RESEARCH PAPER

An experimental model on the protective impact of nanosilymarin on ulcerative colitis induced by TNBS in rats: The inclusion of TLR4 / NF-κB pathway

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ABSTRACT

Objective(s): The present work aimed to assess the protective impacts of nanosilymarin or silymarin on colitis induced by TNBS in rats.

Materials and Methods: Induction of acute colitis was conducted by rectally administering 2 ml of TNBS solution. About 2 hr followed by induction of colitis, the rates were given dexamethasone (2 mg / kg), normal saline, silymarin (50, 100, and 200 mg / kg), and nanosilymarin (50,100, and 200 mg / kg) orally for two weeks. Damage was assessed at the macroscopic and microscopic levels. MPO enzyme activity was measured using biochemical technique and also ELISA kit was used to measure tissue levels of TNF-α and IL-1β and Western blot analysis was utilized to study the expression level of P65 TLR4 and pNF-κB proteins.

Results: According to the results, dexamethasone (2 mg / kg) and nanosilimarin (200 mg / kg) reduced tissue damages than the TNBS group (P<0.001). Moreover, these drugs reduced MPO activity (P<0.001) and levels of TNF-α and IL-1β (P<0.001) in colon tissue than the TNBS group and also the expression of p65 TLR4 and pNF-κB proteins was decreased when in comparison to the TNBS group.

Conclusion: It is proposed that nanosilimarin reduce colon inflammation in TNBS-induced experimental colitis by inhibition of the TLR4 / NFκB molecular pathway.

Keywords: *Colitis, Nanosilymarin, TLR4/NF-κB, TNBS*

How to cite this article

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INTRODUCTION

In inflammatory bowel disease (IBD), the gastrointestinal tract (GI) frequently inflamed due to an abnormal immune response to the intestinal microflora. Inflammatory bowel disease includes two types of intestinal disease divided based on the location of the injury and its severity (mild, moderate, or severe). The first disease is ulcerative colitis (UC), which causes widespread inflammation of the large intestine and often affects the rectum, but may spread to the sigmoid or even the entire colon. The second disease is Crohn's disease (CD) causing transmural ulcers of the gastrointestinal tract, often involving the ileum (1). The cause of IBD is not fully distinguished to date. However, various factors are involved in causing the disease, such as genetics, pathobionts (which are members of the gut microbiota that appear to play a major role in the pathogenesis of IBD) (2). Pattern recognition receptors (PRRs) in intestinal epithelial cells (IEC) identify common signal molecules in gut commensal microflora known as pathogen-associated molecular patterns (PAMP) (3). Among various kinds of pattern recognition receptors (PRRs) are Toll-like receptors (TLRs). These receptors can identify PAMPs (pathogen-associated molecular patterns). This receptor family is related to the antifungal extracellular receptor Toll, which was initially discovered in Drosophila (4). one of the significant

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isoform of the TLR family in mammals is TLR4 that is a critical for detecting different PAMPs like bacterial lipopolysaccharide (LPS) (5). Many ligands such as LPS, some viral proteins, polysaccharides, low-density lipoproteins and heat shock proteins activate this receptor (6). After the activation of TLR4 by PAMPs in the intestine, a conformational change and dimerization has occurred followed by recruiting the myeloid differentiation primary response gene 88 (MyD88) that lead to NF-κB p65 activation and the generation of inflammatory cytokines for instance TNF-α and chemokines (7). As a result, colitis is related to an excessive stimulation of the TLR4/NF-κB signaling pathway (8). Milk thistle gets its name from the white veins that run across its big, thorny leaves. The plant's seeds are used to extract silymarin, one of the active compounds in milk thistle. Antioxidant properties are thought to be present in silymarin. Oral capsules, tablets, and liquid extracts of milk thistle are available. The supplement is mostly used to treat liver problems. silymarin has been proven to have anti-inflammatory activities (9). Nanosilymarin is a silymarin derived product registered as (Sinalive®) as an oral softgel by Nanotechnology Research Center of Mashhad University of Medical Sciences, Mashhad, Iran (IRC:1228225765). Nanosilymarin soft gels contain 80 mg of silymarin in nanomicelle form. Silymarin has a hydrophobic nature and therefore has low oral absorption and bioavailability. Furthermore, a study has revealed greater bioavailability and efficacy of nanosilymarin compared to silymarin powder (10, 11). The goal of this study was to see if nano-silymarin possesses anti-inflammatory effects by inhibiting the TLR4-connected NF-κB signaling pathway in TNBS-induced rat colitis.

