Indian Journal of Experimental Biology
Vol. 63, April 2025, pp. 323-330
DOI: 10.56042/ijeb.v63i04.15399

Effect of St. John's Wort (*Hypericum perforatum* L.) on colonic inflammation and tissue damage in a rat model of TNBS-induced colitis

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Received 30 November 2024; revised 14 February 2025

Chronic inflammatory bowel diseases lack clear aetiology and effective treatments, highlighting the need for novel therapeutic approaches like St. John's Wort. This study investigated the effects of oral St. John's wort (SJW) administration on inflammation, immune responses in the rat colonic mucosa, and blood cytokine levels, using three different doses. One group was separated as a control. For colitis model, a single dose of 2,4,6-trinitrobenzene sulfonic acid (TNBS) was administered directly into the colon. Then, the rats were divided into eight groups: four groups observed for 3 days and four groups observed for 7 days. All groups received SJW exposure in different doses (none, 100, 500, or 1000 mg/kg/day). Serum levels of TNF-alpha, IFN-gamma, IL-4, IL-10 and IL-13 were assessed together with tissue macroscopic/microscopic evaluation and tissue total (anti)oxidant measurements. Macroscopic scoring showed healing rates of colonic mucosa reaching up to 52% in the acute term and 33% in the chronic term. Tissue oxidative stress index was higher both in acute and chronic term of the model, but TNF-alpha, IL-4 and IL-10 were especially prominent in the serum at the chronic term of the disease. They have been regulated by oral SJW treatment; microscopic findings and scoring also supported the beneficial effect SJW. Intracolonic intervention of TNBS induces chronic systemic inflammation, as evidenced by changes in serum cytokine levels. SJW, even when taken orally as a food supplement, can influence cytokine pathways, promote the ulcer healing. This indicates a potential reduction in drug requirements.

Keywords: Inflammatory bowel disease model, Cytokine modulation, Rational drug therapy, Oxidative stress biomarkers, Colonic inflammation, Rat model

In inflammatory bowel disease (IBD), the mucosal tissue of the intestine becomes damaged and inflamed. The exact cause of IBD is unknown, but it likely involves a combination of genes, environment, and immune system dysfunction[1,2]. Cells called neutrophils and macrophages become activated in the inflamed gut tissue of IBD patients. These cells produce excessive amounts of reactive oxygen species (ROS), leading to increased oxidative stress[3]. The uncontrolled activation of the immune system in chronic IBD inflammation also leads to the secretion of various immune response messengers called cytokines. These cytokines, including Tumour Necrosis Factor-alpha (TNF-alpha), Interferon-gamma (IFN-gamma), and Interleukins (IL-2, IL-4, IL-10 and IL-13), are crucial for communication between activated immune cells and other cells in the bowel[4]. Therefore, inhibiting these cytokines could be a valuable strategy for reducing the disease burden on patients or treating IBD.

Hypericum perforatum also referred to as St. John's wort (SJW) shows many effects such as wound healing[5], antidepressant[6,7], anticonvulsant[8], antibacterial[9], antiviral[10], anti-inflammatory[11] and gastroprotective[12]. SJW contains several active components, including hypericin, hyperforine, flavonoids and flavonoid derivates, xanthone derivates and biapigenin[6]. SJW are commonly used in folk medicine in Turkey. It, mainly dissolved in olive oil, is applied over the wounds on skin or taken orally in ulcer treatment. It has been suggested that SJW reduces the area of the surgical wound and increases tissue regeneration[5,13].

In our previous study, Dost *et al.* demonstrated that intraperitoneal administration of SJW extract has a protective effect on colonic tissue due to its antioxidant properties[13]. Furthermore, the current research investigates the effects of orally administered SJW at different doses on serum cytokine responses and histopathological features of the colonic mucosa.

Materials and Methods

Animals

Four month old adult male Wistar albino rats (240±25 g; n=70) were obtained from the Experimental Animal Centre of Aydin Adnan Menderes University, Aydin, Turkey. All animal care and experimental procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Aydin Adnan Menderes University Ethical Committee for the ethical care and use of animals in research (ADU-HADYEK; 2009/009). Rats were kept in conventional room with controlled light (12:12, dark: light), temperature (22±1 °C), relative humidity (40-50%) and ventilation (15 air changes per hour). They had free access to standard laboratory feed and water *ad libitum*. They were allowed to adapt to their environment for 2 weeks prior to the experiments.

