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Original Article

Effect of *Cyperus Rotundus* on Cytokine Gene Expression in Experimental Inflammatory Bowel Disease

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Abstract

Background: The protective effect of the chloroform extract of *Cyperus rotundus* (CHCR) is attributed to its anti-inflammatory and antioxidant activities. Cytokines, important regulators of inflammation and repair, play a key role in the pathogenesis of inflammatory bowel disease (IBD). Targeting these cytokines can effectively ameliorate the symptoms of IBD. The aim of the present study was to unravel the molecular mechanism through cytokine regulation in rats in experimental IBD.

Methods: Sprague Dawley rats were randomly allocated to 5 groups (n=6). Group I served as the normal control. Group II served as the vehicle control and received 50% ethanol intracolonically on day 11 of the study. Group III served as the model control. Group IV and Group V were given standard drug 5-aminosalicylic acid (100 mg/kg) and CHCR (800 mg/kg), respectively, for 18 days once a day orally. Colitis was induced with dinitrobenzene sulfonic acid (180 mg/kg in 50% ethanol) intracolonically in groups III–V on day 11 of the study. On day 18, the rats were euthanized and colon tissues were removed for IL-4, IL-6, IL-12, and IFN-gamma gene expression studies using quantitative RT-PCR.

Results: The expression levels of proinflammatory cytokines IL-4, IL-6, IL-12, and IFN-gamma were upregulated in the model control rats. Pretreatment with 5-aminosalicylic acid (100 mg/kg) and CHCR (800 mg/kg) significantly decreased the fold of the expression of the above cytokines.

Conclusion: CHCR acts as a molecular brake and downregulates the expression of proinflammatory cytokine genes; this is beneficial for reducing the severity of the experimental IBD. Thus, *Cyperus rotundus* is a safe, economical, and effective alternative for the treatment of patients with IBD.

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Keywords ● Inflammatory bowel diseases ● Cyperus rotundus ● Interleukin-4 ● Interleukin-6 ● Interleukin-12 ● Interleukin-18

What's Known

- *Cyperus rotundus* possesses antioxidant and anti-inflammatory properties and has hence been used for the treatment of inflammatory bowel disease (IBD). But its exact mechanism of action was not known.

What's New

- We found that the mechanism of action behind the antioxidant and anti-inflammatory properties of *Cyperus rotundus* is through gene modulation in pro-inflammatory cytokines, viz. IL-4, IL-6, IL-12, and IFN-γ.
- *Cyperus rotundus* is a safe, effective, and economical alternative for the treatment of IBD patients.

Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal disorder comprising of Crohn's disease and ulcerative colitis. It results from an inappropriate intestinal response to intestinal microbes in a genetically susceptible host. Genes encoding innate immune responses, which are triggered by environmental stimuli, are responsible for determining

the susceptibility to IBD. In IBD, there is an imbalance between the effector and the immunity regulatory activities in the intestine, wherein proinflammatory cytokines are predominant. In the active form of IBD, innate immune cells like neutrophils, macrophages, dendritic cells, and natural killer T cells along with adaptive immune cells like B cells and T cells infiltrate into the lamina propria.[1] An excessive infiltration of immune cells to the site of tissue lesions initiates and aggravates inflammation. This elevates the local levels of various proinflammatory cytokines, contributing to disease development by orchestrating the inflammatory process. High cytokine levels imply the overabundance of T cells in patients with IBD. An altered production and signaling of chemokines and cytokines has been implicated in the pathogenesis of IBD. T_H1 responses are characterized by the secretion of IL-1, IL-2, IL-6, IL-12, IL-18, TNF-α, and IFN-γ. Ulcerative colitis exhibits an additional response of defective T_H2 responses characterized by the secretion of IL- 4, IL-5, and IL-10.[2] Increased levels of IL-4, IL- 6, IL-12, and IFN-gamma were found in the intestinal tissue and peripheral blood of patients with IBD.[2]

