

Analgesic activity of cynaropicrin on post-inflammatory irritable bowel syndrome visceral hypersensitivity in a rat model

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Abstract. Visceral hypersensitivity is one of the most common symptoms in patients with post-inflammatory-irritable bowel syndrome (PI-IBS). Enterochromaffin (EC) cells and 5-hydroxytryptamine (5-HT) are important in the development of visceral hyperalgesia, and EC cells are influenced by helper T-cell subtype 1 or 2 cytokine predominant environments. In the present study, the analgesic effect of cynaropicrin and its underlying mechanism on the treatment of trinitrobenzene sulfonic (TNBS)-induced PI-IBS visceral hyperalgesia rats was investigated. The results from the abdominal withdrawal reflex tests and electromyography recordings indicated that treatment with cynaropicrin significantly and dose-dependently alleviated the visceral hyperalgesia of PI-IBS rats ($P < 0.05$). In addition, the increased colonic 5-HT content, colonic tryptophan hydroxylase expression, EC cell number and the cytokine levels, including tumor necrosis factor- α and interleukin-6 in PI-IBS rats were significantly alleviated by cynaropicrin ($P < 0.05$). These data demonstrate that the analgesic activity of cynaropicrin on TNBS-induced PI-IBS visceral hypersensitive rats was mediated via reduction of cytokines levels. Thus, cynaropicrin as a bioactive natural product may offer promising therapeutic avenues for visceral hypersensitivity in IBS.

Introduction

Irritable bowel syndrome (IBS) is a complex disorder that is associated with chronic, functional gastrointestinal (GI) disorder, chronic abdominal pain, altered bowel movements

and affects approximately 10-15% of the world's population. Clinically, there is a subset of IBS patients termed as postinfectious or post-inflammatory IBS (PI-IBS); in these patients, IBS symptoms occur after an initial episode of acute GI infection. PI-IBS is characterized by loose stool with urgency, accelerated colonic transit, and decreased pain threshold (1-3).

The mechanism of visceral hypersensitivity in PI-IBS patients is not fully understood. Recent studies have indicated that altered enterochromaffin (EC) cells and/or 5-HT can result in GI dysmotility, visceral hypersensitivity, and secretomotor abnormalities (4). In addition, it has been thought that receptors of 5-HT such as 5-HT₃ and 5-HT₄ may play an important role in conveying visceral sensation from the GI. These findings suggest that altered EC cells or 5-HT might be one pathophysiologic mechanism contributing to visceral pain in PI-IBS. More than 90% of 5-HT in the body is secreted from EC cells which are located within the mucosal mucosa, and the tryptophan hydroxylase (TPH) which in EC cells is the rate-limiting enzyme in the 5-HT synthesis process (5-7). Moreover, recent studies indicated that CD4⁺ T cells played an important role in the development of colonic EC cell hyperplasia in intestinal infection, and EC cells were influenced by helper T-cell subtype 1 (Th1) or subtype 2 (Th2) cytokine predominant environments (8,9).

Cynaropicrin (Fig. 1) is a sesquiterpene lactone of a guaiano- lide type. Sesquiterpene lactones are the most biologically significant class of secondary metabolites (10). Cynaropicrin has been shown to possess various biological activities and has demonstrated extraordinary pharmacologic properties such as anti-parasitic, anti-spasmodic and anti-inflammatory properties on suppression of NF- κ B (11). Especially, cynaropicrin possesses a marked effect on mucosal injuries, preventing acute gastritis and it is also a promising antispasmodic agent (12). In addition, cynaropicrin is beneficial to the gastrointestinal actions and use to ameliorate dyspeptic symptoms (13). To our knowledge, there are no studies indicating that the administration of cynaropicrin has been tested in a PI-IBS visceral hyperalgesia model.

This study was aimed at evaluating the analgesic activity of cynaropicrin on PI-IBS visceral hyperalgesia. An experimental PI-IBS visceral hyperalgesia rat model was induced by administering trinitrobenzene sulfonic (TNBS) as reported

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previously. Then, TNBS-induced PI-IBS visceral hyperalgesia rats were treated by administration of cynaropicrin, and the effects on visceral sensation, colonic 5-HT content, colonic TPH expression, EC cell number and colonic cytokines levels of TNBS-induced PI-IBS visceral hyperalgesia rats were investigated.