Colitis induction

To induce colitis, rats fasted overnight and underwent bowel cleansing. Under ketamine and xylazine anaesthesia (50 mg/kg and 5 mg/kg, respectively), a single dose of 0.8 mL 37% ethanol (v/v) containing 25 mg 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma) was administered directly into the colon using a polyethylene catheter inserted 8 cm from the anus.

Treatment groups

Animals were divided in to nine groups: Control group (1), no colitis, no treatment; TNBS group (2), colitis was induced by intrarectal administration of 0.8 mL TNBS, followed by a three-day oral administration of 0.5 mL DMSO (the vehicle for dissolved SJW) to the colitis group; SJW treatment groups (3,4,5), colitis was induced by intrarectal administration of 0.8 mL TNBS, then SJW was given orally at different doses (100, 500, or 1000 mg/kg/day) for 3 days; TNBS group (6), colitis was induced by intrarectal administration of 0.8 mL TNBS, followed by a seven-day oral administration of 0.5 mL DMSO (the vehicle for dissolved SJW) to the colitis group; SJW treatment groups (7,8,9), colitis was induced by intrarectal administration of 0.8 mL TNBS, then SJW was given orally at different doses (100, 500, or 1000 mg/kg/day) for 7 days.

At the end of the study, blood was taken from the heart by midline laparotomy, serum was separated and stored for cytokine analysis. The distal 10 cm of colon was removed, cut open by longitudinal incision, and rinsed with saline. Macroscopic scoring of colonic damage was performed using the 0-4 scoring system described as previously published (Table 1A)[14]. After macroscopic examination, proximal half of the intestinal segment from each rat was stored -80ºC for biochemical analyses. The other half was fixed for a histopathological evaluation.

Measurement of serum cytokine levels

Serum levels of TNF-alpha, IFN-gamma, IL-4, IL-10 and IL-13 were quantified using enzyme-linked immuno-sorbent assay (ELISA) kits according to the manufacturer's instructions and guidelines (Biosource International, Camarillo, CA).

Measurement of tissue total oxidant status (TOS) and total antioxidant status (TAS) activity

Tissue levels of TOS and TAS were assessed in colonic homogenates using a novel automated colourimetric method devised by Erel (Rel Assay Diagnostics kits, Mega Tip, Turkey)[15,16]. TOS findings were expressed in micromolar hydrogen peroxide equivalent per liter (μmol H2O2 Eq/L), whereas TAS levels were denoted in millimoles of Trolox equivalent per liter (mmol Trolox Eq/L). The ratio of TOS to TAS constituted the oxidative stress index (OSI) and was computed as follows: OSI (arbitrary unit) = TOS (μmol H2O2 Eq/L /TAS (μmol Trolox Eq/L) × 100[17].

Histological analyses

Distal colon of the rats was fixed in 10% neutral buffered formalin and taken into routine tissue follow-up. After this process, 4 μm thick sections were prepared by rotary microtome from the tissue samples embedded in paraffin blocks. These sections were stained with haematoxylin and eosin (H&E) and evaluated under a light microscope (BX51, Olympus, Tokyo, Japan) at ×10, ×20 and ×40 magnifications. Semi-quantitative scoring was performed based on the criteria used in a previous study by Appleyard and Wallace (Table 1B & 1C)[18]. Because of the short duration of TNBS application, we did not assess muscle thickness in the colon. Appleyard & Wallace evaluated colonic muscle thickening after six weeks of TNBS administration. Our study, however, only

Table 1A — Criteria for macroscopic scoring of colonic mucosa[14]

Macroscopic morphology	Colitis score
No macroscopic changes	0
Mucosal erythema only	1
Mild mucosal oedema, slight bleeding or small erosions	2
Moderate oedema, bleeding ulcers or erosions	3
Severe ulceration, erosions, oedema, and tissue necrosis	4

lasted three and seven days; therefore, we adapted the microscopic scoring methods from previous study (Table 1C).

