Effects of *Hypericum perforatum* extract on rat irritable bowel syndrome

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ABSTRACT

Context: In irritable bowel syndrome (IBS), disturbance of bowel motility is associated with infiltration of inflammatory mediators and cytokines into the intestine, such as neutrophils, myeloperoxidase (MPO), tumor necrosis factor alfa (TNF-α), and lipid peroxide. **Aims:** Regarding promising anti-inflammatory and anti-oxidative effects of *Hypericum perforatum* (HP) extract, besides its anti-depressant effect, this study was designed to evaluate the effects of HP in an experimental model of IBS. **Settings and Design:** IBS was induced by a 5-day restraint stress in rats. The HP extract was administered by gavage in doses of 150, 300, and 450 mg/kg for 26 days. Fluoxetine and loperamide were used as positive controls. Gastric emptying and small bowel and colon transit, besides the levels of TNF-α, MPO, lipid peroxidation, and antioxidant power, were determined in colon homogenates. **Statistical Analysis Used:** Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. **Results:** A significant reduction in small bowel and colonic transit (450 mg/kg), TNF-α, MPO, and lipid peroxidation and an increase in antioxidant power in all HP-treated groups (150, 300, and 450 mg/kg) were seen as compared with the control group. Gastric emptying did not alter significantly when compared with the control group. Treatment with loperamide (10 mg/kg) significantly inhibited gastric emptying and small bowel and colonic transit, while flouxetine (10 mg/kg) decreased gastric emptying, TNF-α, MPO, and lipid peroxidation and increased the antioxidant power of the samples in comparison with the control group. **Conclusions:** HP diminished the recruitment of inflammatory cells and TNF-α following restraint stress not in a dose-dependent manner, possibly via inhibition of MPO activity and increasing colon antioxidant power, without any difference with fluoxetine. The HP extract inhibits small bowel and colonic transit acceleration like loperamide but has minimal effect on gastric emptying.

Key words: *Hypericum perforatum*, irritable bowel syndrome, oxidative stress

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder associated with pain and abdominal discomfort as key symptoms and also bloating and altered bowel habits either as diarrhea or constipation in the absence of detectable structural abnormalities.[1] The pathogenesis of IBS is poorly understood, although factors known to contribute to these alterations include genetics, abnormal gut motor and sensory activity, inflammation, colonic infections, mechanical irritation to the local nerves, luminal factors, stress, and psychological factors.[2] Stress and psychological dysfunction are well-known factors involved in the occurrence of IBS, which is supported by the evidence of using antidepressants as a therapeutic option besides the psychotherapy and cognitive behavioral therapies.[3] There is growing evidence on the role of inflammation and immune activation in the pathogenesis of IBS. Published data show an increased number of activated immune cells, mast cells, and mast cell products in the intestinal mucosa of the terminal ileum and the proximal and distal colon as compared with the controls in IBS patients.[4-6] IBS patients showed higher baseline myeloperoxidase (MPO) activity,

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tumor necrosis factor alfa (TNF-α), interlukin (IL)-1β, IL-6, and more activated neutrophil and eosinophil as compared with healthy controls.[7,8] It is believed that stress-induced colonic inflammation increases cell membrane lipid peroxidation and expression of IL-1α mRNA in mucosal biopsies.[9] Also, stress increases the colonic permeability, facilitating the entry of luminal contents that activate previously sensitized CD4 T cells in the colon, initiating and perpetuating an inflammatory response.[10]

Most of the current drugs available for IBS only relieve the symptoms, and no exact treatment has been found yet.[11] Selective serotonin receptor antagonists,[12] antidepressants,[13,14] selective anticholinergics,[15] alfa adrenergic agonists,[16] probiotics,[17] and antibiotics[18] are some of the currently used pharmaceuticals.

Hypericum perforatum L (HP), family *Hyperiacea*, commonly known as St. John's Wort, is most known for its proven antidepressant effect.[19] The regions of the origin of HP are Europe, West Asia, and North Africa, and the extract of its aerial parts has been used for many medicinal goals.[20] Several bioactive compounds have been detected in the HP extract, including naphthodianthrones (hypericin, pseudohypericin, protohypericin, protopseudohypericin, and cyclopseudohypericin), flavonoids (quercetin, rutin, and luteolin), hyperforin, and tannins.[21] These compounds give the anti-oxidative and anti-inflammatory potentials to this herb.[22-25]

Recently, a meta-analysis showed the efficacy and safety of HP in mild to moderate depressions in comparison with tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs).[19]

Regarding the above findings, we were interested to examine the potential of HP in a stress-induced IBS model in comparison with two previously known effective drugs by different mechanisms.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), trichloroacetic acid (TCA), n-butanol, hexadecyl trimethyl ammonium bromide (HETAB), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), phenol red, malondialdehyde (MDA), ethylene diamine tetra acetic acid (EDTA), and O-dianisidine hydrochloride from Merck Chemical Co. (Tehran, Iran), flouxetine and loperamide from Dr. Abidi Pharmaceutical Co. (Tehran, Iran), rat-specific TNF-α enzyme-linked immunosorbent assay (ELISA) kit from Bender MedSystems GmbH (Vienna, Austria), and *Hypericum perforatum* extract (standardized as

hypericin 0.1 mg/ml) from Pursina Pharmaceutical Co. (Tehran, Iran) were used in this study.