Materials and methods

Materials. Cynaropicrin was obtained from Wako (Tokyo, Japan). Fontana-Masson staining kit and anti-tryptophan hydroxylase antibody were purchased from Abcam (Cambridge, UK). 5-HT enzyme-linked immunosorbent assay (ELISA) kit, 5-HIAA ELISA kit, TNF- α ELISA kit and IL-6 ELISA kit were obtained from MyBioSource, Inc., (San Diego, CA, USA). TNBS and para-chlorophenylalanine (pCPA) were purchased from Sigma-Aldrich (Tokyo, Japan). SABC rabbit IgG kit and DAB coloring reagent kit were obtained from Boster Inc., (Wuhan, China).

Animals. One hundred and twenty male Sprague-Dawley rats (weighing ~ 220 g) were housed under environmentally controlled conditions ($21 \pm 3^\circ\text{C}$ and maintained on a light-dark cycle with the lights on from 6:00 a.m.-7:00 p.m. in sawdust-lined transparent plastic cages with free access to chow pellets and tap water). All experiments were performed in compliance with the Shaanxi administration rules and guidelines for laboratory animals and approved by the Laboratory Animal Ethics Committee at the Shaanxi University of Chinese Medicine (no. 2280109/2015).

Experimental TNBS-induced PI-IBS visceral hyperalgesia in a rat model. After fasting for 24 h, the rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.). A plastic catheter (external diameter approximately 0.95 mm) was inserted into the descending colon at a depth of 8 cm from anus, and then, the rats in the control group were coloretally instilled with 0.9% saline solution, and the PI-IBS visceral hyperalgesia rats were coloretally instilled with TNBS in 50% ethanol (TNBS 5 mg/rat). After TNBS administration for 4 weeks, the animal model of PI-IBS visceral hyperalgesia was determined by measuring visceral pain threshold pressure. The rats with acquired visceral hyperalgesia (pain threshold pressure below 30 mmHg) were selected as the PI-IBS visceral hypersensitive rats (14).

Experimental design. In the present study, the experiments were divided into 2 series. The first series was aimed at investigating whether the cynaropicrin can attenuate visceral hyperalgesia in TNBS-induced PI-IBS rats. This was done by using AWR testing and EMG recording. The second series was aimed at evaluating the effects of cynaropicrin on EC cell number, colonic TPH expression and 5-HT metabolism in PI-IBS rats.

Therefore, 6 groups of 20 rats were used. The normal rats in control group were treated with water (control group, gavage administration), and the PI-IBS visceral hyperalgesia rats in others 5 groups were treated with water (TNBS Group), pCPA (pCPA Group, 150 mg/kg/d, i.p. for 3 days), and cynaropicrin (group 4-6, at the dose of 5, 10, and 20 mg/kg/d, gavage administration for 3 days).

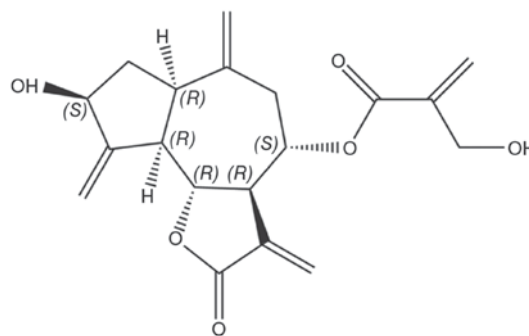


Figure 1. Structure of cynaropicrin.

After the treatment, 10 rats from each group were randomly chosen for AWR testing. Subsequently, the rats were sacrificed for sample collection. A 6 cm long piece of proximal colon (1-2 cm from caecum) was harvested for the evaluation of colonic 5-HT content, colonic TPH expression, EC cell number and the cytokine levels. The rest of 10 rats from each group were just used for EMG recording.

