AhR pathway, which involves the expression of genes such as *CYP1A1*. The insolubility of *indigo* in the intestines hinders its absorption, preventing the induction of *CYP1A1* in the liver. Instead, indigo activates AhR by inducing it in the intestines [22]. Liu et al. [23] provided additional validation for the activation of the AhR pathway by demonstrating upregulation of *CYP1A1* and *Gsta5* following *indirubin* administration. Moreover, *indirubin* exhibited an inhibitory effect on NF-κB/NLRP3/IL-1β as evidenced by the downregulation of NLRP3, NF-κB p65, IL-1β, and *Igkv*.

NF-κB, a transcription factor crucial in inflammation and innate immune responses [24], is induced through the MYD88-dependent pathway of TLR4. Upon recognizing LPS stimulation, TLR4 recruits interleukin-1 receptor-associated kinase 4 (IRAK-4) through interaction with TIR domains like MYD88, initiating downstream signaling involving tumor necrosis factor receptor-associated factor 6 (TRAF6), transforming growth factor-β-activated kinase 1 (TAK1), IKKs, NF-κB, and mitogen-activated protein kinase (MAPK) [25]. Particularly noteworthy is the observation that *indirubin* administration within the

 https://doi.org/10.3831/KPI.2024.27.2.59

Table 1. Preclinical evidence and mechanisms of action

No.	Compound	Study	Model	Inducer	Results	Ref.
1	Indigo	*In vivo* *In vitro*	Male C57BL/6JJmsSlc mice, Caco-2 cell	ND	1. Induce Cyp1a1 and Cyp1a1 mRNA 2. Induce CYP1A1 and CYP1A1 mRNA, AhR is induced to transfer nucleus 3. Strengthening wound closure (inducing cell proliferation and actin polymerization)	[20]
2	Indirubin	*In vivo*	Male BALB/c mice	DSS 2%	1. Decrease NLRP3, IL-1β 2. Decrease of IL-17A mRNA expression and IL-17A secretion 3. Inhibition of NF-κB pathway (down regulation of NF-κB p65, Igkv) 4. Up regulation of Cyp1a1 and Gsta5 5. Supression of lipid peroxidation (decrease HBA and 4-HNE)	[21]
3	IN, indigo, or indirubin	*In vivo*	Male BALB/c mice	DSS 3%	1. Decrease IL-1β, IL-6 levels in serum and tissues 2. Reduce IgG concentration in serum and tissues 3. Reduce IgM concentration in tissues 4. Reduction of TLR4, TLR2, MyD88, p65, p-p65, IKBα, p-IKBα proteins in tissues 5. The dominant bacteria in IN and indigo are *Butyricimonas*, *Eubacterium*, *Coprococcus*, *Ruminococcus* and other probiotics, and in Indirubin, proteobacteria 6. Decrease MPO level in serum and tissue (IN and indirubin) 7. Decrease IgA level in serum (IN and indigo)	[24]
4	Indirubin with isatin	*In vivo*	Male BALB/c mice	DSS 3%	1. Reduction of TNF-α, IL-6 and IFN-γ /increase of IL-10 (singleness decreases, combination largely increases) 2. Reduction of COX-2, iNOS, PGE2, NO 3. Suppression of CD4+ T cell infiltration 4. Increase of Foxp3 expression 5. Decrease of MPO activity 6. Attenuate oxidative stress (decrease MDA /increase SOD, GSH) 7. Suppression cell death (decrease Tunel positive cell ratio, Bax level and cleaved-caspase-3 and increase Bcl-2 level) 8. Inhibition of NF-κB and MAPK signal pathway (decrease p-IκB, p-p38, p-ERK, p-JNK level, inhibition of IκB degradation and NF-κB p65 transfer to nucleus)	[25]
5	Tryptanthrin	*In vivo*	Male SD mice	DSS 5%	1. Suppression of IL-6, TNF-α 2. Decrease of p-STAT3, NF-κBp65 expression level 3. Increase of IκBα	[27]
6	Indirubin	*In vivo* *In vitro*	Male C57BL mice, bone marrow dendritic cell (BMDC)	DSS 2%, LPS	1. Inhibition of MPO activity 2. Suppression of CD80, CD86, CD40 and MHC-II expression 3. Decrease of TNF-α expression and increase of TGF-β expression 4. Increase of Treg proliferation (FOXP3 expression), decrease Th17 ratio, reduction of RORγt 5. Decrease IL-17 and increase IL-10 6. When αVβ8 siRNA treat, reverse 1-3 situation	[30]
7	IN	*In vivo* *In vitro*	Male C57BL/6 mice, naive CD4 T cell	DSS 2%, IL-6, TGF- β, anti-IFN-γ, anti-IL-4	1. Decrease MPO activity 2. Increase T-SOD, CAT, GSH-Px activity 3. Activation of p-AMPK and Nrf-2 4. Inhibition of transcription factor of Th1 and Th17 (decrease IFN-γ, IL-17A, T-bet, RORγt mRNA expression) 5. Decrease pro-inflammatory cytokine (decrease IFN-γ, IL-17A/F, TNF-α and IL-1β) 6. Decrease Th1 and Th17 cell frequency 7. Inhibition of p-STAT1 and p-STAT3 phosphorylation 8. Suppression of Th1 and Th17 cell differentiation (*in vitro*)	[33]

