Original Research

Protective effects of flavonoids isolated from Agrocybe aegirita on dextran sodium sulfate-induced colitis

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ABSTRACT

Mushroom derived phytochemical has become the promising agent to treat inflammatory bowel disease (IBD). Here, we investigated the effect of flavonoids from Agrocybe aegirita (AAF) on dextran sodium sulfate-induced colitis. Our results showed that flavonoids from Agrocybe aegirita had a certain effect on physical signs in mice (improving the weight loss of mice, reducing the DAI index and the spleen index of mice). AAF could also significantly reduce the shortening of the colon, and improve the level of tissue damage and colon inflammation. Besides, AAF could alleviate the colon inflammatory status including reducing the levels of TNF-α and IL-1β and increasing the levels of IL-10. In addition, AAF significantly promoted the growth of goblet cells and enhance the intestinal barrier function (the secretion of mucin in the colon were increased). In conclusion, flavonoids from Agrocybe aegirita has the potential to relieve the DSS-induced colitis in mice and could be a novel therapy for combating with IBD.

1. INTRODUCTION

Agrocybe aegirita, also known as columnar field head mushroom, is a kind of fungus belonging to Basidiomycetes Agaricaceae, mainly distributed in subtropical and north temperate regions [1]. In Guangchang, Jiangxi, the resources of Agrocybe aegirita are very rich, but its value has not been fully explored and utilized, and it is only at the status of consumption. [2] At present, the research on Agrocybe aegirita mainly focuses on Agrocybe aegirita functional products and Agrocybe aegirita polysaccharide [3, 4]. Agrocybe aegirita polysaccharide has been confirmed to possess certain functions in immune regulation, antioxidant, blood sugar regulation, etc [5–7]. However, Agrocybe aegirita is rich in flavonoids, but its biological activity is rarely reported. Plant-derived flavonoids have been studied to have excellent effects in terms of anti-inflammatory activity [8]. Previous research have reported that resveratrol could be biotransformed by intestinal bacteria such as Bacillus cereus and Rhododendron to produce metabolites such as dihydroveratrol and spruce neosides, which have antioxidant, anti-tumor, and cardiovascular protection effects. [9]. This indicates that the anti-inflammatory effect of flavonoids is closely related to the gut microbiota, and it is reasonable to study its anti-inflammatory activity in a colitis model. Therefore, I chose to use the Inflammatory bowel disease (IBD) model to study the anti-inflammatory activity of Agrocybe aegirita.

Inflammatory bowel disease is an idiopathic intestinal inflammatory disease that mainly affects the normal metabolism of the ileum, colon, and rectum. It can be divided into Crohn's disease (CD) and ulcerative colitis (UC) based on the clinical manifestations [10]. The development of IBD can cause symptoms such as diarrhea, abdominal pain, blood in the stool, and malnutrition, which seriously affects the health of patients. At present, the incidence of IBD is increasing rapidly and has become a global health problem [11, 12]. The treatment of IBD is mainly to improve the symptoms of patients and delay the deterioration of IBD, but it cannot be fully cured [13]. The mainstream drugs for the treatment of IBD include immune preparations, biological preparations and hormones [14]. In addition, surgery, fecal bacterial transplantation, and gene therapy are used to treat IBD as well. The efficacy of these treatments varies greatly among different patients, and can even lead to serious side effects. New treatments for colitis now include the use of functional polysaccharides [15], intestinal flora [16], gene therapy [17], etc. Surgery carries certain risks and is prone to recurrence and other problems after surgery. Fecal bacterial transplantation is a new treatment method, and its therapeutic effect varies greatly for different patients, and it is not universal and difficult to apply universally. The cost of gene therapy is too high and some technical difficulties have not been completely broken through. Functional polysaccharides are difficult to purify [18], and there is a big gap for medical requirements. Thence, finding a new therapy for IBD is urgent. IBD animal...
models can generally be divided into chemically induced models, genetically modified models, and spontaneous animal models [19]. Among them, the chemical induction method with the help of DSS has the advantages of convenient operation and high success rate of stable molding. The principle of DSS-induced colitis is to use chemical stimulation to cause apoptosis of colonic epithelial cells, damage to the mucosal barrier structure, increase the permeability of intestinal mucosa, induce inflammatory cell infiltration, and the expression of inflammatory factors to cause colitis [20]. So, this model is often used to study the effect of natural active products on colitis.