Abdominal withdrawal reflex (AWR) test. Ten rats from each group were used for AWR testing by random choice. Each rat was lightly anesthetized with ether, and inserted a balloon into the descending colon, and the end of the balloon was secured at least 1 cm proximal to the anal verge. Then, the rat was housed in a small lucite box (20x8x8 cm) and allowed to wake up and recuperate for 1 h. The colorectal distension was applied in increments of 5 mmHg until a visible contraction of abdominal muscles was observed by an investigator blinded. The Al-chaer's AWR score 3 was defined as the pain threshold pressure here according to the behavioral response of the rat lifting its abdomen off the platform (15).

Electromyography (EMG) recording. The rest of 10 rats from each group were used for EMG recording. After intraperitoneal anesthesia with nembutal, the rats were put into supine position and on the constant temperature mat, keeping the temperature at about 37°C . The surface of hypogastrium was sterilized, then, cut along the surface of the medioventral line, isolating the subcutaneous fascia layer on one side of the rats, exposing the musculus obliquus externus abdominis, then inserting the insulated silver electrode into the left external abdominal oblique muscles, with intervals of 0.5-1 cm. The other end of the electrode was got out of the back subcutaneously. The incision was treated with 1% lidocaine gel for pain relief. After one week recuperation, the rat was lightly anesthetized with ether, and inserted a balloon into the descending colon. The EMG signal was amplified and filtered (50-5,000 Hz). Total three cycles of graded colorectal distention (20, 40, 60, and 80 mmHg; 20 sec duration; 2 min inter-stimulus intervals) were applied to each rat. The changes of the AUC (the area under the curve) during the 20 sec distention period over the preceding 20 sec baseline of each rat were calculated and analyzed by Axconscope software (16,17).

EC cell counting. The method of EC cell counting was performed as previously described (18). Briefly, the colon was harvested and fixed in 4% paraformaldehyde and embedded in paraffin. And then, tissue sections ($6\ \mu\text{m}$ thick, 6 sections for each rat) were

