

*Polygonum tinctorium* leaves suppress sodium dextran sulfate-induced colitis through  
interleukin-10-related pathway

(蓼藍葉はインターロイキン 10 関連経路を介してデキストラン硫酸ナトリウム誘発性大腸炎を抑制する)

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## Abstract

Indigo naturalis, a herbal medicine purified from indigo-containing plants, such as *Strobilanthes cusia*, *Isatis tinctoria*, and *Polygonum tinctorium*, has been reported to be useful in the treatment of ulcerative colitis by activating the aryl hydrocarbon receptor. However, the aryl hydrocarbon receptor pathway causes crucial side effects, such as pulmonary arterial hypertension. Although *P. tinctorium* is one of the plant derivatives of indigo naturalis, it is not identical to it. To date, the pure leaves of *P. tinctorium* have not been reported to ameliorate ulcerative colitis. Therefore, we investigated the effect of pure *P. tinctorium* leaves, which are consumed in some regions, on experimental colitis induced in mice using sodium dextran sulfate. We found that *P. tinctorium* leaves ameliorated weight loss ( $P < 0.01$ ) and pathological inflammatory changes in the colon ( $P < 0.05$ ), enhanced mRNA expression of interleukin-10 ( $P < 0.05$ ), and decreased expression of tumor necrosis factor- $\alpha$  in colonic tissues ( $P < 0.05$ ), as determined using quantitative real-time reverse transcription polymerase chain reaction. The intraperitoneal administration of an aryl hydrocarbon receptor antagonist did not antagonize the inhibition of mucosal destruction, whereas an anti-interleukin-10 receptor antibody did. These results suggest that *P. tinctorium* ameliorate sodium dextran sulfate-induced intestinal inflammation via interleukin-10-related pathway, independent of the aryl hydrocarbon receptor pathway. *P. tinctorium* leaves have the potential to be a new, safe treatment for ulcerative colitis.

Keywords: aryl hydrocarbon receptor pathway; indigo naturalis; interleukin-10; Polygonum tinctorium; ulcerative colitis

## Introduction

The pathogenesis of inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), has not yet been ascertained. However, environmental and genetic factors and immune responses are considered to be the major etiologies of IBD [1,2]. During the past decade, the number of patients with IBD has increased significantly [2], with many cases of refractory disease showing repeated remission and relapse cycles, despite significant progress in medical treatment, such as the use of targeted anti-tumor necrosis factor (TNF)- $\alpha$  antibodies. In addition, the rising cost of medical care is a major problem for patients with IBD.

In China, patients suffering from UC have often been treated with traditional herbal medicines. Previous reports showed that indigo naturalis (IN, also referred to as Qing-Dai), obtained by fermenting plants containing indigo (e.g., *Strobilanthes cusia*, *Isatis tinctoria*, and *Polygonum tinctorium*), is also effective against intractable UC [3,4]. In Japan, a multicenter, double-blind, prospective trial investigated the safety and efficacy of IN in patients with UC and demonstrated the high efficacy of IN and its short-term safety [5]. Indigo-containing preparations, such as IN, are thought to act as ligands for the aryl hydrocarbon receptor (AhR) that induces the production of anti-inflammatory cytokines, such as interleukin (IL)-22 and IL-10, to ameliorate colitis [6]. However, pulmonary arterial hypertension (PAH)

has been reported as a critical side effect of IN treatment [7,8], with reports associating the AhR pathway with PAH [9,10]. Considering its potential safety concerns, IN may be difficult to use clinically as a therapeutic agent.

On the other hand, *P. tinctorium* is an herb that has been known, in Oriental countries, to have anti-bacterial and anti-inflammatory activities since ancient times. Although *P. tinctorium* is one of the plant species providing IN, it is not identical to IN, in terms of its composition and biological activity. Therefore, we hypothesized that pure *P. tinctorium* might have the potential to be used clinically as a therapeutic agent for UC. However, the leaves of *P. tinctorium* have not been reported to ameliorate UC.

In the present study, we aimed to clarify the protective effects of pure *P. tinctorium* leaves in sodium dextran sulfate (DSS)-induced colitis in a mouse model to determine their potential mechanism of action. Considering safety, we used a *P. tinctorium* called “Aomori Blue” that is cultivated without pesticide in the northern-most prefecture of Japan. “Aomori Blue” is not only used as a dye but is also distributed as a safe food ingredient.

