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Original article

Evaluating the Role of Biochanin A in Acetic acid-Induced Colitis in Rats: Involvement of Nitric Oxide Pathway

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Abstract

Background and objectives: Inflammatory bowel disease (IBD) refers to idiopathic chronic and inflammatory bowel disorders such as ulcerative colitis. Considering the lack of definitive treatment and the side effects of existing drugs, finding efficient compounds is needed. Biochanin A has attracted the attention of researchers due to its wide range of medicinal activities. Until now, no study was conducted to evaluate its effects on colitis. Therefore, the aim of this study was to determine the effect of biochanin A on the nitrogen pathway in rats with acetic acid-induced colitis. **Methods:** Male rats were divided into five groups: normal group, negative control group, positive control group, and groups receiving biochanin A (10 and 20 mg/kg). Colitis was induced with 4% acetic acid. Next, the samples were evaluated at different macroscopic and microscopic levels, and biochemical test of superoxide dismutase (SOD) and nitric oxide activity was investigated. **Results:** Macroscopic and microscopic investigations showed that treatment with biochanin A decreased mucosal damage in rats with acetic acid-induced colitis. Biochanin A reduced neutrophil infiltration in the intestinal tissue. It also led to the reduction in nitric oxide and enhancement of SOD in rats. The optimal dose of biochanin A was 20 mg/kg, which had the best effect on reducing inflammation and mucosal lesions in rats. **Conclusion:** Biochanin A, due to its anti-inflammatory effects by reducing nitric oxide and enhancement of SOD and reducing mucosal damage in rats with acetic acid-induced colitis, can be a useful alternative drug for the prevention or treatment of IBD patients.

Keywords: biochanin A; IBD; superoxide dismutase; ulcerative colitis

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Introduction

Inflammatory bowel disease (IBD) is a term for two conditions (Crohn's disease and ulcerative colitis) that are characterized via chronic inflammation of the gastrointestinal tract. Ulcerative colitis is one of the two main forms of IBD and impacts mostly on the rectum and colon. The prevalence of IBD has increased in most regions of the world [1]; therefore, increasing the need for more treatment possibilities [2]. It is identified by continuous ulceration and

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inflammation of the mucosa. It impacts various people worldwide, happening equally between males and females. The exact cause of the disease is unidentified [2,3]. Evidence proposes a genetic predisposition to the illness [4]. Colitis is identified via widespread infiltration of neutrophils, epithelial cell damage, and the formation of crypt abscesses [5].

Infiltration of neutrophils and macrophages into the colon is a significant feature of ulcerative colitis. Activated neutrophils in the intestinal mucous tissue produce reactive oxygen species (ROS) such as superoxide ion, hydroxide radical, and hydrogen peroxide. This leads to the induction of lipid peroxidation, enhancement of the mucosa and blood vessels permeability, and increase of the neutrophil's entry into the mucosal tissue [6]. Oxidative stress seems to play a role in ulcerative colitis. ROS are typically present in colon as a result of cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) activity. Superoxide and hydroxyl radicals are the main mediators of oxidative stress, which play an important role in most diseases [7]. Therefore, the removal of superoxide and hydroxyl radicals is effective in protecting against diseases. The superoxide dismutase (SOD) enzyme activity is decreased in response to the breakdown of superoxide anion produced via lipid peroxidation. Decreased SOD activity may result in some harmful impacts [8]. Acetic acid-induced colitis is normally used in experimental studies to examine novel drugs possibly valuable in IBD treatment [9].

Current anti-inflammatory drugs lead to serious side effects like ulcers and interactions with other medications [10]. Consequently, there is a limitation in using them, particularly in chronic conditions management like colitis. Nature offers various sources for extraction, isolation, and identification of new compounds which might be of advantage in treating various diseases [11]. An example of a promising compound as an anti-inflammatory agent is biochanin A, which is generally extracted from red clover (*Trifolium pretense* L.) [12]. Biochanin A is an isoflavonoid, therefore, similar to other isoflavones, it is used for the treatment of hormonal abnormalities [13]. This compound can play complex roles in regulating body functions by binding to DNA or as a competitive precursor for some enzymes [13]. Antioxidant, anti-inflammatory, anti-infection and anti-cancer properties have been reported for this compound. Biochanin A is present in most commercial compounds containing isoflavonoids [14,15].