Depending on the level of severity, the control of IBD symptoms may require immunosuppressant drugs such as prednisone, infliximab, azathioprine, methotrexate, and 6-mercaptopurine. More commonly, the treatment of IBD requires aminosalicylates. Often, steroids are used to control the flares of the disease and are used as a maintenance drug. Biologicals have also been used intravenously. Severe cases may require surgical operations such as bowel resection, strictureplasty, and colostomy or ileostomy. The treatment of patients with inflammatory diseases is growing more costly by the day; nonetheless, new alternative therapies can be not only effective but also cheap.[3] The alternative medicines meant for treating intestinal diseases focus on controlling pathology and, thus, prevent chronic steroid use or any surgery options.[4] The use of complementary medicine, particularly in the form of herbal therapies, among patients with IBD is widespread in the West as well as in many Asian countries including China and India.[5] Various studies have proved that these substances modulate the immune system, interfere with the proinflammatory pathway through their antioxidant activity, affect cell signaling controlled by the nuclear factor kappaB pathway, affect the levels of various cytokines, and disrupt bacterial flora.[6] Thus, chemokines, which activate and recruit cytokines to an injured site, are target molecules for new drug development in the treatment of chronic inflammatory diseases.

Cyperus rotundus Linn. is commonly known as Musta, mustak, nagarmotha, or nut grass. It is perennial sedge distributed throughout India. The essential oil from *Cyperus rotundus* contains at least 27 components, including sesquiterpene hydrocarbons, epoxides, ketones, monoterpenes, and aliphatic alcohols as well as triterpenes like β-Sitosterol and linoleic acid. The tuber and rhizome are used to treat chronic diarrhea with mucus and other abdominal problems. *Cyperus rotundus* has anthelmintic, antibacterial, and fungicidal activities and has been used for many other complaints.[7] Moreover, it has been reported to have analgesic, central nervous system depressant,[8] anticonvulsant,[9] antidysmenorrhea,[10] antiplatelet,[11] lipid-lowering,[12] and antispasmodic effects.[13]

The studies conducted in our laboratory proved that the anti-inflammatory and antioxidant activities of the chloroform extract of *Cyperus rotundus* (CHCR) were beneficial for reducing the severity of IBD induced by dinitrobenzene sulfonic acid (DNBS) intracolonically administered in rats. In the DNBS-treated group, there was a significant reduction in percent change in body weight, water intake, food intake, superoxide dismutase activity, and colon length and a significant increase in colon weight, malondialdehyde level, myeloperoxidase activity, nitric oxide level, colon mucosa disease index, disease activity index, and serum cortisol level. Aminosalicylate compounds are frequently prescribed drugs for the control of IBD symptoms in humans.[3] Hence, 5-aminosalicylic acid (5-ASA) was given as standard treatment to compare the efficacy of the test drug. Pretreatment with 5-ASA (100 mg/kg), CHCR (600 mg/kg), and CHCR (800 mg/kg) significantly prevented these changes induced by DNBS.[14] So, the next aim was to unravel the molecular mechanism through cytokine regulation in experimental IBD.

Materials and Methods

Plant Material

Tubers of *Cyperus rotundus* were obtained from a commercial supplier in Ahmedabad. The plant material was identified and authenticated by Dr. Satyabrata Maiti, Director, Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand, Gujarat, India. A voucher specimen of the plant materials was deposited in the herbarium of the Department of Pharmacognosy, Anand Pharmacy College, Anand.

Preparation of Chloroform Extracts of Cyperus Rotundus and Phytochemical Tests

The tubers of *Cyperus rotundus* were ground to coarse powder separately and stored at room temperature. For each extraction procedure, 250 g of powdered plant material was extracted with 1000 mL of chloroform in a Soxhlet apparatus for 5–7 hours at 50 ºC. Then, the organic extract was concentrated by evaporation on water bath and thereafter further dried at ambient temperature for 24 hours to obtain a dry extract. All the dried extracts were stored at −20 ºC. The extract of *Cyperus rotundus* was labeled as CHCR. The extract was subjected to qualitative chemical tests to find out the presence or absence of phytoconstituents like alkaloids, carbohydrates, tannins, fats, oils, steroids, saponins, and flavonoids.[15,16]

Animals

Healthy male Sprague Dawley rats (250–300 g, 10–11 weeks' age) were housed in cages with free access to standard rat chow (diet) and water *ad libitum* and acclimatized to the surroundings for 1 week prior to the experiment. The animals were maintained on a light/dark cycle (12/12 hours) at a constant temperature (22±1 ºC) and humidity (55±1). The experimental protocol (Protocol #9012 dated December 26, 2009) was approved by the Institutional Animal Ethical Committee of Anand Pharmacy College as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Toxicity Testing

The safety of the herbal extract was established through subacute toxicity studies as per the OECD guidelines – 407.[17] Three doses of the CHCR extract tested were 200 mg/kg, 600 mg/kg, and 1800 mg/kg.