## Materials and methods

### Mice

Female BALB/c mice (age, 8 weeks; weight, 18–22 g) were purchased from CLEA Japan (Tokyo, Japan) and maintained in a controlled environment (22°C ± 2°C with a 12-h light/dark cycle).

All animal experiments complied with the appropriate guidelines, and were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. The study design was approved by Animal Research Committee of Hirosaki University (Permit number: M16015).

#### Induction of colitis

Colitis was induced in the mice by the addition of 3% DSS (molecular weight, 1000–9000 g/mol; FUJIFILM Wako Pure Chemical, Osaka, Japan) to their drinking water.

#### Administration of *P. tinctorium* leaves

The leaves of *P. tinctorium*, cultivated without pesticide use, were provided by AomoriSangyo Corporation (Aomori, Japan). The mice were fed powdered food (CLEA Rodent Diet CE-2, CLEA Japan) that contained 0.5% by weight of dry powdered *P. tinctorium* leaves for 12 days after the initial DSS administration.

#### Evaluation of colitis activity

The body weights of the mice were measured daily, and at Day 12, the mice were euthanized by cervical dislocation and the colon was isolated. The colon lengths were measured and fixed in 10% formalin, embedded in paraffin, and sliced along the long axis. The specimens were

stained with hematoxylin and eosin (H & E) for histological evaluation, which allowed the measurement of the inflammatory cell infiltration, crypt loss, and mucosal epithelial loss at a height just above the muscularis mucosae. To evaluate colitis activity, we compared the body weight, colon length, and histological changes in two groups: the colitis group (DSS positive [+], *P. tinctorium* negative [□]) (n = 8) and the *P. tinctorium* group (DSS positive [+], *P. tinctorium* positive [+]) (n = 8).

#### Isolation of intestinal epithelial cells and lamina propria

Intestinal epithelial cells (IECs) were purified as described by Mizoguchi et al. [11]. On Day 4, the mice were anesthetized by exposure to isoflurane, and the thoracic cavity was opened. The left ventricle was perfused with 15 mL of 30-mmol/L ethylenediaminetetraacetic acid (EDTA) in Hank's Balanced Salt Solution (HBSS), which was calcium free and magnesium free. Following perfusion, the entire colon (excluding the cecum) was removed, inverted, and placed in a cold tube containing 2 mL of cold 2-mmol/L EDTA in HBSS. The tube was shaken for 20 s using a mini-beadbeater (BioSpec Products, Bartlesville, OK, USA). The suspension was centrifuged at  $15,000 \times g$  (4°C) for 20 min, and the pellet containing IECs was suspended in RNA Later solution (Thermo Fisher Scientific, Waltham, MA, USA). The remaining tissue, which comprised the lamina propria (LP), was placed in an RNA solution.

## Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA contents from the IECs and LPs were isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and were reverse transcribed to cDNA using the iScript Advanced cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The following TaqMan gene expression assays (Thermo Fisher Scientific) were used to quantify the following target genes: *Ifng* (Mm00801778\_m1), *Tgfb1* (Mm01178820\_m1), *TNF* (Mm99999068\_m1), *IL22ra1* (Mm01192943\_m1), *IL22* (Mm0122672\_g1), *IL1b* (Mm00434228\_m1), *Foxp3* (Mm00475156\_m1), *IL10* (Mm00439615\_g1), and *Actb* (Mm00607939\_s1). qRT-PCR was performed using a CFX Connect Real-Time System (Bio-Rad Laboratories) and Brilliant III Ultra-Fast QPCR Master Mix solution (Agilent Technologies, Santa Clara, CA, USA). These results were then normalized to *Actb* RNA expression.

In the qRT-PCR analysis, we compared the expression of mRNA in three groups of mice: the control group (DSS negative [-], *P. tinctorium* negative [-]) (n = 6), colitis group (n = 6), and *P. tinctorium* group (n = 6).

## In vivo administration of chemical antagonist

To confirm AhR involvement, the mice received intraperitoneal injections of an AhR antagonist (CH223191; Selleck Chemicals, Houston, TX, USA; 10 mg/kg) every day from the initial DSS administration. We compared the colon length and histological changes were

evaluated in two groups, namely the colitis + CH223191 group (n = 6) and the *P. tinctorium* + CH223191 group (n = 6).