Data analysis

Experimental values are expressed as the mean ± standard error on the mean (SEM). One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test were used to compare the study groups. *P* values of less than 0.05 were considered statistically significant.

Results

Macroscopic damage score

Macroscopic scoring of colonic damage was performed on day 3 and 7 after TNBS-induced colitis (Fig. 1A). Macroscopic damage score significantly decreased on days 3 ($P = 0.0138$) and 7 ($P < 0.0001$) in treatment groups compared with TNBS groups (Group 2 and 6) (Fig. 1A)

Table 1B — Criteria for histological scoring of damage[18]

Appearance	Score
Loss of mucosal architecture	0, 1, 2 or 3 (absent, mild → severe)
Cellular infiltration	0, 1, 2 or 3 (absent, mild → extensive)
Muscle thickening	0, 1, 2 or 3 (absent, mild → extensive)
Crypt abscesses formation	0 or 1 (absent or present)
Goblet cell depletion	0 or 1 (absent or present)

Table 1C — Modified criteria for histological scoring of damage without muscle thickness

Colitis score	Loss of mucosal architecture	Cellular infiltration	Crypt abscesses	Goblet cell depletion
0	Absent	Absent	Absent	Absent
1	Under 5%	Mild	Present	Present
2	5-10 %	Moderate	Present	Present
3	Above 10%	Extensive	Present	Present

Blood cytokine parameters

Serum TNF-alpha level significantly increased in the SJW (1000 mg/kg/day) group compared with colitis, 100 and 500 mg/kg groups at 3 days ($P = 0.0005$). At the seventh day, TNF-alpha level was higher in colitis group (colitis-7) and SJW significantly decreased this parameter in all treated (100, 500 and 1000 mg/kg/day) groups ($P = 0.0001$) (Fig. 1B & Table 2).

Although IFN-gamma level tends to increase in the treatment groups on the third day, it was not statistically significant. There was no difference between groups on day 7 (Fig. 1C & Table 2).

IL-4 and IL-10 levels did not change on day 3, but level of IL-4 and -10 increased in colitis (colitis-7) group on the seventh day. IL-4 and IL-10 were significant decrease in all treatment groups compared with the colitis group on 7th day ($P = 0.0227$, $P = 0.0129$, respectively) (Fig. 1D, 1E & Table 2). IL-13 level was increased on the seventh day compared with third day, but there was no significant difference between groups (Fig. 1F & Table 2)

Colonic tissue total oxidant and antioxidant parameters

At the third and seventh days, the TOS activity of colitis groups was higher than control groups. The TOS level significantly decreased in the 100 mg/kg SJW