Therefore, we took *Agrocybe aegirita* as the research object, and used modern biotechnology to study the effect of flavonoids of *Agrocybe aegirita* on DSS-induced colitis in mice, and further explore the possible mechanism.

2. MATERIALS AND METHODS

2.1. Materials and reagents

*Agrocybe aegirita* was purchased from Guangchang Industrial Park, Fuzhou, Jiangxi. DSS (molecular weight 9000 Da-12000 Da) was purchased from MP biomedical company. Hematoxylin, eosin dye and AB-PAS staining solution were obtained from Wuhan Sevier Biological Co., Ltd. IL-22, TNF-α and IL-10 enzymes linked immunosorbent assay kit was obtained from Nanjing Formex Biotechnology Co., Ltd.). Rutin standard and other reagents are of analytical grade.

2.2. Animals

Male C57BL/6j mice (18 ± 0.2 g, initial body weight) were obtained from Hunan Slack Scene of Laboratory Animal Co. LTD (Hunan Province, China).

2.3. Preparation of *Agrocybe aegirita* flavonoids

Pretreatment of macroporous resins: 13 macroporous resins were stored in 95% ethanol and then activated. For activation, the resin was firstly soaked in 4% HCl solution for 6 hours, then washed with distilled water to neutrality, and later soaked in 4% NaOH solution for 6 hours. Finally, the resin was washed with distilled water till neutral and stored in distilled water for later use [21].

Preparation of *Agrocybe aegirita* flavonoid extract: pulverize *Agrocybe aegirita* raw material, pass through a 60-mesh sieve, soak in 95% ethanol with a material-to-liquid ratio of 1:8 for 12 hours, repeat twice, degrease and remove small molecules, and evaporate to dryness. Take the dried *Agrocybe aegirita* powder, add 80% ethanol in a 1:20 material-to-liquid ratio, extract at 50°C for 1 h, and repeat the extraction twice; take the supernatant and centrifuge, and store the supernatant in a 4°C refrigerator in the dark [22].

Static adsorption of flavonoids from *Agrocybe aegirita*: take 100 ml of flavonoids from *Agrocybe aegirita*: extract in 5 g of macroporous resin, place it in a constant temperature shaking box at 25°C and 150 rpm and shake for 2 h, and centrifuge to remove the supernatant. Take 100 ml of 80% ethanol for analysis, pour the ethanol into the macroporous resin adsorbed with flavonoids, place it in a constant temperature shaking box at 25°C and 150 rpm/min, shake for 2 h, and centrifuge to remove the precipitate. The obtained supernatant was concentrated to contain a small amount of water, lyophilized, and stored in a -20°C refrigerator in the dark [23].

2.4. Flavonoids Content Determination

Standard curve: 0.2 mg/mL rutin standard substance was prepared with 40% ethanol solution. Draw 0.0 ml, 1.0 ml, 2.0 ml, 4.0 ml, 5.0 ml of rutin standard solution respectively, and diluted to 10 ml volume. 1.0 ml of 5% sodium nitrite solution was added, and mixed solutions were shook well. After standing for 6 minutes, 1.0 ml of aluminum nitrate solution was added and shook well. Consequently, after another 6 minutes standing, 10 ml of 4% NaOH solution was added, and 40% ethanol solution was used to fix the capacity of up to 25 ml. The mixed solution was shook well, and stand for 15 minutes before measured for the absorbance value at 510 nm.

Sample determination: 0.2 mg/mL lyophilized flavonoid sample was prepared with 40% ethanol solution to achieve the sample solution, 1.0 ml of 5% sodium nitrite solution was added to sample solution and shook up. Similarly, after standing for 6 minutes, 1.0 ml of aluminum nitrate solution was added and shook well. Consequently, after another 6 minutes standing, 10 ml of 4% NaOH solution was added, and 40% ethanol solution was used to fix the capacity of up to 25 ml. The mixed solution was shook well, and stand for 15 minutes before measured for the absorbance value at 510 nm [24]. The flavonoids content was calculated according to the standard curve.