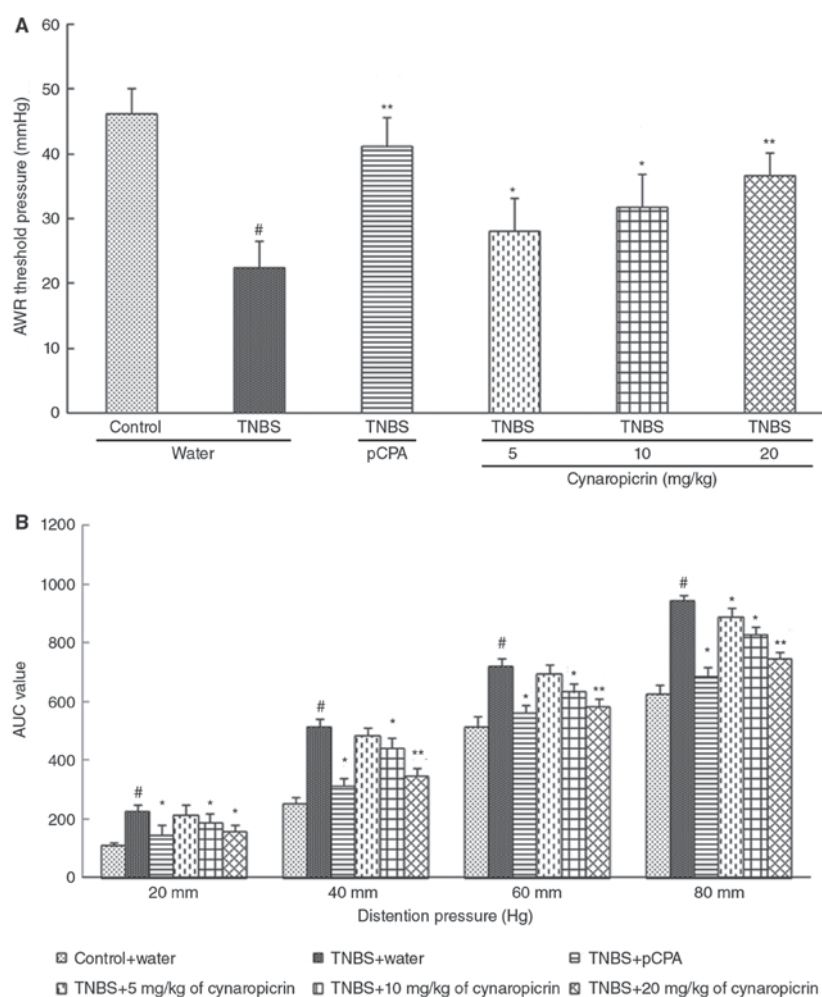


Figure 2. The analgesic effect of cynaropicrin in TNBS-induced PI-IBS visceral hypersensitive rats. (A) The changes on AWR test by cynaropicrin in TNBS-induced PI-IBS visceral hypersensitive rats. (B) The changes on EMG recording by cynaropicrin in TNBS-induced PI-IBS visceral hypersensitive rats. Data are shown as mean \pm SEM (n=10), #P<0.05 vs. control group rats; *P<0.05, **P<0.01, vs. TNBS group (t-test).

deparaffinized and rehydrated for Fontana-Masson staining. The sections were incubated in ammoniacal silver solution (1 h, 60°C), gold chloride solution (0.2%, 30 sec), sodium thiosulfate solution (5%, 2 min, room temperature), and nuclear fast red solution successively. Finally, the sections were dehydrated with alcohol and mounted in synthetic resin. Five random fields at 200x magnifications were counted in each section by a researcher blinded to the treatment; the number of EC cells per mm² of mucosa was quantified using ImageJ NIH software.

Western blot analysis. Western blot analysis was performed as previously described (19). Western blot analysis was used for the detection of tryptophan hydroxylase, and the β -actin was chosen as the loading control. Briefly, the total protein of the colon was extracted and quantified. Then the samples containing 30 μ g of protein were boiled for 5 min and subjected to SDS-PAGE electrophoresis and then transferred to PVDF membranes. The PVDF membranes were incubated in blocking buffer, and then incubated with anti-tryptophan hydroxylase antibody or anti-beta actin antibody for 1 night at 4°C. Subsequently, the PVDF membranes were incubated with secondary antibodies labeled alkaline phosphatase. The immunoblots were detected by western blue and quantified using the ImageJ program.

ELISA. The content of 5-HT and the cytokine levels of TNF- α and IL-6 in the colon tissue were assayed by ELISA (20). The samples were measured according to the manufacturer's protocol. Briefly, the colon tissue was harvested and homogenized. Then the samples were diluted with PBS (0.02 mol/l, pH 7.2). After centrifuging of the samples, the 5-HT content and cytokine levels were measured using ELISA kits. Absorbance at 450 nm in each well was measured using a spectrophotometer.

Statistical analysis. All data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was conducted using SPSS 15.0 Software (SPSS Inc., Chicago, IL, USA). The data of visceral pain threshold pressure were analyzed by comparing the values before and after treatment for each group using a paired t-test, and the differences between before and after treatment in a group using a one-way analysis of variance (ANOVA). After testing for homogeneity of variance, data of EMG recording, EC cell counting, TPH, 5-HT and 5-HIAA in the colon were compared using one-way ANOVA and Student-Newman-Keuls (SNK) method post-hoc testing. P<0.05 was considered to indicate a statistically significant difference.

Results

Analgesic effect of cynaropicrin on TNBS-induced PI-IBS visceral hyperalgesia rats. As shown in Fig. 2A, the pain threshold pressures of the rats (TNBS group, 22.51 ± 4.15 mmHg, $P < 0.05$, $n=10$) administered with TNBS were significantly decreased when compared to that of the rats (control group, 46.27 ± 3.84 mmHg) administered with saline. After treatment with cynaropicrin, pain threshold pressures of the rats in group 4 (28.15 ± 5.09 mmHg, $P < 0.05$ in group 4; 31.85 ± 5.15 mmHg, $P < 0.05$ in group 5; 36.64 ± 3.56 mmHg, $P < 0.01$ in group 6; $n=10$) were significantly and dose-dependently elevated when compared to that of the rats in the TNBS group. After treatment with pCPA, one of the TPH inhibitors that inhibits the production of 5-HT, the pain threshold pressures of the rats in the pCPA group (41.15 ± 4.56 mmHg, $P < 0.01$, $n=10$) were significantly elevated when compared to that of the control group.