METHODS AND MATERIALS

Chemicals

Hexadecyl trimethyl-ammonium bromide (HTAB) and TNBS and O-dianisidine dihydrochloride were bought from Sigma Chemical Co (St Louis, MO). Diethyl ether and formalin solution 35% w/w were bought form Merck (Darmstadt, Germany). Nanosilymarin (Sinalive®) was provided as a present from Nanotechnology Research Center of Mashhad University of Medical Sciences, Mashhad, Iran.

Animals handling and hosting

54 furnished male Wistar rats with the weight of 180± 220 g were achieved from the Animal Center of Pharmacology Department, Medicine School, Tehran University of Medical Sciences, Tehran, Iran. The rats were kept in a controlled environmental situation. Their cages are made of ordinary polycarbonate and the animals were kept at the temperature of 20 to 23 degrees Celsius and relative humidity of 50 to 60 percent. The day / night cycle (12 hr/ 12 hr), as well as free access to tap water and chow pellets in standard plastic bottles was given. The ''Principles of Laboratory Animal Care" were followed in all animal research. (Publication by NIH 82-23, and revised in 1985 and implemented further in 1996). Moreover, the tests were certificated by Tehran University of Medical Sciences ethical committee, number (55-66-02), 30 Sep 2020.

Experimental colitis induction

In a nutshell, the rats were fasted for 24 hr before to colitis induction, although they had unrestricted access to tap water. Diethyl ether was used to gently to anesthetize rats during the experiment. Then, we used 8 cm flexible plastic catheter with a 2 mm outer diameter to insert 2 mL of diluted TNBS solution into the rectum.

Experimental design

54 adult rats were classified into 9 groups which were 6 rats in each group: (It is noteworthy that the rats were selected randomly). The control group: without colitis induction and only received 0.2% tween80 in normal saline daily. The TNBS group: with colitis induction by using TNBS but was not treated and only received 0.2% tween80 in normal saline daily. The dexamethasone group: with colitis induction by using TNBS and also was treated by receiving dexamethasone at a dose of 2 mg / kg daily. The silymarin 50 group: with colitis induction by using TNBS and also was treated by receiving silymarin at a 50 mg / kg dose daily.

The silymarin 100 group: with colitis induction by using TNBS and also was treated by receiving silymarin at a 100 mg / kg dose daily. The silymarin 200 group: with colitis induction by using TNBS and also was treated by receiving silymarin at a dose of 200 mg / kg daily. The nano-silymarin 50 group: with colitis induction by using TNBS and also was treated by receiving nano-silymarin at a 50 mg / kg dose daily. The nano-silymarin 100 group: with colitis induction by using TNBS and also was treated by receiving nano-silymarin at a dose of 100 mg / kg daily.

The nano-silymarin 200 group: with colitis induction by using TNBS and also was treated by receiving nano-silymarin at a dose of 200 mg / kg daily.

On the first day Colitis induction was done and cures were continued for one week after that. The rats were cured with normal saline, sylimarin, nanosilymarin, and dexamethasone by oral

gavage. The silymarin and nanosilymarin doses were selected based on the former work (12).