Table 1. Continued

No.	Compound	Study	Model	Inducer	Results	Ref.
8	IN	*In vivo*	C57/BL6 mice	DSS 2.5%	1. Suppression of GSK3-β mRNA expression 2. Decrease pro-inflammatory cytokine (TNF-α, IL-1β and IL-17a)	[38]
9	Indigo or indirubin	*In vivo*	Male BALB/c mice	DSS 3%	1. Reduction of pro-inflammatory cytokine level included TNF-α, IFN-γ, IL-12, IL-23, IL-17A 2. Attenuation of ROS/RNS production 3. Protection of physical barrier of colitis epithelium (increase ZO-1, occludin and E-cadherin expression) 4. Regulation of colitis permeability (increase MUC2 expression) 5. Regulation of mucosal immune homeostasis 5-1. Decrease neutrophils ($CD11b^+$ $Gr\text{-}1^+$), reduction of regulation neutrophils 5-2. Decrease dendritic cells ($CD11b^+$ $CD11c^+$) and macrophages ($CD11b^+$ $F4/80^+$) 5-3. Increase relative amount of NK cells ($CD335^+$ $CD11b^+$) 5-4. Decrease ILC2, ILC3 6. Regulation of intestinal microbiome (increase *Firmicutes*, *Norank_f_Muribaculaceae*, *Lactobacillus* and *Alloprevotella*)	[45]
10	IN	*In vivo*	Male Sprague-Dawley rats	DSS 4.5%	1. Decrease MPO activity and pro-inflammatory cytokine TGF-β level in serum 2. Recovery of changed microbiome by DSS in colitis (abundantly relative increase of *Firmicutes*, *Actinobacteria*, *Bifidobacteriaceae*, *Ruminococcaceae*, *Uminococcus_1*, *Ruminococcaceae_UCG-005*, *Norank_f__Erysipelotrichaceae*, *Butyricicoccus* and *Bifidobacterium*) (relatively decrease of *Bacteroidetes*, *Escherichia-Shigella* as normal level) 3. Butyric acid (one of the SCFAs): significantly increase in treatment group compare to control group ⇒ Elevated level of GPR41 and GPR43 in treatment group compare to control 4. When removed microbiome by treating antibiotics cocktail, no anti-inflammation effect of IN ⇒ The anti-colitis effect of IN appears to be dependent on the presence of the intestinal microbiome	[46]
11	IN	*In vivo*	Male Kunming (KM) mice	DSS 3%	1. Decrease IL-6, IL-8, TNF-α and increase IL-10 (upregulation of anti-inflammation and down-regulation of pro-inflammation) 2. Recovery of divers microbiome damaged by DSS 3. Recovery of microbiome species changed by colitis: after IN treat, decrease Bacteroidetes, Proteobacteria and increase Firmicutes 4. Increase Peptococcus and decrease Turicibacter	[47]

compounds of IN alleviated colitis, coinciding with a reduction in the expression of proteins related to the TLR4/MYD88/NF-κB pathway [26]. Administration of *indirubin* and *isatin*, either alone or in combination, also led to the inhibition of the MAPK pathway alongside NF-κB [27]. The interplay between signal transducer and activator of transcription 3 (STAT3) and NF-κB involves mutual activation, with IL-6 expression induced in this process [28]. Additionally, *tryptanthrin* reduced the proteins (IL-6, TNF-α, p-STAT3, NF-κBp65) central to these mechanisms [29].

2. Immunomodulation

The immunomodulatory properties of IN are crucial in IBD management. Evidence suggests it can regulate immune responses, potentially suppressing the exaggerated immune reactions observed in IBD patients.

Integrin αvβ8, a cell adhesion molecule expressed by dendritic cells, is crucial in activating transforming growth factor-β (TGF-β) and mediating T-cell differentiation [30]. This deficiency in integrin αvβ8 has been associated with the induction

 https://doi.org/10.3831/KPI.2024.27.2.59

of colitis [31]. In line with this, Zhang et al. [32] reported that *indirubin* induces dendritic cell activation and apoptosis by up-regulating αvβ8 expression. This induction not only facilitates antigen uptake but also inhibits antigen presentation and T-cell activation.

Th17 and Treg cells are differentiated from naive CD4 T cells through the TGF-β signaling pathway. Th17 cells promote inflammatory responses by producing pro-inflammatory cytokines like IL-17, while Treg cells suppress immune responses by generating anti-inflammatory cytokines such as IL-10 and TGF-β [33]. Cytokine expression in Treg cells is regulated through the transcription factor Foxp3 and in Th17 cells through retinoid orphan receptor gamma t (RORγt) [34]. Therefore, maintaining the balance between Th17 and Treg cells is crucial in immune-mediated inflammatory diseases. An increase in the Th17/Treg ratio was reported in DSS-induced IBD mice, and IN regulated this imbalance. In the study by Zhang et al. [32], the administration of *indirubin* to DSS-induced mice increased Foxp3 and IL-10 expression, accompanied by a decrease in RORγt and IL-17 expression. Gao et al. [27] further supported these findings by demonstrating elevated levels of Foxp3 and IL-10 following the single or combined administration of *indirubin* and *isatin*. These results strongly suggest that IN improves UC by modulating the Th17 and Treg cell action balance. Additionally, Xiao et al. [35] expanded on this understanding, revealing that the administration of IN to mice with DSS-induced colitis not only led to a decrease in the frequency of Th1 and Th17 cells but also resulted in the inhibition of the phosphorylation of STAT1 and STAT3, as well as a reduced expression of IFN-γ, IL-17A, T-bet, and RORγt. The observed reduction in expression indicates an immunosuppressive effect by inhibiting Th1 and Th17 differentiation by IN.