Consistent with the results from AWR tests, as shown in Fig. 2B, the results of EMG recording showed that the visceral motor responses to graded colorectal distension of the rats in TNBS group (228.09 ± 18.73 , $P < 0.05$; 516.15 ± 24.77 , $P < 0.05$; 722.58 ± 25.82 , $P < 0.05$; 942.48 ± 21.72 , $P < 0.05$; for the pressures 20, 40, 60, 80 mmHg, $n=10$) were significantly increased when compared to that of the rats in the control group (108.86 ± 12.84 , 252.86 ± 23.16 , 514.35 ± 34.45 , 624.34 ± 28.05 for the pressures 20, 40, 60, 80 mmHg). Also, the visceral motor responses to graded colorectal distension were decreased significantly in the rats treated with pCPA (pCPA group, 146.46 ± 32.7 , $P < 0.05$; 313.57 ± 24.43 , $P < 0.05$; 561.76 ± 25.92 , $P < 0.05$; 687.33 ± 28.05 , $P < 0.05$; for the pressures 20, 40, 60, 80 mmHg, $n=10$) when compared to that of the rats in the TNBS group. Also, the pain threshold pressures of the rats in group 4 (214.3 ± 32.47 ; 484.91 ± 27.75 ; 694.43 ± 30.7 ; 890.06 ± 31.03 , $P < 0.05$; for the pressures 20, 40, 60, 80 mmHg, $n=10$), group 5 (187.32 ± 31.63 , $P < 0.05$; 422.58 ± 34.15 , $P < 0.05$; 632.98 ± 27.67 , $P < 0.05$; 828.42 ± 24.41 , $P < 0.05$; for the pressures 20, 40, 60, 80 mmHg, $n=10$), and group 6 (156.44 ± 24.2 , $P < 0.05$; 348.45 ± 22.87 , $P < 0.01$; 582.72 ± 25.12 , $P < 0.01$; 747.68 ± 22.85 , $P < 0.01$; for the pressures 20, 40, 60, 80 mmHg, $n=10$) were significantly and dose-dependently elevated after treatment with cynaropicrin.

Effects of cynaropicrin on 5-HT content, TPH expression and EC cell number in the colon. The 5-HT content in the colon of the rats in TNBS group (1.88 ± 0.27 ng/mg, $P < 0.05$, $n=10$) was significantly increased when compared to that of the rats from the control group (1.37 ± 0.22 ng/mg) (Fig. 3A). Compared to the rats in the TNBS group, the 5-HT content was significantly reduced in the rats of the pCPA group (1.48 ± 0.22 ng/mg, $P < 0.05$, $n=10$), as well as significantly reduced in group 6 (1.58 ± 0.21 ng/mg in group 6, $P < 0.05$, $n=10$).

TPH expression in the rats of TNBS group (0.628 ± 0.085 , $P < 0.05$, $n=10$) was significantly increased compared to the TPH expression in rats from the control group (0.289 ± 0.092) (Fig. 3B and C). When the data was compared to that of the rats in TNBS group, the TPH expression was significantly reduced in the rats by the treatment with pCPA (0.382 ± 0.074 , $P < 0.05$, $n=10$) and cynaropicrin (0.456 ± 0.087 , $P < 0.05$, $n=10$).

The number of colonic EC cells were significantly increased in the rats of the TNBS group (164.4 ± 10.6 per mm^2 ,

$P < 0.05$, $n=10$) when compared to that of the rats in the control group (78.4 ± 6.2 per mm^2) (Fig. 3D-H). This result suggests that EC cell hyperplasia occurs in PI-IBS visceral hyperalgesic rats. After treatment with pCPA or cynaropicrin, the EC cell hyperplasia was reduced significantly in the rats of the pCPA group (112.4 ± 9.2 per mm^2 , $P < 0.05$, $n=10$) and group 6 (121.5 ± 12.4 per mm^2 , $P < 0.05$, $n=10$).