2.5. Animal experiment

2.5.1. Animal experiment design

Male C57BL6j mice were acclimatized under conditions at 25°C ± 0.5°C and of 50% ± 5% relative humidity, 12/12 h light/dark cycle for 1 weeks before the commencement of the experiment. After acclimation, the mice were divided into 4 groups (n = 12) randomly, including: 1) normal group, 2) model group, 3) low-dose group of flavonoids from *Agrocybe aegirita* (25 mg/kg BW) and 4) high-dose group of flavonoids from *Agrocybe aegirita* (50 mg/kg BW). Details of the experimental design were shown in Figure 1: Mice in Control group were raised with normal water and feed with no other special treatment. Mice in model group were raised normally for 7 days, and later switched to 3% DSS drinking water for 6 days to induce the colitis. And the last day mice were given normal drinking water. Mice in low or high dose flavonoids were similarly raised normally in the first 7 days, and later switched to 3% DSS drinking water for 6 days accompanied with low or high dose flavonoids treatment. And the last day mice were given normal drinking water still accompanied with flavonoids treating [14].
2.5.2. Design of animal feed formula

Flavonoids of Agrocybe aegirita was customized in feed in this experiment. AIN93G feed was customized from Shanghai Fanbo Biotechnology Co., Ltd. The detailed composition of the feed is shown in Table 1. The customized feed of the flavonoids of Agrocybe aegirita is adjusted on the basis of AIN93G purified feed. The low and high dose of Agrocybe aegirita flavonoids were set as 25 mg/kg BW and 50 mg/kg BW respectively [25], according to the doses of flavonoids consumed by adults (2.75-5.5 mg/kg BW).

Table 1  Feed formula table

| Specific ingredients       | Normal purified feed | Flavonoid Customized Feed |
|----------------------------|----------------------|---------------------------|
| casein                     | 20.00%               | 20.00%                    |
| L-cystine                  | 0.30%                | 0.30%                     |
| corn starch                | 39.70%               | 39.667%                   |
| Maltodextrin               | 13.20%               | 13.20%                    |
| sucrose                    | 10.00%               | 10.00%                    |
| cellulose                  | 5.00%                | 5.00%                     |
| Soybean oil                | 7.00%                | 7.00%                     |
| tert-butyl hydroquinone    | 0.0014%              | 0.0014%                   |
| mineral mixture            | 3.50%                | 3.50%                     |
| vitamin mix                | 1.00%                | 1.00%                     |
| Choline Bitartrate         | 0.25%                | 0.25%                     |
| Agrocybe aegirita flavonoids | 0%                  | 0.0334%                   |

2.5.3. Disease Activity Index (DAI Score)

From the 8th day to the end, referring to the literature method [26], the fecal consistency, blood in the stool and weight loss of the mice were scored, and the DAI index was the average of the total scores of the three indicators. The scoring criteria are shown in the table.

\[
DAI = \frac{\text{Stool consistency score} + \text{Fecal blood score} + \text{Body weight Loss score}}{3}
\]

2.5.4. Determination of colon length

After the mice were sacrificed by decapitation, the mice were dissected and the colons were removed and placed on a graph paper to measure the length and record.

Table 2  Scoring criteria for colon histological damage

| Parameter                              | Variety                                      | Score |
|----------------------------------------|----------------------------------------------|-------|
| Inflammatory cell infiltration at the base of the crypt | none                                         | 0     |
| Inflammatory cells infiltrate the muscularis mucosae |                                             | 2     |
| Mucosal invasion, mucosal thickening, edema |                                             | 4     |
| no damage                              |                                              | 0     |
| Epithelial cell reduction               |                                              | 1     |
| Decreased epithelial cells              |                                              | 2     |
| Complete loss of epithelial cells       |                                              | 4     |
| normal                                 |                                              | 0     |
| Partial loss of crypt structure         |                                              | 1     |
| Enlarged crypts or extensive loss of crypts |                                          | 2     |
| no crypt structure                      |                                              | 4     |

2.5.5. Determination of spleen index

The weight of the mouse spleen was weighed and recorded, and the mouse spleen index was calculated according to the following formula: spleen index (%) = spleen weight (g) / mouse weight (g) × 100%.

