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Suppression of inflammatory cytokines expression with bitter melon (*Momordica charantia*) in TNBS-instigated ulcerative colitis

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ABSTRACT

Background and Objective: This study was aimed to elucidate the molecular mechanism of *Momordica charantia (MCh)*, along with a standard drug prednisolone, in a rat model of colitis induced by trinitrobenzene sulfonic acid (TNBS). **Methods:** After the induction of the experimental colitis, the animals were treated with MCh (4 g/kg/day) for 14 consecutive days by intragastric gavage. The colonic tissue expression levels of C-C motif chemokine ligand 17 (CCL-17), interleukin (IL)-1β, IL-6, IL-23, interferon-γ (IFN-γ), nuclear factor kappa B (NF-kB), and tumor necrosis factor-α (TNF-α), were determined at both mRNA and protein levels to estimate the effect of *MCh*. Besides, colonic specimens were analyzed histopathologically after staining with hematoxylin and eosin. **Results:** The body weights from TNBS-instigated colitis rats were found to be significantly lower than untreated animals. Also, the IFN-γ, IL-1β, IL-6, Il-23, TNF-α, CCL-17, and NF-kB mRNA and protein levels were increased significantly from 1.86-4.91-fold and 1.46-5.50-fold, respectively, in the TNBS-instigated colitis group as compared to the control. Both the MCh and prednisolone treatment significantly reduced the bodyweight loss. It also restored the induced colonic tissue levels of IL-1β, IL-6, IFN-γ, and TNF-α to normal levels seen in untreated animals. These results were also supported with the histochemical staining of the colonic tissues from both control and treated animals. **Conclusion**: The presented data strongly suggests that *MCh* has the anti-inflammatory effect that might be modulated through vitamin D metabolism. It is the right candidate for the treatment of UC as an alternative and complementary therapeutics.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are described by chronic relapsing inflammation in the gastrointestinal tract. They are generally divided into two main groups, Crohn's Disease (CD) and Ulcerative Colitis (UC).[1] CD is a disease that can keep the entire digestive tract from mouth to anus in a segmental manner and transmurally, followed by remission and exacerbations.[2,3] On the other hand, UC is a chronic IBD that diffuses from the rectum to the proximal length without leaving any intact parts and diffuses the colon mucosa with remission and exacerbations.[4] Although the etiology remains a mystery, the current view is inflammation caused by inflammatory cells and cytokines via a complex network of interactions.[5]

Cytokines are essential molecules in the initiation and regulation of inflammatory immune responses. For normal colon homeostasis, anti-inflammatory [such as interleukin (IL)-4, IL-10, IL-11, and IL-13] and pro-inflammatory [such as IL-1, IL-2, IL-6, IL-8, IL-12, IL-23, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ] cytokines in the colonic mucosa need to be in balance.[6,7] UC disease is associated with the elevation of inflammatory markers such as IL-1β, IL-6, TNF-α, and IFN-γ.[8–12] Chemokines also play an essential role in the course of inflammation. A significant increase of some chemokines is observed in the intestinal tissues of patients with UC and CD.[13] C-C motif chemokine ligand 17 (CCL-17) is a C-C motif chemokine that is expressed in the thymus and associated with the induction of chemotaxis in T cells.[14] Nuclear factor kappa B (NF-kB) is a nuclear transcription factor and plays a critical role in regulating multiple gene transcription associated with immunity and inflammation.[15–18] It is recognized as one of the dominant players in the pathogenesis of UC.[19]

The utilization of plants and plant-based preparations as complementary and alternative therapeutics has attained popularity throughout the world in recent decades.[20] According to the World Health Organization, it is estimated that 80% of the population in developing countries rely on traditional medicinal plants.[21] *Momordica charantia* (*MCh*) is one such plant used as traditional medicine for various ailments such as tumors, skin diseases, wounds, eczema, scabies, rheumatism, malaria, menstrual problems, diabetes, gastric ulcer, feverish conditions, and intestinal worms.[17,21–30] In our country, *MCh* is widely used for the treatment of UC and peptic ulcer.[31]This study was aimed to elucidate the molecular mechanism of *MCh* in a rat model of colitis instigated by TNBS.

MATERIALS AND METHODS

Plant material

The ripened (orange-yellow) fruits of *MCh* were purchased from local street markets in July 2017 and identified taxonomically by G. Semiz. *MCh* fruits are washed with tap water, stripped of water, chopped into small pieces (approximately 0.5 cm^3 pieces) and placed in a 1 L glass jar. After addition of an equal amount of virgin olive oil, the jar was tightly closed and exposed to sunlight for six weeks. At the end of the incubation period, the resulting marmalade was homogenized by passing through the blender and kept at room temperature in a dark environment. The marmalade was prepared as used by local folks, without involving any chemicals.