To confirm the involvement of IL-10, mice received intraperitoneal injections of mouse anti-IL-10 receptor antibody (anti-IL-10R Ab, Clone 1B1.3A; Bio X Cell, Lebanon, KS, USA; 25 mg/kg) at the start of DSS administration and on Day 5. We compared the colon lengths and histological changes in two groups: the colitis + anti-IL-10R group (n = 6) and the *P. tinctorium* + anti-IL-10R group (n = 6).

#### High-performance liquid chromatography analysis

*P. tinctorium* leaves were extracted by soaking in n-hexane, at 25°C, for 24 h, twice. The extracts were concentrated, dried, dissolved in dimethyl sulfoxide, and subjected to high-performance liquid chromatography (HPLC) to determine the major components. The indigo, indirubin, and tryptanthrin in *P. tinctorium* leaves were analyzed using HPLC with the following conditions: column, COSMOSIL 5PE-MS Packed Column (i.d. 4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan); mobile phase, 40% CH<sub>3</sub>CN; flow rate, 1.0 mL/min; detection 254 nm; and temperature, 25°C. The data were collected by Chromato-PRO (RTC, Tokyo, Japan). The standard reagent for tryptanthrin was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

All the other reagents were purchased from Fujifilm-Wako Pure Chemicals, Co.

## Statistical analysis

All data are presented as means  $\pm$  standard errors of the means (SEMs). The data, when they were not normal probability distribution, were analyzed with a Mann-Whitney U-test using GraphPad Prism (Version 8.2.1, GraphPad Software, San Diego, CA, USA). A p-value of  $<0.05$  was considered statistically significant.

## Results

### *P. tinctorium* leaves ameliorated DSS-induced colitis

Compared with that in the colitis group (n = 8), the animals' weight loss was markedly reduced in the *P. tinctorium* group (n = 8) from Day 9 to Day 12 (Figure 1A). The colons obtained on Day 12 showed that those from the *P. tinctorium* group were longer than those from the colitis group (Figure 1B, 1C). Histological evaluation demonstrated that the crypt and mucosal epithelial losses were lower in the *P. tinctorium* group than in the colitis group (Figure 1D, 1E).