Evaluation of macroscopic damage

Animal's scarification was done by CO_2 tank 24 hr later the last cure. The colon's last 8 cm was ruptured, longitudinally opened, and cleaned by ice cold normal saline. An observer who was not informed of the therapy graded the macroscopic damage appearance using the following scale: 0 = without any macroscopic change, 1 for only erythema of mucosa, 2 for mild mucosal edema, slight erosion, or slight bleeding, 3 for moderate edema, bleeding erosions or ulcers and 4 for severe ulceration, edema, erosions, and tissue necrosis (13). A surgical transparent tape was used to measure the ulcer area, which was positioned on a bright and transparent sheet. The number of cells obscuring the ulcerated region of each specimen was tallied, and each cell on the tape had a 1 mm^2 area. Each tissue specimen's ulcer index was determined by multiplying the ulcer score by the ulcer area. The ulcer index was determined using the formula below. Ulcer area (cm^2) + macroscopic damage score = Ulcer index. Tissue specimens were divided into two pieces, including one half preserved in 10% formalin for histology analysis. Liquid nitrogen was used for freezing of the remaining half of the tissue for biochemical, ELISA and western blot analysis.

Assessing the histological score

Paraffin wax was used to embedded dehydrated tissues and then processed, rupture into four µm-thick slices, and tainted with hematoxylin and eosin (HE). Colonic segments were inspected for indications of inflammation by a histopathologist blinded to that therapy. On a scale of 1–5, the severity of inflammation was assessed (Table 1) (14).

Western blot analysis

Western blot (protein blot or immune blot) is a very powerful and important method for identifying proteins based on the initial antibody-antigen reaction. This technique consists of 5 steps:

Step 1: First the colon tissues segments were homogenized by using lysis buffer (20 mM Tris–HCl pH 8.0, 10 % glycerol, 1 % NP-40, 137 mM NaCl, 10 lg/ml aprotinin, 1 mM phenylmethyl sulfonyl fluoride, 0.5 mM sodium vanadate, and 1 lg/ml leupeptin). Then the laser solution was centrifuged at 4 ° C for 20 min using a 12,500 g centrifuge to collect the supernatant. Finally the supernatant was transferred to a fresh tube to be kept on ice or at -20 ° C. Total protein concentration was estimated utilizing Micro BCA method (Pierce, Rockford, IL, USA).

Step 2: At this stage, the gel was prepared first. After preparing the gel, the samples were heated with the sample buffer for 5 min at 100 ° C and then injected into the gel wells and electrophoresis was performed under reducing circumstances. Finally, after the completion of electrophoresis, SDS-PAGE electrophoresis was resolved equal quantities (100 µg) of protein from each sample.

Step 3: In the transfer step, the separated proteins on the gel were moved to PVDF paper continuously and wet.

Step 4: After the transfer step, PVDF membranes was placed in the blocker solution for one hour at 25° C. 5% non-fat dry milk and 0.1 percent Tween-20 in Tris-buffered saline were used for this purpose. PVDF membranes in solution of anti-NF-κB p65 (1:200 dilution), anti β-actin (1:200 dilution) antibodies, and anti-TLR4 (1:1000 dilution) (Santa Cruz Biotechnology, USA) was placed on a shaker overnight at 4 ° C to incubate. The membrane was rinsed three times with TBST (Tris-buffered saline Tween-20) for 5 min. The PVDF membrane was immersed in goat antirabbit IgG horseradish peroxidase conjugated (1:10,000 dilution) antibody (Santa Cruz Biotechnology, USA) for 1 hr at room temperature to incubate. The membrane was rinsed with TBST three times for 5 min.

Step 5: Through Western blot detection system (WEST–ZOLR Plus, Intron Biotechnology, UK), antibodies on the membrane pursuant to the manufacturer's instructions were detected. Then the bands were visible on X-ray film (Thermo Scientific, USA). (Note that all of these steps were performed in the dark.) Quantity One 1-D analysis software (Bio-Rad, USA) was used to determine the density of protein bands.