GSK3-β, a regulator of various immune system signaling pathways, has been implicated in the control of chronic intestinal inflammation [36, 37]. After GSK3 inhibition, the number of Th17 cells reduced in the intestines of healthy mice [38], and forkhead box P3 (FoxP3) expression was prolonged in Treg cells treated with a GSK3-β inhibitor [39]. Yue et al. [40] demonstrated reduced pro-inflammatory factors (TNF-α, IL-1β, and IL-17a) and decreased GSK3-β expression after administering IN to mice with DSS-induced colitis. Notably, the transcription level of Foxp3 was maintained.

3. Modulation of intestinal microbiota

In recent studies, an imbalance in the intestinal microbiome has emerged as a significant factor contributing to IBD, notably observed in individuals with IBD, where the biodiversity of the intestinal microbiota diminishes. This imbalance includes a decrease in Firmicutes and an increase in Gamma-proteobacteria, resulting in heightened oxidative metabolism, reduced production of short-chain fatty acids (SCFAs), and increased mucin degradation [41]. SCFAs, crucial metabolites produced by intestinal microorganisms through dietary fiber, play a pivotal role in maintaining intestinal homeostasis, with lower SCFA levels associated with an elevated risk of developing IBD [42-44]. Moreover, the gut microbiota's influence extends to intestinal innate and adaptive T cell differentiation and activation, where an imbalance between Th17 and Tregs has been reported in correlation with disruptions in the intestinal microbiota [45, 46]. Consequently, an imbalance in the gut microbiota can lead to immune dysregulation, contributing to IBD, such as UC.

This study specifically aimed to confirm the impact of IN on regulating the intestinal microbiome. Xie et al. [47] noted a decrease in the diversity of the intestinal microbiome and an increase in pathogenic bacteria (Proteobacteria, Verrucomicrobiota) during DSS treatment. Following IN administration, pathogenic bacteria decreased, while beneficial bacteria, including *Firmicutes*, *Norank_f_Muribaculaceae*, *Lactobacillus*, and *Alloprevotella*, increased. In studies conducted by Sun et al. [48] and Liang et al. [49], the diversity and ratio of the intestinal microbiota composition, disrupted by DSS, were effectively restored through IN administration. Furthermore, there was a down-regulation of pro-inflammatory cytokines and an up-regulation of anti-inflammatory cytokines. Sun et al. [48] additionally demonstrated a significant increase in butyrate acid, accompanied by elevated levels of GPR41 and GPR43, which are butyrate acid-related factors and part of the SCFAs. Interestingly, no relief of colitis symptoms was observed when IN was administered to mice with DSS-induced colitis after removing the intestinal microbiome through an antibiotic cocktail treatment. This suggests that the anti-colitis effect of IN appears to depend on the presence of the intestinal microbiome. In the research conducted by Yang et al. [26], the composition of the intestinal microbiota in the DSS group and the *indirubin* group closely resembled that of other groups. However, there was no overlap between the IN and indigo groups in non-metric multidimensional scaling (NMDS) analysis samples and the control

and DSS groups. Notably, compared to *indigo*, *indirubin* exhibited a weaker ability to regulate the composition of the intestinal microbiome.

4. RCT (randomized controlled trials)

A systematic analysis of RCT involving IN in patients with UC is presented. The review assesses study designs, patient populations, and outcomes, offering a comprehensive overview of the current state of clinical research on IN in the context of UC (Table 2).

In a research study by Ben-Horin et al. [50], an eight-week randomized, double-blind trial was conducted with active UC patients in Israel and Greece. The trial used a combination of Curcumin-QingDai, with 41 patients randomly assigned to either the treatment group (CurQD) or the control group (placebo) in a 2:1 ratio. The co-primary outcome, focusing on the percentage of patients meeting both clinical response and Mayo score criteria, revealed a significant disparity: CurQD; 43%: placebo; 8% ($p = 0.033$). Secondary outcomes, assessed through various instruments, demonstrated achievement rates of clinical remission and clinical response as CurQD; 50%: placebo; 8% ($p = 0.01$) and CurQD; 85.7%: placebo; 30.7% ($p < 0.001$), respectively. Additionally, the proportion of patients with a Mayo subscore decrease of more than one point was CurQD; 70%: placebo; 25% ($p = 0.036$). Furthermore, the proportion of patients experiencing a more than 50% decrease in fecal calprotectin levels from the baseline value was CurQD; 46.8%: placebo; 15.4% ($p = 0.08$). While the median calprotectin level significantly decreased in the CurQD group ($p < 0.001$), no noteworthy treatment effect was observed in the placebo group ($p = 0.8$). In the CurQD group, the time for the SCCAI score to decrease by more than three points or for rectal bleeding to cease was 16 days ($p < 0.01$) and 12 ($p < 0.05$), respectively, with no significant results obtained in the control group.