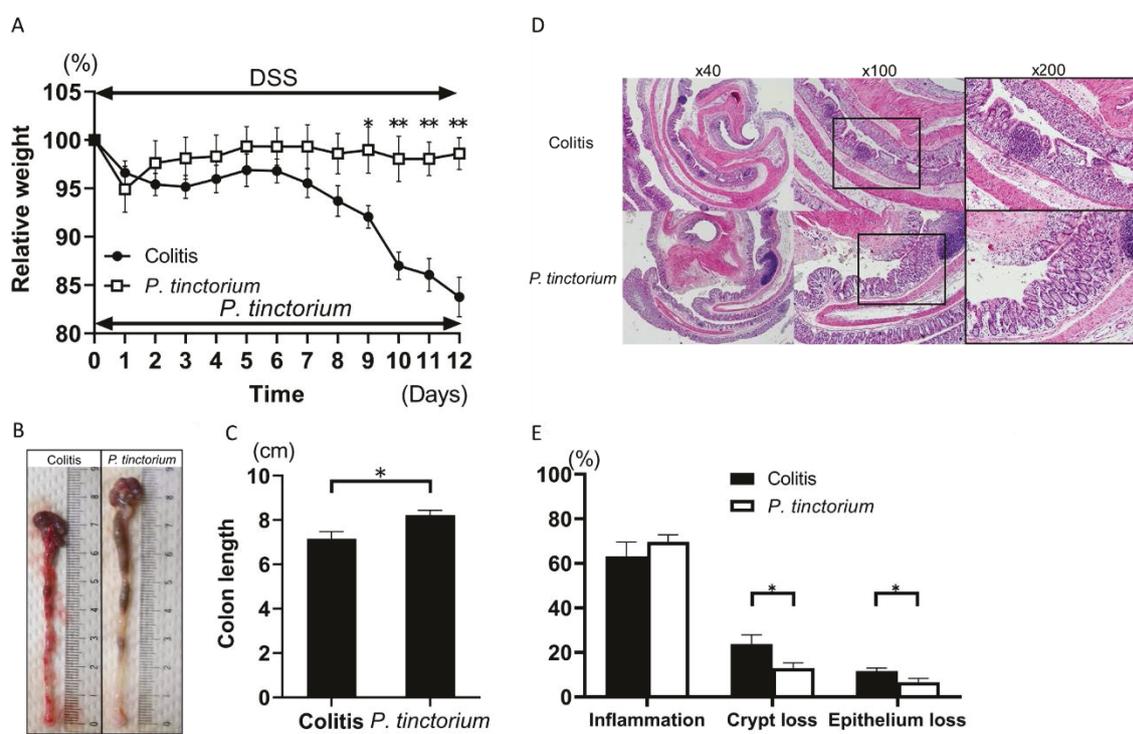
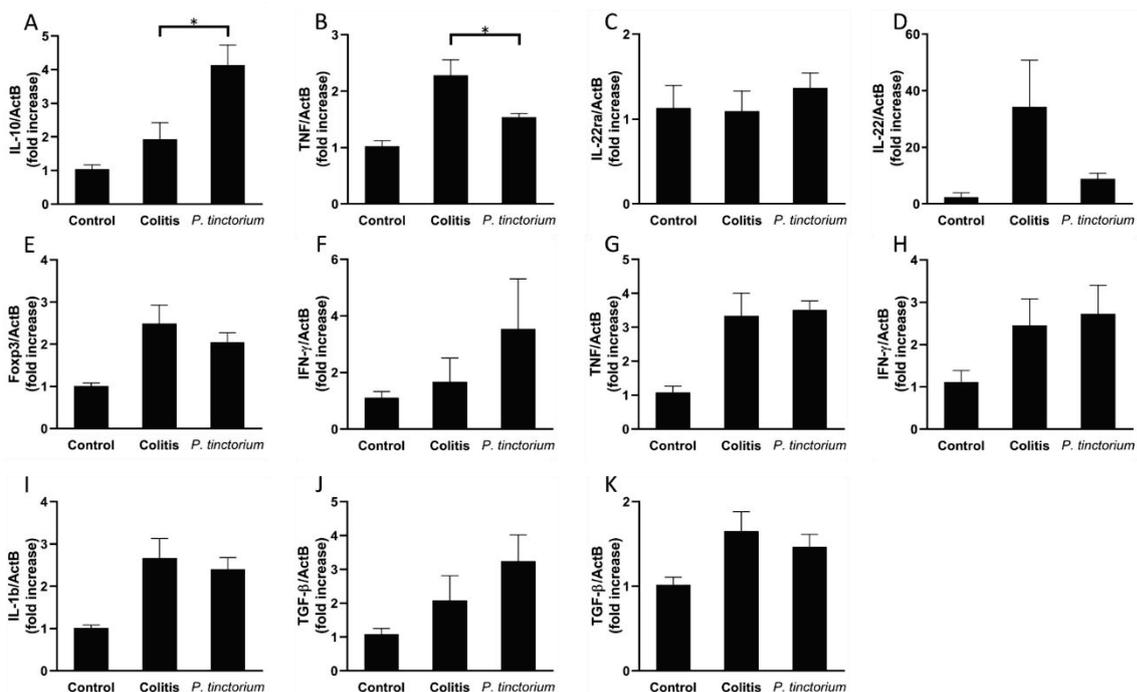


Figure 1. Polygonum tinctorium suppresses dextran sulfate sodium (DSS)-induced colitis.

(A) A graphical representation of the average percent weight loss in the colitis and *P. tinctorium* groups, relative to the weights at the start of DSS treatment. \* $P < 0.05$ ; \*\* $P < 0.01$ . (B) Colons harvested from the mice in the colitis and *P. tinctorium* groups on Day 12. (C) A graphical representation of the colon lengths harvested from the mice in the colitis and *P. tinctorium* groups on Day 12. (D) Histological samples of hematoxylin and eosin-stained colons observed at 40 $\times$ , 100 $\times$ , and 200 $\times$  magnifications. (E) The extent of inflammatory cell infiltration and crypt and mucosal epithelial losses. Data are expressed as the means  $\pm$  standard error of the mean of groups of 8 mice.