2.5.6. Determination of inflammatory factors

Accurately weighed 2 mg of colon tissue, adding 200 μL PBS buffer, and ground to make a 10% PBS homogenate. After high-speed centrifugation, collected the supernatant and measured it according to the methods of TNF-α, IL-10, and IL-22 ELISA kits.

2.5.7. H&E staining

Colon end tissue samples were fixed in 4% buffered paraformaldehyde for 24 hours, then the samples were dehydrated in graded alcohol and embedded in paraffin wax, the sections were cut into a thickness of 4 μm. Subsequent, the samples were stained with hematoxylin and eosin (H&E) for histological analysis. The pathological changes in the gastric tissues were observed under a light microscope. The details of the score of colon damage degree were shown in the table, and the tissue damage score was the sum of the scores of each index [27].

2.5.8. AB-PAS staining

Colon sections were stained with AB-PAS according to the instructions of the AB-PAS staining kit [28]. Paraffin sections were dewaxed and covered with water, and stained with dye solution. The sections were dehydrated by adding anhydrous ethanol, and the sections were sealed with neutral gum. The slice image information was collected under a pathological slice scanner. Image pro plus 6.0 software was used to calculate the area percentage of goblet cells and mucin in the slices.

2.6. Statistical analysis

Statistical analysis was performed by SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 8.0. Significant between groups were
evaluated using ANOVA one way test. All values were expressed as means ± SDs (P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001, showing a significant difference compared with the model group).

3. RESULT

3.1. Effect of *Agrocybe aegirita* flavonoids on dietary intake in mice

By analyzing the dietary intake of each group daily, the average dietary intake of each mouse was calculated. As shown in Figure 2, from the first day to the ninth day, the dietary intake of the mice in each group basically did not differ, and was stable at 3g/day, the difference appeared from the 10th day. The dietary intake of the model group, the low-dose flavonoid group and the low-dose flavonoid group began to decrease, and the decrease in the dietary intake of the model group was greater than that of the other two groups.

3.2. Effect of *Agrocybe aegirita* flavonoids on body weight in mice

Colitis can lead to weight loss. In order to study the effects of flavonoids on DSS-induced colitis in mice, statistical analysis was performed on the weight changes of each group of mice. It can be observed from Figure 3 that the body weight of each group of mice increased continuously from day 1 to day 9, and the weight of mice gradually decreased after DSS treatment. Compared with the model group, both low-dose and high-dose flavonoids could delay the weight loss. On the 14th day, the weight of mice in two flavonoids groups were significantly higher than that in the model group (P $<$ 0.001).

3.3. Effect of *Agrocybe aegirita* flavonoids on spleen index in mice

The spleen is an important immune organ and plays a very important role in the immune regulation of the body. Inflammatory responses are often accompanied by spleen enlargement, resulting in increased weight. As shown in Figure 4, we can see a serious inflammatory reaction in model group spleen comparing with the normal group, While, the spleen index of the high-dose flavonoid group decreased significantly (P $<$ 0.01) comparing with normal group, indicating a relief in this spleen immune action.

3.4. Effect of *Agrocybe aegirita* flavonoids on disease activity index in mice

The DAI index can reflect the severity of colitis in mice, and we scored the mice DAI index during modeling. The results are shown in Figure 5. From Day8 to Day 14, the DAI score of the normal group was always zero. After 3% DSS treatment, the DAI index of the model group, the low-dose flavonoid group and the high-dose flavonoid group increased significantly (P $<$ 0.01). On the 14th day, the DAI index of the model group reached 4 points. While the DAI
index of two flavonoid groups were significantly lower than that of the model group (P < 0.001).

Figure 5 The effect of flavonoids of *Agrocybe aegirita* on the DAI index of mice P*<0.005, **P<0.01, ***P<0.001, showing a significant difference compared with the model group.