Animals

Male Wistar-albino rats, weighing 200–220 g were used. Animals were maintained under standard conditions of temperature (23 ± 1°C), relative humidity (55 ± 10%), and 12/12-h light/dark cycle and had free access to standard pellet diet and tap water. The animals were housed individually in standard polypropylene cages with a wire mesh top. All animals were cared under gridlines set by the institutional review board.

Experimental design

Seven groups of animals containing 18 rats in each group were used. IBS was induced in six groups and the other one was considered as the normal group. The groups receiving stress consisted of control (Cont) group that received normal saline, fluoxetine-treated group (Flx) receiving 10 mg/kg of fluoxetine,[26] loperamide-treated group (Lop) that received 10 mg/kg loperamide,[27] and the remaining three groups were treated with various doses of HP extract (150, 300, and 450 mg/kg) and assigned as HP-150, HP-300, and HP-450, respectively. The higher dose of extract (450 mg/kg) was considered for evaluation of gastric emptying and small bowel and colonic transit. All medications were prepared in a volume of 0.4 ml/200 g of rat body weight and administered intragastrically by gavage.

HP and fluoxetine were administered for 21 days as pretreatment, followed by 5 days during induction of IBS every day before restraining. Loperamide was gavaged four times after 21 days in those receiving normal saline: the first one before induction of IBS and the others during 48 h before dissecting and analyzing small bowel and colonic transit and gastric emptying.

Induction of irritable bowel syndrome

We performed the restraint method for the induction of IBS. Animals were lightly anesthetized with ether and the restraint was performed using plastic restrainers that allowed for a close fit to rats. Stress consisted of 6 h of immobilization starting at 9 am for five consecutive days.[10]

Sample preparation

On the 5th day after stress, each group was divided into three subgroups containing six rats. Phenol red was administered by gavage to overnight-fasted rats in the first two subgroups. Animals in the first subgroup were anesthetized using phenobarbital sodium (50 mg/kg) after 60 min and the other subgroup was anesthetized after 120 min. After laparotomy, the stomach, small intestine, and colon were removed and rinsed in cold saline bath. An overdose of ether inhalation was used to sacrifice all the

rats at the end. Pieces of colons were used for biochemical and immunological evaluations and cut open in an ice bath, cleansed gently with cold saline, and then weighted and homogenized in 10 volume ice cold potassium phosphate buffer (50 mM, pH 7.4). Then, 100 μl of the homogenate was taken for ferric reducing antioxidant power (FRAP) assay and kept at -80°C until analysis. The rest of samples were sonicated and centrifuged for 30 min at 3500 g. Then, the plates were separated and the supernatants were distributed into several microtubes kept at -80°C until analyses. The small intestine was divided horizontally into three equal segments and the stomach and three intestinal segments were used to assess gastric emptying and small bowel transit. In the second part of the experiment, the last subgroup that was not fasted was used to evaluate the colonic transit.

Measurement of gastric emptying and small bowel transit

Phenol red recovery method was used to determine gastric emptying and small bowel transit. Animals received 1 ml of 1.5% methylcellulose solution containing 0.5 mg phenol red by gavage. The stomach and three equal intestinal portions were homogenized in 100 ml of 0.1 N NaOH for 30 s. The suspension was stored at room temperature for 60 min and then 5 ml of the supernatant was added to 0.5 ml of 20% W/V TCA and centrifuged at 3000 g for 20 min. The supernatant was added to 4 ml of 0.5 N NaOH. Finally, the absorbance of the samples was read by an ultraviolet–visible spectrophotometer at 560 nm.[28] A calibration curve was used to measure the concentration of phenol red to calculate the percentage of gastric emptying according to the following formula:

(1-phenol red recovered from test stomach/average phenol red recovered from standard achieved from rats in normal group stomach)*100

To assess small bowel transit, differences between the amount of phenol red in the first and third segments were expressed as a per cent of the total amount recovered from the small intestines.

Colon transit evaluation

In this part of the experiment, fed animals were used to evaluate the fecal excretion. Fecal pellets output in rats was counted for 4 h after the 5th day of 6-h restraint stress.

Assay of tumor necrosis factor alfa

A rat-specific ELISA kit was used to quantify TNF-α in colon tissues. Amount of cytokine was assessed at the final step by measuring the absorbance of the sample in 450 nm as the primary wavelength and 620 nm as the reference wavelength by an ELISA reader as described by the kit brochure. Data were expressed as pg/mg protein of tissue.

Assay of ferric reducing antioxidant power

The definitive antioxidant capability remaining in colon tissue was measured by the ability of the tissue homogenate to reduce Fe^{+3} to Fe^{+2}. Interaction of TPTZ with Fe^{+2} produced a blue color, which was measured at 593 nm, as described in our previous work.[29] Data were expressed as mM ferric ions reduced to ferrous per gram of colon tissue.