Animals

Thirty-five healthy male Wistar rats (twelve-week old ranging 220–250 g body weights) were purchased from the Pamukkale University Animal House. The animals were housed in small polypropylene cages with a 12-h light/12-h dark photocycle in a temperature-controlled room (22 ± 1°C) and were fed commercial rat food with water *ad libitum*. Animal experiments were performed under appropriate ethical administration with veterinary services within licensed projects approved by the Institutional Experimental Animal Ethics Committee (PAUHDEK-2015/18). The animal protocol was intended to minimize pain or discomfort to the animals. Intragastric gavage administration was carried out with cognizant animals, utilizing straight gavage needles fitting for animal size.

Induction of colitis

Colonic inflammation was induced based on the method of El-Salhy[32] with slight modifications. Thirty-five rats divided into five experimental groups as follows:

Group I-Normal control (NC) group: only received distilled water for 14 day.

Group II-Ulcerative colitis control (UCC) group: Anaesthetized rats were treated with rectal catheterization followed by 30 mg/0.1 mL TNBS and 0.5 mL 50% ethanol mixture to induce colitis formation. Animals are expected to be UC at the end of 7 day. At the end of this period, since *MCh* marmalade was prepared in olive oil, the rats were fed with olive oil by intragastric gavage for 14 d to see the solvent's effects. In other words, the goal of the UCC group was to control for olive oil effects in treatment with *MCh*.

Group III-Ulcerative colitis (UC) group: Anaesthetized rats were treated with rectal catheterization followed by 30 mg/0.1 mL TNBS and 0.5 mL 50% ethanol mixture to induce colitis formation. Animals are expected to be UC at the end of 7 day. This group of animals were kept untreated for a further duration of the experiment.

Group IV-*M. charantia* (*MCh*) group: Anaesthetized rats were treated with rectal catheterization followed by 30 mg/0.1 mL TNBS and 0.5 mL 50% ethanol mixture to induce colitis formation. Animals are expected to be UC at the end of 7 day. At the end of this period, rats were fed with 4 g/kg/day of *MCh* marmalade for 14 days by intragastric gavage. The dose given was determined by considering the dose used among the population.

Group V-Positive drug Prednisolone (PR) group: Anaesthetized rats were treated with rectal catheterization followed by 30 mg/0.1 mL TNBS and 0.5 mL 50% ethanol mixture to induce colitis formation. Standard drug prednisolone was given on the same day by oral gavage (2

mg/kg/day, 7 day). This group of animals were kept for the duration of the experiment.

After the last treatment or day, all rats were fasted overnight (16–18 h). Once the animals were sacrificed by cervical dislocation, the colons were removed aseptically and placed on ice-cold physiological saline. Colonic specimens were washed gently with physiological saline to remove fecal residues and then immediately frozen in liquid nitrogen. Blood samples to determine the serum enzymes were taken from the aorta. Portions of colonic tissue samples were fixed in 10% paraformaldehyde solution for histochemical studies.

Histopathological analysis

Histopathological analysis was carried out by a histopathologist from the Faculty of Medicine at Pamukkale University as described elsewhere.[33] Basically, the colon samples were fixed in 10% formalin in phosphate buffer for 24 h, processed routinely for paraffin embedding, sectioned at 3–4 μm, and stained with hematoxylin and eosin (H&E). Histological scoring was based on three parameters as described below: a) severity of inflammation: 0 = no inflammation; 1 = mild; 2 = moderate; 3 = severe; b) depth of inflammatory involvement: 0 = no inflammation; 1 = mucosa; 2 = mucosa and submucosa; 3 = transmural; c) crypt damage: 0 = intact crypts; 1 = loss of the basal one-third; 2 = loss of the basal two-thirds; 3 = entire crypt loss and change of epithelial surface with erosion.[34] Five random fields were evaluated for each section.

Determination of serum AST and ALT and LDH activities

In order to evaluate the toxic potential of the *MCh* marmalade, levels of serum transaminases [Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)] and Lactate dehydrogenase (LDH) were determined using procedures based on the methods described.[35,36] Blood samples were centrifuged for 10 min at 4000 r/min at 4°C to separate serum.

RNA isolation and cDNA synthesis

Total RNA was isolated from about 100 mg colonic samples with the RNeasy lipid tissue universal mini kit (Qiagen) according to the instructions provided by the manufacturer and as optimized in our laboratory.[37] The extracted RNA was quantified by measuring the absorbance at 260/280 nm, and RNA integrity was analyzed by 1% agarose gel. RNA was reverse transcribed using a RevertAid Reverse Transcriptase (ABM).[38]

RT-PCR of mRNAs

Semi-quantitative RT-PCR was carried out using gene-specific primers (Table 1). Beta-actin (ACTB) gene was used to normalize gene expressions. The PCR amplification conditions were applied as described.[20] The PCR products were detected on 1.5% agarose gels, and the bands were visualized using GelQuant Image Analysis Software in the DNR LightBIS Pro Image Analysis System. The relative intensity of the bands was reported relative to ACTB expression. All gene analyses were performed in triplicate and repeated at least three times.