Table 3 Disease Activity Index (DAI) scoring rules

| Parameter       | Variety          | Score |
|-----------------|------------------|-------|
| Stool form      | normal form      | 0     |
|                 | loose stool      | 2     |
|                 | liquefied feces  | 4     |
|                 | no blood in stool| 0     |
| blood in the stool | slight blood in the stool | 2 |
|                 | severe blood in the stool | 4 |
| percent weight loss | 1%-5% | 1 |
|                 | 5%-10%           | 2     |
|                 | 10%-15%          | 3     |
|                 | >15%             | 4     |

3.5. Effect of *Agrocybe aegirita*: flavonoids on colon length in mice

The colon of DSS-induced colitis mice was significantly shortened, and the change in colon length could reflect the severity of inflammation [19], therefore, we measured the mice colon length. The results are shown in Figure 6. The colon length of the model group (4.3 cm) was significantly shorter than that of the normal group (6.4 cm). However, compared with the model group, the length of both the flavonoid low-dose group and the flavonoid high-dose group were statistically increased (P < 0.001).

3.6 Effect of flavonoids from *Agrocybe aegirita* on the secretion level of inflammatory factors in mice

TNF-α and IL-1β are typical pro-inflammatory factors, and IL-10 is a typical anti-inflammatory factor. Colitis is often accompanied by significant changes in these inflammatory factors [29]. In order to study the anti-inflammatory ability of flavonoids from *Agrocybe aegirita*, the secretion levels of these three inflammatory factors in the colon were measured. The results are shown in Figure 7. Compared with the normal group, the secretion levels of TNF-α and IL-1β in the model group were significantly decreased (P < 0.001), and IL-10 in both flavonoid groups were significantly increased (P < 0.001).

3.6. Effect of *Agrocybe aegirita* flavonoids on histopathology in mice

The effects of flavonoids from *Agrocybe aegirita* on the morphology and structure of colon in DSS-induced colitis mice were investigated by HE staining and section histopathological scoring of colon tissue of colitis mice. The results of HE sectioning are shown in Figure 8 (a). The morphological structure of the mice in the normal group was normal, the colonic epithelium was not destroyed and the crypt morphology was normal, and no inflammatory cell infiltration was found. The morphological structure of the mice in the model group changed significantly, which was manifested by severe damage to the colonic epithelium, the crypts basically disappeared or deformed, and a wide range of inflammatory infiltration was observed. Compared with the normal group, crypt damage and partial inflammatory infiltration were still seen in the colon tissue of flavonoids treated mice, but the range of inflammatory infiltration was lower. The colon tissue damage scores are shown in Figure 8 (b). Compared with the model group, flavonoids treatment greatly reduced the tissue damage of colitis mice.

3.7. Effect of *Agrocybe aegirita* flavonoids on goblet cells and mucin in colon tissue

Distal colonic tissue was specifically stained with AB-PAS to evaluate goblet cell growth and mucin secretion in mouse colon. As shown in Figure 9(a), there were almost no goblet cells in the model group. As shown in Figure 9 (b) and (c), Significantly increased amount of goblet cells and mucin secretion were observed in the Agrocybe aegirita flavonoids low-dose and high-dose group, while for flavonoids high-dose group, a large number of goblet cells was found. This phenomenon indicated that flavonoids can probably act on goblet cells to promote mucin secretion, and therefore, improve the intestinal barrier.
4. DISCUSSION