Effects of cynaropicrin on the levels of cytokines in the colon. The levels of TNF- α and IL-6 in the colon of the rats in TNBS group (6.71 ± 0.58 pg/mg, $P < 0.01$, for TNF- α and 8.24 ± 0.77 pg/mg, $P < 0.05$, for IL-6, $n=10$) were all significantly increased when compared to that of the rats in control group (1.12 ± 0.61 pg/mg and 3.77 ± 0.82 pg/mg for TNF- α and IL-6) (Fig. 4). Cynaropicrin significantly improved the levels of the cytokines in the colon of the rats in group 6 (3.66 ± 0.76 pg/mg, $P < 0.01$, for TNF- α and 6.28 ± 0.84 pg/mg, $P < 0.01$, for IL-6, $n=10$).

Discussion

In the present study, the results of AWR tests and EMG recordings indicated a significant analgesic effect on PI-IBS visceral hyperalgesia by cynaropicrin. Meanwhile, the changes of colonic 5-HT content, colonic TPH expression, EC cell number and colonic cytokines levels that induced by cynaropicrin were also indicated here.

As a neurotransmitter, 5-HT plays an important role in the GI tract. 5-HT stimulates the serotonergic receptors such as 5-HT₃ and 5-HT₄ receptors which are located on primary afferent neurons of both splanchnic and vagal fibers, thereby modulating both sensory and motor responses. In previous studies, antagonists such as Alosetron, Granisetron and Odansetron, have been shown to reduce the visceral hypersensitivity and rectal sensitivity in IBS patients, by blocking the transmission of the afferent signal, after occupying the 5-HT₃ receptor combining site (5-7). It is suggested that 5-HT played an important role in the development of visceral hypersensitivity. As an inhibitor, pCPA acts as a selective and irreversible inhibitor of TPH. The results in this study indicated that the colonic 5-HT contents in TNBS-induced PI-IBS visceral hyperalgesia rats were decreased by treatment with pCPA. Similarly, the colonic 5-HT contents in TNBS-induced PI-IBS visceral hyperalgesia rats were decreased by treatment with cynaropicrin. Similar results from AWR testing and EMG recording indicate a significant analgesic effect accompanied by a decrease of 5-HT content on the TNBS-induced PI-IBS visceral hyperalgesia rats treated with pCPA or cynaropicrin.

In addition, results from this study showed that 5-HT content was decreased dramatically and depleted seriously in TNBS-induced PI-IBS visceral hyperalgesia rats by treatment with pCPA. But the results of AWR tests and EMG recordings showed that no differences were found in visceral pain threshold pressure between the rats that were treated with pCPA and the rats in the control group. This result indicated that even below the normal level of 5-HT content may be enough to meet the minimum requirement of invoking the sensory reflex (21-23).

Past studies indicated that the excessive availability of 5-HT mainly come from increases of EC cell number (24), and TPH which in EC cells is the rate-limiting enzyme in the 5-HT synthesis process. Alleviating visceral hyperalgesia