Experimental Design and Treatment

The rats were randomly allocated to 5 groups containing 6 animals each. The sample size was calculated using G-POWER engine software. The animals in all the groups were fasted for 24 hours prior to the study but were given access to water *ad libitum*. Group I served as the normal control group throughout the 18 days of the study period. Group II served as the vehicle control group and received 50% ethanol (Sigma) intracolonically on day 11 of the study. DNBS used to induce experimental IBD was dissolved in 50% ethanol. Group III served as the model control in which colitis was induced with DNBS (120 mg/kg dissolved in 50% ethanol)[18] on day 11 of the study. The animals of Group IV were given the standard drug (5-ASA [Sigma] [100 mg/kg]) for 18 days once a day orally. Group V received CHCR suspended in 0.5% CMC (800 mg/kg) for 18 days once a day orally. Group IV and Group V were also administered DNBS (120 mg/kg dissolved in 50% ethanol) intracolonically on day 11 of the study after overnight fasting to induce colitis.

Sample Collection

On day 18 of the study, the animals were anaesthetized and their abdomen was opened with a midline incision. Colon tissues were removed aseptically, washed directly with PBS (pH 7), and immediately cut in small pieces. The samples were placed in screw cap vials (2 mL) containing 1 mL of RNA later solution and immediately transferred in a liquid nitrogen container for further estimations.

Total RNA Extraction

Total RNA was extracted from the tissue samples following TRI-reagent-based protocol. Mortar pestles were autoclaved in oven at 240 ºC overnight. A frozen tissue sample weighing 100 g was taken in a pre-chilled mortar. It was then powdered by grinding in the mortar with the help of a pestle and occasionally adding liquid nitrogen in the mortar to prevent thawing. Intact total RNA was isolated by pulverizing the tissue into powder and it was kept in a frozen state. Once the tissue was ground to a fine powder, 1 mL of TRI-reagent per 100 mg of tissue was added (taking care that the tissue volume did not exceed 10% of the TRI-reagent volume) and the semi frozen mixture was stirred. The mixture was thawed and transferred into a 2 mL round bottom microcentrifuge tube. The homogenized sample was then incubated at room temperature for 10 minutes and then centrifuged at 13000 rpm for 10 minutes at 4 ºC in refrigerated centrifuge (Eppendorf – 5804R). This settled the tissue debris. Subsequently, 200 µL of chloroform was added per 700 µL of TRI-reagent used and mixed vigorously for 15 seconds. The sample was centrifuged at 13000 rpm for 15 minutes at 4 ºC. The upper aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube and 0.5 mL of isopropanol was added per 700 µL of TRI-reagent used and mixed using inverting tubes. The mixture was kept in the refrigerator at -10 ºC for between 20 minutes and 45 minutes and centrifuged at 13000 rpm for 15 minutes at 4 ºC. The RNA formed a pellet at the bottom of the tube. The supernatant was

removed carefully, and the pellet was washed twice with 75% ethanol, adding at least 1 mL/mL of the TRI-reagent used, followed by vortexing and centrifugation at 11000 rpm for 15 minutes at 4 ºC. The supernatant was removed, and the RNA pellet was air dried. It was then re-suspended in 30 µL of DEPC-treated Milli-Q water and slightly tapped for a few times to dissolve the RNA pellet completely.

Assessment of the Quantity and Quality of the Isolated RNA

The RNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., U.S.A.). The UV absorbance was checked at 260 and 280 nm for the determination of sample concentration and purity. The purity of the RNA was assessed on the basis of optical density (OD) ratio at 260:280. The salt concentration as well as other impurities of the RNA solution was done on the basis of 260: 230. The samples with a ratio 1.8 to 2.0 were processed further.