Table 1: Primer sequences and amplification conditions

Gene	Primer Sequence (5′ -> 3′)	Annealing Temperature (°C)
IFN-γ	F→ GCCGCGTCTTGGTTTTGCAG R→ TACCGTCCTTTTGCCAGTTCCTCCA	65
IL-1β	F→ CATCAGCACCTCTCAAGCAGA R→CATTCTCGACAAGGGGGCTC	63
IL-6	F→TCTCTCCGCAAGAGACTTCC R→TCTTGGTCCTTAGCCACTCC	60
IL-23	F→AAAGGAGGTTGATAGAGGGT R→TCTTAGTAGATCCATTTGTCCC	57
TNF-*a*	F→GCCAATGGCATGGATCTCAAAG R→CAGAGCAATGACTCCAAAGT	59
CCL-17	F→ACCTTCACCTCAGCTTTTGGTACCATG R→GCGTCTCCAAATGCCTCAGCGGGAAGG	68
NF-kB	F→ACCTGGAGCAAGCCATTAGC R→CGGACCGCATTCAAGTCATA	55
ACTB	F→TGCAGAAGGAGATTACTGCC R→CGCAGCTCAGTAACAGTCC	65

Preparation of tissue homogenate

Colon samples were cut to small pieces and granulated in liquid nitrogen by mortar and pestle. Powdered colon samples were weighed (100 mg) into plastic tubes with 10% TCA (2,4,6-trichloroanisole) and 20 mmol/L DTT (dithiothreitol). The mixture was incubated on ice for 1 h and was centrifuged at 12,500 r/min for 20 min at 4°C. Finally, the pellet was dissolved in cold solubilization buffer [7 mol/L urea, 2 mol/L thiourea, 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), protease inhibitor cocktail, and 2 mmol/L DTT]. The amounts of protein were measured with the Bicinchoninic acid (BCA) method using the bovine serum albumin (BSA) standard.[39]

Gel electrophoresis and western blotting

SDS-PAGE and Western blotting analyses were carried out as described previously.[40,41] Briefly, samples (80 μg protein) were resolved on 8.5% polyacrylamide gel with a discontinuous buffer system of Laemmli.[42] The dissociated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane with the Hoefer blotting system (90 V, 90 min at 4°C). Following the transfer, the membranes were blocked with 5% non-fat dry milk in TBST [20 mmol/L Tris-HCl, pH 7.4, 400 mmol/L NaCl, and 0.1% (v/v) Tween 20] for 40 min. Blot was incubated sequentially with primary antibodies (1/1000)

and HRP-conjugated secondary antibodies (1/5000) in TBST containing 5% non-fat for 2 h and 1 h, respectively, while constant shaking at room temperature. Blot were then incubated with Pierce ECL western blotting substrate solution to detect immunoreactive proteins. The bands were visualized using GelQuant Image Analysis Software in the DNR LightBIS Pro Image Analysis System. The protein bands were quantified by measuring band density using Scion Image Version Beta 4.0.2 software.

Statistical analysis

All results were expressed as means, including their Standard Error of Means (SEMs). A comparison between groups was performed using Student's *t*-test, and $P < 0.05$ was selected as the level required for statistical significance. These statistical analyses were carried out using the Minitab 13 statistical software package.

RESULTS

During the treatment protocol, TNBS-instigated UC rats showed significant reductions in body weight compared to the NC group (Table 2). Nevertheless, both *MCh* and PR treated groups showed significant improvements in body weight loss. The effects of *MCh* on the serum transaminases and LDH in control and experimental colitis rats are given in Table 2. Significant differences were observed between the mean transaminases (ALT and AST) and LDH values of the control and UC groups. However, no differences were observed between control and *MCh*-treated rats

The paraffin sections of colonic specimens were subjected to H&E staining for the detection of ulceration and sign of colitis. It was observed that the H&E stained control group had healthy histological structure (Figure 1A). However, histopathological changes such as the presence of diffuse areas of inflammation and loss of cryptic structures in the UC and UCC groups as compared to the NC group (Figure 1B and 1C). Besides, it was found that there was a decrease in the areas of inflammatory cell infiltration in the treatment groups (*MCh* and PR treated) when compared with the UC and UCC groups as shown in Figure 1D and 1E. All treatment groups had significantly lower histological scores than that observed in the UC group (Figure 1F).