In a study performed in 2012 by Qiu et al., it was reported that biochanin A has anti-inflammatory effects by affecting peroxisome proliferator-activated receptors [16]. Also, biochanin A demonstrates anti-inflammatory effects through the suppression of NF-κB as well as the release of inflammatory mediators such as interleukin-1β, nitric oxide, nitric oxide synthase, and prostaglandin E2 in LPS induced via BV2 microglia cells [17]. Biochanin A has also been shown to protect against lipopolysaccharide/D-galactosamine-induced acute liver injury in mice via activation of the Nrf2 pathway. Treatment of mice before lipopolysaccharide/D-galactosamine challenge has led to an increase in SOD levels [18]. Due to the protective effects of biochanin A against inflammation through various mechanisms, the present study was conducted with the aim of evaluating its impact on acetic acid-induced colitis in rats; this is the first study that investigated the effects of biochanin A on ulcerative colitis induced by acetic acid.

Materials and Methods

Ethical considerations

All animals were handled according to the guidelines of the Iranian Council on Animal Care, and the protocol was approved by the Kermanshah University of Medical Sciences Animal Care Committee (Ethic approval code: IR.KUMS.REC.1399.467).

Chemical

Acetic acid, formaldehyde, ketamine, xylazine, and biochanin A were purchased from Merc, Germany. Sulfasalazine, ether, acetone, ethanol, and N-1-naphthylethylenediamine (NED) were obtained from Sigma Chemical, USA.

Animals

In this study, 35 male Wistar rats with a weight range of 200-250 were used. The animals were kept in the animal house under standard conditions for one week in order to adapt to the new conditions, with the temperature of 24±2 °C and the lighting cycle of 12 h light/dark.

The rats were accidentally divided into five groups of seven as follows and received drugs for four days before the induction of colitis.

Group 1: control (animals received distilled water only); group 2: positive control (ulcerative colitis was induced with one mL of 4% acetic acid by rectal route and animals received sulfasalazine orally at a dose of 500 mg/kg for 7 days, 4 days before colitis induction and then 3 days after colitis induction) [19]; group 3: negative control (ulcerative colitis was induced with acetic acid and animals received distilled water for 7 days); group 4: ulcerative colitis was induced with acetic acid and animals received 10 mg of biochanin A per kg of rat weight (63 mg in 6.5 mL) for 7 days, 4 days before the induction of colitis and then 3 days after the induction of colitis [20]; group 5: ulcerative colitis was induced with acetic acid and animals received 20 mg of biochanin A per kg of rat weight (126 mg in 6.5 mL) for 7 days, 4 days before the induction of colitis and then 3 days after the induction of colitis.

Inducing colitis in animals

To induce colitis, 4% acetic acid was used. Before the induction of colitis, the mice were fasted for 24 h and during this time they had only access to water. Then, they were anesthetized by intraperitoneal injection of 10% ketamine and 2% xylazine in a ratio of 9:1.

In the next step, the mice was placed in such a way that the end of the body was slightly higher than the animal's head, and then one mL of 4% acetic acid was slowly injected into the animal's colon within a few seconds. Then, the animal's anus was held with a finger so that the acid does not come out. After 10 min, the animal was put back in the first position and the stomach was gently massaged to help expel the acid. Two h after induction, the administration of drugs continued daily for three days [21-23].

Examining the damage and inflammation of the colon tissue

Macroscopic examination

At the end of the designated days, the rats in different treatment and protection groups were killed by ketamine-xylazine injection and the last seven centimeters of their colons were removed. Then, the size of the wound, hyperemia, and inflammation were evaluated and scored using Gerald's method [24] (Table 1).

Microscopic examination

On the last day of treatment, the last 8 cm of the colon was detached, opened longitudinally and cleaned with normal saline. Microscopic colonic lesions were reported by an observer blinded to the treatments as a semi quantitative scale: 0 = no macroscopic alteration; 1 = only mucosal erythema; 2 = mild bleeding, mild mucosal edema, or mild erosion; 3 = moderate edema, bleeding wounds or erosion; and 4 = severe wound, edema, erosion, and tissue necrosis.