Evaluation of TNF-α and IL-1β levels in Colon tissue

An enzyme-linked immunosorbent assay kit (ELISA) was utilized to evaluate TNF-α and IL-1β levels in colon tissue samples. (It should be noted that the evaluation was performed according to the instructions of the manufacturer (CUSABIO Technology LLC., USA)

In this method, first colorectal tissue samples were homogenized by using ice-cold potassium phosphate buffer (50 mmol/L, pH 6.0) comprising 0.5 percent hexadecyltrimethylammonium bromide. Then the samples were centrifuged for 20 min at 4 ° C. After centrifugation, the supernatants were gathered and kept at 80 ° C. Homogenized specimen supernatant was used for loading the wells precoated with anti-TNF-α monoclonal antibody for 2 hr. After that, for 2 hrs a horseradish peroxidase-

conjugated secondary antibody was used, followed by 30 min of staining with chromogen substrate. Finally, the absorbance was determined at 450 nm.

Biochemical evaluation for MPO

To determine myeloperoxidase (MPO) activity in tissues, the technique explained in previous works was used (15). A 0.1-g slice of colonic tissue was homogenized in potassium phosphate (1 ml) (pH = 6) comprising 5% HTAB in a polytron homogenizer ice bath. For 10 s, the homogenate was sonicated in an ice bath before freezing and thawed three times after adding the buffer to the final amount of 5 ml. Then, the homogenate was centrifuged for 15 min at 4 °C at 15,000 rpm. Utilizing phosphate buffer (pH = 6, 50 mM), the supernatant (0.1 ml) was diluted to a final volume of 3 ml, which contains 0.0005 percent H_2O_2 and 0.167 mg/ml odianisidine dihydrochloride. Ultimately, measuring the absorbance fluctuation of the solution was performed at 460 nm via a UV/VIS spectrophotometer (LSI Model Alfa-1502). In units per gramme of moist colonic tissue, myeloperoxidase activity was measured.

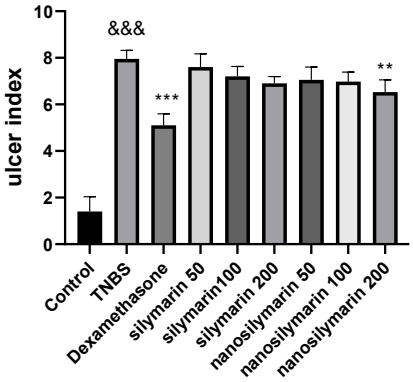

Fig. 1. Effect of TNBS, dexamethasone (2 mg/kg), silymarin (50, 100, 200 mg/kg) and nanosilymarin (50, 100, 200 mg/kg) on ulcer index. Data are expressed as mean ± SEM (n = 6). &&&*P*< 0.001 compared with control group; ***P*<0.01, ****P*<0.001 compared with TNBS group

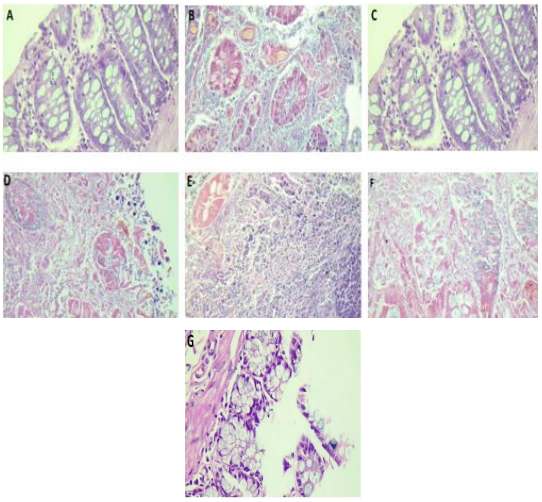

Fig. 2. Histopathological images of tissues in all groups. Hematoxylin and eosin were used to stain colon tissues. Magnifications: × 10. A Control. B TNBS. C Dexamethasone (2 mg/kg). D Silymarin (100 mg/kg). E Silymarin (200 mg/kg). F Nanosilymarin (100 mg/kg). G Nanosilymarin (200 mg/kg)

Statistical analysis

The data were analyzed utilizing GraphPad Prism (Ver.5.04). One-way analysis of variance (ANOVA) was used followed by Tukey's post hoc test for comparing differences between groups and *P*<0.05 was significant. The data were stated as mean ± SEM.