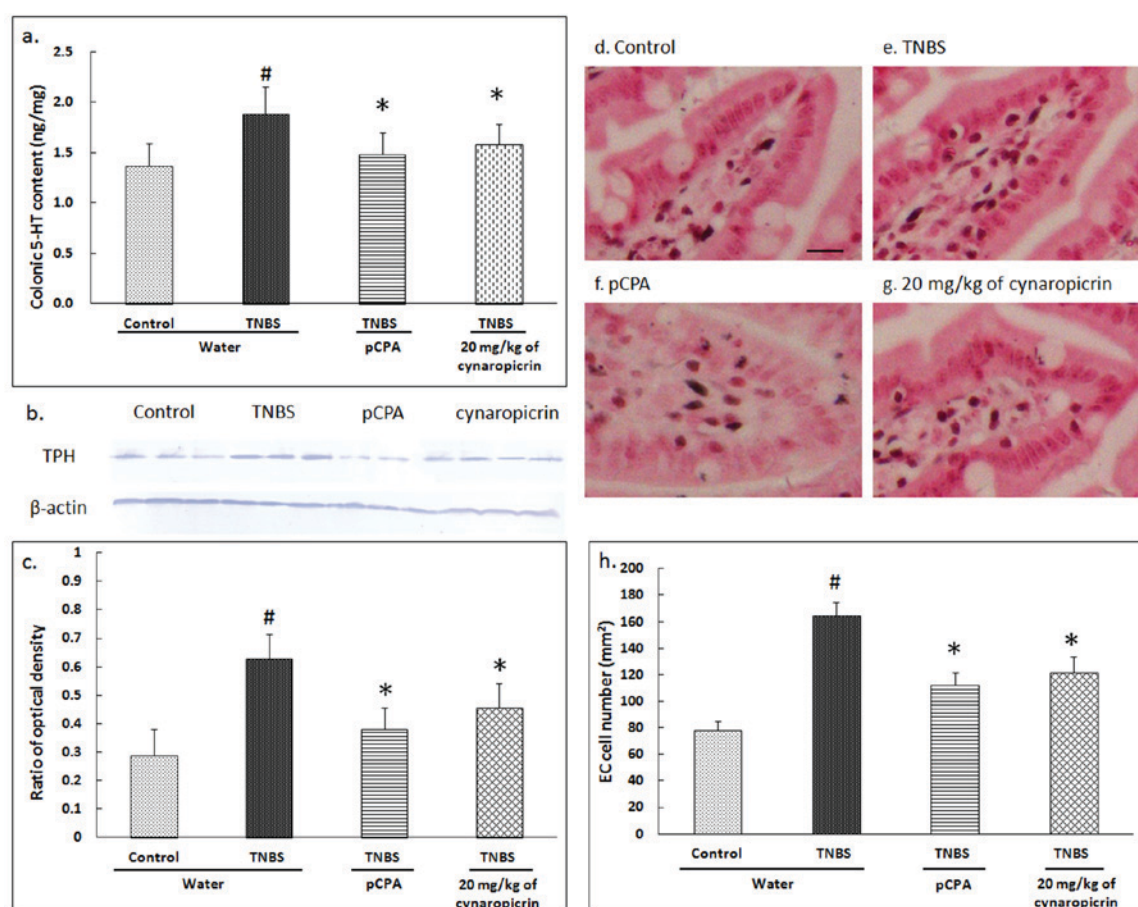


Figure 3. The changes on colonic 5-HT content, TPH expression and EC cell number by cynaropicrin in TNBS-induced PI-IBS visceral hypersensitive rats. (A) Statistical graphs about the colonic 5-HT contents. (B) Western blot analysis of colonic TPH expression. (C) Statistical graph of quantified optical density. d-g show the representative EC cells in colon of these groups. (D) Control group, (E) TNBS group, (F) pCPA group, (G) high dose of cynaropicrin (20 mg/kg) group (Scale bar, 50 μ m). Statistical graph of EC cell density is shown in (H). Data are shown as mean \pm SEM (n=10), [#]P<0.05 vs. control group rats; ^{*}P<0.05 vs. TNBS group (t-test).

may be mediated via decreasing hyperplastic colonic EC cell number. Results from this study indicated that colonic TPH expression and EC cell number were decreased by the treatment with cynaropicrin. The underlying mechanisms of EC cell hyperplasia in PI-IBS are unknown, but they are considered to have close correlation with CD4⁺ T lymphocytes, especially the Th1/Th2 balance. TNF- α has been shown to downregulate CD4⁺ T-cell responses, while deficiency of TNF- α leads to enhanced expansion of CD4⁺ T cells. IL-6 initiates maturation of Th2 cells from Th0 in conjunction with IL-4 (25-27). Therefore, Th1/Th2 balance is influenced by the cytokines. Results from this study, show that the levels of TNF- α and IL-6 in the colon were changed by treatment with cynaropicrin. Therefore, the decreases of colonic TPH expression and EC cell number by cynaropicrin were mediate via the decreases of the cytokines levels.