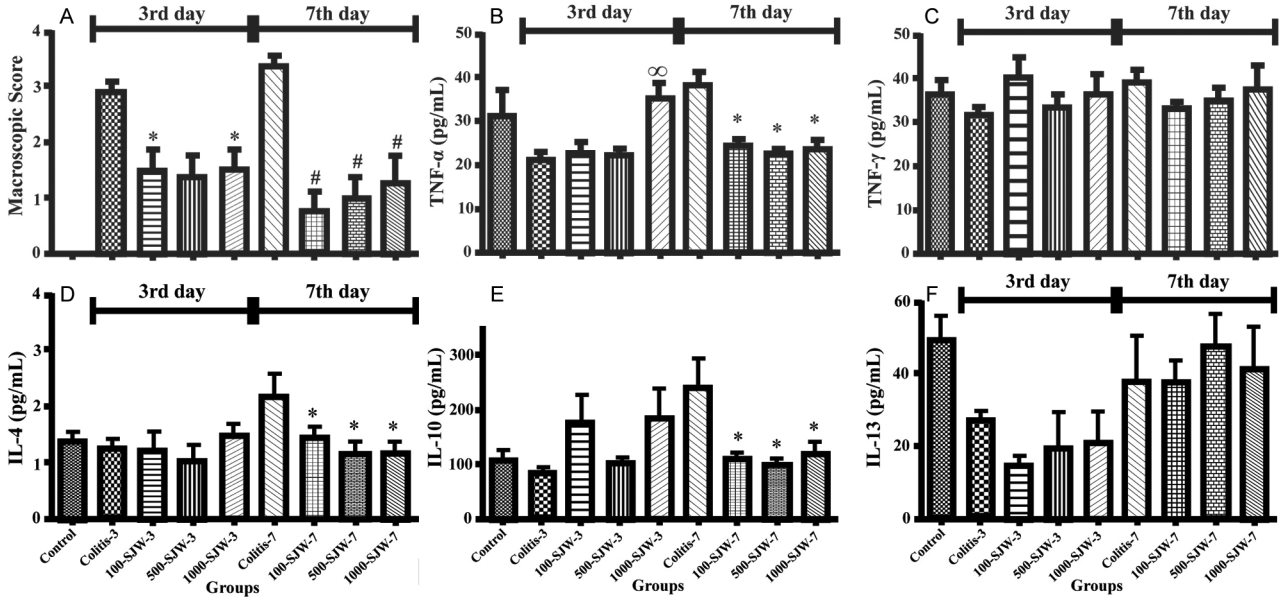

Fig. 1 — Macroscopic damage score and serum cytokine levels of all groups. Data are expressed as Mean ± SEM.

Table 2 — Serum cytokines and tissue TOS and TAS levels of all experimental groups

Groups (mg/kg/day)	TNF-alpha (pg/mL)	IFN-gamma (pg/mL)	IL-4 (pg/mL)	IL-10 (pg/mL)	IL-13 (pg/mL)
Control	31.57±5.90	36.77±2.92	5.32±0.65	109.93±14.39	49.57±6.67
Colitis (3 day)	21.68±1.39	30.85 ±1.82	4.89±0.64	099.69±14.37	27.68±2.57
Colitis (3 day) + SJW 100	22.90±2.55	40.71±4.15	4.87±1.19	178.10±50.54	15.12±1.95
Colitis (3 day) + SJW 500	22.28±1.62	33.69±3.03	6.83±2.81	097.92±11.77	20.11±11.19
Colitis (3 day) + SJW 1000	35.60±3.35*	37.12±4.03	5.07±0.89	130.47±15.31	21.43±8.25
Colitis (7 day)	38.40±3.04	37.97±2.64	7.37±1.26	229.40±49.37	38±12.76
Colitis (7 day) + SJW 100	24.78±1.36**	36.48±2.20	5.71±0.68$^{\#}$	109.93±8.29$^{\#\#}$	38.42±5.90
Colitis (7 day) + SJW 500	22.92±0.57**	36.30±2.67	5.12±0.56$^{\#}$	125.55±30.06$^{\#\#}$	45.68±11.14
Colitis (7 day) + SJW 1000	24.20±1.74**	36.85±4.47	5.83±1.69$^{\#}$	121.33±13.63$^{\#\#}$	41.98±11.38

[Values presented as mean ± SEM from 6-8 rats. TNF-alpha: Tumour Necrosis-alpha; IFN-gamma: Interferon-gamma; IL: Interleukin; TOS: Total Oxidant Status; TAS: Total Antioxidant Status; SJW: St. John's wort. *The SJW (1000 mg/kg/day) group compared with colitis, SJW 100 and 500 mg/kg/day groups at 3 days (P = 0.0005); **The all SJW (1000, 500 and 100 mg/kg/day) groups compared with the colitis group at 7 days (P = 0.0001); $^{\#}$The all SJW (1000, 500 and 100 mg/kg/day) groups compared with the colitis group at 7 days (P = 0.0227); $^{\#\#}$The all SJW (1000, 500 and 100 mg/kg/day) groups compared with the colitis group at 7 days (P = 0.0129)]

Table 3 — OSI index (TOS/TAS×100)

Groups	TOS (H2O2 Equiv./L)	TAS (Trolox Equiv./L)	OSI-index (Arbitrary Unit)
Control	11.20	642.50	1.74
Colitis (3 day)	19.84	514.29	3.86
Colitis (3 day) + SJW 100 mg/kg/day	14.68	684.00*	2.15
Colitis (3 day) + SJW 500 mg/kg/day	34.49	682.50*	5.05
Colitis (3 day) + SJW 1000 mg/kg/day	21.25	718.75*	2.96
Colitis (7 day)	11.20	642.50	1.74
Colitis (7 day) + SJW 100 mg/kg/day	30.18	808.18	3.73
Colitis (7 day) + SJW 500 mg/kg/day	18.70	723.64	2.58
Colitis (7 day) + SJW 1000 mg/kg/day	23.26	578.33**	4.02