RESULTS

Nanosilymarin evaluation on Ulcer Index

TNBS injection led to a remarkable increase in ulcer index (*P*<0.001) (Fig.1). Contrariwise, dexamethasone (2 mg/kg) and nanosilymarin (200 mg/kg) reduced ulcer index (*P*<0.001, *P*<0.01). Besides, silymarin (50, 100, 200 mg/kg) did not decrease ulcer index meaningfully.

Histologic evaluation of nanosilymarin

Histopathologic images of all groups are presented in Figure 2. In control group mucosa, and other tissue layers such as muscularis propria, submucosal, and serosa were unscathed and no proof of inflammation was found; conversely, after intrarectal administration of TNBS, samples indicated intense injuries, necrosis, and penetration of inflammatory cells. Subsequent to the treatment with dexamethasone (2 mg/kg) and nanosilymarin (200 mg/kg), necrosis, ulceration and inflammatory cell infiltration of colon tissue was reduced. By contrast, silymarin (100 and 200 mg/kg) and nanosilymarin (100 mg/kg) did not improve TNBS-induced damages and necrosis.

Effects of nanoSilymarin on TLR-4 and pNF-κB p65 expression

As shown in Fig, 3A, it was observed that TLR-4 and pNF-κB p65 had low expression in control group. However, accordance the colitis induction by manner of intrarectal injection of TNBS, the expression of pNF-κB p65 and TLR4 noticeably rose (*P*<0.001). Healing with dexamethasone (2

mg/kg) and nanosilymarin (200 mg/kg) caused momentous reduction in the expression of pNF-κB p65 and TLR4 (P<0.001 and P<0.01). However, treating with silymarin 200 mg/kg showed no reducing effects on the expression of pNF-κB p65 and TLR4 (Fig. 3).

Nanosilymarin reduced the level of TNF-α and IL-1β

Figure 4 indicates the variations of TNF-α and IL-1β level after TNBS injection as well as after treatment with dexamethasone, silymarin 200mg/kg and nanosilymarin 200mg/kg. The level of IL-1β and TNF-α noticeably increased later colitis induction (P< 0.001). On the contrary, treatment with dexamethasone (2 mg/kg) and nanosilymarin

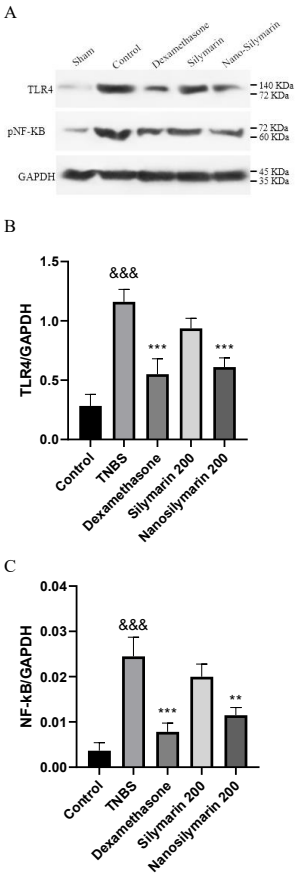

Fig. 3. A Western blot pictures show TLR4 and pNF-κB p65 and β-actin expression in four groups. B Effect of dexamethasone (2 mg/kg) and silymarin (200 mg/kg) and nanosilymarin (200 mg/kg) on TLR4 expression in colon tissue. Data are represented as mean ± SEM (n = 6). &&&p < 0.001 compared to control group and ***P<0.001 compared with TNBS group. C Effect of dexamethasone (2 mg/kg) and silymarin (200 mg/kg) and nanosilymarin (200 mg/kg) on pNF-κB p65 expression in colon tissue. Data are expressed as mean ± SEM (n = 6). &&&P<0.001 compared to control group and **P<0.001 compared with TNBS group