Assay of myeloperoxidase

MPO as a lysosomal peroxidase enzyme is most abundantly expressed in neutrophil granules and is released from neutrophils during the inflammation. MPO activity was measured kinetically by a UV spectrophotometer for 3 min at 460 nm, as described before. MPO activity was reported as u/mg protein of tissue.[30]

Thiobarbituric acid-reactive substance assay

Lipid peroxidation process leads to a variety of aldehydes, and assessing the end products of this process, specifically MDA, is a diagnostic test to evaluate radical production in samples, and the most commonly used test is the TBARS assay. This substance could react with TBA to produce a measurable pink color with maximum absorption at 532 nm. The complete procedure has been described previously.[31] Data were reported as mM/g of tissue.

Total protein of colon homogenate

TP of tissue was measured according to the Bradford method, and the standard curve was obtained from various concentration of BSA as the standard.[32] Results were reported as mg/ml of homogenized tissue.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. *P*-values less than 0.05 were considered significant. Results were expressed as mean ± standard error of the mean (SEM).

RESULTS

Gastric Emptying

Chronic restraint stress caused a significant elevation in gastric emptying in the Cont group when compared with the normal group ($P < 0.001$). Treatment with flouxetine and loperamide caused a significant reduction in gastric emptying in comparison with the Cont group ($P < 0.001$). There was no significant difference between HP-450 and Cont group in gastric emptying. The percentage of gastric emptying HP-450 treated group was significantly higher in comparison with the Flx and Lop groups ($P < 0.001$) [Figure 1].

Small bowel transit

Chronic restraint stress caused a significant elevation in

small bowel transit in the Cont group when compared with the normal group ($P < 0.05$). Small bowel transit did not significantly decrease in the Flx group, while a significant
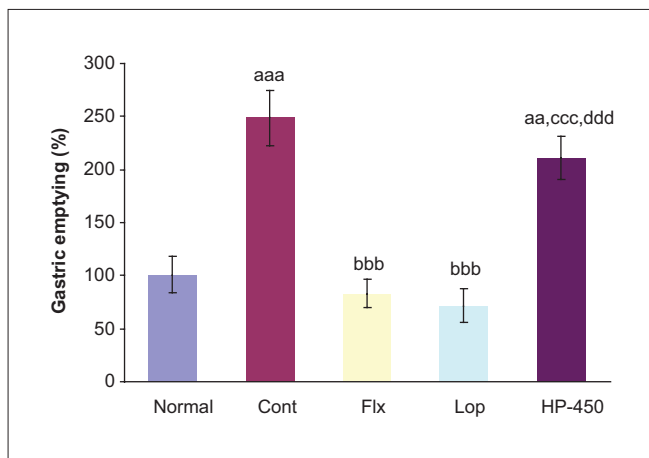

Figure 1: Gastric emptying percentages after 60 min. Values are mean ± SEM. ᵃSignificantly different from normal group at $P < 0.05$. ᵃᵃSignificantly different from the normal group at $P < 0.01$. ᵃᵃᵃSignificantly different from the normal group at $P < 0.001$. ᵇSignificantly different from the control group at $P < 0.05$. ᵇᵇSignificantly different from the control group at $P < 0.01$. ᵇᵇᵇSignificantly different from the control group at $P < 0.001$. ᶜSignificantly different from the fluoxetine group at $P < 0.05$. ᶜᶜSignificantly different from the fluoxetine group at $P < 0.01$. ᶜᶜᶜSignificantly different from the fluoxetine group at $P < 0.001$. ᵈSignificantly different from the loperamide group at $P < 0.05$. ᵈᵈSignificantly different from the loperamide group at $P < 0.01$. ᵈᵈᵈSignificantly different from the loperamide group at $P < 0.001$

decrease in the Lop group was observed in comparison with the Cont group ($P < 0.001$). Small bowel transit was lower in HP-450 as compared with the Cont group ($P < 0.01$), whereas it was higher than that of the Lop group ($P < 0.05$). The HP-450 transit was lower when compared with the Flx group ($P < 0.01$) [Figure 2].

Colon transit

Restraint stress caused a significant elevation in colonic transit in the Cont group in comparison with the normal group ($P < 0.001$). There was no significant difference between the Flx and Cont groups, but the fecal pellet output was significantly reduced in the Lop group ($P < 0.001$). The pellet output was significantly decreased in the HP-450-treated group, which was lower in comparison with the Cont and Flx groups ($P < 0.01$). Pellet output was higher in HP-450 in comparison with the Lop group ($P < 0.01$) [Figure 3].