Table 1. Scoring criteria for tissue damage in rats with colitis (Gerald's method)

Scoring criteria for morphological damage	Score
No damage	0
Local hyperemia, without ulceration	1
Linear ulcer without significant inflammation	2
Linear ulcer with inflammation in one area	3
Two or more areas of wound or inflammation	4
Two or more major areas of inflammation and ulceration or one major area of inflammation and ulceration with a length of more than 1 cm along the length of the colon.	5

Also, the wound area was measured by a transparent surgical tape with a 3M^{TM}Transpore^{TM} scale. The size of each strip cell area was considered 1 square mm. The number of cells which covered the damaged area was counted and the wound area was determined for each colon sample in cm^2. Then the wound index was calculated with the following formula for each colon sample:

$$\text{Wound index} = \text{Wound severity} + \text{Wound area } (cm^2)$$

After counting, colon samples were cut into 2 pieces, one piece was fixed in 10% formalin for histological and immunohistochemical examination and the other piece was frozen in liquid nitrogen for ELISA and biochemical investigation [25].

After separating and fixing the samples in 10% formalin, they were subjected to microscopic examination. A pathologist evaluated the prepared slides in terms of the intensity of inflammation, the depth of the lesion and the severity of fibrosis.

Activity of superoxide dismutase (SOD)

SOD assay was performed as follows: first, a number of isolated colon tissues which were kept at a -20°C freezer were used to measure SOD levels. Next, for every 0.1 g of colon tissue samples, 1 mL of distilled water was added and mixed well with a homogenizer, then was placed in a centrifuge for 20 min at 12,000 rpm at a temperature of 4 °C. Finally, the supernatant mixture was used to perform the SOD test [26].

Measurement of nitric oxide

The content of nitric oxide in supernatant was identified as nitrites. First, a number of isolated colon tissues which were kept in at the -20 °C freezer were used for this test. Next, for every 0.1 g of colon tissue samples, 1 mL of distilled water was added and mixed well with a homogenizer; then places in a centrifuge for 20 min at 12,000 rpm at a temperature of 4°C. Finally, the supernatant mixture was used to perform the nitrite test.

In the first step, 100 microliters of the sample was poured into the test tube or well, and then 50 microliters of sulfonamide was added and incubated for 5 min at room temperature in the dark. After adding and mixing 50 µL of NED reagent, the solution was read with a spectrophotometer at a wavelength of 540 nm.

Histopathological scores

Description and scoring of lesions were performed according to the grading criteria explained previously by El-Akabawya and El-Sherifa [27]. In brief, an assessment of histopathological colonic lesions was performed with consideration for the following parameters: morpho-architectural distortion of crypts, cryptitis, ulceration, glandular atrophy, and submucosal edema. Each of those parameters was scored according to the following scale: 0, absent; 1, mild (present in less than 10% of examined tissue); 2, moderate (present in 10%–50% of examined tissue); and 3, intense (present in over 50% of examined tissue). The final scores for each sample were calculated as the sum of those scores.

Statistical analysis

Data analysis was performed (GraphPad Prism 8.0 graphing and statistics software) by one-way ANOVA along with Tukey's multiple comparisons test. In all tests, $p<0.05$ was considered significant. Data are presented as mean ± standard error of the mean (SEM).

Results and Discussion

In the macroscopic examination of the control group, the colon mucosa was totally normal. In the negative control group, intrarectal administration of acetic acid led to adhesions, ulcers, intestinal wall thickening and severe inflammation.

The data obtained from the macroscopic examination after treating rats with different doses of biochanin A (10 and 20 mg/kg) are presented in Figure 1; the results were compared with the positive control (sulfasalazine). The results indicated that 20 mg/kg of biochanin A significantly reduced inflammation and enhanced wound healing in rats with colitis.

Figure 1. Colon tissue isolated from groups receiving treatments (10 and 20 mg/kg of biochanin A) and controls.

Formula was used to calculate the ulcer index in this research [25].

As shown in Figure 2, the administration of acetic acid led to an enhancement in the macroscopic parameter of the wound index in comparison to the control group. On the other hand, the administration of sulfasalazine and biochanin A led to a significant reduction in the wound index, the greatest reduction was for biochanin A at 20 mg/kg.