[*The all SJW (1000, 500 and 100 mg/kg/day) groups compared with the colitis group at 3 days (P = 0.0041); **The SJW 500 mg/kg/day group compared with the colitis group at 7 days (P = 0. 0.0402). OSI: Oxidative Stress Index; TOS: Total Oxidant Status; TAS: Total Antioxidant Status]

group on day 3 (P = 0.0037), and the SJW (100 and 1000 mg/kg/day) groups on day 7 (P = 0.0259). Tissue oxidative parameters of the groups are given in Table 3. The TAS activity of colitis group was lower than that of the control group at day 3, but was found to be increased at day 7. On the third day, tissue TAS activity in the all treatment (100, 500 and 1000 mg/kg/day) groups were found to be statistically significantly higher than the colitis group (P = 0.0041). On the seventh day, although tissue TAS decreased in the treatment groups compared with colitis (colitis-7) group, there was only significant in the SJW (500 mg/kg/day) group (P = 0.0402).

Oxidative stress index (OSI) is a new indicator parameter of oxidative stress levels. The OSI level was found increased in the colitis groups on the third and seventh days. The OSI level decreased in the SJW (100 and 1000 mg/kg/day) groups, but only 500 mg/kg/day group was increased compared with the colitis group on days 3 and 7 (Table 3).

Histopathological evaluation

The histopathological examination of control and colitis groups (at days 3 and 7) are given in Fig. 2, 3 & 4. Microscopic scoring of histopathological lesions are shown in Table 4. Results of pathological evaluation was consistent with the macroscopic appearance. This examination revealed mucosal inflammation. Multiple ulcerations were also noticed. In the submucosa, multifocal areas of inflammation and ulceration were present and condition was oedematous. Ulcer did not exceeded the muscularis mucosa on the 3rd day but showed progress to muscularis propria on the 7th day.

Discussion

IBD patients suffer from diarrhea, abdominal pain, and bleeding[2,19]. Medications like corticosteroids, aminosalicylates, antibiotics, immunosuppressive drugs, and monoclonal antibodies can limit these inflammatory symptoms[1,2]. However, conventional treatments can cause side effects, prompting patients to seek alternative options. Some plant-based food supplements have been proposed and used to help IBD patients, potentially decreased the dose of conventional medications as adjuvant treatment. Previously, our laboratory research found that administering SJW