DNase Treatment and cDNA Synthesis

To remove contaminating DNA, the samples were treated with DNase purchased from Fermentas. Next, cDNA was prepared via the reverse transcriptase PCR (RT-PCR) method using an Omniscript cDNA synthesis kit (Qiagen, U.S.A.). The required mixture containing template RNA solution, primer solutions, reaction buffer, RevertAid RT enzyme (purchased from Fermentas), dNTP Mix, and RiboLock RNase inhibitor was vortexed and centrifuged briefly. Subsequently, it was incubated for 60 minutes at 42 ºC in a MiniCycler Thermal cycler (Bio-Rad). The reaction was terminated by heating at 70 ºC for 10 minutes.

Gene Expression by Quantitative Real-Time PCR Assay

Amplification of the cDNA was done using real-time PCR to measure the fold of the expression of IL-4, IL-6, IL-12, and IFN-γ genes in each group. Real-time PCR reactions were performed in triplicate with specific Oligo primer pairs using a QuantiTect SYBR Green PCR kit purchased from Qiagen according to the manufacturer's recommendations in optical 96-well plates. The primers were commercially synthesized with Ocimum Biosolutions, Hyderabad (table 1). The specificity of the primers was checked using NCBI blast software (*http://www.ncbi.nlm.nih.gov/BLAST/*). The amplification was carried out in a final reaction volume of 50 µL containing 2X QuantiTect SYBR Green PCR master mix, 5 pmol of each gene-specific primer, and 1 µL of cDNA template. The PCR amplification profile was as follows: 95 ° C for 5 minutes followed by 50 cycles of denaturation at 95 ºC/30 s, gene-specific annealing temperature/30 s, and extension at 72 ºC/35 s and final extension of 72 ºC/5 min. The data were collected at step 3 of stage 2. Annealing temperatures per primer set were determined empirically. Real-time analysis of PCR amplification was performed with an Applied Biosystems 7500 (U.S.A.), and data were generated using Sequence Detection software (SDS V. 1.3.1). The fold of expression for all the samples was normalized with endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the calibrator used was the model control. Sequence detection software was used to calculate the fold of expression. Melting curves for each PCR reaction were generated to ensure the purity of the amplified product.

Table 1: Primer sequences of the selected genes

Name of primer	Sequences (5'-3')
IL-4F	CCCTGTTCTGCTTTCTCATA
IL-4R	CCGAGAACCCCAGACTTGTT
IL-6F	GCCAGAGTCATTCAGAGCAATACTG
IL-6R	TTGGGATATCAGGTTTCTGGATGG
IL-12F	GGGTCCGGTTTGATGATGTCCCTG
IL-12R	GGAGAAACGGTGACCCTCACCT
IFN-gamma F	TATGGAAGGAAAGAGCCTCC
IFN-gamma R	TCTGTGGGTTGTTCACCTCG
GAPDH F	CGGAGTCAACGGATTTGGTCGTAT
GAPDH R	AGCCTTCTCCATGGTGGTGAAGAC

C_T cycle is the number of cycles detected as fluorescence signal when amplification crosses the threshold value.

dC_T=C_T cycle of test sample-C_T cycle of endogenous control or C_T target-C_T endo.

ddC_T=difference between Avg. dC_T value of a target sample and the average dC_T value for the corresponding calibrator sample or ddC_T=Avg. dC_T (test or target)- avg.dC_T (calibrator sample).

This value is used to calculate the expression fold value. The expression fold value=2–ddC_T.

Statistical Analysis

The results were expressed as mean±standard error of the mean (SEM). The data were analyzed using the one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test. Also, 95% level of significance ($P<0.05$) was used for the statistical analyses. For the data on the gene expression studies, statistical analysis was performed using PRIMER 6.1 statistical software.

Results

Phytochemical Tests

The chloroform extract obtained was dark brown in color. It was a sticky, thick, smooth mass with 0.84% yield. In addition, the extract showed positive tests for steroids.