The effect of *MCh* marmalade on the mRNA levels of inflammatory cytokines/chemokines/transcription factors (such as IFN-γ, IL-1β, IL-6, IL-23, TNF-α, CCL-17, and NF-kB) was also determined throughout in this study (Table 3). IFN-γ, IL-1β, IL-6, Il-23, TNF-α, CCL-17, and NF-kB mRNA levels were increased significantly, from 1.38-2.23-fold, in the UC group compared to the control, as shown in Table 3. On the other hand, treatment with *MCh* reduced mRNA expressions significantly, from 2.12-3.54-fold, concerning the UC rats (Table 3). Group V rats treated with the standard drug (PR) also shown decreased levels in IFN-γ, IL-1β, IL-6, Il-23, TNF-α, CCL-17, and NF-kB, as shown in Table 3.

To determine the extent of the ameliorative effect of *MCh* marmalade at the protein level of the selected genes, proteins were initially resolved on polyacrylamide gels and then analyzed on immunoblots probed with different antibody preparations (Figure 2). The densitometric scanning of western blot results showed that the protein levels of inflammatory cytokines/chemokines/transcription factors (IFN-γ, IL-1β, IL-6, IL-23, TNF-α, CCL-17, and NF-kB) were increased significantly, from 1.46-5.50-fold, in the UC group compared to the NC group (Table 4). On the contrary, treatment with *MCh* reduced protein levels of these cytokines/chemokines/transcription factors significantly, from 1.31-3.22-fold, concerning the UC rats (Table 4). Besides, the treatment with *MCh* and PR reduced the protein levels significantly compared with the UC group, as shown in Figure 2.

DISCUSSION

Genetic, environmental, and especially immunological factors are thought to play an essential role in the pathogenesis of ulcerative colitis. Therefore, drugs targeting immuno-inflammatory pathways have been used in the treatment of UC for the last 15 years. Although many new drugs have been developed for this purpose, aminosalicylates and corticosteroids are the most commonly used drugs in the treatment of UC. However, alternative plant-based therapies are also used by the public in the treatment of the disease. Plants have natural potentials to promote healing mechanism with fewer side effects.[21,43] The simplicity of establishing experimental UC model in rats has enabled the investigation of various agents in the treatment of this disease. In our country, *M. charantia* is widely used in the treatment of peptic ulcer and UC.[24,32]

In this study, molecular mechanisms of the potential therapeutic effect of *MCh* in TNBS-instigated colitis model were investigated. The most important and useful aspects of the TNBS-instigated colitis model is the similarity of inflammation to IBD.[44,45] Moreover, the changes in body weight and weight loss in the TNBS-instigated colitis model are shown to arise as similar to human UC.[46,47] In our study, it was observed that there was a significant decrease in mean body weight in the TNBS-instigated experimental UC group compared to the NC group in accordance with the literature. In addition, both *MCh* and PR treatment not only restored body weight loss but also causes a significant increase in body weight, which might be considered as a sign of recuperation (Table 2).