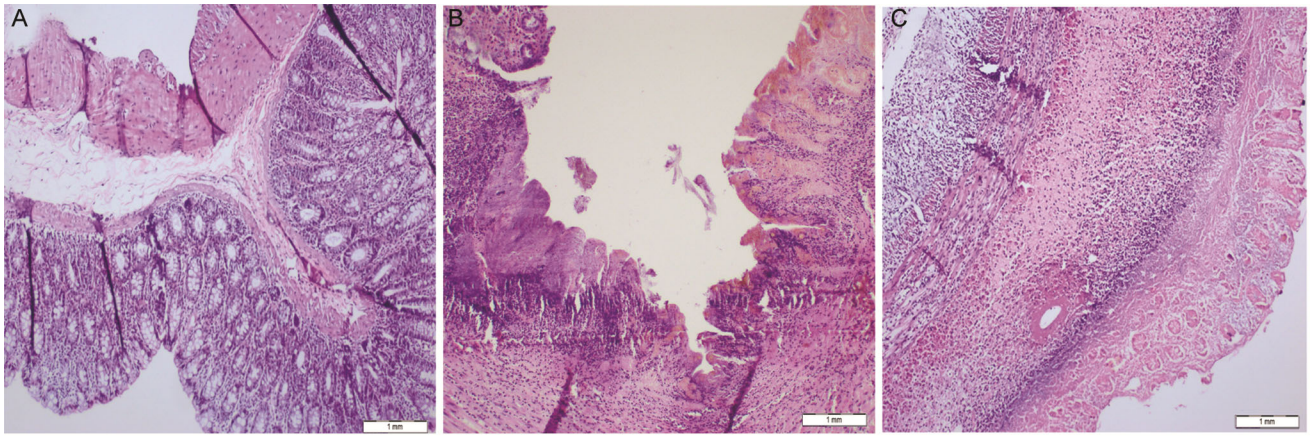

Fig. 2 — (A) Normal colonic mucosa of rat (control group); (B) Histopathological evaluation of distal colon at the third day after TNBS administration. The ulcer has not exceeded the muscularis mucosa (necrotic structure and development of colitis; colitis-3); (C) Histopathological evaluation of distal colon at the seventh day after TNBS administration. The ulcer has exceeded the muscularis propria (worse development of colitis than on day 3; colitis-7). [×100, H&E]

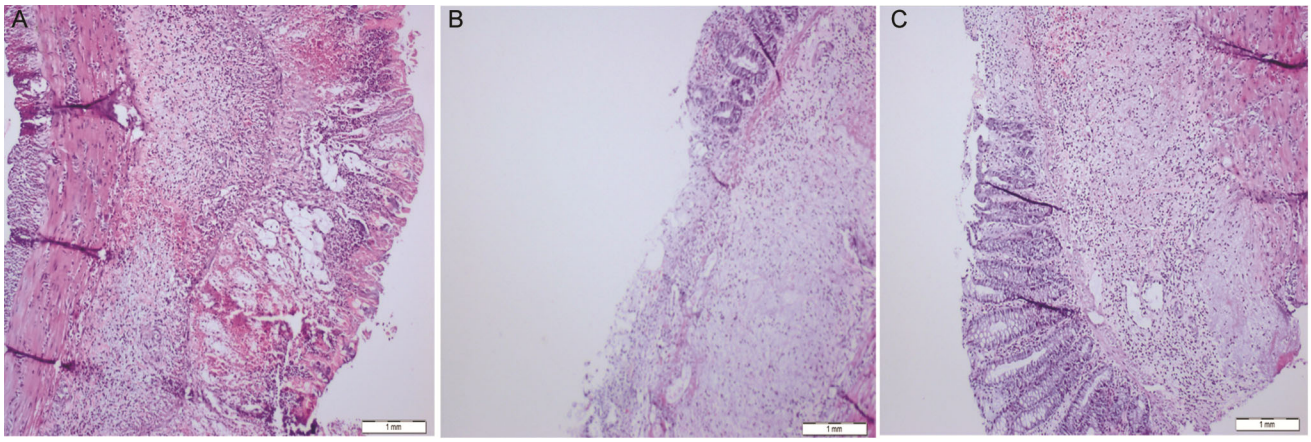

Fig. 3 — Third day after St. John's wort (SJW) administration. (A) 100 mg/kg/day-ulcer existing in the submucosa surface (100-SJW-3); (B) 500 mg/kg/day-ulcer existing in the submucosa, mild epithelial regeneration (500-SJW-3); (C) 1000 mg/kg/day-ulcer existing in the submucosa, mild epithelial regeneration (1000-SJW-3). [×100, H&E]