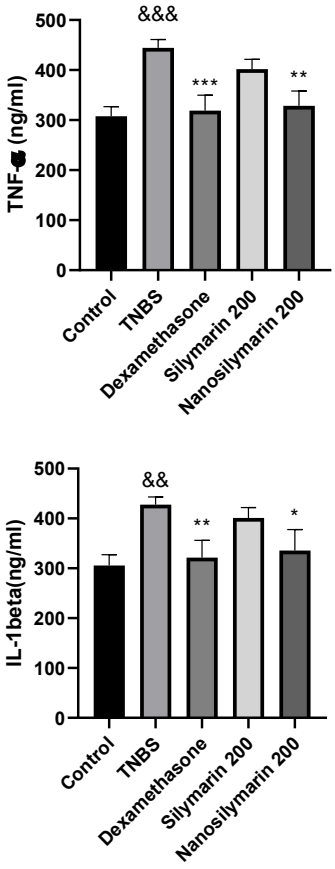

Fig. 4. Colonic tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) levels in rats with TNBS–induced colitis and relative treatments. Data are represented as mean ± SEM (n= 6). &&&P< 0.001 compared to control group. *P<0.05, **P<0.01 and ***P< 0.001 compared to TNBS group

(200 mg/kg) reduced TNF-α and IL-1β level (P< 0.01, P<0.05, and P< 0.001); however, administration of silymarin 200 mg/kg showed no impression on the level of TNF-α and IL-1β compared to TNBS group.

Nanosilymarin reduced MPO level

Evaluations represented that MPO activity noticeably was elevated later colitis induction in TNBS group compared to the control group (P< 0.001). In contrast, MPO activity prominently was decreased, following cure with dexamethasone (2 mg/kg) and nanosilymarin (200 mg/kg) (P< 0.01 and P< 0.001). silymarin 200 mg/kg did not decrease MPO activity in comparison to TNBS group (Fig. 5).

DISCUSSION

According to the results, induction of colitis in rats by using TNBS rectal administration led to bleeding and necrosis in the colon tissue and macroscopic, microscopic lesions, and biochemical vicissitudes.

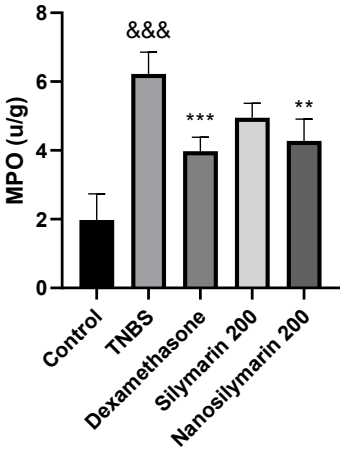

Fig. 5. Activity of MPO enzyme in animals with TNBS–induced colitis and relative treatments. Data are represented as mean ± SEM (n =6). &&&*P*< 0.001 compared to control group. ***P*< 0.01 and ****P*< 0.001 compared to TNBS group

Treatment of these rats with dexamethasone at a dose of 2 mg / kg or nanosilymarin at a dose of 200 mg / kg meaningfully reduced these lesions and biochemical vicissitudes. Induction of colitis enhanced tissue damage such as degeneration of goblet cells and infiltration of inflammatory cells. Conversely, cure with nanosilymarin and dexamethasone improves these lesions. TLR4 and pNF-κB p65 expressions, TNF-α and IL-1β levels, and the activity of MPO enzyme were also increased after TNBS injection, while treatment with nanosilymarin and dexamethasone resulted in reduction of those markers.

IBD is a group of diseases in which the intestines become inflamed for a variety of reasons. Ulcerative colitis (UC) and Crohn's disease (CD) are the most common ones. There are differences between the two diseases, including the location of the lesion and the type of inflammatory changes. In patients with CD, the site of inflammation may be anywhere in the gastrointestinal tract, from the mouth to the anus. Conversely, in the UC, the colon is usually the only place that is affected. However, in some people with UC, the last part of the small intestine called the ileum may also become inflamed (16).