Toxicity Study

No unusual behavioral changes, disturbance in locomotor activity, any untoward clinical signs, sign of intoxication, and mortality were observed at the dose levels tested in the sub-acute toxicity test. From day 0 to day 28, nonsignificant changes were observed in the body weights of the male and female rats fed with different doses of the extract as compared to the male and female rats of the control group. The food and water intake was not affected by treatment with CHCR. Observations of gross pathology immediately after the dissection of the rats in all the groups were found to be normal, lacking in any apparent pathological abnormalities. The external appearance of the organs (heart, liver, lungs, and kidneys) did not show any alterations as compared to that in the control group. Treatment with the extract did not affect hematological and biochemical parameters, renal and hepatic function tests, serum glucose level, and lipid profile. The histopathological findings were similar in both control and treated rats, indicating that the administration of CHCR at all 3 dose levels did not result in any adverse toxicological effect on these organs. The no-observed-adverse-effect level (NOAEL) for CHCR was 1800 mg/kg body weight/d, administered orally for 28 days in the rats under the conditions of this study.

Expression of IL-4, IL-6, IL-12, and IFN- γ

IL-4 mRNA was overexpressed in the inflamed colon tissue of the disease control group on analysis of inflammatory cytokine production using real-time RT-PCR. Treatment with 5-ASA and CHCR significantly lowered mRNA expression for cytokine IL-4 (table 2).

We found an increased amount of IL-6 genes in the colon samples of the DNBS-treated rats. Cytokine levels after the treatment with standard drug and CHCR decreased when compared to the levels in the rats treated with DNBS alone (table 2).

DNBS led to an enhanced expression of mRNA of IL-12 and aggravated colon injury. Treatment with 5-ASA and CHCR prevented this target from modulating the progression of colitis (table 2).

Significant upregulation of IFN- γ mRNA expressions in the DNBS model group, which was attenuated by 5-ASA and CHCR as seen by their levels of their transcripts, seems favorable (table 2).

Discussion

Oxidative stress upregulates the transcription of IL-4.[19] Our results showed that the levels of intracellular reactive oxygen species affected IL-4 gene expression. There was also a significant reduction in the expression of this cytokine, which may be explained by the antioxidant effects of CHCR and the downregulation of the transcription of IL-4 mRNA in the treated groups. Thus, an increased production of IL-4 in IBD contributed to disease pathogenesis and the test drug suppressed its expression, thereby reversing the condition.

In IBD, T_H1 lymphocytes increase the production of IL-6 and initiate cytotoxic, apoptotic, and acute phase responses. IL-6 and soluble IL-6 receptor have been implicated in the inflammation of IBD.[6] IL-6 stimulates neutrophil chemotaxis and is associated with necrosis in the colon, which, in turn, leads to tissue destruction. IL-6 plays an important role in the SAMP1/Yit murine model of Crohn's-like ileitis.[20] There is an upregulation of proinflammatory IL6:STAT3 biological network when pediatric patients with active IBD are diagnosed and treated. Targeting this pathway can effectively reduce mucosal inflammation. When patients with Crohn's disease and ulcerative colitis were diagnosed,

Table 2: Fold of expression values of various genes

Groups	Normal	50% Ethanol	Model	5-Aminosalicylic Acid (100 mg/kg)	Chloroform Extract of *Cyperus Rotundus* (800 mg/kg)	P value
IL-4	0.342±0.03	0.31±0.02	1.0±0.01*	0.255±0.01#	0.591±0.01#	<0.001
IL-6	0.443±0.01	0.3±0.01	1.0±0.03*	0.23±0.02#	0.098±0.02#	<0.001
IL-12	0.161±0.01	0.163±0.03	1.0±0.02*	0.13±0.01#	0.424±0.01#	<0.001
IFN-gamma	0.24±0.01	0.051±0.01	1±0.01*	0.23±0.03#	0.591±0.01#	<0.001

The data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and are presented as treated colitis relative to untreated colitis using the delta–delta Ct method for comparing the relative fold of expression differences. Data are represented as mean±SEM. Statistical analysis was carried out using PRIMER statistical software and the one-way analysis of variance, followed by the Dunnett post hoc test. *Significantly different from the normal group at P≤0.001. #Significantly different from the model group at P≤0.001

they showed increased serum IL-6 and activated STAT3 and remained elevated to the same extent during treatment as that in patients with active Crohn's disease.[21] In male children, the IL-6 promoter region sequence (IL6 -174) affects age at onset of Crohn's disease.[22] From these data, it is apparent that IL-6 may contribute to enhanced susceptibility to injury and contribute to the immunopathogenesis of this disorder. CHCR treatment modulated IL-6 cytokine mRNA expression due to its cytoprotective, antiapoptotic, and anti-inflammatory effects in the experimentally induced IBD model.