*P. tinctorium* enhanced IL-10 expression in colonic LP of mice with DSS-induced colitis. Next, we analyzed the mRNA expressions of inflammatory or anti-inflammatory cytokines which

are important in pathophysiology of IBD [2] and concern mechanism of indigo naturalis including IL-22 or IL-10 [6]. The qRT-PCR analysis showed that IL-10 mRNA expression in the LP of the *P. tinctorium* group (n = 6) was significantly enhanced compared with that in the LP of the colitis group (n = 6) (Figure 2A). Conversely, TNF- $\alpha$  mRNA expression in the LP of the *P. tinctorium* group was lower than that in the LP of the colitis group (Figure 2B). The mRNA expression levels of IL22ra in IECs and IL22 and Foxp3 in the LP were similar between the colitis and *P. tinctorium* groups (Figure 2C-2E). Moreover, there were no significant differences in the mRNA expression levels of several inflammatory cytokines, such as IFN- $\gamma$  and TNF- $\alpha$  in IECs and IFN- $\gamma$  and IL-1 $\beta$  in LP (Figure 2F-2I), between the colitis and *P. tinctorium* groups. The mRNA expression levels of the anti-inflammatory cytokine TGF- $\beta$  in the IECs and LP of the colitis group were similar to those in the IECs and LP of the *P. tinctorium* group (Figure 2J, 2K).



## Figure 2. mRNA expressions in IECs and LP

*Polygonum tinctorium* increases the expression of IL-10 mRNA and decreases the expression of TNF- $\alpha$  mRNA in the LP. On Day 4 after the induction of colitis, colon cells were separated into IECs and LP, the total RNA was extracted, and mRNA expression was analyzed using qRT-PCR. (A) LP expression of IL-10. (B) LP expression of TNF- $\alpha$ . (C) IEC expression of IL22ra. (D) LP expression of IL-22. (E) LP expression of Foxp3. (F) IEC expression of IFN- $\gamma$  (G) IEC expression of TNF- $\alpha$ . (H) LP expression of IFN- $\gamma$  (I) LP expression of IL-1 $\beta$  (J) IEC expression of TGF- $\beta$  (K) LP expression of TGF- $\beta$  these data are compared between the colitis and *P. tinctorium* groups and are expressed as means  $\pm$  SEMs of groups of 6 mice; \*P < 0.05.

IECs, intestinal epithelial cells; IL, interleukin; IFN, interferon; LP, lamina propria; mRNA, messenger ribonucleic acid; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; SEM, standard error of the mean; TGF, transforming growth factor; TNF, tumor necrosis factor

AhR antagonist did not antagonize the protective effect of *P. tinctorium* leaves in DSS-induced colitis

In animals receiving intraperitoneal administration of the AhR antagonist, the mean body weight in the colitis + CH223191 group (n = 6) was significantly less than that in the *P. tinctorium* + CH223191 group (n = 6) by Day 11 (Figure 3A). Additionally, the mean colon

length in the *P. tinctorium* + CH223191 group tended to be longer than that in the colitis + CH223191 group, although the difference was not significant (Figure 3B, 3C). Notably, histological evaluations of crypt and mucosal epithelial loss showed that the effects of *P. tinctorium* leaves were significant, even when the mice received an AhR antagonist (Figure 3D, 3E).