Uchiyama et al. [51] investigated 42 patients with mild to moderately active UC, randomly assigning them to the IN group (23 patients) or the placebo group (19 patients). After treatment administration, the Lichtiger index of the IN group significantly improved ($p = 0.001$), while no change was observed in the placebo group ($p = 0.359$). The proportion of patients whose Lichtiger index decreased by more than 30% compared to baseline was 82.6% and 26.5%, respectively ($p = 0.0003$), and the proportion of patients whose Lichtiger index decreased by more than 50% compared to baseline was 60.9% and 5.3%, respectively ($p = 0.0002$). The IN group recorded a significantly higher rate than the placebo group. During the two-week experiment, five people (21.7%; 5/23) in the IN group and one person (5.26%; 1/19) in the placebo group experienced mild headaches, and no severe adverse reactions such as pulmonary hypertension and intussusception occurred.

Furthermore, Naganuma et al. [52] conducted a multicenter randomized controlled trial examining the efficacy of IN in 86 patients with active UC in Japan. Patients were randomly assigned to four groups and administered 62.5 mg, 125 mg, or 250 mg of IN or placebo, four capsules twice daily, for eight weeks (total of 0.5 g, 1 g, or 2 g of IN). After an eight-week clinical trial, the primary endpoint, clinical response, was 0.5 g IN; 69.6% ($p = 0.0002$), 1.0 g IN; 75.0% ($p = 0.0001$), 2.0 g IN; 81.0% ($p < 0.0001$), placebo; 13.6%, confirming a dose-dependent linear trend for IN. Examining the secondary endpoint, clinical remission, a higher treatment effect was found in the patient group administered 1 g IN daily than in the patient group administered 2 g IN daily (0.5 g IN; 26.1% [$p = 0.0959$], 1.0 g IN; 55.0% [$p = 0.0004$], 2.0 g IN; 38.1% [$p = 0.0093$], placebo; 4.5%). Mucosal healing also showed similar results (0.5 g IN; 56.5% [$p = 0.0045$], 1.0 g IN; 60.0% [$p = 0.0032$], 2.0 g IN; 47.6% [$p = 0.0217$], placebo; 13.6%). Patients receiving IN daily had greater improvements in Mayo scores compared to patients receiving placebo (0.5 g IN; –2.83 [$p = 0.0024$], 1.0 g IN; –3.64 [$p = 0.0002$], and 2.0 g IN; –3.42 [$p = 0.0004$]). The MTWSI score was also significantly improved in the IN group compared to the placebo group (0.5 g IN; –4.67 [$p < 0.0001$], 1.0 g IN; –5.46 [$p < 0.0001$], 2.0 g IN; –6.4 [$p < 0.0001$]). At week eight, the proportion of patients with fecal calprotectin levels less than 150 μg/g and the proportion of patients with fecal immunochemical blood levels less than 100 ng/mL were respectively 60.0% ($p = 0.0022$); 57.6% ($p = 0.0051$); 36.8% ($p = 0.1245$); 10.5%, and 38.1% ($p = 0.0670$); 40.0% ($p = 0.0648$); 44.4% ($p = 0.0265$); 10.0%. During eight weeks of treatment, no serious side effects requiring hospitalization occurred. 10-20% of patients receiving 0.5 to 2.0 g of IN daily for eight weeks experienced mild liver dysfunction, which was reversible. The patients recovered, and, except for one patient (1 g IN group) who terminated early due to nausea, there were no serious side effects requiring hospitalization.