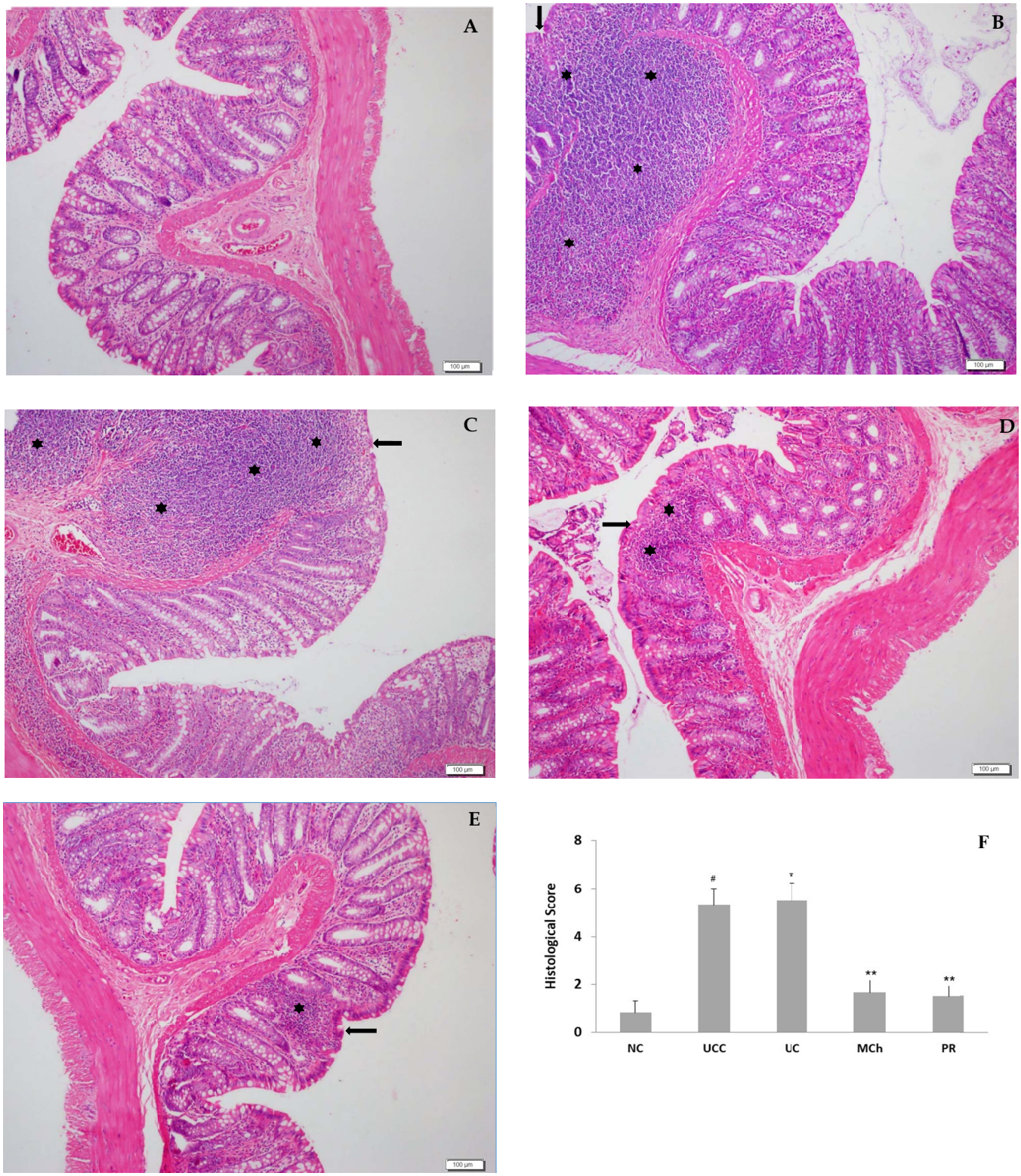

Figure 1: Histopathological images of colon tissues stained with H&E. (A) The appearance of normal colonic mucosa in the normal control group. (B and C) Inflamed ulcerative colitis mucosa in colitis control and colitis groups, associated with mucosal layer destruction, crypt damage and intense inflammatory cell infiltration in the mucosa and submucosa. Colitis tissue treated with (D) *MCh* (4 g/kg/day) and (E) PR (2 mg/kg/day) showing improved histopathological signs of colon damage including the decrease of infiltration areas in the lamina propria layer of mucosa and loss of crypts seen less than colitis group. (★: infiltration areas, ➞ : loss of crypts). (F) Histopathological analysis showed tissue degradation. $^{\#}P < 0.001$ *vs.* the control group; $^{*}P < 0.01$, $^{**}P < 0.001$ *vs.* the model group.

As shown in Table 2, blood serum AST, ALT and LDH activities were increased in the colitis group compared to the control rats. The elevated activities of these serum marker enzymes are indicative of loss of functional integrity in liver.[48–50] The absence of any increase in the levels of these enzymes in the treatment groups implies that *MCh* does not pose any toxicity, particularly to tissues such as erythrocytes, heart muscle, liver and lungs.

Microscopic assessment of H&E stained sections showed healthy histological structure in the control group. The mucosal surface was covered with a single-layer prismatic epithelium containing multiple goblet cells. When H&E stained sections from UC and UCC groups were examined, histopathological changes, such as the presence of diffuse areas of inflammation and loss of cryptic structures, were detected as reported by different researchers.[35,51–53] In addition, thinning of the mucosal layer, erosion and deterioration were observed in the surface epithelium covering the mucosa. However, when UC and UCC groups were compared with the treated with *MCh* and PR treated group, it was found that there was a decrease in the areas of inflammatory cell infiltration. The observed anti-inflammatory and healing effects of *M. charantia* may be attributed to hindering leukocyte infiltration and preventing edema as reported by other studies.[54–56]

IL-6 produced in response to TNF and IL-1 is one of the crucial cytokines acting in the inflammatory response and cancer pathogenesis.[57,58] It is known that IL- 6 and IL-1 released by active macrophages are increased in UC, CD and experimental colitis models.[59–62] Similarly, other studies have shown that levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are elevated in IBD and are directly proportional to the severity of inflammation and levels of these cytokines.[1,21,63,64] The present findings that the *MCh* treatment reduced IL-1β and IL-6 mRNA and protein levels 2.10- and 2.90-fold and 2.62- and 2.78-