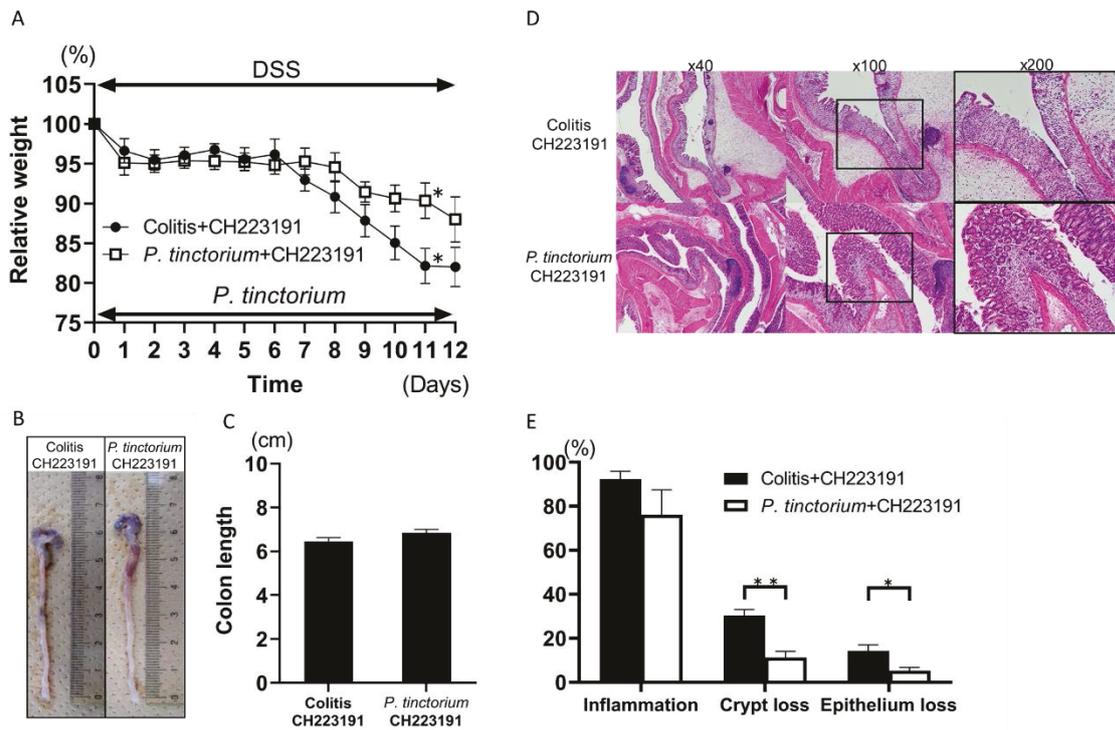


Figure 3. Weight loss, colon length, and extent of inflammatory cell infiltration and crypt and mucosal epithelial losses between the colitis and *Polygonum tinctorium* groups after administration of an AhR antagonist.

Intraperitoneal administration of an AhR antagonist did not attenuate the mucosal improvement effect of *P. tinctorium* leaves. (A) AhR antagonist was intraperitoneally administered to the colitis and *P. tinctorium* groups every day from the start of DSS administration. The average percent weight loss in the two groups of mice is compared with the weights at the start of DSS treatment. (B) The colon samples from both groups, harvested on Day 12. (C) Graphical representation of the colon lengths of both groups on Day 12. (D) histological samples of the colon tissue stained with H&E and observed under 40×, 100×, and 200× magnifications. (E) Graphical representation of the extent of inflammatory cell infiltration and crypt and mucosal epithelial losses; data are expressed as means ± SEM in groups of 6 mice; \*P < 0.05 and \*\*P < 0.01

AhR, aryl hydrocarbon receptor; DSS, sodium dextran sulfate; H&E, hematoxylin and eosin; SEM, standard error of the mean

IL-10-neutralizing Ab counteracted the ameliorating effect of *P. tinctorium* leaves in DSS-induced colitis

After intraperitoneal administration of anti-IL-10R, the weight loss in the *P. tinctorium* + anti-IL-10R group was similar to that in the colitis + anti-IL-10R group. One of the six mice in the colitis + anti-IL-10R group was unable to survive until Day 12. In the *P. tinctorium* + anti-IL-10R group (n = 6) and the colitis + anti-IL-10R group (n = 5), weight loss was exacerbated

after the second administration of anti-IL-10R Ab (Figure 4A). The colon lengths were similar between the *P. tinctorium* + anti-IL-10R and colitis + anti-IL-10R groups after the administration of anti-IL-10R Ab (Figure 4B, 4C). Additionally, histological evaluation demonstrated that anti-IL-10R Ab administration to the *P. tinctorium* + anti-IL-10R group exacerbated inflammatory cell infiltration and increased crypt and mucosal epithelial loss; these inflammatory changes were similar to those observed in the colitis group (Figure 4D, 4E).