DISCUSSION

Despite the emergence of various biological agents and small

 https://doi.org/10.3831/KPI.2024.27.2.59

Table 2. Randomized controlled trials

Disease	Patients	Treatment intervention	Period	Outcome	Results	Ref.
Ulcerative colitis	41	CurQD (curcumin 500 mg, Qindai 500 mg) 3 capsule (curcumin 1.5 g, qingdai 1.5 g)/ oral	8 weeks	[Co-primary outcome] - Clinical response (SCCAI, a decrease ≥ 3 point) - Mayo score (a decrease ≥ 3 point & a decrease ≥ 30% from baseline) [Secondary outcomes] 1. Clinical remission (SCCAI, ≤ 2) 2. Clinical response 3. Mayo subscore (a decrease ≥ 1 point from baseline) 4. Fecal calprotectin level (a decrease ≥ 50% from baseline) 5. The median calprotectin levels 6. The onset of the reduction in SCCAI of ≥ 3 points 7. The onset of no rectal bleeding	[Co-primary outcome] 43 (12/28) : 8 (1/13) p = 0.033 [Secondary outcomes] (CurQD : placebo) 1. 50 (14/28) : 8 (1/13) p = 0.01 2. 85.7 (24/28) : 30.7 (4/13) p < 0.001 3. 70 : 25 p = 0.036 4. 46.8 (13/28) : 15.4 (2/13) p = 0.08 5. Significantly reduce (p < 0.001) : no significant (p = 0.8) 6. Day 16 (p < 0.01) : no significant 7. Day 12 (p < 0.05) : no significant	[48]
Ulcerative colitis	42	The IN powder 5 capsule (500 mg)/ oral	2 weeks	1. Lichtiger index 2. Lichtiger index decreased by more than 30% compared to baseline 3. Lichtiger index decreased by more than 50% compared to baseline	1. Significantly reduce (p = 0.001) : no significant (p = 0.359) 2. 82.6% and 26.5%, respectively (p = 0.0003) 3. 60.9% and 5.3%, respectively (p = 0.0002) 5 people in the IN group and 1 person in the placebo group experienced mild headaches	[49]
Ulcerative colitis	86	62.5 mg, 125 mg, or 250 mg IN 4 capsules twice daily for 8 weeks (total of 0.5 g, 1 g, or 2 g of IN)/oral	8 weeks	[Primary outcome] 1. Clinical response [Secondary outcomes] 1. Clinical remission 2. Mucosal healing 3. Mayo score 4. MTWSI score 5. Fecal calprotectin level 6. Fecal immunochemical blood	[Primary outcome] (0.5 g IN : 1.0 g IN : 2.0 g IN : placebo) 1. 69.6% (p = 0.0002) : 75.0% (p = 0.0001) : 81.0% (p = 0.0001) : 13.6% [Secondary outcomes] (0.5 g IN : 1.0 g IN : 2.0 g IN : placebo) 1. 26.1% (p = 0.0959) : 55.0% (p = 0.0004) : 38.1 (p = 0.0093) : 4.5% 2. 56.5% (p = 0.0045) : 60.0% (p = 0.0032) : 47.6% (p = 0.0217) : 13.6% 3. Mayo scores compared to placebo; –2.83 (p = 0.0024) : –3.64 (p = 0.0002) : –3.42 (p = 0.0004) 4. MTWSI scores compared to placebo; –4.67 (p < 0.0001) : –5.46 (p < 0.0001) : –6.4 (p < 0.0001) 5. 60.0% (p = 0.0022) : 57.6% (p = 0.0051) : 36.8% (p = 0.1245) : 10.5% 6. 38.1% (p = 0.0670) : 40.0% (p = 0.0648) : 44.4% (p = 0.0265) : 10.0% 10-20% of patients experienced mild liver dysfunction (reversible) except for one patient (1 g IN group) due to nausea No serious side effects requiring hospitalization	[50]

molecule drugs for inflammatory diseases, a significant number of patients do not respond to these new drugs, experience side effects, or have difficulty receiving treatment due to issues such as cost [50]. IN, which is proposed as a new drug candidate for these diseases, is a multi-ingredient herbal medicine containing both organic and inorganic compounds and is effective in suppressing inflammation, tumors, bacteria, and psoriasis [10]. In this study on DSS-induced colitis, IN improved the DAI score, increased colon length, alleviated mucosal damage, and reduced macrophage infiltration, thereby improving overall colitis lesions. In addition, the anti-inflammatory efficacy of IN has been proven in various RCT studies [50-54].

In this study, the anti-inflammatory mechanism of IN for UC was summarized. The three main compounds of IN, *indirubin*, *indigo*, and *tryptanthrin*, were mainly used as a single ingredient. *Indirubin* regulates various inflammatory pathways by activating the AhR pathway by expressing genes such as *CYP1A1*. In addition, inhibition of the MAPK pathway was also observed along with NF-κB inhibition by reducing the expression of proteins related to the TLR4/MYD88/NF-kB pathway. Upregulating the cell adhesion molecule integrin αvβ8, an important factor related to T cell-mediated immunity, induced DC activation and apoptosis and antigen absorption to suppress antigen presentation and T cell activation. In addition, after *indirubin* administration, the expression of Foxp3 and IL-10 increased, and the expression of RORγt and IL-17 decreased, suggesting that *indirubin* suppresses the immune response by regulating the Treg/Th17 ratio. Furthermore, the intestinal microbiota analysis revealed an overlap in the composition of the *indirubin* group and the DSS group in terms of intestinal microbiota, suggesting that the anti-inflammatory mechanism of *indirubin* in colitis is primarily associated with suppressing cellular immunity rather than regulating intestinal microbiota. Like *indirubin*, *indigo* also showed AhR pathway activation and a clearer recovery of the intestinal microbiota composition damaged by DSS than *indirubin*. *Tryptanthrin* showed a decrease in IL-6/STAT3/NF-κB related proteins (IL-6, TNF-α, p-STAT3, NF-κBp65). In addition, IN extract reduced the expression of Th1-related IFN-γ and T-bet, which are involved in inflammatory responses other than Th17, and decreased the expression of GSK3-β and decreased proinflammatory factors (TNF-α, IL-1β, and IL-17a). In addition, it has been reported that the imbalance of the intestinal microbiome, which has recently been attracting attention as a cause of IBD, can be improved through IN. After IN administration, intestinal microbiota diversity was restored, pathogenic bacteria decreased, and beneficial bacteria such as *Firmicutes*, *Norank_f_Muribaculaceae*, *Lactobacillus*, and *Alloprevotella* increased. Considering that the effect was suppressed when IN was administered after removing the intestinal microbiome through antibiotic cocktail treatment, the anti-colitis mechanism of IN appears to be executed dependent on the intestinal microbiome.