Tumor necrosis factor alpha

Restraint stress caused a significant elevation in TNF-α in the Cont group when compared with the normal group ($P < 0.001$). TNF-α significantly decreased in the Flx group and in the HP-treated groups in comparison with the Cont group ($P < 0.001$). There was no significant difference in decreasing TNF-α between the three HP-treated groups when compared with the Flx group [Figure 4].
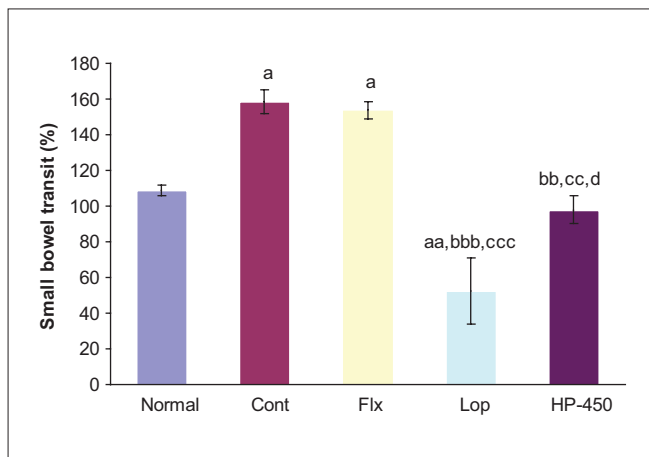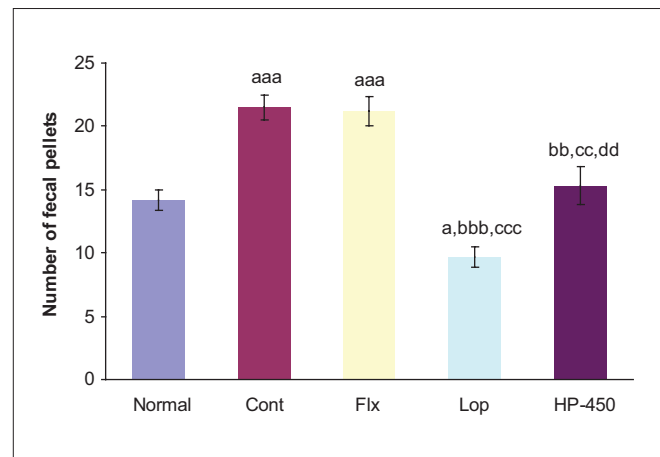

Figure 2: Small bowel transit percentages after 120 min. Values are mean ± SEM. ᵃSignificantly different from the normal group at $P < 0.05$. ᵃᵃSignificantly different from the normal group at $P < 0.01$. ᵃᵃᵃSignificantly different from the normal group at $P < 0.001$. ᵇSignificantly different from the control group at $P < 0.05$. ᵇᵇSignificantly different from the control group at $P < 0.01$. ᵇᵇᵇSignificantly different from the control group at $P < 0.001$. ᶜSignificantly different from the fluoxetine group at $P < 0.05$. ᶜᶜSignificantly different from the fluoxetine group at $P < 0.01$. ᶜᶜᶜSignificantly different from the fluoxetine group at $P < 0.001$. ᵈSignificantly different from the loperamide group at $P < 0.05$. ᵈᵈSignificantly different from the loperamide group at $P < 0.01$. ᵈᵈᵈSignificantly different from the loperamide group at $P < 0.001$

Figure 3: Fecal pellet excretion after 4 h. Values are mean ± SEM. ᵃSignificantly different from the normal group at $P < 0.05$. ᵃᵃSignificantly different from the normal group at $P < 0.01$. ᵃᵃᵃSignificantly different from the normal group at $P < 0.001$. ᵇSignificantly different from the control group at $P < 0.05$. ᵇᵇSignificantly different from the control group at $P < 0.01$. ᵇᵇᵇSignificantly different from the control group at $P < 0.001$. ᶜSignificantly different from the fluoxetine group at $P < 0.05$. ᶜᶜSignificantly different from the fluoxetine group at $P < 0.01$. ᶜᶜᶜSignificantly different from the fluoxetine group at $P < 0.001$. ᵈSignificantly different from the loperamide group at $P < 0.05$. ᵈᵈSignificantly different from the loperamide group at $P < 0.01$. ᵈᵈᵈSignificantly different from the loperamide group at $P < 0.001$
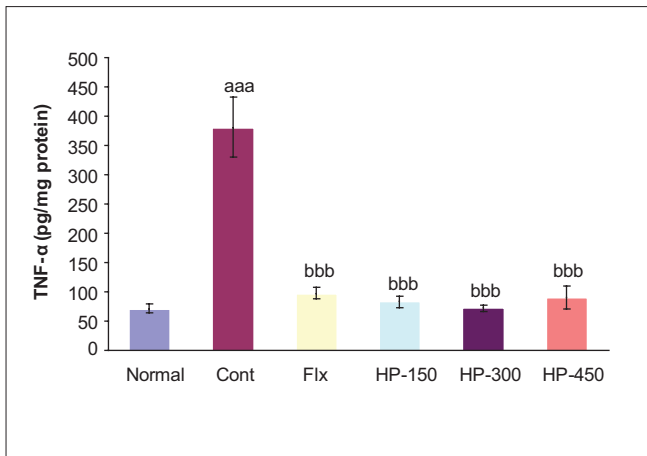
Figure 4: Tumor necrosis factor-alfa level in the colon. Values are mean ± SEM. [a]Significantly different from the normal group at $P < 0.05$. [aa]Significantly different from the normal group at $P < 0.01$. [aaa]Significantly different from the normal group at $P < 0.001$. [b]Significantly different from the control group at $P < 0.05$. [bb]Significantly different from the control group at $P < 0.01$. [bbb]Significantly different from the control group at $P < 0.001$. [c]Significantly different from the fluoxetine group at $P < 0.05$. [cc]Significantly different from the fluoxetine group at $P < 0.01$. [ccc]Significantly different from the fluoxetine group at $P < 0.001$. [d]Significantly different from the loperamide group at $P < 0.05$. [dd]Significantly different from the loperamide group at $P < 0.01$. [ddd]Significantly different from the loperamide group at $P < 0.001$
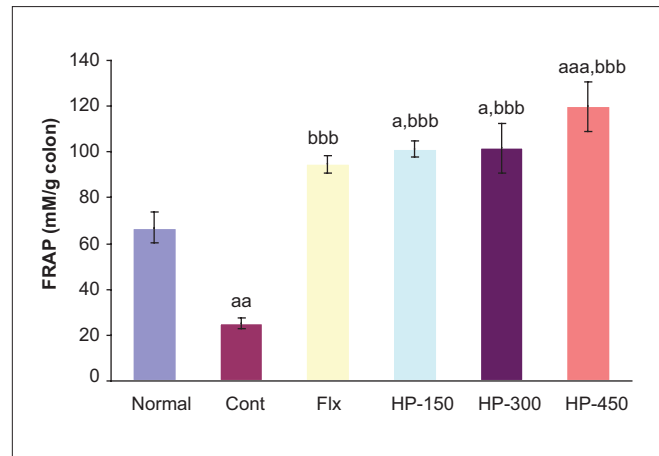
Figure 5: Ferric reducing anti-oxidant power of the colon. Values are mean ± SEM. [a]Significantly different from the normal group at $P < 0.05$. [aa]Significantly different from the normal group at $P < 0.01$. [aaa]Significantly different from the normal group at $P < 0.001$. [b]Significantly different from the control group at $P < 0.05$. [bb]Significantly different from the control group at $P < 0.01$. [bbb]Significantly different from the controlgroup at $P < 0.001$. [c]Significantly different from the fluoxetine group at $P < 0.05$. [cc]Significantly different from the fluoxetine group at $P < 0.01$. [ccc]Significantly different from the fluoxetine group at $P < 0.001$. [d]Significantly different from the loperamide group at $P < 0.05$. [dd]Significantly different from the loperamide group at $P < 0.01$. [ddd]Significantly different from the loperamide group at $P < 0.001$