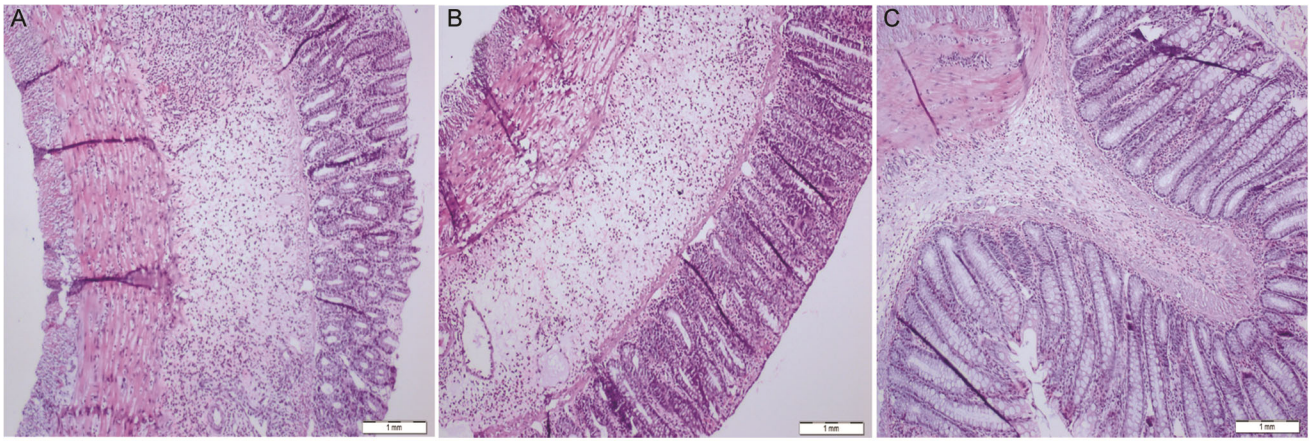

Fig. 4 — Seventh day after St. John's wort (SJW) administration. (A) 100 mg/kg/day-epithelial regeneration, submucosal edema and chronic inflammation (100-SJW-7); (B) 500 mg/kg/day-epithelial regeneration, submucosal edema and chronic inflammation (500-SJW-7); (C) 1000 mg/kg/day-mild submucosal edema and mild inflammation (the best healing ratio among the seventh day groups; 1000-SJW-7). [×100, H&E]

Table 4 — Microscopic scoring of histopathological lesions in all groups

Groups	Loss of mucosal architecture	Cellular infiltration	Crypt abscesses	Goblet cell depletion
Control	0	1	0	0
Colitis (3 day)	3	2	1	1
Colitis (7 day)	3	3	1	1
Colitis (3 day) + SJW 100mg/kg/day	1	2	1	1
Colitis (3 day) + SJW 500mg/kg/day	1	2	1	1
Colitis (3 day) + SJW 1000mg/kg/day	1	1	1	1
Colitis (7 day) + SJW 100mg/kg/day	1	2	1	1
Colitis (7 day) + SJW 100mg/kg/day	1	2	1	1
Colitis (7 day) + SJW 100mg/kg/day	1	1	0	1

[SJW: St. John's wort]

intraperitoneally decreased oxidants in colonic tissue[13]. In this current study, we further investigated the effects of SJW, but this time administered orally in three different doses. Oral administration is preferred by patients for daily intake of supplements. Similar to our current study, Liu *et al*. have compared the rhubarb's oral and intraperitoneal routes effects, because the high molecular weight of polysaccharides of rhubarb may make it difficult for absorption by the digestive tract of rats through oral administration[20]. Additionally, the digestive system presents challenges for delivering medications. Stomach acid, pancreatic and gallbladder enzymes, and the liver (first-pass effect) can easily break down many chemicals, even beneficial ones. Factors like gastrointestinal *p*H, transit time, and temperature can act as barriers, affecting the bioavailability of chemicals. Therefore, this study aimed to assess the effects of orally administered SJW in three different doses and revealed that SJW has a beneficial effect in reducing the severity of clinical symptoms, promoting macroscopic healing of the colonic mucosa, similar to previous study[13].

Histological evaluation supports these findings. At day 3, ulcers were confined to the mucosa, not extending beyond the muscularis mucosa. By day 7, ulcers had progressed to involve the muscularis propria. Appleyard & Wallace assessed colonic muscle thickening after six weeks of TNBS administration [18]. However, our study only lasted three and seven days; therefore, we modified the microscopic scoring from previous paper, excluding muscle thickness (Table 1C). Treatment with different SJW doses at acute stage induced mild epithelial regeneration, the amelioration rate was up to 52% in this stage of macroscopic score. Microscopic score in the high doses SJW group decreased from 7 to 4 in the acute phase. Additionally, the high dose of SJW partially reduced submucosal edema and inflammation in the colonic tissue, decreasing the microscopic score from 8 to 3 and achieving a macroscopic healing rate of up to 33% in chronic phase. We assume this beneficial effect will reduce the need for corticosteroids and other conventional medicines as an adjuvant treatment.