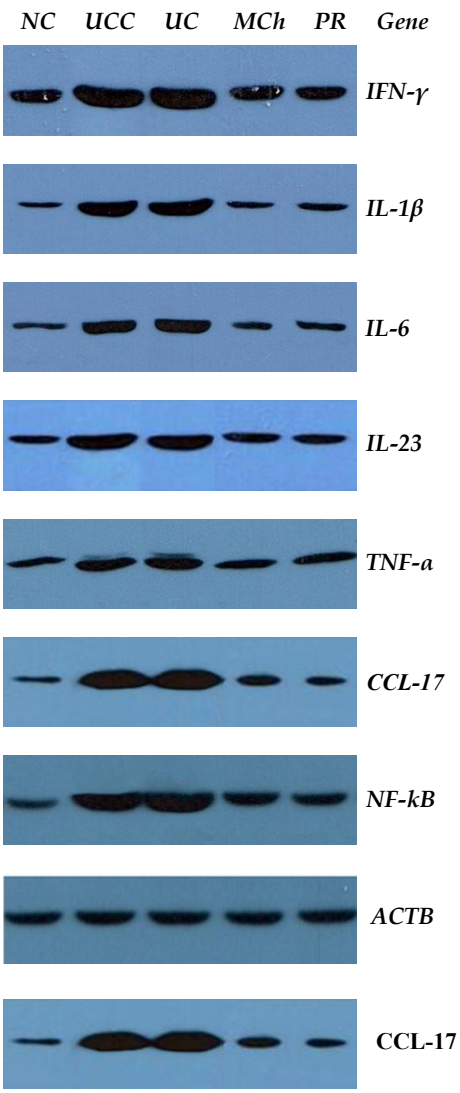

Figure 2: Quantification of proteins in rat colon tissues from various groups by western blot analysis. Rats were treated and proteins were extracted as described in Materials and Methods section. The proteins were separated by SDS-PAGE and western blot analysis was performed as described. Each lane contained 100 μg protein. Proteins were detected using chemiluminescent substrate and bands were visualized and recorded using a DNR Light IS Pro Image Analysis System.

Table 2: Blood serum LDH, AST, and ALT enzyme activities in control, colitis, and treated rats

Groups	Enzyme Activities (U/L)			Body Weights (g)	
	LDH	AST	ALT	Begin	End
Normal control	608 ± 96	36 ± 08	30 ± 6	236 ± 30	235 ± 30
UC control	1503[a] ± 125	82[a] ± 12	85[a] ± 13	226 ± 7	221 ± 11
UC	1516[a,b] ± 166	85[a,b] ± 15	85[a] ± 13	228 ± 30	204 ± 28[a]
MCh-treated	610[a,b] ± 90	25[a,b] ± 02	23[a,b] ± 02	238 ± 27	249 ± 28[a,b]
PR-treated	580[a,b] ± 67	37[a,b] ± 05	35[a,b] ± 07	257 ± 17	266 ± 24[a,b]

[a]Significantly different from the respective control value $P < 0.05$

[b]Significantly different from the respective UC control value $P < 0.05$

fold, respectively, further support the anti-inflammatory action of *MCh*.

TNF-α is vital in the host's normal response to viral, bacterial and parasitic infections, but insufficient or overproduction is harmful to the host.[65] In IBD, TNF-α act as the main constituent and increased colonic TNF-α expression in TNBS-instigated colitis model is well documented.[18,21,63,66,67] Increased activation of innate and adaptive immune system cells in IBD causes increased cytokines of TNF-α, IL-1β and IL-23/Th17 pathway.[68] IL-23 is a member of a small family of pro-inflammatory cytokines and plays a vital role in the pathogenesis of many immune-mediated inflammatory diseases, including IBD.[69–72] Numerous studies have shown that IL-23 is essential for the development of IBD.[73–75] Significant reductions in levels of TNF-α, IFN-γ and IL-6 pro-inflammatory cytokines have been identified in IL-23 deficiency.[72–74] The current study manifested that *MCh* treatment significantly reduced the TNBS-instigated TNF-α inductions in rats, which is a well-known and significant player of systemic inflammation.

Another cytokine, interferon-gamma (IFN-γ) secreted by Th1 lymphocytes, is involved in the pathogenesis of colitis.[18,76,77] When intestinal epithelial cells are damaged, IFN-γ aggregates in the damaged intestinal mucosa to participate in epithelial immune response.[18,78] Anti-IFN-γ antibody treatment significantly reduced the damage in the TNBS-colitis model.[79] In our study, colonic IL-1β, IL-6, IL-23, TNF-α, and IFN-γ protein and mRNA expression levels were significantly higher in the colitis group compared to the control group in accordance with the literature. The treatment with *MCh* fruit marmalade has also optimally reduced the TNBS-induced levels of inflammatory mediators, IL-1β, IL-6, IL-23, TNF-α, and IFN-γ, which are comparable to those achieved by the standard drug (PR) treatment.