Total antioxidant power as ferric reducing antioxidant power

FRAP in the Cont group was significantly lower than that in the normal group ($P < 0.01$). FRAP values were significantly increased in the Flx group in comparison with the Cont group ($P < 0.001$). Also, a significant increase was observed in all HP groups when compared with the Cont group ($P < 0.001$). There was no significant difference in the HP groups when compared with the Flx group. And, there was no significant difference between the HP groups [Figure 5].

Myeloperoxidase activity

MPO activity in the Cont group was significantly higher than that of the normal group ($P < 0.001$). Treatment with Flx decreased the MPO activity in comparison with the Cont group ($P < 0.001$). MPO activity decreased in all HP groups, which was significantly lower than that of the Cont group ($P < 001$). There was no difference between HP-treated and Flx groups. Also, there was no significant difference between the HP groups [Figure 6].

Lipid peroxidation level as thiobarbituric acid-reactive substance

TBARS was significantly higher in the Cont group when compared with the normal group ($P < 0.001$). Its level was significantly decreased in the Flx group as compared with the Cont group (0.01). TBARS decreased in the HP groups

in comparison with the Cont group ($P < 0.05$). TBARS was the same in the Flx and HP groups. There was no significant difference between the HP groups [Figure 7].

DISCUSSION

The present study has been carried out to evaluate the effects of HP on an established stress-induced experimental model of IBS using gastric emptying and small bowel and colon transit to assess whole gastrointestinal motor function and, moreover, to also investigate the effect of this extract on bowel inflammatory and oxidative stress biomarkers in comparison with loperamide and fluoxetine as approved psychological and symptomatic therapies were used for comparison with HP. The results showed that HP is able to reduce small bowel and colonic transit, TNF-α, MPO, and lipid peroxidation, and to improve the mucosal antioxidant capability to confronting the oxidative stress, which activated after 5-day restraint stress in control rats. However, HP was not able to significantly improve the stress-induced increase in gastric emptying.