In addition to the macroscopic evaluation, this study investigated serum cytokine levels, which differs from our previous study[13]. It has been stated that TNF-alpha is highly important mediators in ulcerative colitis[19]. Yousefi-Ahmadipour *et al*. detected high level of pro-inflammatory cytokine TNF-alpha and anti-inflammatory cytokine IL-10 on the 9th day of colitis [21]. This study in agreement with our results that TNF-alpha and IL-10 in serum were more increased at the 7th day of our study, than on the 3rd day. Considering that, macroscopic scoring of the 7th day of TNBS induction is higher than the 3rd day due to ulcer progression to the muscularis propria on the 7th day, this phase of the animal model may be called the chronic term. It has been shown that tissue nitric oxide activity was strongly increased in the colitis at day 3 and decreased to normal level at day 7[13]. This results though that different mediators are responsible for the tissue inflammation at the beginning and the late phase of the disease.

We hypothesize that measuring serum levels of TNF-alpha, IF-gamma, IL-4, IL-10, and IL-13 later in the disease course, when ulcers penetrate deeper, might be more informative. As tissue damage progresses, released cytokines could potentially enter the bloodstream, leading to more prominent systemic effects in the chronic phase. It has been shown that IL-4 level has decreased in isolated peritoneal macrophage of rats[20], did not change in colonic supernatant of male BALB/c mice[2] on the 6th day of TNBS colitis; but in our study we found its level increased in serum. Therefore, by measuring serum cytokine levels after TNBS-induced colitis, this

animal model allows us to investigate systemic inflammation particularly after the 7th day of the study. Colitis induction did not trigger serum IF-gamma levels significantly in both acute and chronic term in our study. This result is also consistent with a previous study, which suggested that IF-gamma may not play an important role in TNBS induced colitis in mice[22]. Both IF-gamma level and its mRNA expression were unchanged in colonic supernatant of male BALB/c mice on the 6th day of TNBS colitis[2]. Contrary, when the macrophages were isolated from the peritoneal exudates of TNBS colitis of rats, IF-gamma has been found high at the 6th day of colitis[20]. This result suggested that IF-gamma secretion related to tissue macrophage, not available in the serum.

It has been shown that IL-13, Th2 secretion factor, both level and mRNA expression were significantly high in colonic supernatant of male BALB/c mice on the 6th day of TNBS colitis[2]. Similar to IF-gamma, its level was not detectable in serum. Since it is an anti-inflammatory cytokine, it could be involved in our study's 33% mucosal healing observed on day 7. SJW has in regulating cytokines in the late term of the disease. Results of SJW-treated groups on serum cytokines were inconclusive at the first 3 day of the disease; but clearly serum levels of cytokines are regulated on a well-developed disease model in chronic phase without dose-dependently, even 100 mg/kg per day was effective.

Meanwhile, OSI is taken from the colonic tissue was elevated in both acute and chronic term of this study. OSI has been calculated as the parameters of oxidative balance of body[23]. We have shown that tissue OSI has been decreased in both acute and chronic term by SJW 100 and 1000 mg/kg doses. Interestingly 500 mg/kg increased OSI. This dose of SJW increased TOS level on the 3rd day and it seems, TAS consumed when the disease progress to the 7th day, therefore, an increment of OSI has been determined. Tissue oxidant and antioxidants has to be in a balance at the body, as we mentioned before, several different mediators can join the different steps of the disease. We, here, only were determined total oxidants and total antioxidant level of the tissue. Individual measurements of the inflammatory mediators and their regulation by the dose of SJW can further understand this dose response of the tissue.

Conclusion

Intracolonic intervention of TNBS may trigger a systemic inflammation on the 7th day, as evidenced by changes in serum cytokine levels. This study suggests that. This finding could be beneficial for patients seeking alternative management options for IBD in their daily lives. This should be taken into account when considering reductions in drug use.

Acknowledgements

This work was supported by Research Fund of the Aydin Adnan Menderes University, (Project Number: TPF-10026). We are appreciative of Indena S.p.A (Italy) for *Hypericum perforatum* extract.

Conflict of interest

The authors declare that they have no competing interests.

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