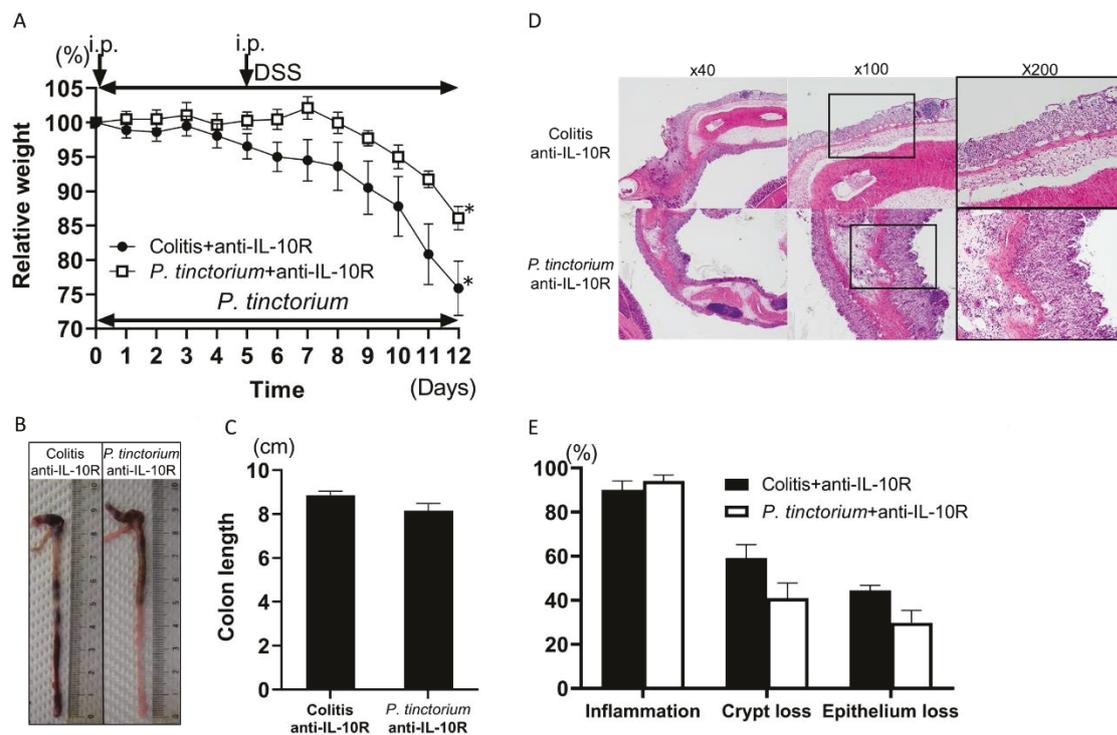


Figure 4. Weight loss, colon length, and extent of inflammatory cell infiltration and crypt and mucosal epithelial cell losses between the colitis and *Polygonum tinctorium* groups after administration of an anti-IL10R Ab.

Intraperitoneal administration of an anti-IL10R Ab attenuated the mucosal improvement effect of *P. tinctorium*. At the start of DSS initiation and on Day 5, the anti-IL10R Ab was

intraperitoneally administered to the colitis and *P. tinctorium* groups. (A) The average percent weight loss in the two groups is compared with the weights at the start of DSS treatment. (B) Colon samples from both groups, harvested on Day 12. (C) Graphical representation of the colon lengths of both groups on Day 12. (D) Histological samples of the H&E-stained colon tissue observed under 40×, 100×, and 200× magnifications. (E) Graphical representation of the extent of inflammatory cell infiltration and crypt and mucosal epithelial losses; data are expressed as means ± SEM of groups of 5–6 mice; \*P < 0.05

Ab, antibody; DSS, sodium dextran sulfate; H&E, hematoxylin and eosin; i.p., intraperitoneal administration of Ab; SEM, standard error of the mean.

Components of *P. tinctorium* leaves

Since *P. tinctorium* contains multiple constituents, quantitative analysis was performed using HPLC, revealing the amounts of tryptanthrin, indirubin, and indigo in *P. tinctorium* to be 28.11, 14.23, and 4.6 ng/g, respectively.

## Discussion

To the best of our knowledge, this is the first report suggesting that *P. tinctorium* leaves have a protective effect against DSS-induced mucosal destruction and revealing the main mechanism involved in IL-10-related pathway, but not the AhR pathway.

UC is a disease in which the intestinal mucosal epithelium is repeatedly damaged, and its etiology is not fully understood. Despite advances in medical therapy and treatment outcomes that have decreased the proportion of patients undergoing surgery [12,13], the occurrence of refractory cases and the high cost of treatment have become social problems [14,15]. The high efficacy of IN in cases of refractory UC has been demonstrated in many studies, to date [4,16].

Moreover, indigo and indirubin (the active ingredients of IN) act as ligands for the AhR pathway and stimulate innate lymphoid cell 3 in the intestinal mucosa to induce IL-22 and promote healing of mucosal damage [17,18].