The role of the central nervous system (CNS) factors in the pathogenesis of IBS is strongly suggested by the clinical association of emotional disorders and stress with symptom exacerbation and therapeutic response to therapies that act on the cerebral cortical sites such as antidepressants. Therefore, IBS is considered as a stress-related disorder.
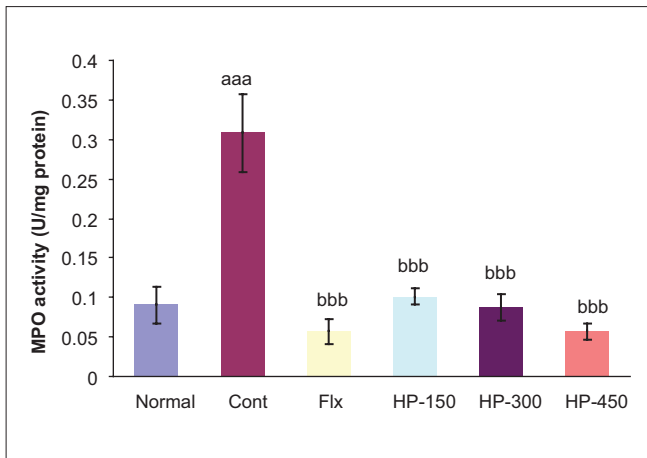
Figure 6: Myeloperoxidase (MPO) activity in the colon. Values are mean ± SEM. [a]Significantly different from the normal group at P <0.05. [aa]Significantly different from the normal group at P <0.01. [aaa]Significantly different from the normal group at P <0.001. [b]Significantly different from the control group at P <0.05. [bb]Significantly different from the control group at P <0.01. [bbb]Significantly different from the control group at P <0.001. [c]Significantly different from the fluoxetine group at P <0.05. [cc]Significantly different from the fluoxetine group at P <0.01. [ccc]Significantly different from the fluoxetine group at P <0.001. [d]Significantly different from the loperamide group at P <0.05. [dd]Significantly different from the loperamide group at P <0.01. [ddd]Significantly different from the loperamide group at P <0.001
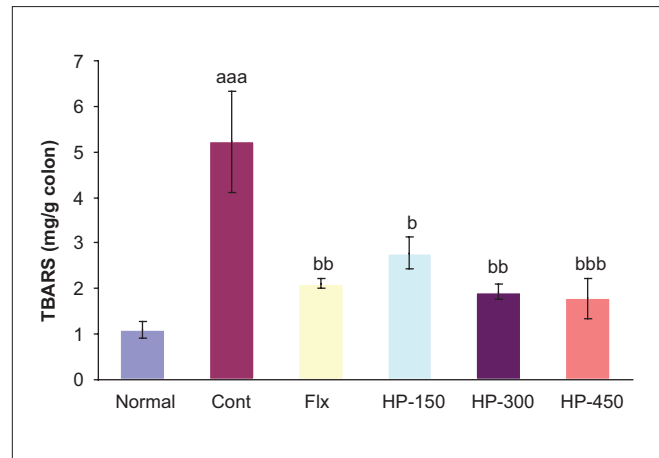

Figure 7: Lipid peroxidation as thiobarbituric acid-reactive substance in the colon. Values are mean ± SEM. [a]Significantly different from the normal group at P <0.05. [aa]Significantly different from the normal group at P <0.01. [aaa]Significantly different from the normal group at P <0.001. [b]Significantly different from the control group at P <0.05. [bb]Significantly different from the control group at P <0.01. [bbb]Significantly different from the control group at P <0.001. [c]Significantly different from the fluoxetine group at P <0.05. [cc]Significantly different from the fluoxetine group at P <0.01. [ccc]Significantly different from the fluoxetine group at P <0.001. [d]Significantly different from the loperamide group at P <0.05. [dd]Significantly different from the loperamide group at P <0.01. [ddd]Significantly different from the loperamide group at P <0.001

In response to stress, corticotropin releasing hormone (CRH) is released from the paraventricular nucleus (PVN) of the hypothalamus that stimulates secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH stimulates releasing cortisol from the adrenocortex. Also, CRH stimulates the sympathetic cardiovascular nervous system. On the other hand, it increases colonic motility via parasympathetic stimulation of the sacral nerve. In IBS, psychosocial or interoceptive stress is likely to induce CRH release, which results in the augmentation of colonic motility, visceral perception, and anxiety. Various effects that are seen with CRH work via two distinct CRH receptors (CRHR). Anxiety, visceral nociception, inflammation, and increased colonic motility are the responses to CRHR1 stimulation, whereas known responses to CRHR2 stimulation are anxiolysis, reduced visceral perception, delayed gastric emptying, and inflammatory response suppression. Known pro-inflammatory mechanism of CRH is via increasing the intestinal permeability and mast cell degranulation.[33]

Growing studies confirm the presence of a mild inflammation of the mucosa in IBS patients' intestine. Probably, psychosocial stress leads to this inflammation, which combined with excess gastrointestinal tract sensitization.[9] Recent studies indicated an increase in lymphocyte infiltration (e.g., CD3-positive and CD25-positive cells and mast cells) and elevated plasma cytokines, although histamine and proteases were found in IBS patients.[4] It was shown that IBS patients have an increased plasma IL-6, which was correlated positively with the ACTH level, while TNF-α was present in normal levels.[34]

We have set up a known model of stress-induced IBS model in rats. There are *in vivo* evidences that physical stress such as immobilization leads to weight loss and increased bowel transit, resulting in diarrhea and disturbances in barrier integrity, which allow more luminal antigens to pass through the mucosal barrier and, consequently, stimulate interstitial macrophages and dendritic cells in the lamina propria.[35] Ponferrada *et al.* showed that acute (6 h) and subacute (5 days), but not subchronic (10 days), exposure to immobilization stress resulted in barrier dysfunction and inflammatory responses. They suggested that immobilization stress in an acute or subacute period will develop a diarrhea, with evidences of involved immune response like IBS in humans.[10]

Besides the inflammatory response stimulation, stress has been shown to increase the intestinal membrane permeability,[36,37] allowing bacteria and other inflammatory macromolecules to pass through the mucosal layer, resulting in accumulation of inflammatory cells such mast cells, macrophages, polymorphonuclears, and lymphocytes in the muscularis and lamina propria.[38] The presence of these cells and their pro-inflammatory mediators leads to more

sensitization and nociception in Auerbach's plexus, which results in visceral hypersensitivity (motor abnormalities and abdominal pain). Barbara *et al*. demonstrated a 30% increase in lymphocyte infiltration in the colonic mucosa of IBS patients.[5] Dunlop *et al*. have shown increased intestinal permeability in patients with diarrhea-predominant IBS, and suggested an association between mucosal barrier dysfunction and immune activation in this subgroup of IBS patients.[39] In this study, we used MPO activity to assess leukocyte infiltration. Our results showed that MPO activity increased in 5-day exposure to immobilization stress, and its activity decreased significantly in the Flx and HP groups in a non dose-dependent manner.