In the microscopic examination of the control group, there was a normal histological appearance of four layers (mucosa, submucosa, muscular and serous); the surface epithelium, glands, mucous lining and different submucosa and muscular layers did not change. Scattered lymphatic tissue with single or multiple lymph follicles could be seen in the mucous lining. Mucosal folds and goblet cells were normal in number and distribution (Figure 3).

In the acetic acid group, variable areas of severe damage (necrosis) and widespread inflammation were observed. Several areas lacked epithelium and glands and showed submucosal edema and severe leukocyte and reticulocyte infiltration. Intestinal glands (Liber Komen) were destroyed and in the less damaged areas, dilated glands and severe decrease was observed in goblet cells. The muscularis mucosae was destroyed or swollen

and changed in nature. In the acetic acid group, relative increase of the annular and longitudinal muscle layer and the increase of the thickness of the connective tissue of the serous layer were observed.

In the sulfasalazine and acetic acid group, there was moderate to mild damage in small and limited areas, but in most areas, the tissue profile was similar to the control group, but a significant difference was observed compared to the acetic acid group only ($p<0.01$).

In the areas without damage, the surface epithelium and the Lieberkühn glands were normal with more goblet cells. The mucous lining was thin with leukocyte cells and no reticulocyte infiltration. In the submucosa, there were few leukocytes and reticulocytes which were less than the acid group.

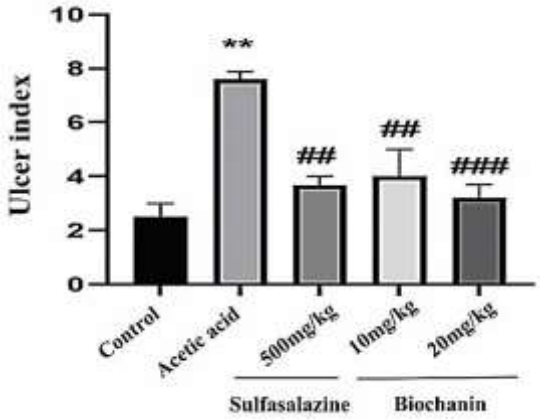

Figure 2. Changes in the average ulcer index in the tested groups; the results are expressed as Mean±SEM of three separate tests. ###: $p<0.001$, ##: $p<0.01$ in comparison to the acetic acid group; **: $p<0.01$ compared to the control group