From our results, oral consumption of *P. tinctorium* leaves could inhibit the weight loss, colon shortening, and mucosal damage caused by DSS-induced colitis. Notably, this study demonstrates a new mechanism through which *P. tinctorium* leaves inhibit mucosal damage: enhancement of IL-10 expression and inhibition of TNF- $\alpha$  expression, without inducing IL-22. In addition, the treatment with anti-IL-10R antibody lessened the protective effect of *P. tinctorium* in vivo. The protective effect of *P. tinctorium* on mucosal damage that was maintained during treatment with AhR-antagonist suggests that the mechanism of inhibiting

the protective effect is AhR-independent. However, colon shortening was decreased following treatment with the AhR-antagonist. These results suggest that the AhR pathway partially influences the protective effect of *P. tinctorium* leaves.

The main components of IN are indigo and indirubin (2.0% and 0.13% or more, respectively) [19]. Although IN also contains tryptanthrin and other alkaloids, the ratios of some of these components are reportedly lower than those in other indigo-containing plants [20]. In *P. tinctorium* leaves, the ratios of indigo, indirubin, and tryptanthrin extracted with n-hexane showed tryptanthrin to be the most abundant component, followed by indirubin (about one-half), and then indigo (about one-sixth). These data suggest that tryptanthrin or other alkaloids, not indigo or indirubin, are responsible for the protective effect through IL-10-related pathway. Previous reports demonstrated that tryptanthrin act as therapeutic agent through production IL-10 in RA model mice [21], and that tryptanthrin ameliorates dextran sodium sulfate-induced colitis [22]. Tryptanthrin might be one of the key components of *P. tinctorium* leaves.

In this study, the mice receiving *P. tinctorium* leaves were protected from DSS-induced mucosal damage through the upregulation of IL-10. Conversely, blocking the IL-10 receptor restricted this protective effect, demonstrating that the protective effect of *P. tinctorium* leaves is associated with the upregulation of IL-10. The major source of IL-10 is CD4-positive regulatory T-cells that express Foxp3. However, the cells that are stimulated by *P. tinctorium*

leaves to upregulate IL-10. remain unclear. Previous reports have shown that kaempferol, 3,5,4'-trihydroxy-6,7-methylenedioxyflavone-O-glycosides, and their aglycones from indigo upregulate IL-10 in cultured macrophages [23,24]. Another study showed that IN induces the expansion of CD4-positive, Foxp3-negative, IL-10 producing T-cells, known as type I regulatory T-cells [6]. In our study, the expression of Foxp3 mRNA was not upregulated in the *P. tinctorium*-treated mice, suggesting that *P. tinctorium* might upregulate macrophage-derived IL-10 or CD4-positive, Foxp3-negative, IL-10-producing T cell-derived IL-10. In DSS-induced colitis, mucosal inflammation is known to develop via loss of intestinal epithelial cells as a steppingstone [25]. Loss of intestinal epithelial cells leads to mucosal inflammation by entry of luminal bacteria. The inflammation occurs in the absence of T-cells and B-cells [26], suggesting that this model of colitis is the best for research of innate immune systems including intestinal epithelial cells and macrophages. As for IL-10-related pathway in innate immune systems, Toll-like receptors-mediated IL-10 expression by macrophages play an important role to protect from mucosal injury. Therefore, mucosal macrophages may be a key player in the IL-10-related protective effect of *P. tinctorium* leaves. On the other hands, IL10R-signaling pathway in intestinal epithelial cells including JAK/STAT pathway is also important for gut mucosal homeostasis [27]. To confirm the opinions, further studies are needed.

This study had some limitations. Notably, all the components of *P. tinctorium* leaves were not identified, in detail, and only one type of *P. tinctorium* leaves (Aomori Blue) was used. It is

unknown whether one particular ingredient included in *P. tinctorium* leaves or a particular proportional mixture shows the protective effect. We demonstrated the effect of *P. tinctorium* leaves involving IL-10 pathway in vivo, while the protein level of IL-10 was not shown.

In conclusion, *P. tinctorium* leaves were found to ameliorate DSS-induced intestinal inflammation via IL-10-related pathway. The protective effect of *P. tinctorium* leaves on mucosal damage is independent of the AhR pathway, which is known to cause PAH. Therefore, *P. tinctorium* leaves are a potential new, safe treatment for UC.

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## Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

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