There are evidences about the anti-inflammatory effects of the HP extract.[40,41] However, few studies evaluate this effect for fluoxetine.[42] The probable mechanism for this effect can be the influence of antidepressants on CRH releasing;[43] however, more evidences are needed to determine the definite mechanisms. Although antidepressant medications have several physiologic effects, suggesting that they may be beneficial in IBS, in addition to their mood-elevating effects. The beneficial effects of these compounds in the treatment of IBS appear to be independent of their effects on depression.[44,45] However, Butterweck *et al*. showed that, similar to imipramine, the HP extract decreased plasma ACTH and corticosterone after 2 weeks of daily treatment. They also showed that treatment with hypericin (0.2 mg/kg) given daily by gavage for 8 weeks significantly decreases CRH mRNA levels by 16–22% in the PVN.[46] As discussed later, CRH has a known effect on increasing the intestinal permeability that is usually seen in patients with diarrhea-predominant IBS.[39] Thus, it might provide a mechanism for activating the mucosal immune system,[7] as reflected by increased MPO activity in colonic samples.

There are some insights into the presence of inflammatory cytokines in IBS. Psychologic stress and anxiety can significantly increase the release of pro-inflammatory cytokines. Moreover, patients with psychiatric disorders have a higher chance of developing postinfectious IBS.[47,48] Liebregts and coworkers observed a significant positive association of TNF-α with anxiety and, unlike others, they suggested that the observed association of TNF-α and anxiety is linked to cytokine-induced hipothalamic–pituitary–adrenal axis activation, which consequently may modulate psychologic symptoms.[7] Dinan *et al*. indicated that increased serum IL-1, IL-6, and TNF-α levels synergistically stimulate the hypothalamic pituitary adrenal axis. Therefore, a positive feedback worsens the first stress effect.[34] Furthermore, a polymorphism in the TNF-α gene seems to be more incident in IBS patients, which encodes more production of this cytokine.[49] Our results indicated

a significant increase in TNF-α in colon samples after 5-day exposure to immobilization stress and pretreatment with HP extract. Meanwhile, fluoxetine reduced TNF-α and HP did not act dose-dependently. Liebregts and coworkers reported a significant increase of TNF-α release from peripheral macrophages, and suggested it as a more useful tool to identify cellular immune responses than that of plasma levels,[7] although in contrary to Dinan *et al.,* who did not observe any increase in the serum TNF-α of IBS patients.[34]

In our literature review, there were some investigations about the presence of oxidative stress in IBS. O'Sullivan and coworkers found an increase in iNOS expression in IBS patients.[50] Ding *et al*.[51] showed an increase in the expression of peroxiredoxins, a member of a new investigated family of antioxidant enzymes, which is one of the host defenses in response to oxidative stress, while quantifying the oxidative stress components is a valuable method for determining the severity of inflammation. Thus, we used TBARS and FRAP assays to appraise lipid peroxidation and antioxidant capability in colon samples of 5-day-immobilized stress-induced IBS in rats.

HP has been found to inhibit the activity or expression of radical producer enzymes, free radical scavenging, and metal ion chelating.[22,23,52] Interestingly, 5-day stress reduced the antioxidant power of the colon, indicating that activation of oxidative stress leads to the conclusion that a pick-up is observed in its level in the Flx and HP groups; later, the maximum effect was seen in the HP-450 group.

Lipid peroxidation refers to an oxidative reaction of unsaturated fatty acids with reactive oxygen species (ROS); if the concentration of radical species is high enough, chain reaction of radicals happens and if this reaction is not terminated fast enough, it results in cell damage. Our result illustrated a significant reduction in lipid peroxidation in the Flx and HP groups. The lowest TBARS was seen in HP-450. In the HP-450 group, TBARS dropped to its normal value, indicating that HP inhibits lipid peroxidation in a dose-dependent manner. Benedı *et al*. reported such a dose-dependent effect of the HP extract in inhibition of rat brain cortex lipid peroxidation.[23]

Stress has some effects on gastrointestinal motility, such as delayed gastric emptying or impaired gastric accordance, as mentioned later, and CRH is one of the stress-related neuropeptides that plays an important role in mediating delayed gastric emptying induced by stress.[53] Martinez *et al*. reported that injection of CRH intracisternaly inhibited gastric emptying.[54] Its probable mechanism to delay the gastric emptying is through the sympathetic nervous system via the activation of CRHR2 in the brain stem.[53]