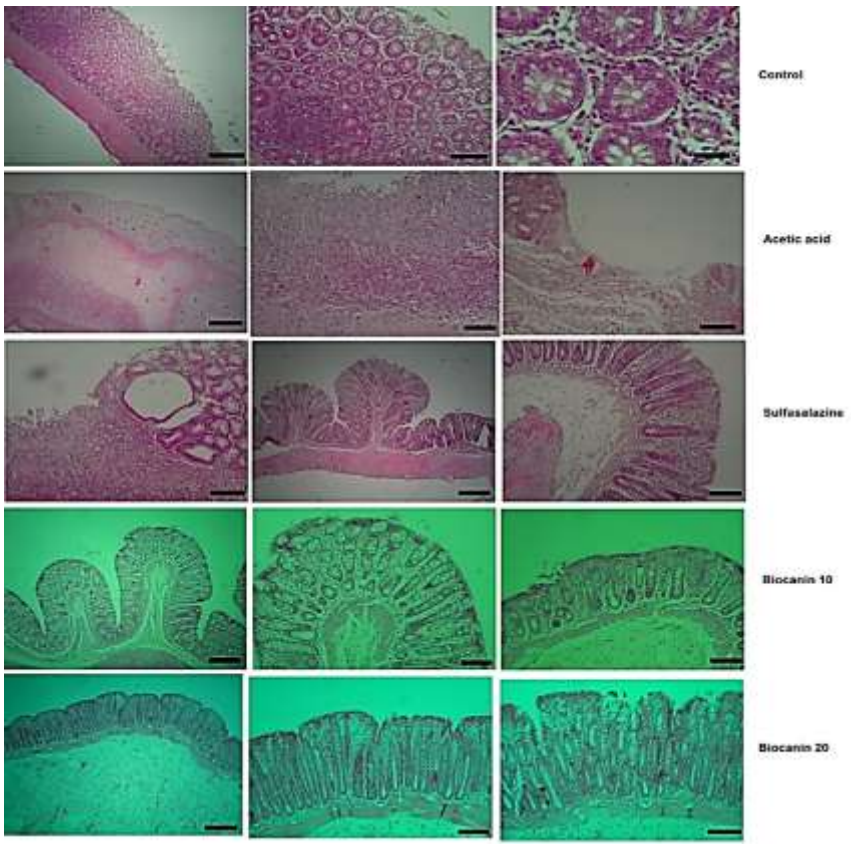

Figure 3. Microscopic view of colon histopathology in control and treatment groups (acetic acid, sulfasalazine, biochanin A 10 and 20 mg/kg). In the microscopic examination of the control group, there was a normal histological appearance of four layers. In the acetic acid group, variable areas of severe damage (necrosis) and widespread inflammation were observed. In the sulfasalazine and acetic acid group, there was moderate to mild damage in small and limited areas, but in most areas, the tissue profile was similar to the control group, but a significant difference was observed compared to the acetic acid group only ($p<0.01$). The group of biochanin A (10 mg/kg) and acetic acid had limited wound areas. In the group of biochanin A (20 mg/kg) and acetic acid, in some areas of the mucosa, there were ulcers and lacked surface and glandular epithelium. H&E staining; scale bar: 150 µm

The group of biochanin A (10 mg/kg) and acetic acid had limited wound areas (without surface epithelium and glands). Most of the areas were normal and with fewer changes compared to the acid group. The mucosa was less thick with slightly enlarged and shorter Lieberkühn glands than the control group with more abundant and larger goblet cells. The mucous lining had low leukocyte infiltration and no reticulocyte infiltration. The submucosa had partial edema and lacked leukocytes and reticulocytes and inflammatory symptoms. The thickness of the muscle layer was significantly increased.

In the group of biochanin A (20 mg/kg) and acetic acid, in some areas of the mucosa, there were ulcers and lacked surface and glandular epithelium. In this group, relative infiltration of leukocytes and reticulocytes were observed, but they were less and more limited than in the acid group. Also, the thickness of the mucus and the length of the glands were increased, but the goblet cells were less, the submucosa had little edema, and no reticulocyte and inflammatory symptoms were seen. There was an increase in the thickness of the muscle layer.

In the term of pathological scores, the highest score of intestinal lesions was observed in acetic acid control group, followed by biochanin A 10 mg/kg groups, respectively (Table 2). In comparison between treated groups with biochanin A, the most protective effects were observed with the administration of 20 mg/kg.

According to Figure 4, it was observed that the level of SOD in the acetic acid group had a considerable reduction in comparison to the control group (***, $p<0.001$). Sulfasalazine was considered as the positive control and after treatment with this drug, the activity of SOD increased significantly, so that the enzyme activity level was almost similar to the control group. In the investigation of the effects of biochanin A, it was observed that the effect of this compound was concentration-dependent, and biochanin A 20 mg/kg showed a significant increase compared to biochanin A 10 mg/kg.

Acetic acid was used as the positive control in this research. The purpose of using a positive control is to ensure the formation of colitis by the released NO. As demonstrated in Figure 5, acetic acid led to a considerable enhancement in nitrite secretion in comparison to the control group, while the addition of sulfasalazine and biochanin A led to a significant decrease in nitrite concentration. The protective effects of biochanin A reached its maximum level at 20 mg/kg and decreased the amount of nitrite to an approximate amount of 10 mg/kg.

Ulcerative colitis is an idiopathic chronic inflammatory disease of the gastrointestinal tract that causes inflammation and ulcers in the large intestine (colon). This disease also has complications such as diarrhea, abdominal pain and cramps, blood in stool, loss of appetite and weight loss. It has no definitive treatment, but to deal with the symptoms and damage of this disease, there are various drugs such as sulfasalazine.