Considerable data point to a prominent role for IL-12 produced from activated macrophages in the development of T_H1 CD4+ T cells in the intestinal mucosa. IL-12 is secreted in the mucosal tissue of patients with Crohn's disease, contributing to the characteristic predominance of T_H1 cytokine response of the mucosal immune system. There is a strong synergistic action with IL-12 to trigger interferon-γ production, which may contribute to the perpetuation of the inflammatory process in patients with Crohn's disease.[23] The intestinal inflammation in patients with IBD is due to an increased expression of IL-12 and IL-17 mRNA. Thus, to treat IBD, we can target IL-12 or IL-17 inhibition.[24] Treatment with anti-IL-12 antibody significantly prevented the development of colitis in rodents. The role of IL-12 behind mucosal inflammation in mice was further substantiated in the study where the TNF ARE knockout mice did not develop intestinal inflammation when intercrossed with IL-12 knockout mice.[25] Clinical remission in patients with Crohn's disease can be induced and maintained by the administration of monoclonal antibodies blocking the IL-12/p40 subunit.[26] 5-ASA and CHCR treatments prevented the colon injury caused by IL-12.

Vigorous IFN-*γ* production by T cells can be detected in colonic mucosal tissue cultures and intestinal lamina propria mononuclear cells from patients with IBD. Several studies have reported the role of IFN-*γ* in the pathogenesis of this disease, which augments the inflammatory response and damages the mucosal barrier.[27] IFN-γ promotes the inflammation in animal models of IBD.[25] In dextran sodium sulfate (DSS)-induced colitis model also, the levels of IFN-γ were high, which further released other chemokines. Chronic intestinal inflammation was partially, but significantly, ameliorated using IFN-γ antibodies in the DSS-induced colitis murine model.[28] Our treatment directly abrogated IFN-γ production.

The experimental group treated with vehicle (i.e., 50% ethanol) used for dissolving DNBS showed results similar to those in the normal control animals.

The results demonstrated in our study are in accordance with various studies and lend support to the immune theories behind IBD. The DNBS model closely mimics the modifications observed in the colonic epithelium of patients with IBD. The DNBS-treated rats exhibited severe erosion with an enhanced expression of IL-4, IL-6, IL-12, and IFN-γ cytokines mRNA. Treatment with the investigational extract had an influence on mRNA levels and, thus, modulated the colitis-induced cytokine responses. Accordingly, the protective effects were due to the downregulation of the genes of proinflammatory cytokines, affecting the immune system and inflammatory processes. The positive results suggest that CHCR serves as an important "molecular brake" to dampen local inflammation by addressing immune responses involved in IBD, thereby rarefying the progression of the symptoms of colitis.

Conclusion

Treatment with CHCR led to a direct inhibition of gene expression for proinflammatory cytokines like IL-4, IL-6, IL-12, and IFN-γ in the colon tissues of the rats in the present study. Thus, the possible mechanism of the ameliorating effect might be attributed to active principles such as natural steroids and terpenoids alone or in combination exhibiting antioxidant and anti-inflammatory effects via the downregulation of proinflammatory cytokines. The current study provides ample scientific and ethnopharmacological evidence regarding the potential of the extract as a new promising therapeutic approach to abrogate mucosal inflammation in IBD. Since the plant is easily available, the treatment would be less costly. Furthermore, the plant extract can be formulated to evaluate the efficacy in clinical studies. Thus, *Cyperus rotundus* can be considered a novel, safe, effective, and economical alternative for the treatment of IBD.

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Conflict of Interest: None declared.

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