In inflammation, blood chemokines lead to the passage of leukocytes into the tissue and accumulation and activation at the site of inflammation.[80] CCL-17 is a C-C chemokine and expressed in the thymus.[14] While CCL-17 shows upregulation in stress, injury or inflammation, overexpression is seen in autoimmune disorders including UC and CD.[13,81,82] In human CCL-17 is induced by IFN-γ and TNF-α stimulation.[13] CCL-17 was the highest increased gene (5.5-fold and 2.23-fold for protein and mRNA level, respectively) in our study and returned to almost control values with the *MCh* treatment.

Table 3. The expression level of the selected genes in the control, colitis, and treatment groups

Genes	NC	UCC	UC	NC *vs.* UC	MCh	PR	UC *vs.* MCh
IFN-γ	100 ± 3	151[a] ± 4	151[a] ± 6	1.51x ↑	56.34[a,b] ± 5	56.1[a,b] ± 2	2.68x ↓
CCL-17	100 ± 6	220[a] ± 4	223[a,b] ± 5	2.23x ↑	63.0[a,b] ± 4	60.0[a,b] ± 7	3.54x ↓
TNF-α	100 ± 5	137[a] ± 6	141[a,b] ± 4	1.41x ↑	63.8[a,b] ± 7	65.1[a,b] ± 5	2.21x ↓
Il-23	100 ± 3	134[a] ± 2	138[a,b] ± 5	1.38x ↑	65.6[a,b] ± 4	53.4[a,b] ± 7	2.12x ↓
IL-1β	100 ± 5	178[a] ± 8	183[a,b] ± 5	1.83x ↑	62.6[a,b] ± 7	56.5[a,b] ± 3	2.92x ↓
IL-6	100 ± 2	151[a] ± 5	158[a,b] ± 4	1.58x ↑	75.2[a,b] ± 3	77.2[a,b] ± 6	2.10x ↓
NF-kB	100 ± 3	169[a] ± 1	172[a,b] ± 4	1.72x ↑	61.8[a,b] ± 2	61.3[a,b] ± 2	2.78x ↓

NC: normal control; UCC: ulcerative colitis control; UC: ulcerative colitis; MCh: *M. charantia* treated; PR: prednisolone treated.

Control was taken as 100%

[a]Significantly different from the respective control value $P < 0.05$

[b]Significantly different from the respective UC control value $P < 0.05$

Table 4. Relative expressions of the selected genes at the protein level in the control, colitis and treatment groups

Proteins	NC	UCC	UC	NC *vs.* UC	MCh	PR	UC *vs.* MCh
IFN-γ	100 ± 7	201[a] ± 9	198[a,b] ± 8	1.98x ↑	103[a,b] ± 5	97[a,b] ± 6	1.91x ↓
CCL-17	100 ± 8	555[a] ± 11	549[a,b] ± 9	5.50x ↑	170[a,b] ± 6	117[a,b] ± 7	3.22x ↓
TNF-α	100 ± 4	143[a] ± 9	146[a,b] ± 8	1.46x ↑	111[a,b] ± 7	107[a,b] ± 8	1.31x ↓
IL-23	100 ± 8	221[a] ± 8	205[a,b] ± 10	2.05x ↑	118[a,b] ± 5	103[a,b] ± 7	1.74x ↓
IL-1β	100 ± 4	282[a] ± 8	317[a,b] ± 13	3.16x ↑	114[a,b] ± 7	159[a,b] ± 4	2.78x ↓
IL-6	100 ± 5	227[a] ± 10	241[a,b] ± 5	2.40x ↑	92[a,b] ± 7	121[a,b] ± 8	2.62x ↓
NF-kB	100 ± 6	257[a] ± 10	305[a,b] ± 6	3.06x ↑	186[a,b] ± 4	144[a,b] ± 9	1.64x ↓

NC: normal control; UCC: ulcerative colitis control; UC: ulcerative colitis; MCh: *M. charantia* treated; PR: prednisolone treated.