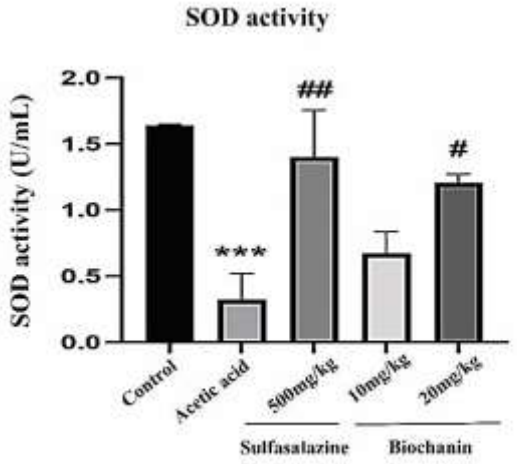

Figure 4. The impact of various concentrations of biochanin A on the activity of SOD enzyme. The results are expressed as mean±SEM of three separate experiments. ##, $p<0.01$ and #, $p<0.05$ in comparison to the acetic acid group; ***, $p<0.01$ in comparison to the control group

Table 2. Histopathological scores of colon in different groups

	Inflammation				Depth of lesion				Fibrosis			
	None	Mild	Medium	Severe	None	Mild	Medium	Severe	None	Mild	Medium	Severe
Acetic acid				++++				++++				+++
Sulfasalazine		++				+			+			
***Biochanin A* (10 mg/kg)**			+++				+++					+
***Biochanin A* (20 mg/kg)**		++				++				+		

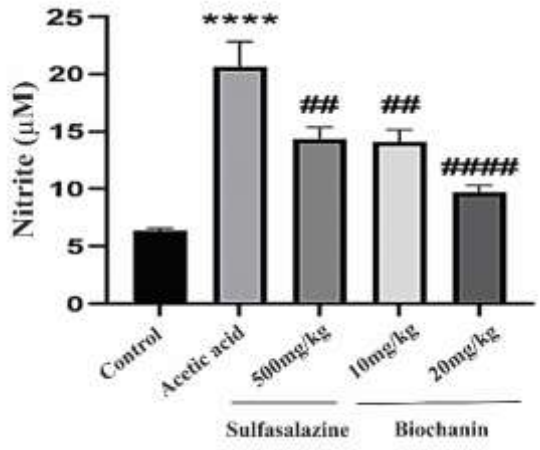

Figure 5. The impact of various concentrations of biochanin A on nitrite levels; Results are presented as Mean±SEM of three separate tests. ##: p<0.01 and ####: p<0.001 in comparison to the acetic acid group; ****: p<0.001 in comparison to the control group

Side effects happen in 31% of patients taking sulfasalazine and include vomiting, nausea, headache, rash, agranulocytosis, fever, pancreatitis, hepatitis, nephritis, and male infertility. Immunomodulators are also associated with considerable side effects, such as pancreatitis, fever, rash, arthralgia, diarrhea, and nausea [28].

Infiltration of neutrophils and macrophages into the colon is a key feature of ulcerative colitis. Activated neutrophils in the intestinal mucous tissue produce and secrete reactive oxygen species (ROS) which cause lipid peroxidation, enhance the permeability of the mucosa and blood vessels, and elevate the neutrophils entry into the mucosal tissue. By affecting the expression of cytokine genes and enzymes involved in the inflammatory response, ROS leads to the destruction of the intestinal wall and cause ulcers, bleeding and diarrhea. Therefore, using antioxidant agents can prevent recurrence [6]. Considering the lack of definitive treatment and several side effects of existing drugs, finding more efficient and safer compounds is needed. One of these compounds with a natural origin is biochanin A.

Biochanin A is an isoflavone mainly found in red clover and like other isoflavones, is used in the treatment of hormonal abnormalities [13]. This compound can have complex roles in regulating body functions by binding to DNA or as a competitive precursor for some enzymes. Antioxidant, anti-inflammatory, anti-infection and anti-cancer properties have been observed for biochanin A, and it is also effective in treating diabetes and reducing blood lipids [13].

Superoxide and hydroxyl radicals are mediators of oxidative stress, which play an important role in most diseases. Therefore, the removal of superoxide and hydroxyl radicals is effective in protecting against diseases. The SOD enzyme activity decreases in response to the breakdown of anion produced by lipid peroxidation. Decreased SOD activity may result in several harmful effects. In the research conducted by Haj Rezaei and his colleagues, the protective effects of biochanin A on ethanol-induced stomach ulcer in rats were investigated. The group treated with biochanin A showed an increase in SOD activity and a decrease in fat peroxidation levels [8]. Biochanin A has also been shown to protect against lipopolysaccharide/D-galactosamine-induced acute liver injury in mice by activating the Nrf2 pathway. Treatment of mice before lipopolysaccharide/D-galactosamine challenge led to an increase in SOD levels [18].