IN may offer potential benefits in treating IBD compared to well-established medications such as mesalazine, balsalazide, and olsalazine. These conventional medications alleviate inflammatory symptoms and slow the progression of IBD but do not effectively control beneficial bacteria or restore intestinal function [55-57]. However, IN's clinical efficacy and safety profiles lack extensive validation compared to the well-established medications.

The side effects reported in the studies of IN included headache and nausea, which were minimal in two of the three studies. In the remaining study, early termination due to worsening colitis occurred in four out of 28 patients in the treatment group and appeared in six out of 13 control subjects. In the same study, one case was reported where liver levels increased 10 times the upper limit of normal. Recently, many cases of pulmonary hypertension have been reported in patients taking green tea for UC [58-60]. These cases have a common rapid improvement after stopping taking IN. Although the exact mechanism between IN and the development of pulmonary arterial hypertension (PAH) has not yet been elucidated, expected hypotheses include that indigo and *indirubin* are AhR ligands [22, 23]. Indigo contains serotonin, which can act as a pulmonary vasoconstrictor. There are claims that it is involved in the occurrence of PAH due to its similar structure [60, 61] Although IN is a promising drug candidate for inflammatory diseases, its side effects may hinder its development. Therefore, to develop new drugs for IN, follow-up research is necessary to uncover the mechanism of developing severe side effects, such as pulmonary hypertension, related to IN and to find ways to improve them.

Furthermore, research on IN has predominantly focused on these three active ingredients, neglecting other compounds in IN. IN contains indirubin, tryptanthrin, indigo, and other organic and inorganic ingredients. Therefore, utilizing IN as a therapeutic agent necessitates genetic, developmental, and innovative research to understand the genetics, development, efficacy, and mechanisms of action of its various ingredients [62].

 https://doi.org/10.3831/KPI.2024.27.2.59

CONCLUSION

This study highlights the potential of IN as a promising therapeutic effect for IBD. Preclinical evidence and clinical trials demonstrate that IN has anti-inflammatory and immunomodulatory effects, improving disease activity and promoting mucosal healing. However, concerns about potential side effects warrant further investigation. Overall, IN emerges as a multifaceted candidate for IBD treatment, emphasizing the need for continued research to optimize its efficacy and safety profile.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

FUNDING

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number : HF22C007234).

ORCID

Hyeonjin Kim, https://orcid.org/0000-0001-5380-9610
Soohyun Jeong, https://orcid.org/0000-0002-5274-0698
Sung Wook Kim, https://orcid.org/0000-0001-5394-0280
Hyung-Jin Kim, https://orcid.org/0009-0006-8311-1276
Dae Yong Kim, https://orcid.org/0000-0001-9532-809X
Tae Han Yook, https://orcid.org/0000-0001-6379-7596
Gabsik Yang, https://orcid.org/0000-0002-9158-6531

REFERENCES

1. Le Berre C, Honap S, Peyrin-Biroulet L. Ulcerative colitis. Lancet. 2023;402(10401):571-84.
2. Pastorelli L, De Salvo C, Mercado JR, Vecchi M, Pizarro TT. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. Front Immunol. 2013;4:280.
3. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology. 2014;146(6):1489-99.
4. Yang Y, Zhang Y, Song J, Li Y, Zhou L, Xu H, et al. Bergamot polysaccharides relieve DSS-induced ulcerative colitis via regulating the gut microbiota and metabolites. Int J Biol Macromol. 2023;253(Pt 7):127335.
5. Ordás I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. Lancet. 2012;380(9853):1606-19.
6. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med. 2009;361(21):2066-78.
7. Feuerstein JD, Moss AC, Farraye FA. Ulcerative colitis. Mayo Clin Proc. 2019;94(7):1357-73.
8. Park J, Cheon JH. Incidence and prevalence of inflammatory bowel disease across Asia. Yonsei Med J. 2021;62(2):99-108.
9. da Silva BC, Lyra AC, Rocha R, Santana GO. Epidemiology, demographic characteristics and prognostic predictors of ulcerative colitis. World J Gastroenterol. 2014;20(28):9458-67.
10. Sun Q, Leng J, Tang L, Wang L, Fu C. A comprehensive review of the chemistry, pharmacokinetics, pharmacology, clinical applications, adverse events, and quality control of indigo naturalis. Front Pharmacol. 2021;12:664022.
11. Chen LH, Wang XJ, Liao W, Xiao B, Tan LY, Long JG. Extraction of indirubin from indigo naturalis and its antioxidant activity. J Jishou Univ (Nat Sci Ed). 2013;34(1):72-6.
12. Jie C, Luo Z, Chen H, Wang M, Yan C, Mao ZF, et al. Indirubin, a bisindole alkaloid from *Isatis indigotica*, reduces H1N1 susceptibility in stressed mice by regulating MAVS signaling. Oncotarget. 2017;8(62):105615-29.
13. Chang SJ, Chang YC, Lu KZ, Tsou YY, Lin CW. Antiviral activity of Isatis indigotica extract and its derived indirubin against Japanese encephalitis virus. Evid Based Complement Alternat Med. 2012;2012:925830.
14. Mak NK, Leung CY, Wei XY, Shen XL, Wong RN, Leung KN, et al. Inhibition of RANTES expression by indirubin in influenza virus-infected human bronchial epithelial cells. Biochem Pharmacol. 2004;67(1):167-74.
15. Lin CW, Tsai FJ, Tsai CH, Lai CC, Wan L, Ho TY, et al. Anti-SARS coronavirus 3C-like protease effects of Isatis indigotica root and plant-derived phenolic compounds. Antiviral Res. 2005;68(1):36-42.
16. Farias-Silva E, Cola M, Calvo TR, Barbastefano V, Ferreira AL, De Paula Michelatto D, et al. Antioxidant activity of indigo and its preventive effect against ethanol-induced DNA damage in rat gastric mucosa. Planta Med. 2007;73(12):1241-6.
17. Tsai YC, Lee CL, Yen HR, Chang YS, Lin YP, Huang SH, et al. Antiviral action of tryptanthrin isolated from *Strobilanthes cusia* leaf against human coronavirus NL63. Biomolecules. 2020;10(3):366.
18. Chiang YR, Li A, Leu YL, Fang JY, Lin YK. An in vitro study of the antimicrobial effects of indigo naturalis prepared from Strobilanthes formosanus Moore. Molecules. 2013;18(11):14381-96.