Control was taken as 100%

[a]Significantly different from the respective control value $P < 0.05$

[b]Significantly different from the respective UC control value $P < 0.05$

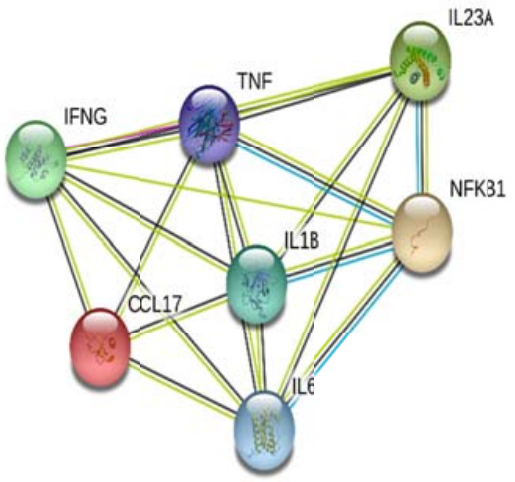

Figure 3: A typical association network of IL-1β, IL-6, IL-23, IFN-γ, TNF-α, NF-kB, and CCL-17 in STRING. IL: interleukin; IFN-γ: interferon γ; TNF: tumor necrosis factor α; NF-kB: nuclear factor kappa B

NF-kB is a protein complex that controls transcription, cytokine production associated with immunity and inflammation and cell viability[15,83]. It is found in almost all animal cell types and is involved in the regulation of cellular responses to many stimuli, such as stress.[84,85] In the literature, experimental studies are emphasizing the importance of NF-kB in the pathogenesis of IBD.[86] Our results were in agreement with the literature, wherein an up-regulation of NF-kB mRNA and protein levels in experimental colitis,[86] whose mRNA levels were decreased to even lower than the untreated control level with *MCh* treatment.

Inflammation is the physiological response against infectious agents, which is also involved in the pathophysiologies of many diseases.[18,87] The inflammatory stimuli cause activation of NF-kB signaling, and it acts as a transcription factor for different pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12, IL-23, TNF-α, and IFN-γ.[18,88] Therefore, suppression of NF-kB signaling would be one of the therapeutic approaches to alleviate inflammation. In our experiment, we administered *MCh* orally after induction of colitis with TNBS and evaluated the therapeutic effect of *MCh*. Experimental colitis model induced with TNBS caused the loss of body weight, histological changes of the colon, and finally, the change of the tissue mRNA and protein levels of inflammatory cytokines (IL-1β, IL-6, IL-23, IFN-γ, TNF-α, and CCL-17). In conclusion, mRNA expressions, histological analyses and changes in protein levels of genes associated with the disease show that *MCh* exhibited the protective effect on TNBS-instigated ulcerative colitis by inhibiting inflammation via NF-kB mediated inflammatory responses and this suppression effect at least associated with the expression of some pro-inflammatory cytokines, including IL-1β, IL-6, IL-23, IFN-γ, TNF-α, and CCL-17.

We have further analyzed the protein-protein interaction network of IL-1β, IL-6, IL-23, IFN-γ, TNF-α, and CCL-17 (Figure 3).[89] It was found that these proteins have more interactions among themselves and involved in cytokine and chemokine mediated signaling pathway as expected. On the other hand, functional annotations of these proteins interestingly yielded another pathway, namely regulation of calcidiol 1-monooxygenase activity. Five out of the seven proteins investigated here were found to be involved in positive regulation of calcidiol 1-monooxygenase. It is also called 25-hydroxyvitamin D_3 1-alpha-hydroxylase or cytochrome P450 27B1 (CYP27B1) and involved in the synthesis of active vitamin D_3 [1a,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$)].[90] It is known that $1,25(OH)_2D_3$ inhibits the production of inflammatory cytokines.[91,92] Studies have shown that induction of colonic CYP27B1, enhancing the local production of $1,25(OH)_2D_3$, is a protective mechanism for colonic inflammation.[93,94] Collectively, these data suggest that *MCh* may exert its anti-inflammatory action through inducing the production of $1,25(OH)_2D_3$ so as to protect the mucosal barrier and decrease colonic inflammation though it might be an exaggerated inference but highly probable in the light of current literature. Further studies are required for a better understanding of the role of either CYP27B1 or $1,25(OH)_2D_3$ in the pathogenesis of UC.

Our data presented here demonstrated compelling pieces of evidence that the *MCh* marmalade is an efficient alternative and complementary therapeutics for the treatment of ulcerative colitis by alleviating the inflammation in colonic tissues. It suppressed the inflammation induced by TNBS and ameliorated the tissue damage, which was positively reflected in the preservation of animals' body weight and lower histochemical scores along with a reduction in inflammatory cytokine/chemokine productions. Additionally, *MCh* marmalade effects were in many characteristics equivalent to those attained by the standard drug (PR) treatment.

CONCLUSION

In conclusion, *M. charantia* is an essential alternative therapeutic or prophylactic agent for ulcerative colitis due to its low cost and lack of side effects as well as its ease of use. However, it should be kept in mind that individual dose adjustment is essential, and further pharmacokinetics and pharmacodynamics investigations are required to prevent possible drug and diet interactions. Besides, further supportive evaluation is required to elucidate the exact

mechanism of *MCh* as an anti-inflammatory agent in the management of IBD.

Conflict of Interest

The authors declared that they have no conflict of interest.

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