TNBS (trinitrobenzene sulfonic acid) is used extensively to induce chronic and acute colitis in large intestine of rats. The colitis induced by this method has similar properties to human colitis. In this experiment, we used anal administration of TNBS to cause colitis in rats. When colitis is induced, damage occurs in mucosa and under mucosa, and this damage activates inflammatory mediators and TLR4/NF-κB signaling pathway (17).

In a leading study, TNBS (trinitrobenzene sulfonic acid) was used in order to the induction of colitis in the large intestine of rats, destroying the structure of rat colon tissue and causing the infiltration of inflammatory cells (neutrophils) and edema in colon tissues. On the other hand, nanosilymarin reduced histological symptoms such as reducing inflammation, edema and neutrophil infiltration.

There are several pathways involved in IBD pathogenesis. The TLR4 / NF-kB signaling pathway is one of them. TLR4 is a transmembrane protein (a member of the toll-like receptor family). Its activation leads to an NF-kB intracellular signaling pathway that produces inflammatory cytokines and activates the innate immune system. In this pathway, TLR4 first activates myeloid differentiation protein (MyD88), which is an adapter-activating molecule in the TLR signaling pathway. MyD88 then activates NF-κB transcription factor phosphorylation. The phosphorylated form of NF-κB (pNF-κB) is transported to the nucleus thus initiating the transcription of inflammatory cytokine genes like TNF-α, IL-1β and IL-6, which eventually leads to the onset or progression of IBD (2).

Studies show that NF-κB has an important role in IBD. NF-κB is highly activated during disease, as can be seen in rat colitis model. NF-κB has a family of five members, such as p50 and p65. These proteins are homo and heterodimers, in most cells p50 / p65 heterodimer is the main NF-κB complex. If there is not any inflammatory situation, it is inactivated inside cytoplasm p50 / p65 and forms a ternary complex with inhibitory protein IκBα. IκBα inhibitor protein is phosphorylated by enzyme IκB kinase when inflammatory stimulated. With this event, NF-κB p50 / p65 enters nucleus and binds to specific sequences in gene promoter and activates gene transcription. As a result, NF-κB signaling pathway is a dominant mechanism for regulating inflammatory responses by controlling transcription of genes. Therefore, for IBD, activation of NF-κB signaling pathway is very important and inactivation of this pathway reduces inflammation, as well as many drugs such as Sulfasalazine, infliximab that treat IBD (18, 19). Treatment with nanosilymarin resulted in reduction of pNF-κB p65, and TLR4 proteins in the large intestine tissue.

After induction of colitis, the enzyme myeloperoxidase (a proteolytic enzyme weighing 140 kDa and present in neutrophil granulocytes) is released, causing oxidative damage to colon tissue.

The amount of tissue damage is significantly related to the activity of this enzyme (6). This study revealed that cure with nanosilymarin decreased the level of myeloperoxidase activity.

IL-1β and TNF-α are proinflammatory mediators and have a main role in the IBD pathogenesis. (16). This study showed that cure with nanosilymarin reduced TNF-α and IL-1β expressions in colon tissue.

Silymarin (SM), obtained from the plant Silybum marianum using extraction methods, has a variety of flavonolignans, the main of which is silybin. Silymarin is an antioxidant complex that is often used for its antioxidant properties for protective functions (20). Based on the explanations that has been mentioned in the introduction, the results of various studies indicated the anti-inflammatory impacts of nanosilymarin in animal models (21, 22).

CONCLUSION

As a result, TNBS was injected into the rectum of rats to induction of colitis which caused macroscopic and microscopic lesions. Base on the present work, treatment with nanosilimarin reduced these lesions, as well as inflammatory cytokines like IL-1β, TNF- α, and IL-6. In conclusion treatment with nanosilimarin reduced the symptoms of colitis and improved the disease.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

It is confirmed that no known conflicts of interest exist related to this publication. Moreover, this work had no considerable financial support affecting its outcomes.

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