19. Plitzko I, Mohn T, Sedlacek N, Hamburger M. Composition of Indigo naturalis. Planta Med. 2009;75(8):860-3.
20. Chang HN, Huang ST, Yeh YC, Wang HS, Wang TH, Wu YH, et al. Indigo naturalis and its component tryptanthrin exert anti-angiogenic effect by arresting cell cycle and inhibiting Akt and FAK signaling in human vascular endothelial cells. J Ethnopharmacol. 2015;174:474-81.
21. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol. 2014;104:15.25.1-14.
22. Shimizu T, Takagi C, Sawano T, Eijima Y, Nakatani J, Fujita T, et al. Indigo enhances wound healing activity of Caco-2 cells via activation of the aryl hydrocarbon receptor. J Nat Med. 2021;75(4):833-9.
23. Liu Z, Zhang JR, Huang YX, Li XY, Zhu HP, Yang RY, et al. Transcriptomic analysis reveals the regulatory mechanism underlying the indirubin-mediated amelioration of dextran sulfate sodium-induced colitis in mice. Pharm Biol. 2023;61(1):1082-93.
24. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer. 2002;2(4):301-10.
25. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42(2):145-51.
26. Yang QY, Ma LL, Zhang C, Lin JZ, Han L, He YN, et al. Exploring the mechanism of indigo naturalis in the treatment of ulcerative colitis based on TLR4/MyD88/NF-κB signaling pathway and gut microbiota. Front Pharmacol. 2021;12:674416.
27. Gao W, Zhang L, Wang X, Yu L, Wang C, Gong Y. The combination of indirubin and isatin attenuates dextran sodium sulfate induced ulcerative colitis in mice. Biochem Cell Biol. 2018;96(5):636-45.
28. He G, Karin M. NF-κB and STAT3 - key players in liver inflammation and cancer. Cell Res. 2011;21(1):159-68.
29. Wang Z, Wu X, Wang CL, Wang L, Sun C, Zhang DB, et al. Tryptanthrin protects mice against dextran sulfate sodium-induced colitis through inhibition of TNF-α/NF-κB and IL-6/STAT3 pathways. Molecules. 2018;23(5):1062.
30. Travis MA, Sheppard D. TGF-β activation and function in immunity. Annu Rev Immunol. 2014;32:51-82.
31. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, et al. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. Nature. 2007;449(7160):361-5.
32. Zhang T, Peng H, Li Y, Zhou X, Pu W, Zhang Y, et al. Indirubin regulates T cell differentiation by promoting αVβ8 expression in bone marrow-derived dendritic cells to alleviate inflammatory bowel disease. Phytother Res. 2023;37(1):89-100.
33. Lee GR. The balance of Th17 versus Treg cells in autoimmunity. Int J Mol Sci. 2018;19(3):730.
34. Chen Z, Lin F, Gao Y, Li Z, Zhang J, Xing Y, et al. FOXP3 and RORγt: transcriptional regulation of Treg and Th17. Int Immunopharmacol. 2011;11(5):536-42.
35. Xiao HT, Peng J, Wen B, Hu DD, Hu XP, Shen XC, et al. Indigo naturalis suppresses colonic oxidative stress and Th1/Th17 responses of DSS-induced colitis in mice. Oxid Med Cell Longev. 2019;2019:9480945.
36. Beurel E, Michalek SM, Jope RS. Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3). Trends Immunol. 2010;31(1):24-31.
37. Hofmann C, Dunger N, Schölmerich J, Falk W, Obermeier F. Glycogen synthase kinase 3-β: a master regulator of toll-like receptor-mediated chronic intestinal inflammation. Inflamm Bowel Dis. 2010;16(11):1850-8.
38. Beurel E, Yeh WI, Michalek SM, Harrington LE, Jope RS. Glycogen synthase kinase-3 is an early determinant in the differentiation of pathogenic Th17 cells. J Immunol. 2011;186(3):1391-8.
39. Graham JA, Fray M, de Haseth S, Lee KM, Lian MM, Chase CM, et al. Suppressive regulatory T cell activity is potentiated by glycogen synthase kinase 3{beta} inhibition. J Biol Chem. 2010;285(43):32852-9.
40. Yue LI, Shuting W, Runyuan Z, Dongmei F, Dike Z, Fengbin L, et al. Efficacy of active ingredients in Qingdai on ulcerative colitis: a network pharmacology-based evaluation. J Tradit Chin Med. 2023;43(1):124-33.
41. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 2012;13(9):R79.
42. Akhtar M, Chen Y, Ma Z, Zhang X, Shi D, Khan JA, et al. Gut microbiota-derived short chain fatty acids are potential mediators in gut inflammation. Anim Nutr. 2021;8:350-60.
43. Shin Y, Han S, Kwon J, Ju S, Choi TG, Kang I, et al. Roles of short-chain fatty acids in inflammatory bowel disease. Nutrients. 2023;15(20):4466.
44. Lee C, Kim S, Kim B, Holzapfel WH, Hyun CK. Disturbance of lipid metabolism in germ-free mice transplanted with gut microbiota of DSS-induced colitis mice. PLoS One. 2023;18(2):e0280850.
45. Kabat AM, Srinivasan N, Maloy KJ. Modulation of immune development and function by intestinal microbiota. Trends Immunol. 2014;35(11):507-17.
46. Yan JB, Luo MM, Chen ZY, He BH. The function and role of the Th17/Treg cell balance in inflammatory bowel disease. J Immunol Res. 2020;2020:8813558.
47. Xie J, Tian S, Liu J, Huang S, Yang M, Yang X, et al. Combination therapy with indigo and indirubin for ulcerative colitis via