Inflammatory bowel disease is an intestinal disease with unclear pathogenesis and difficult to cure completely. The number of patients with colitis is increasing year by year, owing to changes in lifestyle and eating habits. According to statistics, as of 2017, there were about 6.8 million patients with colon disease in the world \[30\]. This is a big challenge to the medical and health system. Therefore, how to alleviate IBD has always been a hot topic. Flavonoid active substances are secondary metabolites in plants and belong to important class of natural active products. Agrocybe aegirita is rich in a variety of active ingredients, such as polysaccharides, amino acids, flavonoids, etc. Related reports show that flavonoids have certain anti-inflammatory and antioxidant activities \[31, 32\]. Compared with human ulcerative colitis, DSS-induced colitis in mice has certain similarities in pathological manifestations, including hematochezia, weight loss, massive loss of colonic epithelial cells, and infiltration of inflammatory factors \[33\]. Compared with plant cultivation, Agrocybe aegirita cultivation is relatively low, and only needs a suitable environment and fungus bag, and can be reused \[34\]. Therefore, a large amount of flavonoids can be obtained by increasing the cultivation of Agrocybe aegirita. This lays the foundation for the mass production and application of Agrocybe aegirita. In this study, 3% DSS drinking water was used to establish an acute colitis model for study. The mice in Agrocybe aegirita flavonoids group were physiologically normal and there was no normal colon tissue morphology, while the weight of the mice in the model group decreased rapidly, with severe blood in the stool. Besides, appearing tissue damages such as loss of crypt morphology, mucin secretion decrease and inflammatory infiltration in the colon tissue. It follows that Agrocybe aegirita flavonoids can improve the symptoms of colitis mice, reduce the impact of inflammation, and protect colon tissue, and a dose-dependent manner can also be speculated.

Inflammatory factors refer to various cytokines involved in the inflammatory response, which are mainly endogenous polypeptides secreted by immune cells. Studies have shown that colitis and the rise of pro-inflammatory factors are related to the decline of anti-inflammatory factors \[35, 36\]. TNF-α and IL-1β are typical...
pro-inflammatory factors that can induce the deterioration of intestinal inflammation and lead to tissue damage. IL-10 is an inflammatory inhibitor that participates in inflammatory responses and can inhibit the release of inflammatory mediators and regulate cell differentiation and growth [14–16]. In this study, we used ELISA kits to measure the contents of TNF-α, IL-1β and IL-10 in serum. The results showed that the contents of TNF-α and IL-1/β in colon increased significantly after DSS induction, and IL-10 content decreased significantly. Compared with the model group, the decrease of IL-10 and rise of TNF-α and IL-1/β shows Agrocybe aegirita flavonoids can recover colitis by regulating inflammatory factors. The Agrocybe aegirita flavonoids significantly inhibited the expression of TNF-α and IL-1/β, while significantly promoted the expression of IL-10, indicating that Agrocybe aegirita flavonoids can recover colitis by regulating inflammatory factors. According to research, when flavonoids play an anti-inflammatory effect, they do not directly participate in the inflammatory response, but generally use some intermediate metabolites to play an anti-inflammatory effect, such as the use of intestinal flora to decompose flavonoids to produce small flavonoid molecules [40]. Goblet cells in the gut and their secreted mucus are the main components of the intestinal mucus layer and play an important role in the intestinal barrier [41]. Mucin in the intestine is a type of glycoprotein secreted by goblet cells. It mainly uses its special binding site to prevent bacteria from contacting and combining with the intestinal epithelium, so bacteria are wrapped in mucus, which is beneficial to intestinal clearance. Apoptosis of goblet cells is mainly due to the invasion of inflammatory cytokines, leading to damage to the intestinal barrier [42, 43]. In this study, AB-PAS was used for specific staining to evaluate goblet cell apoptosis and mucin secretion. Compared with the normal group, the number of goblet cells in the colon of mice in the model group was severely decreased, and mucin secretion was almost absent. Compared with the model group, the number of goblet cells of flavonoids in Agrocybe aegirita was significantly higher than that in the model group, as well as mucin secretion indicating that the Agrocybe aegirita flavonoids played a critical role in maintaining the integrity of the intestinal barrier.

5. CONCLUSION

It can be concluded from the above results and discussions that the flavonoids of Camellia sinensis have a certain improvement effect on DSS-induced colitis. This is mainly supported by the facts that Camellia sinensis delayed the weight loss trend of mice, reduced the DAI index, and alleviated the shortening and damage of the colon in mice. In addition, the flavonoids of Agrocybe aegirita decreased the expression of pro-inflammatory factors and increased the expression of anti-inflammatory factors in the colon of mice. Apart from the cytokine levels, Agrocybe aegirita flavonoids also had a positive effect on the intestinal barrier, reflected by the facts that it stabilized the proliferation of goblet cells, improved the mucin secretion and repaired the colonic barrier damage. In conclusion, Agrocybe aegirita flavonoids can be used as a potential natural active substance for treating colitis.

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