Based on the relation between SOD levels and oxidative stress, in this study, we investigated biochanin A effects on SOD levels. It was found that biochanin A led to a significant increase in SOD level compared to the sulfasalazine group, which indicates the probable antioxidant effect of this compound.

In a study conducted by Jun and his colleagues, the inhibitory effects of several isoflavonoids on nitric oxide production were investigated. In the formation of nitric oxide, biochanin A showed a significant inhibition (62% at 50 mM concentration). It seems that the 5- and 7-dihydroxyl group in the A ring of isoflavones is a key functional group that is responsible for the great suppressive activity of biochanin A in nitric oxide production [29]. In our study, we investigated the effects of biochanin A on nitric oxide levels in rats with acetic acid-induced colitis. The results showed that biochanin A has a greater ability to trap nitrite radicals compared to sulfasalazine.

Due to the great similarity between the model of acetic acid-induced colitis in animals and human colitis, we induced this model of colitis by administration of acetic acid in rats [30]. Induction of colitis by acetic acid is one of the acceptable models, and is a proper example for

studying intestinal inflammation, the pattern of cytokine secretion, adhesion and the use of drugs that affect the immune system [31]. In the acetic acid model, the function of the intestinal epithelial layer is affected, so the role of mast cells is evident in this case. Administration of 4% rectal acetic acid in rats causes scattered inflammation, abrasion and destruction of the wound and increases the weight of the colon tissue in macroscopic examination. According to microscopic and pathological studies, in the acetic acid-induced colitis model, activation of inflammatory and pro-inflammatory cytokines, increased tissue permeability and increased MPO enzyme activity have been observed [32].

Wound severity was used as a simple and useful criterion for the effectiveness of drugs in this method, which can be easily graded by observing erythema, scratches, wounds and tissue necrosis. Since none of the two parameters of wound area and wound severity alone show us a comprehensive evaluation of the effectiveness of the combination, the ulcer index parameter, which is the sum of the two mentioned parameters, is used to complete the macroscopic evaluation [33,34]. According to the results of this study, the ulcer index in the treatment groups has significantly decreased compared to the acetic acid group. In order to confirm the positive effect of biochanin A on ulcer index, pathological studies were conducted, which show inflammatory changes in the colon tissue.

The results of this study indicated that the therapeutic indicators in the biochanin A groups were reduced compared to the acetic acid group, so that in the biochanin A and sulfasalazine groups, only mild damage in the mucosa with partial edema under the mucosa and mild infiltration of neutrophils were observed. While in the acetic acid group, necrosis across the membrane along with abundant infiltration of neutrophil inflammatory cells and significant edema in the submucosa layer were observed. These observations show that the induction of colitis by acetic acid has common tissue aspects with ulcerative colitis in humans and includes mucosal edema, infiltration of neutrophils into the mucosa and submucosa, and ulceration.

The anti-inflammatory effect of biochanin A at both macroscopic and microscopic levels showed that it can be as effective as sulfasalazine, which is the drug of choice for the treatment of the ulcerative colitis.

Conclusion

According to this study, biochanin A has anti-inflammatory effects and reduces neutrophil infiltration in the intestinal tissue. It also led to the reduction in nitric oxide and enhancement of SOD in the colon tissue of rats. Moreover, based on the results obtained from macroscopic and microscopic investigations, treatment with biochanin A decreased mucosal damage in rats with acetic acid-induced colitis. Taken together, biochanin A can be a useful alternative drug for prevention or treatment of patients with IBD. The optimal dose of biochanin A was 20 mg/kg, which had the best effect on reducing inflammation and mucosal lesions in rats.

Acknowledgments

None

Author contributions

Mohammad Reza Morovati and Maryam Ghanbari-Movahed were involved in the conception and design of the study; Mohammad Hosein Frazaei acquired and analyzed data; Atefe Tahani contributed to drafting the article; Sedigheh Asgary contributed to designing of study; Samira Shirooie designed the study and was involved in the analysis of data and critical revision.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

IBD: inflammatory bowel disease; iNOS: inducible nitric oxide synthase; ROS: reactive oxygen species; SOD: superoxide dismutase