reinforcing intestinal barrier function. Oxid Med Cell Longev. 2023;2023:2894695.
48. Sun Z, Li J, Dai Y, Wang W, Shi R, Wang Z, et al. Indigo naturalis alleviates dextran sulfate sodium-induced colitis in rats via altering gut microbiota. Front Microbiol. 2020;11:731.
49. Liang YN, Yu JG, Zhang DB, Zhang Z, Ren LL, Li LH, et al. Indigo naturalis ameliorates dextran sulfate sodium-induced colitis in mice by modulating the intestinal microbiota community. Molecules. 2019;24(22):4086.
50. Ben-Horin S, Salomon N, Karampekos G, Viazis N, Lahat A, Ungar B, et al. Curcumin-QingDai combination for patients with active ulcerative colitis: a randomized, double-blinded, placebo-controlled trial. Clin Gastroenterol Hepatol. 2024;22(2):347-56.e6.
51. Uchiyama K, Takami S, Suzuki H, Umeki K, Mochizuki S, Kakinoki N, et al. Efficacy and safety of short-term therapy with indigo naturalis for ulcerative colitis: an investigator-initiated multicenter double-blind clinical trial. PLoS One. 2020;15(11):e0241337.
52. Naganuma M, Sugimoto S, Mitsuyama K, Kobayashi T, Yoshimura N, Ohi H, et al. Efficacy of indigo naturalis in a multicenter randomized controlled trial of patients with ulcerative colitis. Gastroenterology. 2018;154(4):935-47.
53. Lin YK, See LC, Huang YH, Chi CC, Hui RC. Comparison of indirubin concentrations in indigo naturalis ointment for psoriasis treatment: a randomized, double-blind, dosage-controlled trial. Br J Dermatol. 2018;178(1):124-31.
54. Lin YK, Chang SH, Yang CY, See LC, Lee BH, Shih IH. Efficacy and safety of indigo naturalis ointment in treating atopic dermatitis: a randomized clinical trial. J Ethnopharmacol. 2020;250:112477.
55. Iacucci M, de Silva S, Ghosh S. Mesalazine in inflammatory bowel disease: a trendy topic once again? Can J Gastroenterol. 2010;24(2):127-33.
56. Baker DE. Safety of balsalazide therapy in the treatment of inflammatory bowel disease. Rev Gastroenterol Disord. 2005;5(3):135-41.
57. Li J, Pu Y, Li S, He B, Chen J. Orally administrated olsalazine-loaded multilayer pectin/chitosan/alginate composite microspheres for ulcerative colitis treatment. Biomacromolecules. 2023;24(5):2250-63.
58. Kubota K, Imai Y, Okuyama T, Ishiyama Y, Ueno S, Kario K. Dramatically improved severe pulmonary arterial hypertension caused by Qing-Dai (Chinese herbal drug) for ulcerative colitis. Int Heart J. 2023;64(2):316-20.
59. Inoue Y, Ishihara A, Mori T, Horio S, Yoshizane T, Arai M, et al. Development of pulmonary arterial hypertension following long-term Qing-Dai use for ulcerative colitis. J Cardiol Cases. 2023;27(5):218-21.
60. Misumi K, Ogo T, Ueda J, Tsuji A, Fukui S, Konagai N, et al. Development of pulmonary arterial hypertension in a patient treated with Qing-Dai (Chinese herbal medicine). Intern Med. 2019;58(3):395-9.
61. Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X, et al. Appetite-suppressant drugs and the risk of primary pulmonary hypertension. International Primary Pulmonary Hypertension Study Group. N Engl J Med. 1996;335(9):609-16.
62. Zhang Q, Xie J, Li G, Wang F, Lin J, Yang M, et al. Psoriasis treatment using indigo naturalis: progress and strategy. J Ethnopharmacol. 2022;297:115522.