Unlike the cynaropicrin, the analgesic effect in TNBS-induced PI-IBS visceral hyperalgesia rats by treatment of pCPA is mediated via selective and irreversible inhibition of TPH. Many serotonergic receptors have been found on various immune cells such as B and T lymphocytes, monocytes, macrophage, and dendritic cells (28). EC cell hyperplasia is considered to have close correlation with T lymphocytes. Therefore, 5-HT content can influence the

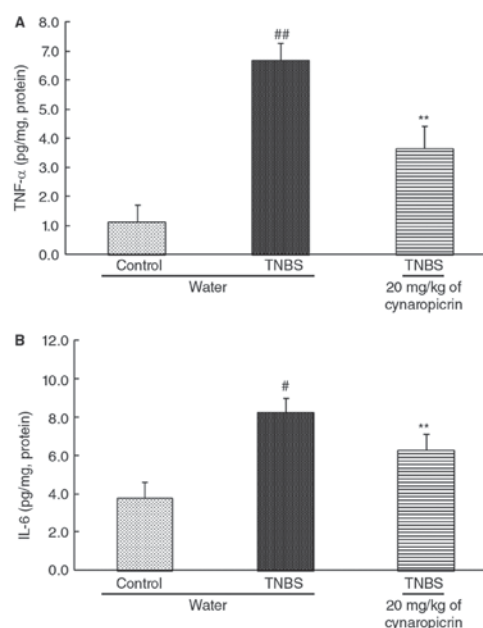


Figure 4. The changes on the levels of cytokines by cynaropicrin in TNBS-induced PI-IBS visceral hypersensitive rats. (A) Statistical graphs about the level of TNF- α . (B) Statistical graphs about the level of IL-6. Data are shown as mean \pm SEM (n=10), [#]P<0.05, ^{##}P<0.01 vs. control group rats; ^{**}P<0.01 vs. TNBS group (t-test).

EC cell hyperplasia. Consequently, the phenomenon of the EC cell number decrease by treatment with pCPA may be mediated via reducing colonic 5-HT content. The TPH inhibitors that were developed for the selective inhibition of 5-HT biosynthesis are expected to be used in the treatment of GI diseases such as IBS and IBD. Besides its well characterized function as a neurotransmitter, 5-HT has been reported to be a potent immunoregulator (29-34). 5-HT has been reported to be a potent regulator of cytokine secretion in different kinds of cells. Human monocytes release different cytokines and chemokines, mainly via 5-HT₃, 5-HT₄ and 5-HT₇ activation (35). Past studies also showed that 5-HT increase production of the pro-inflammatory cytokine IL-6 in mature Dendritic cells (DCs) via 5-HT₃, 5-HT₄ and 5-HT₇ (8,9). And DCs are known to produce different chemokines thereby regulating the traffic of Th1 and Th2 cells into inflamed tissue (36). Results from this study showed that pCPA significantly improved the levels of the cytokines in the colon of the PI-IBS rats. It is not hard to understand that pCPA, as an inhibitor of TPH, improved the levels of the cytokines in the colon via reducing colonic 5-HT content. The shortcoming of most of these inhibitors is that the blockade of the enzyme or the 5-HT depletion produced is relatively short-lasting. And the side effects of some TPH inhibitors such as pCPA have been impeded by the central adverse effects of inhibition of brain 5-HT synthesis with consequent affective disorders. Unlike the pCPA, cynaropicrin reduced colonic 5-HT content was mediated via the decreases of the cytokines levels and thus reducing putative side effects.

In conclusion, this study demonstrated that cynaropicrin can attenuate visceral hyperalgesia on TNBS-induced PI-IBS visceral hyperalgesia rats. The analgesic activity of cynaropicrin on TNBS-induced PI-IBS visceral hypersensitive rats was mediated via reducing the cytokines levels. Thus, cynaropicrin as a promising bioactive natural product will offer therapeutic avenues for visceral hypersensitivity in IBS.

Acknowledgements

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