Kalin and coworkers demonstrated that restraint stress increased CRH mRNA in PVN.[55] Ochi *et al.* showed that in a continuous stress model, gastric emptying delayed in the first day whereas it was accelerated on days 3 and 5 and, moreover, they illustrated that adrenalin and noradrenalin were increased in the initial first day in association with gastric emptying. In addition, in the late phase (3–5 days), ghrelin, an appetite-regulating factor, worked in a recovery from the delay in gastric emptying due to neural activation in the early phase.[56] Our results showed the same effects as those reported by Ochi and coworkers for IBS rats kept in a cage filled with water. Our result demonstrated that 5-day-restraint stress resulted in a significant increase in gastric emptying while pretreatment with fluoxetine and treatment with loperamide inhibited this subacute stress-induced acceleration of gastric emptying, but results from the HP-450 group showed that pretreatment with HP did not have any positive effect on inhibiting the increased gastric emptying in this model.

Colonic transit in the proximal colon is significantly accelerated in diarrhea-predominant IBS patients.[57] Stress-induced acceleration of colonic transit has the same mechanism, in which stress affects central and peripheral CRH receptors stimulation and vagal efferent and peripheral 5-HT3 receptors stimulation.[58] Some studies were designed to investigate whether stress-induced colonic transit acceleration is via central or peripheral signaling. Lenz and colleagues identified that intracerebroventricular injection of CRH accelerates colonic transit in rats, which are abrogated with vagotomy and atropine.[59] Although Habib *et al.* showed that intracerebroventricular injection of CRHR1 antagonists reduced the autonomic responses to stress,[60] peripheral injection did not influence these responses, but others showed that peripheral administration of CRHR1 antagonists abolished the effect of CRH on colon transit.[61] In addition to cerebral CRHR1, this receptor was also expressed in Auerbach's plexus; hence, CRH can increase colon motility via direct stimulation of CRHR1 in the enteric nervous system. Nakade and colleagues demonstrated that CRH released in response to restraint stress and stimulated central CRHR1 resulting in 5-HT release from the proximal colon to the lumen, released 5-HT stimulate 5-HT3 receptors and activation of this receptor increased colonic transit.[58]

The present study demonstrates that subacute restraint stress significantly increases colonic transit. Pretreatment with fluoxetine had no effect on the same while loperamide excessively inhibited colonic transit to the extent that the difference between Lop and the normal group was significant. Analyzing HP-450 data demonstrated that colonic transit reduced to normal values in this group.

There are some known mechanisms in which HP extract or its pharmacologically active component influence the stress-induced colonic transit acceleration. Hypericin potently inhibits CRHR1 stimulation at low concentrations (IC_{50} = 300 nM).[62] Moreover, Butterweck *et al.*[47] reported that 8-week treatment with hypericin given daily by gavage significantly decreased the levels of CRH mRNA in the PVN and also 5-HT1A receptor mRNA in the hippocampus. Others showed that amentoflavone could bind to brain benzodiazepine receptors with an affinity comparable to diazepam and also that amentoflavone significantly inhibited binding at 5-HT1D, 5-HT2C, and dopamine D3 receptors. In addition, quercitrin inhibited ATP-induced conductance and biapigenin inhibited both the acetylcholine and the ATP-induced conductance.[63]

There were a few clinical trials that were designed to evaluate the beneficial effects of HP extract in IBS,[64,65] whereas some controversies exist in their results. For instance, Yuri and coworkers suggested that HP is less-effective than placebo for IBS.[65] Of course, some differences exist in the composition of the extracts isolated in various studies. For instance, Melzer *et al.* reported distinct hyperforin content from 0.5 mg/unit to 24.87 mg/unit in 33 commercially available products in the German market.[66] However, more conclusive data are needed to assess the correlation between hyperforin concentration and efficacy.[63] Butterweck *et al.* also showed that removal of either hyperforin or hypericin did not alter the pharmacological activity of the whole extract,[67,68] indicating that HP is a mixture of several constituents with pharmacologic activity. The Iranian source of HP that was used in this study exists commercially as Hypiran drop for the management of migraine.

Supporting the present results, a recent study confirmed disturbances in endogenous melatonin concentration in IBS patients that recovered by administration of exogenous melatonin via anxiolytic, anti-inflammatory, anti oxidative and motility regulatory effects of on gastrointestinal tract.[69] Further supports come from other systematic reviews indicating benefit of anti oxidative herbs in management of inflammatory bowel disease.[70]

CONCLUSION

In conclusion, subacute restraint stress leads to a low-grade inflammatory IBS presented with increased neutrophil infiltration, TNF-α, lipid peroxidation, and reduced antioxidant power of colon tissue other than increased motor function of stomach and small bowel. We compared HP with fluoxetine, a conventional therapy for this disorder, and loperamide, an anti-diarrheal agent, in controlling

bowel motility. Fluoxetine-treated rats showed less gastric emptying, but this therapeutic agent had no beneficial effect in lowering the bowel transit increment. In contrast, HP inhibited small bowel and colon transit acceleration, but minimally affected gastric emptying. In addition, either pretreatment with fluoxetine or HP decreased recruitment of inflammatory cells, TNF-α, and oxidative stress within the colon tissue.

ACKNOWLEDGMENTS

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